NEW INSIGHTS INTO THE PROGNOSTIC VALUE OF THE TUMOR-STROMA RATIO IN PATIENTS WITH BREAST CANCER

Kiki M.H. Vangangelt

Colofon

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

ISBN: 978-94-6375-641-9

Illustrator: Marion Vangangelt
Lay-out: Vera van Ommeren | www.persoonlijkproefschrift.nl
Printing: Ridderprint | www.ridderprint.nl

Printing of this thesis was financially supported by Uitgeverij Jaap, Erbe Nederland B.V., Norgine B.V., Pfizer B.V., Servier Nederland Farma B.V., Chipsoft B.V., Sysmex Nederland B.V. and Blaak&Partners.

The research described in this thesis was financially supported by the Genootschap Keukenhof voor de vroege opsporing van kanker, Lisse, The Netherlands.

NEW INSIGHTS INTO THE PROGNOSTIC VALUE OF THE TUMOR-STROMA RATIO IN PATIENTS WITH BREAST CANCER

Proefschrift

ter verkrijging van de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.M.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 16 september 2020 klokke 13:45 uur

door

Kiki Margaretha Hubertina Vangangelt

geboren te Maastricht

in 1989

Promotor
Prof. dr. R.A.E.M. Tollenaar

Copromotor
Dr. W.E. Mesker

Leden promotiecommissie
Prof. dr. J.A van der Hage
Prof. dr. E. van der Wall, Universitair Medisch Centrum Utrecht, Utrecht
Prof. dr. P.J. van Diest, Universitair Medisch Centrum Utrecht, Utrecht

TABLE OF CONTENTS

1

Introduction and outline

EPIDEMIOLOGY OF BREAST CANCER

Breast cancer is the leading cause of cancer-related death in women. In 2016, almost 1.7 million women globally were diagnosed with breast cancer. In the same year, more than half a million women died due to this disease (1). Survival rates of breast cancer patients have improved over the last decade, mainly due to improvements in organized screening, early diagnosis and treatment modalities (2).

PROGNOSTIC MARKERS IN STANDARD CLINICAL CARE AND NEW PROGNOSTICATORS

Breast cancer is a heterogeneous disease with different morphological and biological features. This leads to differences in clinical behavior and response to treatment. Tailor-made treatment is a promising strategy to improve prognosis. Prognostic markers are important to identify patients with a high or low risk of disease relapse and cancer-related death. By identifying patients with a low risk of recurrences, patients can be spared from adjuvant treatment. This will result in decreased overtreatment and harmful side effects, such as heart failure and cognitive dysfunction (3). On the other hand, selecting patients with an aggressive type of breast cancer will decrease the risk of undertreatment and thereby the risk of recurrence or breast cancer-related death.

An online tool frequently used in daily practice for the estimation of prognosis and expected adjuvant treatment benefit in patients diagnosed with early invasive breast cancer primarily treated with surgery is the PREDICT (4). The prognostic parameters used in this tool are based on features of the tumor cells and patient characteristics. The PREDICT includes age, post-menopausal status, estrogen receptor (ER) status, human epidermal growth factor receptor 2 (HER2) status, Ki67 status, tumor size, lymph node involvement, differentiation grade and mode of detection. This prediction tool helps clinicians and patients in decision-making about adjuvant therapies by calculating the expected 5-, 10- and 15-year overall survival. Moreover, the online program provides the beneficial value of post-operative treatment options (chemotherapy, endocrine therapy, trastuzumab and bisphosphonates) (5, 6). During the last decades, a great amount of research has

been performed to develop new prognostic biomarkers and tests. Gene expression profiling tests, such as the MammaPrint (70-gene profile) and Oncotype DX Breast Cancer Assay (20-gene profile) are well investigated (7, 8). The Dutch national guidelines recommend these tests in case of doubt about the indication of adjuvant chemotherapy for patients older than 35 years diagnosed with invasive carcinoma of no special type (NST).

In 2011, Hanahan et al. published an important update in *Cell* on the role of the tumor microenvironment in cancer development. The authors determined that the tumor microenvironment plays a pivotal role in tumorigenesis (9). Although increasing efforts have focused on the research of the tumor microenvironment, no markers of the microenvironment have been implemented in standard clinical care in the Netherlands yet. Cancer cells and the tumor microenvironment are in a complex interplay and evolve continuously during tumor progression (10). Cancer cells recruit and activate non-neoplastic cells, such as fibroblasts, the extracellular matrix, cells evolved in a vascular network and immune cells (11). The non-neoplastic cells secrete proteins which contribute in tumor progression, such as vascular endothelial growth factor, stromal cell-derived factor 1, platelet-derived growth factor and transforming growth factor-β. Also, cancer-associated fibroblasts are thought to be strongly involved in cancer progression (12). Immune cells are an important component of the tumor microenvironment and have either an antitumorigenic or protumorigenic effect on cancer development. A prognostic marker involving the tumor microenvironment are tumor-infiltrating lymphocytes (TILs). TILs show to have prognostic and predictive value in breast cancer patients (13-17), but are not integrated into standard clinical care yet.

Another tumor microenvironment derived prognostic marker, which is the main topic of this thesis, is the tumor-stroma ratio (TSR). Assessment of this parameter is quick, easy to perform and inexpensive. The scoring is performed on routine hematoxylin and eosin (H&E) stained tissue slides with a conventional light microscope (18). The TSR represents the proportion of stroma versus tumor cells in the most stroma-abundant field of a primary tumor. Mesker et al. first described this scoring method in 2007 (18). Since then, this method is validated in various solid tumors by different research groups (19-42). Most studies demonstrate that

cancer patients with a stroma-high tumor have a worse clinical outcome compared to patients with a stroma-low tumor. **Chapter 2** discusses the studies published on the prognostic value of the TSR in breast cancer patients with special attention to the effect on clinical outcome in patients with triple-negative tumors. This review also provides an insight into the methods used for the TSR assessment and the rationale behind the importance of tumor-associated stroma.

Further research presented in this thesis aims (1) to optimize the prognostic impact of the TSR in subgroups of breast cancer patients and (2) to investigate the prognostic impact of the TSR in combination with other tumor-related parameters.

Clinically relevant subgroup analyses in a heterogeneous disease

Breast cancer is a heterogeneous disease and can be divided into different subgroups. Firstly, a subdivision can be made based on the histological type, of which invasive carcinoma of NST is the most common subgroup. The diversity in histological aspects is already translated into this specific subgroup, as the World Health Organization (WHO) describes invasive carcinoma of NST as a group of tumors which do not possess specific characteristics to be classified in a particular histological type. This is in contrast to lobular carcinomas, which are the second most common histological group (43). The various histological subtypes are associated with different outcomes. For example, papillary tumors have better outcomes compared to invasive lobular carcinomas (44).

Subgroups can also be based on ER, progesterone receptor (PR) and HER2 status. These parameters have a prognostic and predictive value and are therefore assessed in routine clinical care (44). Furthermore, breast cancer can also be divided into four molecular subtypes based on gene expression; luminal A, luminal B, HER2-enriched and basal-like tumors.

Tumor grade is part of the standard evaluation of breast cancer tissue and is a robust prognostic parameter used in clinical decision-making and online tools such as the PREDICT. The tumor grade is classified into three groups (low, intermediate and high) based on the pathological evaluation of tubule and gland formation on H&E slides, nuclear polymorphism and mitotic counts (45).

Chapter 3 elaborates on the prognostic impact of the TSR in clinically relevant subgroups of breast cancer patients. In this chapter, the effect of TSR assessment,

categorized in stroma-low and stroma-high, on breast cancer-specific survival and recurrence-free survival is evaluated in the largest cohort published so far.

Older women with breast cancer

A major risk factor for breast cancer development in women is aging (46). At the moment, the majority of women are older than 65 years at the time of diagnosis, and the incidence will increase as the general population is aging (46-48). The significant improvement in survival rates in younger women with breast cancer in the last 30 years has not been observed in older patients (49). Disease-specific mortality is often underestimated in older patients (50). This may suggest that older patients may be undertreated, as at the moment, few patients over the age of 70 receive chemotherapy. More accurate identification of disease aggressiveness in the older patient is necessary to improve the selection of patients who will benefit from extensive adjuvant therapy in order to reduce the gap in survival rates between younger and older patients with breast cancer.

Tumor biology in older patients is different compared to their younger counterparts. The tumors of older patients have shown to possess lower proliferation rates, to be genetically more stable and more often ER-positive (51). Furthermore, differences between younger and older patients with breast cancer are observed in the extracellular matrix and products secreted by senescent fibroblasts (52). Research showed that the molecular profile of the tumor microenvironment is age-dependent. For instance, induced stromal features associated with a senescence-associated secretory profile and autophagy which promote tumorigenesis are observed in older patients with triple-negative tumors (53).

In **chapter 4,** the differences in the amount of intra-tumoral stroma with the increase of age are evaluated by the assessment of the TSR. Moreover, the prognostic value of the TSR in older patients with breast cancer is assessed.

Tumor-positive axillary lymph nodes

The presence of lymph node metastasis are an important prognostic factor for predicting long-term clinical outcome (54). Axillary lymph node dissection (ALND) was standard therapy in patients with tumor-positive lymph nodes before the introduction of the sentinel node biopsy (SNB). Recent studies show that not all patients with tumor-positive lymph nodes need an ALND or adjuvant radiotherapy.

Downsizing therapy to prevent overtreatment is desirable, for example, to minimize unnecessary side effects, such as invalidating lymphoedema of the arm after ALND. **Chapter 5** evaluates the prognostic impact when adding TSR assessment of the tumor-positive lymph nodes to TSR assessment of the primary tumor alone. This might lead to a better stratification of high-risk patients and finally to improved decision-making concerning treatment.

Immune infiltration in breast cancer

On the one hand, immune cells are an important component of the tumor microenvironment. The immune system can control tumor progression, but on the other hand, the tumor cells can acquire modalities to escape the host immune system through their genetically unstable appearance (55, 56). Immune infiltration in breast cancer is related to prognosis and treatment response. For example, the presence of regulatory T cells (Tregs) is associated with a poor prognosis (57).

In **chapter 6,** the prognostic value of the immune status of tumors combined with the TSR is evaluated. The immunological markers included in the immune status are markers which play a role in tumor control and escape; cytotoxic T lymphocytes (CTLs), Tregs, classical human leukocyte antigen (HLA) class I (HLA-A, HLA-B and HLA-C), non-classical HLA class I (HLA-E and HLA-G) and natural killer (NK) cells.

These immunological markers are selected based on biological rationale and interactions; classical HLA class I presents tumor-associated antigens on the cell surface of the tumor and CTLs recognize the presented tumor-associated antigens (58). Tumor cells can downregulate HLA expression to escape recognition by CTLs, but make them more prone to NK cell recognition (59). Expression of non-classical HLA class I can inhibit the function of NK cells (59-61). Furthermore, tumor cells can attract and activate Tregs and thereby contribute to tumor progression (62).

Finally, **chapter 7** summarizes and discusses the published research in this thesis and describes future perspectives. The summary in Dutch is presented in **Chapter 8**. **Chapter 9** provides a list of publications, curriculum vitae and acknowledgements.

REFERENCES

1. Global Burden of Disease Cancer C, Fitzmaurice C, Akinyemiju TF, Al Lami FH, Alam T, Alizadeh-Navaei R, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2016: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol. 2018.
2. Malvezzi M, Carioli G, Bertuccio P, Boffetta P, Levi F, La Vecchia C, et al. European cancer mortality predictions for the year 2019 with focus on breast cancer. Ann Oncol. 2019;30(5):781-7.
3. Khan NF, Mant D, Carpenter L, Forman D, Rose PW. Long-term health outcomes in a British cohort of breast, colorectal and prostate cancer survivors: a database study. Br J Cancer. 2011;105 Suppl 1:S29-37.
4. https://breast.predict.nhs.uk. 21-05-2019.
5. Wishart GC, Azzato EM, Greenberg DC, Rashbass J, Kearins O, Lawrence G, et al. PREDICT: a new UK prognostic model that predicts survival following surgery for invasive breast cancer. Breast Cancer Res. 2010;12(1):R1.
6. Candido Dos Reis FJ, Wishart GC, Dicks EM, Greenberg D, Rashbass J, Schmidt MK, et al. An updated PREDICT breast cancer prognostication and treatment benefit prediction model with independent validation. Breast Cancer Res. 2017;19(1):58.
7. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. New Engl J Med. 2016;375(8):717-29.
8. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. New Engl J Med. 2004;351(27):2817-26.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
10. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer. 2004;4(11):839-49.
11. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature. 2013;501(7467):346-54.
12. Hawinkels LJ, Paauwe M, Verspaget HW, Wiercinska E, van der Zon JM, van der Ploeg K, et al. Interaction with colon cancer cells hyperactivates TGF-beta signaling in cancer-associated fibroblasts. Oncogene. 2014;33(1):97-107.
13. Dieci MV, Conte P, Bisagni G, Brandes AA, Frassoldati A, Cavanna L, et al. Association of tumor-infiltrating lymphocytes with distant disease-free survival in the ShortHER randomized adjuvant trial for patients with early HER2+ breast cancer. Ann Oncol. 2019;30(3):418-23.
14. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26(2):259-71.

15. Loi S, Drubay D, Adams S, Pruneri G, Francis PA, Lacroix-Triki M, et al. Tumor-Infiltrating Lymphocytes and Prognosis: A Pooled Individual Patient Analysis of Early-Stage Triple-Negative Breast Cancers. J Clin Oncol. 2019;37(7):559-69.
16. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol. 2014;25(8):1544-50.
17. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40-50.
18. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
19. Courrech Staal EF, Smit VT, van Velthuysen ML, Spitzer-Naaykens JM, Wouters MW, Mesker WE, et al. Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies. Eur J Cancer. 2011;47(3):375-82.
20. de Kruijf EM, van Nes JG, van de Velde CJ, Putter H, Smit VT, Liefers GJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat. 2011;125(3):687-96.
21. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. Ejso. 2012;38(4):307-13.
22. Dekker TJA, van de Velde CJH, van Pelt GW, Kroep JR, Julien JP, Smit VTHBM, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Tr. 2013;139(2):371-9.
23. Gujam FJ, Edwards J, Mohammed ZM, Going JJ, McMillan DC. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. Br J Cancer. 2014;111(1):157-65.
24. Roeke T, Sobral-Leite M, Dekker TJA, Wesseling J, Smit V, Tollenaar R, et al. The prognostic value of the tumour-stroma ratio in primary operable invasive cancer of the breast: a validation study. Breast Cancer Res Treat. 2017;166(2):435-45.
25. West NP, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, et al. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. Br J Cancer. 2010;102(10):1519-23.
26. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Ann Oncol. 2014;25(3):644-51.

27. Vogelaar FJ, van Pelt GW, van Leeuwen AM, Willems JM, Tollenaar RA, Liefers GJ, et al. Are disseminated tumor cells in bone marrow and tumor-stroma ratio clinically applicable for patients undergoing surgical resection of primary colorectal cancer? The Leiden MRD study. Cell Oncol (Dordr). 2016;39(6):537-44.
28. Aurello P, Berardi G, Giulitti D, Palumbo A, Tierno SM, Nigri G, et al. Tumor-Stroma Ratio is an independent predictor for overall survival and disease free survival in gastric cancer patients. Surgeon. 2017;15(6):329-35.
29. Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013;24(1):179-85.
30. Wang K, Ma W, Wang JB, Yu L, Zhang XM, Wang ZB, et al. Tumor-Stroma Ratio Is an Independent Predictor for Survival in Esophageal Squamous Cell Carcinoma. J Thorac Oncol. 2012;7(9):1457-61.
31. Mesker WE, Liefers GJ, Junggeburt JM, van Pelt GW, Alberici P, Kuppen PJ, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients. Cell Oncol. 2009;31(3):169-78.
32. Chen Y, Zhang L, Liu W, Liu X. Prognostic Significance of the Tumor-Stroma Ratio in Epithelial Ovarian Cancer. Biomed Res Int. 2015;2015:589301.
33. Hansen TF, Kjaer-Frifeldt S, Lindebjerg J, Rafaelsen SR, Jensen LH, Jakobsen A, et al. Tumor-stroma ratio predicts recurrence in patients with colon cancer treated with neoadjuvant chemotherapy. Acta Oncol. 2018;57(4):528-33.
34. Li H, Yuan SL, Han ZZ, Huang J, Cui L, Jiang CQ, et al. Prognostic significance of the tumor-stroma ratio in gallbladder cancer. Neoplasma. 2017;64(4):588-93.
35. Liu J, Liu J, Li J, Chen Y, Guan X, Wu X, et al. Tumor-stroma ratio is an independent predictor for survival in early cervical carcinoma. Gynecol Oncol. 2014;132(1):81-6.
36. Lv Z, Cai X, Weng X, Xiao H, Du C, Cheng J, et al. Tumor-stroma ratio is a prognostic factor for survival in hepatocellular carcinoma patients after liver resection or transplantation. Surgery. 2015;158(1):142-50.
37. Niranjan KC, Sarathy NA. Prognostic impact of tumor-stroma ratio in oral squamous cell carcinoma - A pilot study. Ann Diagn Pathol. 2018;35:56-61.
38. Pongsuvareeyakul T, Khunamornpong S, Settakorn J, Sukpan K, Suprasert P, Intaraphet S, et al. Prognostic evaluation of tumor-stroma ratio in patients with early stage cervical adenocarcinoma treated by surgery. Asian Pac J Cancer Prev. 2015;16(10):4363-8.
39. Scheer R, Baidoshvili A, Zoidze S, Elferink MAG, Berkel AEM, Klaase JM, et al. Tumor-stroma ratio as prognostic factor for survival in rectal adenocarcinoma: A retrospective cohort study. World J Gastrointest Oncol. 2017;9(12):466-74.
40. Xi KX, Wen YS, Zhu CM, Yu XY, Qin RQ, Zhang XW, et al. Tumor-stroma ratio (TSR) in non-small cell lung cancer (NSCLC) patients after lung resection is a prognostic factor for survival. J Thorac Dis. 2017;9(10):4017-26.

41. Zhang XL, Jiang C, Zhang ZX, Liu F, Zhang F, Cheng YF. The tumor-stroma ratio is an independent predictor for survival in nasopharyngeal cancer. Oncol Res Treat. 2014;37(9):480-4.
42. Zhang T, Xu J, Shen H, Dong W, Ni Y, Du J. Tumor-stroma ratio is an independent predictor for survival in NSCLC. Int J Clin Exp Pathol. 2015;8(9):11348-55.
43. Lakhani SR EI, Schnitt SJ, Tan PH, Van De Vijver MJ, editors. WHO Classification of Tumours of the Breast. Lyon: France International Agency for Research on Cancer; 2012.
44. Turashvili G, Brogi E. Tumor Heterogeneity in Breast Cancer. Front Med (Lausanne). 2017;4:227.
45. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991;19(5):403-10.
46. Beard JR, Officer A, de Carvalho IA, Sadana R, Pot AM, Michel JP, et al. The World report on ageing and health: a policy framework for healthy ageing. Lancet. 2016;387(10033):2145-54.
47. Rosenberg PS, Barker KA, Anderson WF. Estrogen Receptor Status and the Future Burden of Invasive and In Situ Breast Cancers in the United States. J Natl Cancer Inst. 2015;107(9).
48. DeSantis CE, Ma J, Goding Sauer A, Newman LA, Jemal A. Breast cancer statistics, 2017, racial disparity in mortality by state. CA Cancer J Clin. 2017;67(6):439-48.
49. Bastiaannet E, Liefers GJ, de Craen AJ, Kuppen PJ, van de Water W, Portielje JE, et al. Breast cancer in elderly compared to younger patients in the Netherlands: stage at diagnosis, treatment and survival in 127,805 unselected patients. Breast Cancer Res Treat. 2010;124(3):801-7.
50. van de Water W, Markopoulos C, van de Velde CJ, Seynaeve C, Hasenburg A, Rea D, et al. Association between age at diagnosis and disease-specific mortality among postmenopausal women with hormone receptor-positive breast cancer. JAMA. 2012;307(6):590-7.
51. Benz CC. Impact of aging on the biology of breast cancer. Crit Rev Oncol Hematol. 2008;66(1):65-74.
52. Lodi M, Scheer L, Reix N, Heitz D, Carin AJ, Thiebaut N, et al. Breast cancer in elderly women and altered clinico-pathological characteristics: a systematic review. Breast Cancer Res Treat. 2017;166(3):657-68.
53. Brouwers B, Fumagalli D, Brohee S, Hatse S, Govaere O, Floris G, et al. The footprint of the ageing stroma in older patients with breast cancer. Breast Cancer Res. 2017;19(1):78.
54. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2015;26 Suppl 5:v8-30.

55. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006;6(10):715-27.
56. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004;22:329-60.
57. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol. 2006;24(34):5373-80.
58. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother. 2004;53(10):904-10.
59. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol. 2007;17(6):459-68.
60. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol. 2002;3(11):999-1005.
61. Marin R, Ruiz-Cabello F, Pedrinaci S, Mendez R, Jimenez P, Geraghty DE, et al. Analysis of HLA-E expression in human tumors. Immunogenetics. 2003;54(11):767-75.
62. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A. 2001;98(20):11521-6.

2

The prognostic value of the tumor-stroma ratio in primary breast cancer with special attention to triple negative tumors: a review

C.J.H. Kramer
K.M.H. Vangangelt
G.W. van Pelt
T.J.A. Dekker
R.A.E.M. Tollenaar
W.E. Mesker

Breast Cancer Research and Treatment, 2019 Jan;173(1):55-64

ABSTRACT

Purpose

There is a strong need to improve the prognostication of breast cancer patients in order to prevent over- and undertreatment, especially when considering adjuvant chemotherapy. Tumor stroma characteristics might be valuable in predicting disease progression.

Methods

Studies regarding the prognostic value of the tumor-stroma ratio (TSR) in breast cancer were evaluated.

Results

A high stromal content was related to a relatively poor prognosis. The most pronounced prognostic effect of this parameter seemed to be observed in the triple-negative breast cancer subtype.

Conclusions

TSR assessment might represent a simple, fast and reproducible prognostic factor at no extra costs, and could be incorporated into routine pathological diagnostics. Despite these advantages, robust clinical validation of this parameter has yet to be established in prospective studies.

INTRODUCTION

According to the European cancer statistics for 2018, the estimated number of new breast cancer cases is 522.500 and the estimated number of breast cancer related-deaths is 137.700 (1). Breast tumors are classified into four molecular subtypes, namely luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched and basal-like (2, 3). The triple-negative breast cancer (TNBC) belongs to the basal-like phenotype in the vast majority, which is an aggressive form of breast cancer with a shorter relapse-free period (RFP) and relative survival compared to luminal A and B (4, 5). However, gene-expression analyses have shown that this group is notoriously heterogeneous, with some molecular subtypes even associated with a relatively favorable prognosis (5). Approximately 16% of all breast cancer cases are represented by TNBC (6).

In recent years, extensive research has been performed to discover new prognostic biomarkers and determine optimal prognostication schemes for breast cancer patients. Molecular tests, such as the 70-gene signature (MammaPrint, Agendia BV, The Netherlands) and the 21-gene assay (Oncotype DX, Genomic Health, United States) have shown to improve clinical decision making in early-stage breast cancer of certain molecular and clinical subtypes, such as estrogen receptor (ER)-positive or HER2-negative breast cancer (7, 8). These molecular markers are now endorsed into routine clinical practice, according to the American Society of Clinical Oncology Clinical Practice guideline, to reduce the administration of adjuvant chemotherapy and prevent overtreatment (9).

Despite the fact that alterations in the tumor microenvironment have been recognized as important drivers of tumor progression, the tumor environment has not been integrated in routine clinical decision making yet. A parameter which translates the amount of tumor-associated stroma is the tumor-stroma ratio (TSR), which has been extensively described as a rich source of prognostic information for various solid cancer types (10-38). The TSR was first described as a prognostic factor in breast cancer in 2011 by De Kruijf et al. and has been validated in numerous studies (12-15, 17).

For TSR assessment, the amount of tumor-associated stroma is determined on routine hematoxylin and eosin (H&E) stained slides of the primary tumor tissue.

Each tumor is assigned to either the stroma-high or stroma-low category based on a set cut-off value (10).
In this review, literature investigating the effect of the TSR as a prognostic factor in female breast cancer is discussed with a special interest in the prognostic effect in TNBC patients.

RATIONALE

The influence of the tumor-associated stroma on epithelial tumor progression is mostly derived from functional in vitro studies. Similarly, those in vitro studies have demonstrated events in the stromal compartment that occur during carcinogenesis and could contribute to tumor progression. The production of growth factors and proteases by cancer cells initiate changes in the stromal environment (39). Those alterations lie within remodeling of the matrix, recruitment of fibroblasts, the migration of immune cells and angiogenesis, all contributing to tumor progression (40). Cancer-associated fibroblasts (CAFs) contribute to carcinogenesis through the development of unique functions, including an amplified extracellular matrix (ECM) production, higher proliferation rate and the secretion of several cytokines, like vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF1) and platelet-derived growth factor (PDGF), leading to angiogenesis (40). Transforming growth factor-β (TGF-β) is another factor that is thought to be strongly involved in the tumor-promoting effects of CAFs as described in colon cancer by Hawinkels et al. (41). Those behavioral modifications lead to an elevated expression of enzymes, like matrix metalloproteinases (MMPs), resulting in remodeling and deposition of the ECM, with concurrently the release of pro-angiogenic factors (42).
The ECM is frequently disorganized in tumors. One of the most important mechanisms in the ECM contributing to tumor progression is collagen crosslinking. Due to crosslinking collagen by lysyl oxidase (LOX), the ECM of the tumor becomes more stiff, leading to increased focal adhesions and enhanced PI3K signaling, thereby indirectly ensuring tumor progression (43). Besides the fact that alterations in the tumor niche lead to progression directly, the tumorigenesis can also be strengthened indirectly due to the aforementioned production of

pro-angiogenic factors by CAFs and immune cells. Thus, during the process of tumorigenesis, changes occur in the organization of stromal cells, contributing both directly and indirectly to tumor growth and progression.

Previous studies investigating gene-expression profiles in stromal cells have demonstrated gene signatures related to clinical outcome and response to treatment in breast cancer (44, 45). Clinical application of these signatures was impractical and a definitive indication was never discovered. However, these studies did provide a strong indication that valuable clinical information was ignored by solely focusing on the epithelial compartment. As the stromal processes that are reflected by these assays likely have a quantitative relationship with the amounts of stromal tissue within the tumor, quantitative stromal parameters might equally express prognostic information just by morphology alone (45).

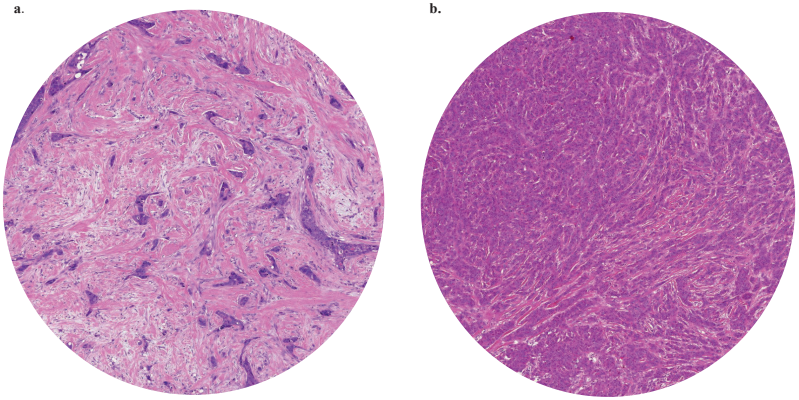
METHODS USED FOR TSR ASSESSMENT

In literature, two methods are described for TSR assessment in breast cancer. The visual scoring method utilized by Mesker et al. and the automated point counting method, a semi-automated approach, utilized by West et al. (10, 18).

Visual eyeballing

Mesker et al. and others determined the TSR by visual eyeballing (10, 12-17). The microscopic determination of the amount of stroma in the primary tumor is performed on routine H&E stained slides. A 2.5x or 5x objective is used to determine the most stroma-abundant area on the slide. In this area, image-fields with tumor cells at all borders of the image are used to determine the amount of stroma, using a 10x objective. The stroma percentage is estimated in increments of 10% per image-field, considering the highest scored stroma percentage as decisive. A stroma percentage ≤50% is categorized as stroma-low and a stroma percentage >50% is categorized as stroma-high, based on the statistical determination, initially performed on colon cancer and subsequently verified for breast cancer (figure 1) (10, 18). Considerable segments of necrosis or *in situ* tumors were excluded in the evaluation of the TSR by neglecting them in the analysis (12, 14).

FIGURE 1. Microscopic evaluation of the tumor-stroma ratio on hematoxylin and eosin stained sections of breast tumors with a 10x objective categorized in stroma-high tumors (>50% stroma) and stroma-low tumors (≤50% stroma) by visual eyeballing. **a.** Stroma-high **b.** Stroma-low.



Semi-automated point counting

West and colleagues have objectified the measurement by evaluating the tumor tissue slides in colon carcinoma using 300 random measurement points validated for breast cancer by Downey et al. (18, 46, 47). Four-micrometer-thick H&E stained sections are scanned using a 20x objective and subsequently two areas without large segments of necrosis are selected with a digital slide viewer. In this method, the two sampled 9 mm^2 areas are in the tumor-leading edge, as well as in the non-leading edge. The group utilizes a grid with a sample of 300 random points, superimposed on the selected area. Under each of the 300 points, the histopathology is categorized in 'tumor', 'stroma' or 'unclassified' (necrosis, blood vessels, inflammation, etc.). The ultimate TSR is the proportion of 'stroma' under the 300 points, compared with all points per section. In other words, the TSR is the number of points, categorized as 'stroma' divided by the total number of points, categorized as 'tumor' and 'unclassified' (18, 46, 47). Downey et al. used 0.49 (i.e. 49%) as a cut-off value in their study in 2014, with ^{3}0.49 being stroma-high and <0.49 stroma-low, based on

statistical analysis (46). However, in another study, cut-off values of 0.31 for OS and 0.46 for DFS are used for categorizing the TSR (47).
The inter-observer variation of these two methods, determined by the Cohen's kappa coefficient (K) or intraclass correlation coefficient (ICC), lies in the range of 0.68-0.85, indicating substantial to good agreement between observers in both methods (table 1).

THE TUMOR-STROMA RATIO IN BREAST CANCER PATIENTS

The first study on the TSR in breast cancer was published by De Kruijf et al. (12). The TSR was estimated by visual eyeballing according to the method described by Mesker et al. (10). The authors showed that the TSR was an independent prognostic parameter in 574 breast cancer patients with invasive breast tumors without distant metastasis (pT1-4, pN0-3, M0). Stroma-high tumors were associated with a worse RFP (HR 1.97, 95% CI 1.47-2.64, $p < 0.001$) and overall survival (OS) (HR 1.50, 95% CI 1.18-1.91, $p = 0.001$) analyzed with multivariate Cox regression analysis (table 1) (12). Vangangelt et al. analyzed the prognostic value of the TSR in a subset of the cohort of De Kruijf et al. in combination with the immune status of tumors. Determination of classical human leukocyte antigen (HLA) class I, HLA-E, HLA-G, natural killer cells and/or regulatory T cells in addition to the TSR showed to have an even stronger prognostic effect (16).
Dekker et al. investigated the prognostic value of the amount of stroma determined by visual eyeballing in 403 premenopausal node-negative breast cancer patients (cT1-3) (14). These patients were selected from the perioperative chemotherapy trial (POP trial, 10854) (48). This study supported the earlier finding of the TSR as an independent prognostic parameter for disease-free survival (DFS) (HR 1.85, 95% CI 1.33-2.59, $p < 0.001$) in favor of stroma-low tumors and borderline statistical significance for OS (HR 1.60, 95% CI 1.00-2.57, $p = 0.050$) (14).
Gujam et al. assessed the TSR on the H&E slides of 361 patients with invasive carcinoma of no special type (NST) (T1-3, N0->3, grade I-III) and subsequently found a correlation between stroma-high tumors and a poor 15-year cancer-specific survival (CSS) (HR 2.12, 95% CI 1.37-3.29, $p = 0.001$) in the multivariate survival analysis (15). Downey et al. dispute this finding in their work by analyzing the

TABLE 1. Detailed overview of studies on the prognostic value of the tumor-stroma ratio in the main study population and triple-negative breast cancer patients.

	Author, year	Sample Size	Percentage of stroma-high tumors	Population	Method	Inter-observer variation	Outcome	Most favorable prognosis
Results of the main study population (except triple-negative breast cancer as main cohort or subgroup)								
General BC	De Kruijf et al., 2011 (12)	574	68%	pT1-4, pN negative-positive, grade I-III	VS	K = 0.85	OS: HR 1.50, 95% CI 1.18-1.91, $p = 0.001$ RFP: HR 1.97, 95% CI 1.47-2.64, $p < 0.001$	Stroma-low
General BC	Dekker et al., 2013 (14)	403	40%	cT1-3, N0, grade I-III	VS	K = 0.804	OS: HR 1.60, 95% CI 1.00-2.57, $p = 0.050$ DFS: HR 1.85, 95% CI 1.33-2.59, $p < 0.001$	Stroma-low
General BC	Roeke et al., 2017 (17)	737	38%	T1-3, N negative–positive, grade I-III	VS	K = 0.68	OS: HR 1.56, 95% CI 1.18-2.05, $p = 0.002$ RFS:HR 1.35, 95% CI 1.01-1.81, $p = 0.046$ DMFS: HR 1.52, 95% CI 1.12-2.06, $p = 0.008$	Stroma-low
Invasive carcinoma of NST	Gujam et al., 2014 (15)	361	30%	T1-3, N0->3, grade I-III	VS	ICC = 0.83	CSS: HR 2.12, 95% CI 1.37-3.29, $p = 0.001$	Stroma-low
Estrogen receptor positive BC	Downey et al., 2014 (46)	118		N0-3, grade I-III	APC	K = 0.70	OS: HR 0.2-0.7, $p = 0.008$ RFS: HR 0.1-0.6, $p = 0.006$	Stroma-high
Inflammatory BC	Downey et al., 2015 (47)	45		N0-3, grade I-III	APC		OS: $p = 0.53$ DFS: $p = 0.66$	No difference

TABLE 1. Continued.

	Author, year	Sample Size	Percentage of stroma-high tumors	Population	Method	Inter-observer variation	Outcome	Most favorable prognosis
Results of triple-negative breast cancer as main study population or subgroup								
TNBC	Moorman et al., 2012 (13)	124	40%	pT1-4, pN0-3, grade I-III	VS	K = 0.74	OS: HR 3.00, 95% CI 1.08-8.32, p = 0.034 RFP: HR 2.39, 95% CI 1.07-5.29, p = 0.033	Stroma-low
TNBC	De Kruijf et al., 2011 (12)	82	56%	pT1-4, pN negative-positive, grade I-III	VS		OS: HR 1.87, 95% CI 1.07-3.26, p = 0.028 RFP: HR 2.92, 95% CI 1.36-6.32, p = 0.006	Stroma-low
TNBC	Dekker et al., 2013 (14)	69			VS		DFS: HR 2.71, 95% CI 1.11-6.61, p = 0.028	Stroma-low
TNBC	Roeke et al., 2017 (17)	77	26%		VS		OS: p = 0.221	No difference
Invasive carcinoma of NST TNBC	Gujam et al., 2014 (15)	151	24%	T1-3, N0->3, grade I-III	VS		CSS: p = 0.151	No difference

Abbreviations: APC = automated point counting, BC = breast cancer, CSS = cancer-specific survival, DFS = disease-free survival, DM = distant metastasis, DMFS = distant metastasis-free survival, ICC = intraclass correlation coefficient, K = Cohen's kappa value, NST = no special type, OS = overall survival, RFP = relapse-free period, RFS = recurrence-free survival, TNBC = triple-negative breast cancer, VS = visual scoring

stromal content with semi-automated point counting (46). They showed that a high tumor-stroma content in 118 women with ER-positive invasive breast tumors (grade I-III) was independently associated with a better OS and relapse-free survival (RFS) (95% CI 0.2-0.7, $p = 0.008$ and 95% CI 0.1-0.6, $p = 0.006$, respectively) (46). After their first study, Downey and colleagues investigated the stromal content in 45 patients with inflammatory breast cancer, a rare and aggressive form of breast cancer, using the semi-automated point counting method (47, 49). However, no statistically significant difference was observed for this series (OS $p = 0.53$, DFS $p = 0.66$) (47).

Roeke et al. (T1-3, N0-2, grade I-III) validated by visual TSR assessment that a high stromal content was a prognostic factor for worse OS (HR 1.56, 95% CI 1.18-2.05, $p = 0.002$), distant-metastasis-free survival (DMFS) (HR 1.52, 95% CI 1.12-2.06, $p = 0.008$) and RFS (HR 1.35, 95% CI 1.01-1.81, $p = 0.046$) in their study of 737 patients with primary operable invasive breast cancer (17). Unlike the work of Downey et al., patients with ER-positive stroma-high tumors were associated with a worse OS (HR 1.43, 95% CI 1.04-1.99, $p = 0.030$) (17).

THE TUMOR-STROMA RATIO IN TRIPLE-NEGATIVE BREAST CANCER

For the applicability of the TSR as a prognostic parameter in TNBC patients, a study has been performed by Moorman et al. in 2012. They analyzed the TSR in a retrospective cohort study consisting of TNBC patients (pT1-4, pN0-3, grade I-III) (n = 124) (13). The amount of stroma was evaluated by visual eyeballing. Multivariate Cox regression analysis showed that the TSR was an independent prognostic factor for both RFP (HR 2.39, 95% CI 1.07-5.29, $p = 0.033$) and OS (HR 3.00, 95% CI 1.08-8.32, $p = 0.034$), in favor of stroma-low tumors. The 5-year RFP and OS for patients with stroma-low tumors compared to stroma-high tumors were 85% and 89% versus 45% and 65%, respectively (13).

Subgroup analysis of 82 TNBC in the cohort of De Kruijf et al. supported the results of Moorman and colleagues that patients with stroma-high tumors had a significant shorter RFP (HR 2.92, 95% CI 1.36-6.32, $p = 0.006$) and OS (HR 1.87, 95% CI 1.07-3.26, $p = 0.028$) (12). After 5 years of follow-up, 81% of the TNBC

patients with stroma-low tumors were relapse-free compared to 56% of patients with stroma-high tumors (12).
Among the 403 patients in the cohort of Dekker and colleagues, 69 patients were diagnosed with TNBC. A separate analysis of patients with stroma-high TNBC validated a 2.71 greater risk of developing a recurrence compared to patients with stroma-low TNBC (DFS: HR 2.71, 95% CI 1.11-6.61, $p = 0.028$) (14).
However, in the study of Gujam et al., the percentage of tumor stroma was not found to be an independent prognostic factor for cancer-specific survival in 151 TNBC patients ($p = 0.151$) (15). Likewise, Roeke et al. were not able to prove this correlation either ($p = 0.221$) (table 1) (17).

THE TUMOR-STROMA RATIO IN OTHER SUB-GROUPS

De Kruijf et al., Gujam et al. and Roeke et al. described the role of the TSR in other subgroups. The results of De Kruijf et al. showed an independent prognostic value of the TSR in patients who only received local therapy ($p < 0.001$), adjuvant chemotherapy ($p = 0.038$) or adjuvant endocrine therapy ($p = 0.024$) (12). The latter was confirmed by Roeke et al. ($p = 0.001$) (17). The same results were seen in patients with TNBC who received only local therapy ($p = 0.006$).
In non-TNBC patients ($p = 0.013$), ER-positive patients ($p = 0.030$) and HER2-negative tumors the TSR was also of independent prognostic value (12, 17). This was not the case for ER-negative and PR-negative breast tumors (17). In node-negative tumors the TSR was also proved to be statistically significant for CSS and OS ($p = 0.002$ and $p = 0.003$, respectively) in two different studies (15, 17). Table 2 presents a summary of these results.

DISCUSSION OF CURRENT LITERATURE

Extensive research has been performed to determine prognostic biomarkers for patient prognosis. Molecular tests, as the MammaPrint and Oncotype DX, have seemed to be valuable for the improvement of clinical decision making in early-stage breast cancer (7, 8). These tests will possibly be endorsed into routine

clinical practice to reduce the administration of adjuvant chemotherapy and prevent overtreatment (9). However, the disadvantages of the aforementioned molecular testing are the relatively high cost and the far more unknown influence of tumor heterogeneity. More specifically, intermingled non-tumor tissue may have a profound influence on the test results (50).

The TSR has shown to be of prognostic value in addition to the traditional prognostic markers which are implemented in standard clinical care, for example, TNM stage, receptor status and HER2 expression, in breast cancer with a robust inter-observer variability. In supplementary table 1 and supplementary table 2 the effect of the TSR in addition to the most important traditional prognostic markers is shown for the entire study population and triple-negative tumors, respectively.

So far, seven studies regarding the TSR have been performed in the field of breast cancer, of which five have shown a significant association between high tumor stroma content and a poor prognosis (12-15, 17). However, the results of both studies of Downey and colleagues were not in line with the other five (46, 47). As Downey et al. have determined the TSR with semi-automated point counting instead of visual eyeballing and have utilized different cut-off values in both studies, it may be concluded that a standardized estimation of the TSR is essential for a robust method, which can be applicable for patient management. The method of determining the TSR differed considerably, resulting in underestimating the heterogeneity (51). In contrast with previous studies, where the ultimate TSR category is based on the highest stroma rate in the sample, Downey and colleagues only scored an area of 9 mm^2 at the edge of the tumor (10, 46, 51, 52).

Although the difference in results can be attributed to this inconsistency, the different breast cancer subgroups regarding basic characteristics must be taken into consideration as well. The applicability in the subtypes, namely TNBC, ER-positive and inflammatory breast cancer, may differ and subsequently the individual relevance of the TSR has to be determined in breast cancer subgroups, as is previously performed by Roeke and colleagues (17). For example, in lobular carcinomas, the question is raised on how to determine which part is tumor induced stroma or tumor supportive stroma. This should be further determined in larger cohorts. Concerning TNBC, five studies have investigated this subgroup, of which three studies have shown significant results (12-15, 17). The results of these three

TABLE 2. The results of the multivariate Cox regression analysis on the prognostic value of the tumor-stroma ratio in different subgroups of breast tumors described in literature (data on the main cohort of publication and triple-negative tumors are presented in table 1). Stroma-low is used as a reference value.

Subgroups	**De Kruijf et al., 2011**				**Gujam et al., 2014**				**Roeke et al. 2017**			
	Recurrence-free period				Cancer-specific survival				Overall survival			
	n (% stroma-high)	HR	95% CI	*p*-value	n (% stroma-high)	HR	95% CI	*p*-value	n (% stroma-high)	HR	95% CI	*p*-value
Treatment												
Only local therapy (no systemic therapy)	244 (66)	2.06	1.42-2.97	<0.001								
Only adjuvant chemotherapy	88 (68)	1.83	1.04-3.25	0.038								
Only adjuvant endocrine therapy	27 (29)	2.59	1.13-5.91	0.024						2.02	1.34-3.07	0.001
Only local therapy in TNBC		4.12	1.49-11.39	0.006								
Hormone receptor and/or HER2 status												
Non-TNBC		1.50	1.09-2.07	0.013								
ER-positive										1.43	1.04-1.99	0.030
ER-negative and PR-negative												No statistically significant difference (data not shown)
HER2-negative												Results comparable with results of ER-positive tumors (data not shown)
Tumor stage												
Lymph node-negative					54 (26)	3.11	1.53-6.33	0.002		1.90	1.24-2.90	0.003

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, PR = progesterone receptor, TNBC = triple-negative breast cancer

studies are rather promising regarding the prognostic effect of the TSR (12-14). However, two other studies have not validated this prognostic effect despite the favorable results showed earlier. As mentioned by Roeke et al., this discordance could be contributed to the relatively low amount of stroma-high tumors in the TNBC subgroup (17). The similar reason could be the cause for the effect of the TSR in TNBC patients in the study of Gujam et al. (15). Another explanation could be that the histological type of TNBC plays a role.

Although different studies researched the prognostic value of TSR, little is known about the composition of the stroma. Even when using conventional light microscopy, vast differences in stromal morphology can be appreciated, which are surely reflective of enormous differences in stromal functionality. Molecular analyses have identified multiple molecular markers that are associated with varying degrees of stromal activation (53-55). These findings might allow us to distinguish activated, highly tumor-promoting stromal tissues from non-activated or only mildly active stromal tissues. Future studies investigating stromal activation might therefore solely focus on specific highly active subsets of stromal tissues as opposed to counting all stromal tissues equally, thereby further refining this parameter. For instance, as shown in a previous publication by the identification of PA28 as a marker of stromal activation (53).

Similarly, Ahn et al. investigated the stromal composition of breast cancer tissue. Besides the TSR, the dominant histological stroma type (collagen, fibroblast or lymphocytes) offers additional prognostic information. Five- and 10-year RFS rates were most favorable in the lymphocytic stroma type, followed by the fibroblast and collagen type. The latter was associated with the most aggressive tumor and consequently poorest prognosis (56). Interestingly, Ahn et al. observed a trend between TNBC and a predominantly lymphocytic stroma type, with 56.1% of the samples classified as ‘lymphocytic’. Considering TNBC has a relatively poor prognosis, the observed trend between TNBC and a predominantly lymphocytic stroma type, with a favorable prognosis, is striking. Leon-Ferre and colleagues showed similar results in early-stage TNBC in which the presence of low tumor-infiltrating lymphocytes (TILs) contributes to a poor prognosis (57).

Considering the aforementioned generally promising prognostic effect in TNBC, this subgroup is the most obvious candidate for further exploration of the TSR.

Currently, adjuvant systemic chemotherapy is advocated for all patients that present with operable TNBC due to the aggressive nature of this tumor subgroup. Regarding TNBC, unlike other molecular subtypes, there is no Food and Drug Administration (FDA) approved targeted therapy yet. Forasmuch as both the aggressive nature of the subtype as the devoid of therapeutic options, supplementary research is necessary. For the development of curative therapeutics in TNBC, stromal targets have to be determined. Given the fact that TNBC predominantly consists of lymphocytic stroma, according to Ahn and colleagues, the possible target might lie within this stroma. The quantity of programmed death-ligand 1 (PD-L1), expressed on tumor cells, could be prognostic as well. Tomioka et al. have shown that low TILs, in combination with high PD-L1 expression, predicts an unfavorable prognosis. Within the abundant lymphocytic stroma in TNBC, PD-L1 could operate as a target for therapeutic options (58). Thus, in further research, in addition to a standardized estimation of the TSR, the biology or quality of the stroma should be taken into account as well, in both general breast cancer and especially in TNBC patients to clarify the paradox and subsequently to lay a foundation regarding targeted therapy. Lastly, it should be noted that although previous studies demonstrated prognostic value in the past, these studies have always been performed as part of retrospective studies by researchers and pathologists with a specific interest in stromal tissues. Breast cancer is a heterogeneous disease, and for this reason, additional larger retrospective studies could add valuable information about the prognostic value of TSR in specific subgroups as well. Moreover, no prospective feasibility studies have been performed, and as such, it remains to be seen whether the broad application of this parameter would lead to reproducible test results. Current research efforts in this direction are, however, ongoing.

CONCLUSIONS

The current breast cancer prognostication schemes do not adequately predict patient prognosis. This leads to both over- and undertreatment with adjuvant chemotherapy. To better predict tumor biology and prevent unwarranted chemotherapy, additional prognostic parameters are necessary. The TSR can be a valuable biomarker for determining patient prognosis. The scoring can easily be performed by the

pathologist during routine pathological examination of H&E stained slides in less than a minute and without additional costs, as it is a quick, simple method with a high reproducibility. The field of tumor stroma provides promising perspectives, although standardization of the methodology is desired. There is a trend toward high stromal content and a poor prognosis, being most applicable in TNBC. The TSR, in this case, could be used to predict both disease progression and patient prognosis.

ACKNOWLEDGEMENTS

Funding information: This work was supported by Genootschap Keukenhof voor de Vroege Opsporing van Kanker, Lisse, The Netherlands. No grant number applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. Eur J Cancer. 2018.
2. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006;295(21):2492-502.
3. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70.
4. Engels CC, Kiderlen M, Bastiaannet E, Mooyaart AL, van Vlierberghe R, Smit VT, et al. The clinical prognostic value of molecular intrinsic tumor subtypes in older breast cancer patients: A FOCUS study analysis. Mol Oncol. 2016;10(4):594-600.
5. Perou CM. Molecular stratification of triple-negative breast cancers. Oncologist. 2011;16 Suppl 1:61-70.
6. de Kruijf EM, Bastiaannet E, Ruberta F, de Craen AJ, Kuppen PJ, Smit VT, et al. Comparison of frequencies and prognostic effect of molecular subtypes between young and elderly breast cancer patients. Mol Oncol. 2014;8(5):1014-25.
7. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. N Engl J Med. 2016;375(8):717-29.
8. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004;351(27):2817-26.
9. Krop I, Ismaila N, Andre F, Bast RC, Barlow W, Collyar DE, et al. Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline Focused Update. J Clin Oncol. 2017;35(24):2838-47.
10. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
11. Courrech Staal EF, Smit VT, van Velthuysen ML, Spitzer-Naaykens JM, Wouters MW, Mesker WE, et al. Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies. Eur J Cancer. 2011;47(3):375-82.
12. de Kruijf EM, van Nes JG, van de Velde CJ, Putter H, Smit VT, Liefers GJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat. 2011;125(3):687-96.
13. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. Eur J Surg Oncol. 2012;38(4):307-13.

14. Dekker TJ, van de Velde CJ, van Pelt GW, Kroep JR, Julien JP, Smit VT, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Treat. 2013;139(2):371-9.
15. Gujam FJ, Edwards J, Mohammed ZM, Going JJ, McMillan DC. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. Br J Cancer. 2014;111(1):157-65.
16. Vangangelt KMH, van Pelt GW, Engels CC, Putter H, Liefers GJ, Smit V, et al. Prognostic value of tumor-stroma ratio combined with the immune status of tumors in invasive breast carcinoma. Breast Cancer Res Treat. 2017.
17. Roeke T, Sobral-Leite M, Dekker TJA, Wesseling J, Smit V, Tollenaar R, et al. The prognostic value of the tumour-stroma ratio in primary operable invasive cancer of the breast: a validation study. Breast Cancer Res Treat. 2017;166(2):435-45.
18. West NP, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, et al. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. Br J Cancer. 2010;102(10):1519-23.
19. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Ann Oncol. 2014;25(3):644-51.
20. Vogelaar FJ, van Pelt GW, van Leeuwen AM, Willems JM, Tollenaar RA, Liefers GJ, et al. Are disseminated tumor cells in bone marrow and tumor-stroma ratio clinically applicable for patients undergoing surgical resection of primary colorectal cancer? The Leiden MRD study. Cell Oncol (Dordr). 2016;39(6):537-44.
21. Aurello P, Berardi G, Giulitti D, Palumbo A, Tierno SM, Nigri G, et al. Tumor-Stroma Ratio is an independent predictor for overall survival and disease free survival in gastric cancer patients. Surgeon. 2017;15(6):329-35.
22. Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013;24(1):179-85.
23. Wang Z, Liu H, Zhao R, Zhang H, Liu C, Song Y. [Tumor-stroma ratio is an independent prognostic factor of non-small cell lung cancer]. Zhongguo Fei Ai Za Zhi. 2013;16(4):191-6.
24. Mesker WE, Liefers GJ, Junggeburt JM, van Pelt GW, Alberici P, Kuppen PJ, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients. Cell Oncol. 2009;31(3):169-78.
25. Wu J, Liang C, Chen M, Su W. Association between tumor-stroma ratio and prognosis in solid tumor patients: a systematic review and meta-analysis. Oncotarget. 2016;7(42):68954-65.
26. Chen Y, Zhang L, Liu W, Liu X. Prognostic Significance of the Tumor-Stroma Ratio in Epithelial Ovarian Cancer. Biomed Res Int. 2015;2015:589301.

27. Hansen TF, Kjaer-Frifeldt S, Lindebjerg J, Rafaelsen SR, Jensen LH, Jakobsen A, et al. Tumor-stroma ratio predicts recurrence in patients with colon cancer treated with neoadjuvant chemotherapy. Acta Oncol. 2018;57(4):528-33.
28. Li H, Yuan SL, Han ZZ, Huang J, Cui L, Jiang CQ, et al. Prognostic significance of the tumor-stroma ratio in gallbladder cancer. Neoplasma. 2017;64(4):588-93.
29. Liu J, Liu J, Li J, Chen Y, Guan X, Wu X, et al. Tumor-stroma ratio is an independent predictor for survival in early cervical carcinoma. Gynecol Oncol. 2014;132(1):81-6.
30. Lv Z, Cai X, Weng X, Xiao H, Du C, Cheng J, et al. Tumor-stroma ratio is a prognostic factor for survival in hepatocellular carcinoma patients after liver resection or transplantation. Surgery. 2015;158(1):142-50.
31. Niranjan KC, Sarathy NA. Prognostic impact of tumor-stroma ratio in oral squamous cell carcinoma - A pilot study. Ann Diagn Pathol. 2018;35:56-61.
32. Pongsuvareeyakul T, Khunamornpong S, Settakorn J, Sukpan K, Suprasert P, Intaraphet S, et al. Prognostic evaluation of tumor-stroma ratio in patients with early stage cervical adenocarcinoma treated by surgery. Asian Pac J Cancer Prev. 2015;16(10):4363-8.
33. Scheer R, Baidoshvili A, Zoidze S, Elferink MAG, Berkel AEM, Klaase JM, et al. Tumor-stroma ratio as prognostic factor for survival in rectal adenocarcinoma: A retrospective cohort study. World J Gastrointest Oncol. 2017;9(12):466-74.
34. Wang K, Ma W, Wang J, Yu L, Zhang X, Wang Z, et al. Tumor-stroma ratio is an independent predictor for survival in esophageal squamous cell carcinoma. J Thorac Oncol. 2012;7(9):1457-61.
35. Xi KX, Wen YS, Zhu CM, Yu XY, Qin RQ, Zhang XW, et al. Tumor-stroma ratio (TSR) in non-small cell lung cancer (NSCLC) patients after lung resection is a prognostic factor for survival. J Thorac Dis. 2017;9(10):4017-26.
36. Zhang R, Song W, Wang K, Zou S. Tumor-stroma ratio(TSR) as a potential novel predictor of prognosis in digestive system cancers: A meta-analysis. Clin Chim Acta. 2017;472:64-8.
37. Zhang T, Xu J, Shen H, Dong W, Ni Y, Du J. Tumor-stroma ratio is an independent predictor for survival in NSCLC. Int J Clin Exp Pathol. 2015;8(9):11348-55.
38. Zhang XL, Jiang C, Zhang ZX, Liu F, Zhang F, Cheng YF. The tumor-stroma ratio is an independent predictor for survival in nasopharyngeal cancer. Oncol Res Treat. 2014;37(9):480-4.
39. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer. 2004;4(11):839-49.
40. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature. 2013;501(7467):346-54.
41. Hawinkels LJ, Paauwe M, Verspaget HW, Wiercinska E, van der Zon JM, van der Ploeg K, et al. Interaction with colon cancer cells hyperactivates TGF-beta signaling in cancer-associated fibroblasts. Oncogene. 2014;33(1):97-107.

42. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol. 2000;2(10):737-44.
43. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell. 2009;139(5):891-906.
44. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, et al. Stromal gene expression predicts clinical outcome in breast cancer. Nat Med. 2008;14(5):518-27.
45. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. Nat Med. 2009;15(1):68-74.
46. Downey CL, Simpkins SA, White J, Holliday DL, Jones JL, Jordan LB, et al. The prognostic significance of tumour-stroma ratio in oestrogen receptor-positive breast cancer. Br J Cancer. 2014;110(7):1744-7.
47. Downey CL, Thygesen HH, Sharma N, Shaaban AM. Prognostic significance of tumour stroma ratio in inflammatory breast cancer. Springerplus. 2015;4:68.
48. Clahsen PC, van de Velde CJ, Julien JP, Floiras JL, Delozier T, Mignolet FY, et al. Improved local control and disease-free survival after perioperative chemotherapy for early-stage breast cancer. A European Organization for Research and Treatment of Cancer Breast Cancer Cooperative Group Study. J Clin Oncol. 1996;14(3):745-53.
49. Robertson FM, Bondy M, Yang W, Yamauchi H, Wiggins S, Kamrudin S, et al. Inflammatory breast cancer: the disease, the biology, the treatment. CA Cancer J Clin. 2010;60(6):351-75.
50. Acs G, Kiluk J, Loftus L, Laronga C. Comparison of Oncotype DX and Mammostrat risk estimations and correlations with histologic tumor features in low-grade, estrogen receptor-positive invasive breast carcinomas. Mod Pathol. 2013;26(11):1451-60.
51. Mesker WE, Dekker TJ, de Kruijf EM, Engels CC, van Pelt GW, Smit VT, et al. Comment on: The prognostic significance of tumour-stroma ratio in oestrogen receptor-positive breast cancer. Br J Cancer. 2015;112(11):1832-3.
52. Downey CL, Simpkins SA, Holliday DL, Jones JL, Jordan LB, Kulka J, et al. Reponse to: comment on, 'Tumour-stroma ratio (TSR) in oestrogen-positive breast cancer patients'. Br J Cancer. 2015;112(11):1833-4.
53. Dekker TJ, Balluff BD, Jones EA, Schone CD, Schmitt M, Aubele M, et al. Multicenter matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) identifies proteomic differences in breast-cancer-associated stroma. J Proteome Res. 2014;13(11):4730-8.
54. Witkiewicz AK, Dasgupta A, Sotgia F, Mercier I, Pestell RG, Sabel M, et al. An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers. Am J Pathol. 2009;174(6):2023-34.
55. Paulsson J, Sjoblom T, Micke P, Ponten F, Landberg G, Heldin CH, et al. Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. Am J Pathol. 2009;175(1):334-41.

56. Ahn S, Cho J, Sung J, Lee JE, Nam SJ, Kim KM, et al. The prognostic significance of tumor-associated stroma in invasive breast carcinoma. Tumour Biol. 2012;33(5):1573-80.
57. Leon-Ferre RA, Polley MY, Liu H, Gilbert JA, Cafourek V, Hillman DW, et al. Impact of histopathology, tumor-infiltrating lymphocytes, and adjuvant chemotherapy on prognosis of triple-negative breast cancer. Breast Cancer Res Treat. 2018;167(1):89-99.
58. Tomioka N, Azuma M, Ikarashi M, Yamamoto M, Sato M, Watanabe KI, et al. The therapeutic candidate for immune checkpoint inhibitors elucidated by the status of tumor-infiltrating lymphocytes (TILs) and programmed death ligand 1 (PD-L1) expression in triple negative breast cancer (TNBC). Breast Cancer. 2018;25(1):34-42.

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE 1. Prognostic value of the tumor-stroma ratio in addition to traditional prognostic tumor characteristics in the main study cohort discussed in the literature calculated by Cox regression analysis.

Tumor characteristics	**De Kruijf et al., 2011**						**Dekker et al., 2013**						**Gujam et al., 2014**					
	General breast cancer cohort						General breast cancer cohort						Invasive carcinoma of no special type					
	Recurrence-free period						Disease-free survival						Cancer-specific survival					
	Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Tumor size	2.49	1.71-3.64	<0.001	1.86	1.24-2.79	0.009	3.17	1.37-7.36	0.024	2.72	0.99-7.47	0.150	2.17	1.54-3.07	<0.001			0.142
Lymph node involvement	3.06	2.38-3.95	<0.001	2.66	2.03-3.49	<0.001							1.97	1.51-2.56	<0.001	1.97	1.46-2.66	<0.001
Tumor grade	2.02	1.33-3.08	0.001	1.71	1.09-2.70	0.022	1.85	1.26-2.72	0.006	1.32	0.82-2.13	0.440	1.85	1.30-2.60	<0.001	1.72	1.18-2.51	0.005
Histological type	1.24	0.83-1.85	0.291															
ER status	1.05	0.81-1.36	0.725				0.87	0.60-1.26	0.454				0.52	0.34-0.79	0.002			0.240
PR status	0.96	0.74-1.24	0.744				0.83	0.60-1.16	0.275				0.44	0.32-0.82	0.006			0.184
HER2 status	1.21	0.78-1.88	0.401				1.27	0.83-1.97	0.275				1.44	0.88-2.35	0.145			
Ki67 expression	1.00	0.71-1.42	0.994				2.06	1.30-3.27	0.002	1.73	1.02-2.92	0.042						
Lymphovascular invasion													2.07	1.39-3.09	<0.001			0.864
Angiogenesis							1.21	0.81-1.80	0.349									
Tumor-stroma ratio	1.62	1.23-2.13	0.001	1.97	1.47-2.64	<0.001	1.69	1.23-2.31	0.001	1.85	1.33-2.59	<0.001	1.89	1.26-2.82	<0.001	2.12	1.37-3.29	0.001

SUPPLEMENTARY TABLE 1. Continued.

Tumor characteristics	Downey et al., 2014						Downey et al., 2015						Roeke et al., 2017					
	Estrogen receptor-positive breast cancer						Only inflammatory breast cancer						General breast cancer					
	Relapse-free survival						Disease-free survival						Recurrence-free survival					
	Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Tumor size													1.61	0.40-6.52	0.670	1.33	0.30-5.84	0.709
Lymph node involvement													4.41	2.68-7.26	<0.001	4.58	2.51-8.36	<0.001
Tumor grade													2.73	1.74-4.30	<0.001	1.78	1.06-2.99	0.028
Histological type													0.97	0.61-1.55	0.910			
ER status													0.53	0.38-0.74	<0.001	0.82	0.52-1.28	0.375
PR status													0.59	0.44-0.79	<0.001	0.78	0.54-1.12	0.183
HER2 status													1.09	0.77-1.54	0.633	1.04	0.73-1.48	0.819
Ki67 expression																		
Lymphovascular invasion																		
Angiogenesis																		
Tumor-stroma ratio		0.1-0.6	0.006						0.66				1.26	0.95-1.67	0.113	1.35	1.01-1.81	0.046

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, PR = progesterone receptor

The reference group used in the univariate and multivariate Cox regression analyses was different between the included studies. In this table, only the traditional prognostic markers are shown. In the original papers, more parameters were included in de multivariate Cox regression analysis. Tumor size; pT1 versus pT3/4 (De Kruijf et al.), cT1 versus cT3 (Dekker et al.), ≤20 mm versus >50 mm (Gujam et al.) and ≤20 mm versus >50 mm (Roeke et al.). Lymph node involvement; pN-negative versus pN-positive (De Kruijf et al.), 0 versus >3 involved lymph nodes (Gujam et al.) and pN0 versus pN3 (Roeke et al.). Tumor grade; grade I versus grade III. Histological type; invasive carcinoma of no special type versus others (De Kruijf et al.) and invasive carcinoma of no special type versus lobular carcinoma (Roeke et al.). ER status; negative versus positive. PR status; negative versus positive. HER2 status; no overexpression versus overexpression (De Kruijf et al.) and negative versus positive (Dekker et al. and Roeke et al.). Ki67 expression; negative versus positive (De Kruijf et al.) and low versus high (Dekker et al.). Angiogenesis; low microvessel density versus high microvessel density. Lymphovascular invasion; no versus yes. Tumor-stroma ratio; stroma-low versus stroma-high.

SUPPLEMENTARY TABLE 2. Prognostic value of TSR in addition to traditional prognostic tumor characteristics in the triple-negative breast cancer population described in the discussed literature calculated by Cox regression analysis.

Tumor characteristics	De Kruijf et al., 2011						Moorman et al., 2012						Dekker et al., 2013					
	Recurrence-free period						Relapse-free period						Disease-free survival					
	Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Tumor size	3.53	1.03-12.08	0.131										10.17	2.29-45.11	0.009	6.19	1.04-36.96	0.075
Lymph node involvement	2.30	1.61-4.57	0.017	1.88	0.89-3.96	0.096	3.38	1.27-9.00	0.010									
Tumor grade	1.72	0.80-3.69	0.163										0.84	0.12-6.22	0.478			
Histological type																		
Ki67 expression	0.70	0.31-1.61	0.403										2.44	0.33-17.91	0.382			
Lymphovascular invasion							2.46	1.19-5.07	0.012									
Angiogenesis													1.53	0.61-3.84	0.364			
Tumor-stroma ratio	3.19	1.49-6.83	0.003	2.92	1.36-6.32	0.006	2.93	1.37-6.26	0.004	2.39	1.07-5.29	0.033	2.21	1.004-4.84	0.049	2.71	1.11-6.61	0.028

The reference group used in the univariate and multivariate Cox regression analysis was different between the included studies. In this table, only traditional prognostic markers are shown. In the original papers, more parameters were included in de multivariate Cox regression analysis. Tumor size; pT1 versus pT3/4 (De Kruijf et al.), T1 versus T3 (Dekker et al.) and ≤20 mm versus >50 mm (Gujam et al.). Lymph node involvement; pN-negative versus pN-positive (De Kruijf et al.), pN0 versus pN2/3 (Moorman et al.) and 0 versus >3 involved lymph nodes (Gujam et al.). Tumor grade; grade I/II versus grade III (De Kruijf et al.), grade I versus grade III (Gujam et al. and Dekker et al.). Ki67 expression; negative versus positive (De Kruijf et al.) and low versus high (Dekker et al.). Lymphovascular invasion; no versus yes. Angiogenesis; low microvessel density versus high microvessel density. Tumor-stroma ratio; stroma-low versus stroma-high.

2

3

The prognostic value of the tumor-stroma ratio is most discriminative in patients with grade III or triple-negative breast cancer

K.M.H. Vangangelt
A.R. Green
M.F. Heemskerk
D. Cohen
G.W. van Pelt
M. Sobral-Leite
M.K. Schmidt
H. Putter
E.A. Rakha
R.A.E.M. Tollenaar
W.E. Mesker

Int J Cancer. 2020 Apr 15;146(8):2296-2304

ABSTRACT

Purpose

The tumor-stroma ratio (TSR) was evaluated as a promising parameter for breast cancer prognostication in clinically relevant subgroups of patients.

Methods

The TSR was assessed on hematoxylin and eosin stained tissue slides of 1794 breast cancer patients from the Nottingham City Hospital. An independent second cohort of 737 patients from the Netherlands Cancer Institute-Antoni van Leeuwenhoek was used for evaluation.

Results

In the Nottingham Breast Cancer series, the TSR was an independent prognostic parameter for recurrence-free survival (RFS) (HR 1.35, 95% CI 1.10-1.66, $p = 0.004$). The interaction term was statistically significant for grade and triple-negative status. Multivariate Cox regression analysis showed a more pronounced effect of the TSR for RFS in grade III tumors (HR 1.89, 95% CI 1.43-2.51, $p < 0.001$) and triple-negative tumors (HR 1.86, 95% CI 1.10-3.14, $p = 0.020$). Comparable hazard ratios and confidence intervals were observed for grade and triple-negative status in the ONCOPOOL study. The prognostic value of TSR was not modified by age, tumor size, histology, estrogen receptor status, progesterone receptor status, human epidermal growth factor receptor 2 status or lymph node status.

Conclusions

In conclusion, patients with a stroma-high tumor had a worse prognosis compared to patients with a stroma-low tumor. The prognostic value of the TSR was most discriminative in patients with grade III tumors and triple-negative tumors. The TSR was not modified by other clinically relevant parameters making it a potential factor to be included for improved risk stratification.

INTRODUCTION

Breast cancer mortality rates are declining in most European countries due to early detection and improved treatment options (1). Optimizing risk stratification to prevent undertreatment and overtreatment by personalizing therapy is thereby essential.

In the last decade, the interplay of tumor cells and its microenvironment has gained increased interest. The tumor microenvironment, also known as tumor-associated stroma, consists of immune cells, fibroblasts, pericytes and endothelial cells in an extracellular matrix. The tumor microenvironment plays an active role in creating an environment that favors the tumor cells; increased motility of cells, suppression of the immune response, remodeling of the extracellular matrix and angiogenesis (2-6).

A promising prognostic parameter based on the tumor-associated stroma is the tumor-stroma ratio (TSR). The TSR reflects the amount of tumor stroma to the cancer cells, which is determined on routinely retrieved hematoxylin and eosin (H&E) stained tissue slides used for pathological assessment of surgically removed breast tissue. TSR assessment is easy, quick and without additional costs. Previous research demonstrated the prognostic value of the TSR in different types of invasive solid tumors, including breast cancer (7-32). Most of these studies validated a worse prognosis for patients with stroma-high tumors.

Breast cancer is a heterogeneous disease, which makes subgroup analysis essential. Kramer et al. reviewed literature published on the prognostic value of TSR in the general breast cancer population and different clinically important subgroups (33). Here, we set out to validate the effect of the TSR and further expand its utility in the clinically relevant subgroups for breast cancer prognostication. This is an essential step toward prospective validation and clinical implementation, such as the addition of the TSR to the frequently used online prediction tool PREDICT.

MATERIAL AND METHODS

Study population

The Nottingham Breast Cancer series from Nottingham City Hospital (UK)

The study population consists of women of ≤70 years with primary invasive breast cancer without distant metastases, diagnosed and treated primarily with surgery in the Nottingham City Hospital between 1993 and 2002 (n = 1809). This cohort was retrospectively assembled. Patients were included if digital H&E slides of the primary breast tumors and follow-up data were available. Exclusion criteria were breast cancer in medical history and/or neo-adjuvant treatment.

The ONCOPOOL study from the Netherlands Cancer Institute-Antoni van Leeuwenhoek (the Netherlands)

A total of 737 women treated primarily with surgery for invasive non-metastasized breast cancer between 1990 and 1999, included in the ONCOPOOL study at The Netherlands Cancer Institute-Antoni van Leeuwenhoek hospital, were analyzed in this study. The included patients were part of the larger ONCOPOOL database of European primary breast cancer patients. Details on data management and patient selection were described previously (14, 34). Survival data, estrogen receptor (ER) status and progesterone receptor (PR) status are updated since the previous publication on tumor-stroma ratio according to the last publication using the ONCOPOOL study (14, 35).

All patient data were used in an anonymized manner and handled according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies"). The Nottingham Breast Cancer Series was approved by the Nottingham Research Ethics Committee 2 under the title "Development of molecular genetic classification of breast cancer".

Assessment of the tumor-stroma ratio

In the Nottingham Breast Cancer series, the TSR was visually assessed on digital H&E stained slides of the primary breast tumor via CaseViewer 2.2 for Windows (3DHISTECH Ltd., Budapest, Hungary), a digital application for the evaluation of microscopic images. The original 4 µm routine H&E stained slides were

scanned into high-resolution (0.19 µm/pixel) digital images at 20x magnification using 3DHistech Panoramic 250 Flash II scanner (3DHISTECH Ltd., Budapest, Hungary). First, the whole tissue slide was visually evaluated for the orientation of the most stromal rich field. Second, the most stroma-abundant area was annotated using a circle with an area of 3.1 mm^2. This microscopic field is comparable with the surface selected with a 10x objective of most light microscopes and corresponds with the magnification used previously (36). All slides were double scored in a blinded fashion (KV, WM). A third observer (DC) was consulted if consensus could not be reached. The tissue slide with the highest stroma percentage was decisive in cases where multiple slides were available per patient. Stromal areas suspected for post-biopsy effects were excluded from TSR assessment.

The TSR assessment on tumor tissue of patients included in the ONCOPOOL study was assessed using visual microscopy on conventional H&E slides (14).

The TSR was scored by the method of Mesker et al. in both cohorts (7). A percentage of ≤50% stroma was categorized as stroma-low and >50% stroma was categorized as stroma-high (supplementary figure 1).

Statistical analyses

Statistical analyses were performed using IBM SPSS statistics (version 23 for Windows). The recurrence-free survival (RFS), the primary endpoint, was defined as the time between the date of diagnosis and local, regional or distant recurrence. Patients who died without a recurrence were censored. Breast cancer-specific survival (BCSS), the secondary endpoint in the Nottingham Breast Cancer series, was defined as the time from date of diagnosis and breast cancer-specific death. The BCSS was not available for the ONCOPOOL study. Therefore, in this cohort, the overall survival (OS) was used as the second endpoint. The OS was defined as the time from diagnosis to death from any cause.

The X^2 test was used to evaluate the difference between categorical variables in stroma-low and stroma-high groups. Fisher's exact test was performed if less than five patients were included per category and Fisher-Freeman-Halton when the table was larger than 2x2. The Kaplan-Meier method and the log-rank test were performed. Cohen's kappa coefficient was used to test interobserver variability.

The Cox regression model was used to perform univariate and multivariate analyses. In the multivariate Cox regression analysis of the Nottingham Breast Cancer series, the TSR and confounders were entered; age at diagnosis (continuous), grade (I,II or III), size (≤2 cm and >2 cm), histological type (invasive carcinoma of no special type (NST), lobular carcinoma, tubular carcinoma and others), ER status (negative or positive), PR status (negative or positive) and human epidermal growth factor receptor 2 (HER2) status (negative or positive). These analyses were also performed with triple-negative status as a variable instead of ER status, PR status and HER2 status. Also, lymph node status was entered in the multivariate Cox regression in addition to standard confounders as described above, as lymph node status is not a confounder, but a clinically important parameter. A p-value <0.05 was considered as statistically significant. The univariate and multivariate Cox regression analyses of the ONCOPOOL study were also performed as described in the original report of Roeke et al., to check reproducibility. For the evaluation of the prognostic value of the TSR for clinically relevant subgroups, the interaction term was introduced in the Cox regression analysis. This was corrected for clinically relevant confounders, as described above.

RESULTS

Patients

The Nottingham Breast Cancer series

A total of 2385 H&E slides of 1809 patients were assessed for the TSR. The slides of 15 (0.8%) patients were not eligible for TSR scoring due to the poor quality of the tissue. The Cohen's kappa coefficient was 0.61 between two observers, which corresponds with a substantial to a good level of agreement. Due to the digital learning curve, slides with an incongruent value were individually assessed by the same observers for a second time (blinded from their first scores). Cohen's kappa coefficient in the total cohort increased up to 0.87, which corresponds with an almost perfect level of agreement. The H&E slides of 37 patients were discussed with a third observer. A final agreement for the TSR was reached in all cases. A total of 1794 patients were suitable for statistical analysis. The median age at the time of diagnosis was 55 years (range 23–70 years), and the median follow-up

period was 11 years (range 0-18 years). Table 1 provides an overview of patient and tumor characteristics.

The ONCOPOOL study

The ONCOPOOL study included 737 women with breast cancer and was previously analyzed for the prognostic value of the TSR (14). The median age at inclusion was 54 (range 23-71 years). The median follow-up was 12 years (range 0-24 years). Patient, tumor and treatment characteristics are shown in supplementary table 1.

TABLE 1. Overview of the stratification of age and tumor characteristics of the patients included in the Nottingham Breast Cancer Series.

		Stroma-low		**Stroma-high**		
	n	n = 681	%	n = 1113	%	*p*-value
Age (in years)						
<40	144	71	10.4	73	6.6	0.006
40-<50	385	151	22.2	234	21.0	
50-<60	636	247	36.3	389	35.0	
≥60	628	212	31.1	416	37.4	
Missing	1	0	0.0	1	0.1	
Tumor size (in cm's)						
≤2	1146	505	74.2	641	57.6	<0.001
>2-<5	625	169	24.8	456	41.0	
≥5	21	6	0.9	15	1.3	
Missing	2	1	0.1	1	0.1	
Lymph node involvement						
No	1127	452	66.4	675	60.6	0.015
Yes	664	227	33.3	437	39.3	
Missing	3	2	0.3	1	0.1	
Grade						
I	279	105	15.4	174	15.6	0.606
II	733	272	39.9	461	41.4	
III	781	303	44.5	478	42.9	
Missing	1	1	0.1	0	0	
Histological type						
Invasive carcinoma of NST	1129	450	66.1	679	61.0	0.117

TABLE 1. Continued.

		Stroma-low		Stroma-high		
	n	n = 681	%	n = 1113	%	*p*-value
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	
ER status						
Negative	331	151	22.2	180	16.2	0.001
Positive	1463	530	77.8	933	83.8	
PR status						
Negative	708	282	41.4	426	38.3	0.262
Positive	1067	390	57.3	677	60.8	
Missing	19	9	1.3	10	0.9	
HER2 status						
Negative	1573	594	87.2	979	88.0	0.645
Positive	221	87	12.8	134	12.0	
Triple-negative tumors						
No	1546	560	82.2	986	88.5	0.001
Yes	235	115	16.9	120	10.8	
Missing	13	6	0.9	7	0.6	
Chemotherapy						
No	699	255	37.4	444	39.9	0.577
Yes	292	115	16.9	177	15.9	
Missing	803	311	45.7	492	44.2	
Hormonal therapy						
No	455	182	26.7	273	24.5	0.112
Yes	778	274	40.2	504	45.3	
Missing	561	225	33.0	336	30.2	

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, NST = no special type, PR = progesterone receptor

The prognostic value of the TSR

In the total study population of the Nottingham Breast Cancer series, 681 (38%) patients were categorized in the stroma-low group and 1113 (62%) patients in the stroma-high group. Table 1 shows the statistically significant differences between both stroma categories. Age, tumor size, lymph node involvement, ER status and triple-negative tumors were significantly different between both stromal categories. The Kaplan-Meier analysis and the log-rank test for RFS showed a statistically significant different outcome between patients with a stroma-low and stroma-high tumor in favor of patients with stroma-low tumors (supplementary figure 2). The TSR was an independent prognostic parameter in favor of patients with stroma-low tumors for both RFS and BCSS when adjusted for different sets of confounders (table 2 and table 3)

Since the ONCOPOOL study was updated, the prognostic value of the TSR was evaluated again. The analyses showed that patients with a high stromal content tumor had a worse survival in the total cohort as well as in subgroups. The results from the multivariate Cox regression analysis of the updated database were comparable with those of the original observations; RFS HR 1.35, 95% CI 1.01-1.79, $p = 0.040$ versus HR 1.35, 95% CI 1.01-1.81, $p = 0.046$ and OS HR 1.46, 95% CI 1.13-1.88, $p = 0.003$ versus HR 1.56, 95% CI 1.18-2.05, $p = 0.002$, respectively (data not shown). When the TSR was adjusted confounders, the OS showed a statistically significant difference in favor of stroma-low tumors. The results for the RFS were borderline statically significant (supplementary table 2 and supplementary table 3)

TABLE 2. Univariate and multivariate analysis of the Nottingham Breast Cancer Series calculated by Cox regression analysis.

	Recurrence-free survival							Breast cancer-specific survival					
	Univariate analysis			*Multivariate analysis*				*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age													
	1793	0.99	1.98-1.00	0.129	1.00	0.99-1.01	0.680	0.99	0.98-1.00	0.222	1.01	0.99-1.02	0.388
Tumor size (in cm's)													
≤2	1146			<0.001			<0.001			<0.001			<0.001
>2	646	2.17	1.81-2.61		1.73	1.42-2.11		2.41	1.93-3.00		1.70	1.35-2.15	
Grade													
I	279			<0.001			<0.001			<0.001			<0.001
II	733	1.52	1.07-2.15		1.24	0.83-1.84		2.96	1.66-5.29		2.32	1.34-4.37	
III	781	2.91	2.09-4.06		2.08	1.37-3.15		7.01	4.01-12.28		4.67	2.46-8.88	
Histological type													
Invasive carcinoma of NST	1129			<0.001			0.012			<0.001			0.083
Lobular carcinoma	155	0.93	0.67-1.30		1.30	0.91-1.85		0.90	0.61-1.32		1.44	0.94-2.19	
Tubular carcinoma	275	0.53	0.38-0.72		0.98	0.70-1.43		0.34	0.22-0.53		0.93	0.56-1.55	
Others	235	1.16	0.90-1.50		1.51	1.16-1.97		0.98	0.72-1.34		1.41	1.02-1.95	
ER status													
Negative	331			<0.001			0.296			<0.001			0.801
Positive	1463	0.64	0.52-0.80		1.16	0.88-1.54		0.50	0.39-0.63		1.04	0.76-1.43	
PR status													
Negative	708			<0.001			0.011			<0.001			0.005
Positive	1067	0.61	0.51-0.73		0.74	0.59-0.93		0.50	0.40-0.62		0.67	0.51-0.89	

TABLE 2. Continued.

		Recurrence-free survival						Breast cancer-specific survival					
		Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
HER2 status													
Negative	1573			<0.001			<0.001			<0.001			0.002
Positive	221	2.09	1.66-2.64		1.70	1.32-2.18		2.27	1.74-2.96		1.56	1.17-2.08	
TSR													
Stroma-low	681			<0.001			0.004			<0.001			0.001
Stroma-high	1113	1.46	1.19-1.78		1.35	1.10-1.66		1.60	1.25-2.04		1.51	1.18-1.95	

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, NST = no special type, PR = progesterone receptor, TSR = tumor-stroma ratio

TABLE 3. Results of the independent prognostic value of the tumor-stroma ratio adjusted for only confounders, confounders including triple-negative status or confounders combined with lymph node status, calculated with multivariate Cox regression analysis in the Nottingham Breast Cancer series.

	Recurrence-free survival	Breast cancer-specific survival
Confounders	HR 1.35, 95% CI 1.10-1.66, $p = 0.004$	HR 1.51, 95% CI 1.18-1.95, $p = 0.001$
Confounders including triple-negative status	HR 1.34, 95% CI 1.09-1.64, $p = 0.006$	HR 1.47, 95% CI 1.15-1.90, $p = 0.002$
Confounders combined with lymph node status	HR 1.35, 95% CI 1.10-1.66, $p = 0.004$	HR 1.51, 95% CI 1.17-1.94, $p = 0.002$

The TSR stratified by clinically important subgroups

In the Cox regression analysis, the interaction term was introduced to evaluate the prognostic effect in different clinically important subgroups.

In the Nottingham Breast Cancer series, the interaction term showed a statistically significant *p*-value for grade ($p < 0.001$ and $p = 0.002$) and triple-negative status ($p = 0.040$ and $p = 0.026$) for RFS and BCSS, respectively. No statistically significant results for RFS and BCSS were observed if stratified for age, tumor size, histology, ER status, PR status, HER2 status and lymph node status. The prognostic value of the TSR calculated by multivariate Cox regression analysis showed the most discriminative effect of the TSR in grade III tumors compared to grade I and grade II tumors, and in triple-negative tumors compared to nontriple-negative tumors, for RFS and BCSS (table 4). Kaplan-Meier analysis and log-rank test for RFS of the TSR stratified by grade and triple-negative status showed a statistically significant difference between subgroups (figure 1 and figure 2)

The ONCOPOOL study was used to validate the survival effects in grade III tumors and triple-negative tumors. The interaction term for grade ($p = 0.122$) and triple-negative status ($p = 0.343$) was not significant for RFS. The HRs of the prognostic effect of the TSR for RFS were most discriminative in grade III tumors compared to grade I and grade II. If stratified by triple-negative status, the HR of the TSR in nontriple-negative tumors was lower compared to triple-negative tumors, but this

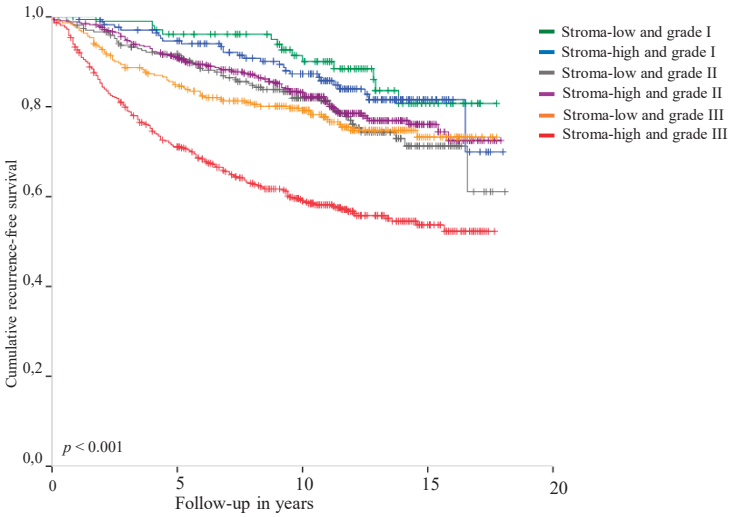
was not statistically significant. The interaction term was not statistically significant if stratified by age, tumor size, histology, ER status, PR status, HER2 status and lymph node status (supplementary table 4)

TABLE 4. Results of the tumor-stroma ratio stratified by clinically important prognostic parameters in the Nottingham Breast Cancer series and the multivariate Cox regression analysis per clinically relevant subgroups with a statistically significant difference.

TSR stratified by group	Subgroups	Recurrence-free survival	Breast cancer-specific survival
Age		$p = 0.881$	$p = 0.874$
Tumor size		$p = 0.422$	$p = 0.209$
Grade		$p < 0.001$	$p = 0.002$
	Grade I	HR 1.16, 95% CI 0.58-2.29, $p = 0.670$	HR 6.34, 95% CI 0.81-49.95, $p = 0.079$
	Grade II	HR 0.78, 95% CI 0.55-1.10, $p = 0.152$	HR 0.83, 95% CI 0.54-1.30, $p = 0.422$
	Grade III	HR 1.89, 95% CI 1.43-2.51, $p < 0.001$	HR 1.86, 95% CI 1.35-2.57, $p < 0.001$
Histological type		$p = 0.684$	$p = 0.951$
ER status		$p = 0.088$	$p = 0.101$
PR status		$p = 0.861$	$p = 0.532$
HER2 status		$p = 0.205$	$p = 0.851$
Triple-negative status		$p = 0.040$	$p = 0.026$
	Nontriple-negative status	HR 1.21, 95% CI 0.97-1.51, $p = 0.095$	HR 1.27, 95% CI 0.96-1.67, $p = 0.092$
	Triple-negative status	HR 1.86, 95% CI 1.10-3.14, $p = 0.020$	HR 2.24, 95% CI 1.24-4.07, $p = 0.008$
Lymph node status		$p = 0.995$	$p = 0.432$

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, PR = progesterone receptor

FIGURE 1. Kaplan-Meier analysis for recurrence-free survival of patients included in the Nottingham Breast Cancer Series stratified by tumor-stroma ratio combined with grade.



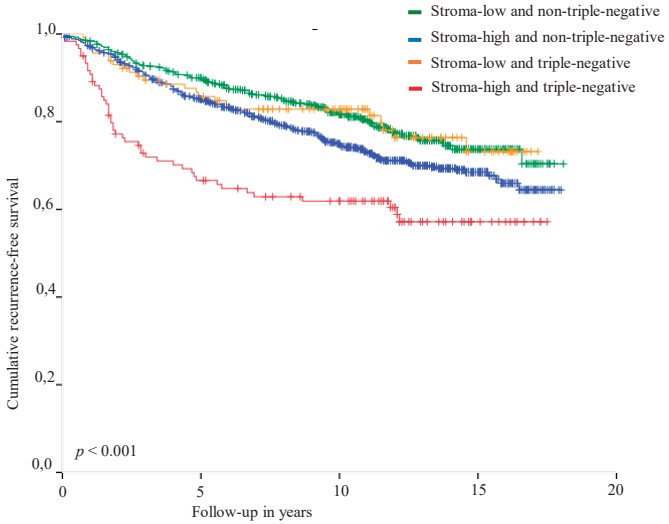
Numbers at risk

Stroma-low/grade I	105	96	66	14	3
Stroma-high/grade I	174	154	113	15	3
Stroma-low/grade II	272	229	143	22	3
Stroma-high/grade II	461	385	255	49	7
Stroma-low/grade III	303	239	162	34	4
Stroma-high/grade III	478	320	197	42	5

DISCUSSION

In our study, we evaluated the prognostic value of the TSR in, to the best of our knowledge, the largest cohort published on the prognostic value of the TSR in breast cancer. The number of patients offered the opportunity to perform analyses of clinically relevant subgroups for breast cancer prognostication and treatment. First, patients with a stroma-high tumor had a worse prognosis compared to patients with a stroma-low tumor. Second, the results of the Nottingham Breast Cancer series showed that the prognostic effect of the TSR was most discriminative in grade III tumors, compared to grade I and grade II tumors, and in triple-negative tumors, compared to nontriple-negative tumors. In the ONCOPOOL study, the HRs

FIGURE 2. Kaplan-Meier analysis for recurrence-free survival of patients included in the Nottingham Breast Cancer Series stratified by tumor-stroma ratio combined with triple-negative status.



Numbers at risk

Stroma-low/non-triple negative	560	469	306	52	9
Stroma-high/non-triple negative	986	781	505	94	13
Stroma-low/triple negative	115	91	63	17	1
Stroma-high/triple negative	120	74	57	12	2

and confidence intervals of the TSR stratified by grade and triple-negative status were comparable with the Nottingham Breast Cancer series. The interaction term showed no statistically significant effect for RFS if stratified by grade or triple-negative status. A possible explanation of the lack of statistical significance is the moderate number of events.

Third, the prognostic effect of the TSR was not modified by age, tumor size, histology, ER status, PR status, HER2 status and lymph node status. This means that the prognostic value of the TSR in these clinically relevant subgroups does not differ from the prognostic value of the total cohort.

No former published literature has evaluated the prognostic value of the TSR by introducing the interaction term. Therefore, the results are not completely comparable. However, previous research showed higher HRs for the TSR in patients with triple-negative tumors as overviewed by Kramer et al. (33). The effect of the TSR stratified by grade has not been previously described.

The next step toward the clinical implementation of the TSR is to investigate the discriminating prognostic value of the TSR additional to the commonly used online PREDICT tool, which helps oncologists and patients in a shared decision-making toward personalized therapy (37, 38). Therefore, retrospective data will be analyzed and a prospective study such as the UNITED (Uniform Noting for International application of the Tumor-stroma ratios as Easy Diagnostic Tool) study needs to be performed (39). The UNITED study is a prospective international multicenter study initiated by our research group. The study aim is to validate and prepare the TSR for implementation in standard clinical care in patients with colon cancer. Implementation of TSR assessment in standard clinical care has advantages compared to other potential biomarkers as this method is easy to perform, takes less than two minutes and requires no additional costs. Therefore, a comparable study for breast cancer would be desirable in the next step toward clinical implementation. Inter-observer and intra-observer reliability of the TSR assessment on digital slides in colon cancer is also evaluated in the UNITED study. The TSR assessment is explained to pathologists and residents via e-learning and test sets.

The Nottingham Breast Cancer series is the first study in which the TSR is digitally assessed on breast cancer tissue by the method of Mesker et al. (7). For the digital assessment, a field of 3.1 mm^2 was used for final TSR scoring, which corresponds with conventional light microscopy used in our previous research. Van Pelt et al. described that the diameters of the different conventional light microscopes are between 2.54 and 3.80 mm^2. However, this has not led to any major differences in the final score (36). One hundred percent of the slides were double scored in a blinded fashion by two observers (KV,WM), instead of the customary 30% double scoring, because of the possible learning curve of scoring digitally for the first time. The Cohen's kappa coefficient increased from 0.61 at first assessment to 0.87 in the second assessment of the slides with an incongruent value at the first time. In our opinion, observers who perform digital TSR assessment for the first time

need to be aware of a learning curve. If this stage is passed, the TSR scoring on digital slides seems to be reliable and therefore gives a good perspective for further digital assessment.

Furthermore, the intra-tumoral stroma contains valuable prognostic information and may, therefore, be an important source for the development of new stroma based therapeutic agents. A major component of the tumor-associated stroma and thus a promising therapeutic target are CAFs. At the moment, CAFs are still difficult to target due to the lack of specific cell surface targets, as they are heterogenous in phenotype and function. An important recent finding is the identification of CD10 and GPR77 as surface markers on CAFs in breast cancer. $CD10^{+}GPR77^{+}$ CAFs are predictive for response to chemotherapy and patient survival, particularly in breast tumors with a high grade (40). The authors showed that the disease-free survival of breast cancer patients with a high $CD10^{+}GPR77^{+}$ CAF infiltration was significantly shorter. The disease-free survival of patients with grade I and grade II tumors were independent of $CD10^{+}GPR77^{+}$ CAF infiltration (6). These results are interesting as we found that the prognostic value of the TSR is most discriminative in grade III tumors compared with grade I and grade II tumors. Whether CAF subtypes differ between stroma-low and stroma-high tumors is not known at this moment and requires further research.

Moreover, Ahn and colleagues concluded that, especially in patients with grade III tumors, the dominant stroma type was an independent risk factor for disease-free survival in favor for patients with lymphocyte dominant stroma (41). Therefore, evaluation of the stromal composition would be interesting, for instance by dividing the stromal compartment in dominant stroma type; collagen, fibroblast or lymphocyte.

Advantages of this study are the large cohort size and long follow-up period. A limitation of this study is the time period in which patients are included and as a consequence the changes in treatment modalities. In the studied patient groups, proportionally less patients received hormonal therapy than in current treatments. However, previously published research, including the ONCOPOOL study, showed that the TSR was of prognostic value in patients with hormone receptor-positive tumors who received hormonal therapy (9,14). This may suggest that the prognostic value of the TSR can be translated to current hormonal treatment strategies. Also,

the introduction of Trastuzumab has positively influenced clinical outcome. Therefore, a large more recent retrospective study, in which the change in treatment modalities and a decent follow-up period are considered, and/or a prospective cohort study should be performed to validate the TSR in the next step toward clinical implementation.

CONCLUSIONS

The results showed that the prognostic effect of the TSR is most discriminative in grade III tumors, compared to grade I and grade II tumors, and in triple-negative tumors, compared to nontriple-negative tumors. Furthermore, the prognostic value of the TSR was not modified by age, tumor size, histology, ER status, PR status, HER2 status and lymph node status. This makes this parameter a potential factor to be included to improve risk stratification. Validating the TSR in a prospective study could further improve clinical decision making using the PREDICT tool.

ACKNOWLEDGEMENTS

Funding information: This work was supported by Genootschap Keukenhof voor de Vroege Opsporing van Kanker, Lisse, The Netherlands. No grant number applicable. M. Sobral-Leite received a scholarship from Science without Borders-CAPES.

Conflict of interest: The authors declare that there is no conflict of interest.

REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. Eur J Cancer. 2018.
2. Tchou J, Conejo-Garcia J. Targeting the tumor stroma as a novel treatment strategy for breast cancer: shifting from the neoplastic cell-centric to a stroma-centric paradigm. Adv Pharmacol. 2012;65:45-61.
3. Troester MA, Lee MH, Carter M, Fan C, Cowan DW, Perez ER, et al. Activation of host wound responses in breast cancer microenvironment. Clin Cancer Res. 2009;15(22):7020-8.
4. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315(26):1650-9.
5. Dekker TJ, Balluff BD, Jones EA, Schone CD, Schmitt M, Aubele M, et al. Multicenter matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) identifies proteomic differences in breast-cancer-associated stroma. Journal of proteome research. 2014;13(11):4730-8.
6. Chen X, Song E. Turning foes to friends: targeting cancer-associated fibroblasts. Nat Rev Drug Discov. 2019;18(2):99-115.
7. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
8. Courrech Staal EF, Smit VT, van Velthuysen ML, Spitzer-Naaykens JM, Wouters MW, Mesker WE, et al. Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies. Eur J Cancer. 2011;47(3):375-82.
9. de Kruijf EM, van Nes JG, van de Velde CJ, Putter H, Smit VT, Liefers GJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat. 2011;125(3):687-96.
10. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. Ejso. 2012;38(4):307-13.
11. Dekker TJA, van de Velde CJH, van Pelt GW, Kroep JR, Julien JP, Smit VTHBM, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Tr. 2013;139(2):371-9.
12. Gujam FJ, Edwards J, Mohammed ZM, Going JJ, McMillan DC. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. Br J Cancer. 2014;111(1):157-65.
13. Vangangelt KMH, van Pelt GW, Engels CC, Putter H, Liefers GJ, Smit V, et al. Prognostic value of tumor-stroma ratio combined with the immune status of tumors in invasive breast carcinoma. Breast Cancer Res Treat. 2017.

14. Roeke T, Sobral-Leite M, Dekker TJA, Wesseling J, Smit V, Tollenaar R, et al. The prognostic value of the tumour-stroma ratio in primary operable invasive cancer of the breast: a validation study. Breast Cancer Res Treat. 2017;166(2):435-45.
15. West NP, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, et al. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. British journal of cancer. 2010;102(10):1519-23.
16. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2014;25(3):644-51.
17. Vogelaar FJ, van Pelt GW, van Leeuwen AM, Willems JM, Tollenaar RA, Liefers GJ, et al. Are disseminated tumor cells in bone marrow and tumor-stroma ratio clinically applicable for patients undergoing surgical resection of primary colorectal cancer? The Leiden MRD study. Cell Oncol (Dordr). 2016;39(6):537-44.
18. Aurello P, Berardi G, Giulitti D, Palumbo A, Tierno SM, Nigri G, et al. Tumor-Stroma Ratio is an independent predictor for overall survival and disease free survival in gastric cancer patients. Surgeon. 2017;15(6):329-35.
19. Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Annals of oncology : official journal of the European Society for Medical Oncology. 2013;24(1):179-85.
20. Wang K, Ma W, Wang JB, Yu L, Zhang XM, Wang ZB, et al. Tumor-Stroma Ratio Is an Independent Predictor for Survival in Esophageal Squamous Cell Carcinoma. J Thorac Oncol. 2012;7(9):1457-61.
21. Mesker WE, Liefers GJ, Junggeburt JM, van Pelt GW, Alberici P, Kuppen PJ, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients. Cellular oncology : the official journal of the International Society for Cellular Oncology. 2009;31(3):169-78.
22. Chen Y, Zhang L, Liu W, Liu X. Prognostic Significance of the Tumor-Stroma Ratio in Epithelial Ovarian Cancer. Biomed Res Int. 2015;2015:589301.
23. Hansen TF, Kjaer-Frifeldt S, Lindebjerg J, Rafaelsen SR, Jensen LH, Jakobsen A, et al. Tumor-stroma ratio predicts recurrence in patients with colon cancer treated with neoadjuvant chemotherapy. Acta oncologica (Stockholm, Sweden). 2018;57(4):528-33.
24. Li H, Yuan SL, Han ZZ, Huang J, Cui L, Jiang CQ, et al. Prognostic significance of the tumor-stroma ratio in gallbladder cancer. Neoplasma. 2017;64(4):588-93.
25. Liu J, Liu J, Li J, Chen Y, Guan X, Wu X, et al. Tumor-stroma ratio is an independent predictor for survival in early cervical carcinoma. Gynecol Oncol. 2014;132(1):81-6.
26. Lv Z, Cai X, Weng X, Xiao H, Du C, Cheng J, et al. Tumor-stroma ratio is a prognostic factor for survival in hepatocellular carcinoma patients after liver resection or transplantation. Surgery. 2015;158(1):142-50.

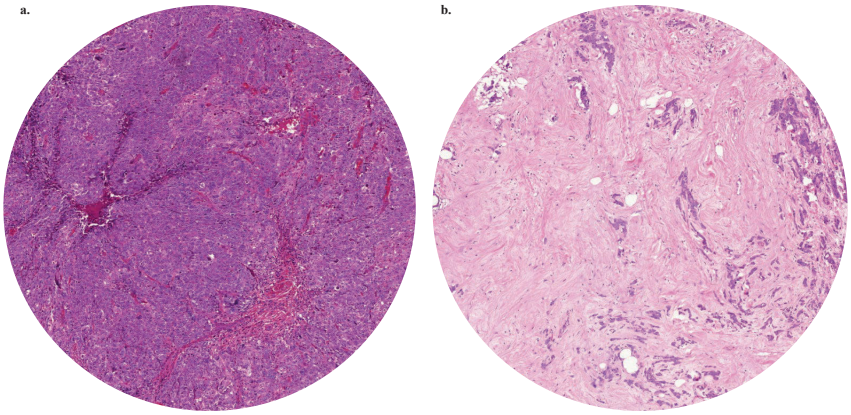
27. Niranjan KC, Sarathy NA. Prognostic impact of tumor-stroma ratio in oral squamous cell carcinoma - A pilot study. Ann Diagn Pathol. 2018;35:56-61.
28. Pongsuvareeyakul T, Khunamornpong S, Settakorn J, Sukpan K, Suprasert P, Intaraphet S, et al. Prognostic evaluation of tumor-stroma ratio in patients with early stage cervical adenocarcinoma treated by surgery. Asian Pac J Cancer Prev. 2015;16(10):4363-8.
29. Scheer R, Baidoshvili A, Zoidze S, Elferink MAG, Berkel AEM, Klaase JM, et al. Tumor-stroma ratio as prognostic factor for survival in rectal adenocarcinoma: A retrospective cohort study. World J Gastrointest Oncol. 2017;9(12):466-74.
30. Xi KX, Wen YS, Zhu CM, Yu XY, Qin RQ, Zhang XW, et al. Tumor-stroma ratio (TSR) in non-small cell lung cancer (NSCLC) patients after lung resection is a prognostic factor for survival. J Thorac Dis. 2017;9(10):4017-26.
31. Zhang XL, Jiang C, Zhang ZX, Liu F, Zhang F, Cheng YF. The tumor-stroma ratio is an independent predictor for survival in nasopharyngeal cancer. Oncol Res Treat. 2014;37(9):480-4.
32. Zhang T, Xu J, Shen H, Dong W, Ni Y, Du J. Tumor-stroma ratio is an independent predictor for survival in NSCLC. Int J Clin Exp Pathol. 2015;8(9):11348-55.
33. Kramer CJH, Vangangelt KMH, van Pelt GW, Dekker TJA, Tollenaar R, Mesker WE. The prognostic value of tumour-stroma ratio in primary breast cancer with special attention to triple-negative tumours: a review. Breast Cancer Res Treat. 2018.
34. Blamey RW, Hornmark-Stenstam B, Ball G, Blichert-Toft M, Cataliotti L, Fourquet A, et al. ONCOPOOL - a European database for 16,944 cases of breast cancer. Eur J Cancer. 2010;46(1):56-71.
35. Sobral-Leite M, Van de Vijver K, Michaut M, van der Linden R, Hooijer GKJ, Horlings HM, et al. Assessment of PD-L1 expression across breast cancer molecular subtypes, in relation to mutation rate, BRCA1-like status, tumor-infiltrating immune cells and survival. Oncoimmunology. 2018;7(12).
36. van Pelt GW, Kjaer-Frifeldt S, van Krieken J, Al Dieri R, Morreau H, Tollenaar R, et al. Scoring the tumor-stroma ratio in colon cancer: procedure and recommendations. Virchows Arch. 2018.
37. Wishart GC, Azzato EM, Greenberg DC, Rashbass J, Kearins O, Lawrence G, et al. PREDICT: a new UK prognostic model that predicts survival following surgery for invasive breast cancer. Breast Cancer Res. 2010;12(1):R1.
38. Candido Dos Reis FJ, Wishart GC, Dicks EM, Greenberg D, Rashbass J, Schmidt MK, et al. An updated PREDICT breast cancer prognostication and treatment benefit prediction model with independent validation. Breast Cancer Res. 2017;19(1):58.
39. Smit M, van Pelt G, Roodvoets A, Meershoek-Klein Kranenbarg E, Putter H, Tollenaar R, et al. Uniform Noting for International Application of the Tumor-Stroma Ratio as an Easy Diagnostic Tool: Protocol for a Multicenter Prospective Cohort Study. JMIR Res Protoc. 2019;8(6):e13464.

40. Su S, Chen J, Yao H, Liu J, Yu S, Lao L, et al. CD10(+)GPR77(+) Cancer-Associated Fibroblasts Promote Cancer Formation and Chemoresistance by Sustaining Cancer Stemness. Cell. 2018;172(4):841-56 e16.
41. Ahn S, Cho J, Sung J, Lee JE, Nam SJ, Kim KM, Cho EY. The prognostic significance of tumor-associated stroma in invasive breast carcinoma. Tumour Biol. 2012;33(5):1573-80.

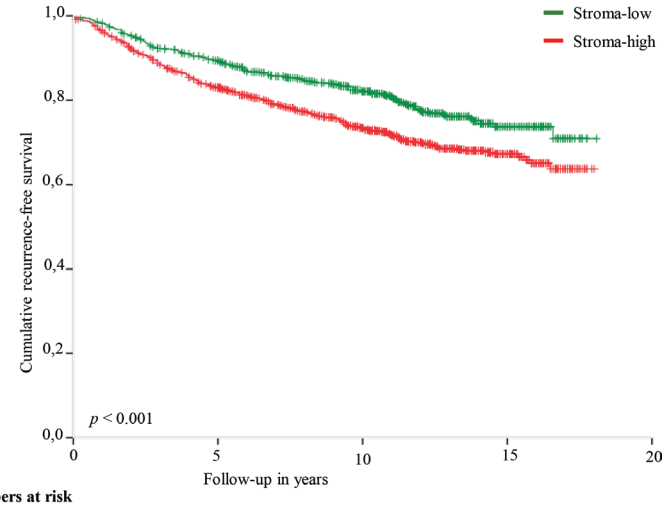
SUPPLEMENTARY DATA

SUPPLEMENTARY FIGURE 1. Representative tissue selection for tumor-stroma ratio assessment.

a. Stroma-low **b.** Stroma-high.



SUPPLEMENTARY FIGURE 2. Kaplan-Meier analysis for recurrence-free survival of patients included in the Nottingham Breast Cancer Series stratified by tumor-stroma ratio.



Numbers at risk

Stroma-low	681	565	372	70	9
Stroma-high	1113	858	564	105	15

SUPPLEMENTARY TABLE 1. Overview of the stratification of age, tumor characteristics and treatment options of patients included in the ONCOPOOL study.

		Stroma-low		Stroma-high		
	n	n = 454	%	n = 283	%	*p*-value
Age (in years)						
<40	63	49	10.8	14	4.9	0.012
40-49	206	134	29.5	72	25.4	
50-59	259	147	32.4	112	39.6	
≥60	209	124	27.3	85	30.0	
Histological type						
Invasive carcinoma of NST	621	386	85.0	235	83.0	0.484
Lobular carcinoma	69	38	8.4	31	11.0	
Tubular carcinoma	32	22	4.8	10	3.5	
Others	15	8	1.8	7	2.5	
Grade						
I	159	101	22.2	58	20.5	0.274
II	255	146	32.2	109	38.5	
III	216	142	31.3	74	26.1	
Missing	107	65	14.3	42	14.8	
Tumor size (in cm's)						
≤2	479	314	69.2	165	58.3	0.005
>2-≤5	252	135	29.7	117	41.3	
>5	6	5	1.1	1	0.4	
Nodal status						
Negative	416	257	56.6	159	56.2	0.145
Positive	315	191	42.1	124	43.8	
Missing	6	6	1.3	0	0	
ER status						
Negative	127	87	19.2	40	14.1	0.142
Positive	606	365	80.4	241	85.2	
Missing	4	2	0.4	2	0.7	
PR status						
Negative	234	149	32.8	85	30.0	0.442
Positive	496	302	66.5	194	68.6	
Missing	7	3	0.7	4	1.4	

SUPPLEMENTARY TABLE 1. Continued.

		Stroma-low		Stroma-high		
	n	n = 454	%	n = 283	%	*p*-value
HER2 status						
Negative	573	357	78.6	216	76.3	0.462
Positive	151	91	20.0	60	21.2	
Missing	13	6	1.3	7	2.5	
Chemotherapy						
No	573	346	76.2	227	80.2	0.204
Yes	164	108	23.8	56	19.8	
Hormonal therapy						
No	369	229	50.4	140	49.5	0.798
Yes	368	225	49.6	143	50.5	

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, NST = no special type, PR = progesterone receptor

SUPPLEMENTARY TABLE 2. Univariate and multivariate Cox regression analyses of the ONCOPOOL study. The tumor-stroma ratio is adjusted for confounders.

	Recurrence-free survival							**Overall survival**					
	Univariate analysis				*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age													
	733	1.00	0.98-1.01	0.482	1.00	0.98-1.02	0.978	1.04	1.03-1.05	<0.001	1.04	1.03-1.06	<0.001
Tumor size (in cm's)													
≤2	479			<0.001			0.098			<0.001			0.028
>2	258	1.77	1.35-2.32		1.31	0.95-1.79		1.65	1.30-2.09		1.37	1.04-1.82	
Grade													
I	159			<0.001			0.022			<0.001			0.001
II	255	1.86	1.22-2.86		1.63	1.04-2.56		1.64	1.13-2.38		1.65	1.10-2.46	
III	216	2.51	1.64-3.85		2.00	1.22-3.28		2.47	1.71-3.56		2.24	1.46-3.43	
Histological type													
Invasive carcinoma of NST	621			0.657			0.464			0.461			0.326
Lobular carcinoma	69	0.99	0.63-1.56		1.32	0.74-2.35		0.93	0.62-1.40		1.11	0.63-1.97	
Tubular carcinoma	32	0.69	0.32-1.47		0.92	0.39-2.15		0.93	0.53-1.63		1.02	0.52-2.00	
Others	14	0.61	0.19-1.91		0.41	0.10-1.67		0.40	0.13-1.26		0.16	0.02-1.15	
ER status													
Negative	127			<0.001			0.542			<0.001			0.470
Positive	606	0.55	0.40-0.76		0.87	0.55-1.37		0.54	0.41-1.71		0.87	0.59-1.27	

SUPPLEMENTARY TABLE 2. Continued.

	Recurrence-free survival						Overall survival					
	Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
PR status													
Negative	234			<0.001			0.271			<0.001			0.016
Positive	496	0.60	0.45-0.79		0.81	0.55-1.18		0.51	0.40-0.64		0.67	0.49-0.93	
HER2 status													
Negative	573			0.572			0.896			0.017			0.033
Positive	151	1.10	0.79-1.53		1.02	0.72-1.47		1.40	1.06-1.84		1.39	1.03-1.88	
TSR													
Stroma-low	454			0.093			0.085			0.016			0.029
Stroma-high	283	1.26	0.96-1.66		1.30	0.96-1.76		1.34	1.06-1.69		1.35	1.03-1.77	

Abbreviations: ER = estrogen receptor, HER2= human epidermal growth factor receptor 2, NST = no special type, PR = progesterone receptor, TSR= tumor-stroma ratio

SUPPLEMENTARY TABLE 3. Results of the independent prognostic value of the tumor-stroma ratio adjusted for confounders, confounders including triple-negative status and confounders combined with lymph node status, calculated with multivariate Cox regression analysis in the ONCOPOOL study.

	Recurrence-free survival	**Overall survival**
Confounders	HR 1.30, 95% CI 0.96-1.76, $p = 0.085$	HR 1.35, 95% CI 1.03-1.77, $p = 0.029$
Confounders including triple-negative status	HR 1.30, 95% CI 0.96-1.75, $p = 0.091$	HR 1.34, 95% CI 1.02-1.74, $p = 0.033$
Confounders combined with lymph node status	HR 1.28, 95% CI 0.95-1.73, $p = 0.112$	HR 1.33, 95% CI 1.02-1.74, $p = 0.037$

SUPPLEMENTARY TABLE 4. Results of the tumor-stroma ratio stratified by clinically important prognostic parameters in the ONCOPOOL study and the multivariate Cox regression analysis per clinically relevant subgroup with a statistically significant difference in the Nottingham Breast Cancer series.

TSR stratified by group	Subgroups	Recurrence-free survival	Overall survival
Age		$p = 0.496$	$p = 0.840$
Tumor size		$p = 0.816$	$p = 0.823$
Grade		$p = 0.122$	$p = 0.414$
	Grade I	HR 0.92, 95% CI 0.39-2.35, $p = 0.992$	HR 0.83, 95% CI 0.38-1.81, $p = 0.631$
	Grade II	HR 1.06, 95% CI 0.66-1.70, $p = 0.806$	HR 1.30, 95% CI 0.83-2.02, $p = 0.257$
	Grade III	HR 1.86, 95% CI 1.18-2.93, $p = 0.008$	HR 1.61, 95% CI 1.08-2.41, $p = 0.020$
Histological type		$p = 0.838$	$p = 0.620$
ER status		$p = 0.445$	$p = 0.222$
PR status		$p = 0.982$	$p = 0.387$
HER2 status		$p = 0.646$	$p = 0.910$
Triple-negative status		$p = 0.343$	$p = 0.255$
	Nontriple-negative status	HR 1.25, 95% CI 0.91-1.73, $p = 0.176$	HR 1.28, 95% CI 0.96-1.72, $p = 0.093$
	Triple-negative status	HR 1.54, 95% CI 0.64-3.66, $p = 0.333$	HR 1.75, 95% CI 0.83-3.66, $p = 0.140$
Lymph node status		$p = 0.423$	$p = 0.097$

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor, PR = progesterone receptor

4

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

K.M.H. Vangangelt
C.J.H. Kramer
E. Bastiaannet
H. Putter
D. Cohen
G.W. van Pelt
E.A. Rakha
A.R. Green
R.A.E.M. Tollenaar
W.E. Mesker

Breast Cancer Res Treat. 2020 Jan;179(1):37-45

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.

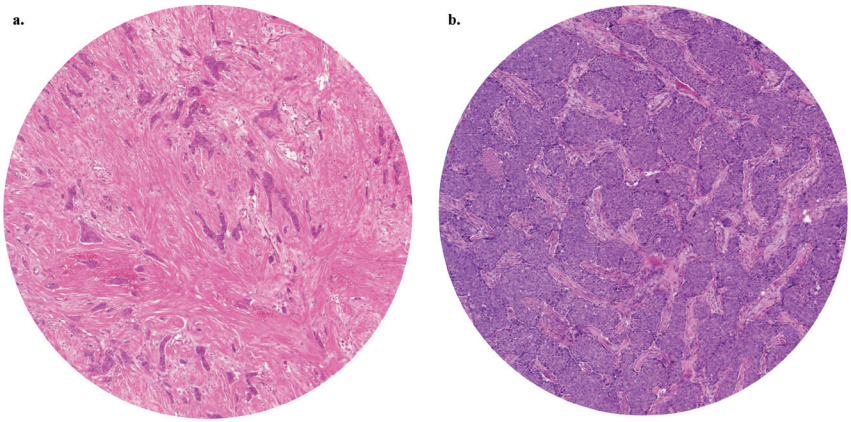
For standard clinical care all resected tumors were assessed by a pathologist, according to the currently applied pathological standards. The clinical data from the Nottingham Breast Cancer series were anonymized and the study was approved by the Nottingham Research Ethics Committee 2 under the title '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).

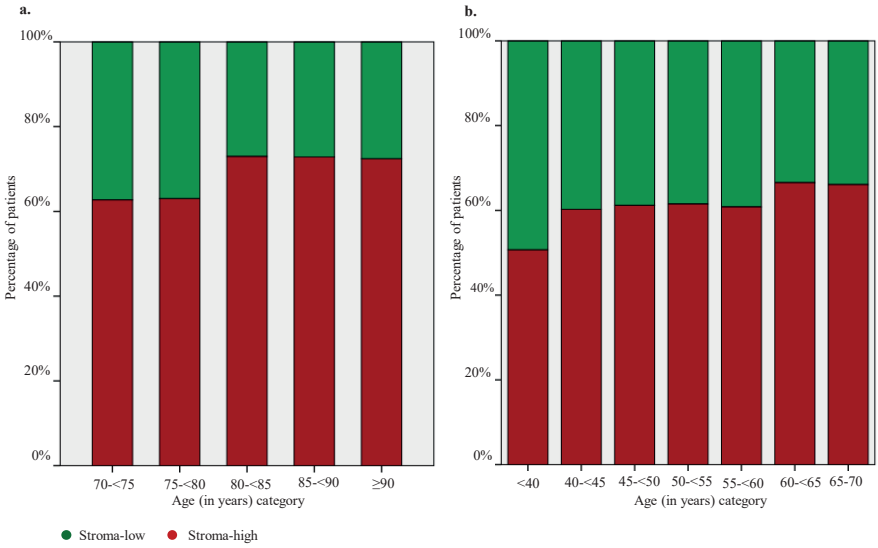
Linear regression was adjusted for tumor size, histology, ER status, PR status, HER2 status, TN status and grade in the FOCUS study and the Nottingham Breast Cancer Series (B 0.031, 95% CI 0.006-0.057, $p = 0.016$ and B 0.034, 95% CI 0.015-0.054, $p < 0.001$, respectively). These results showed that 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

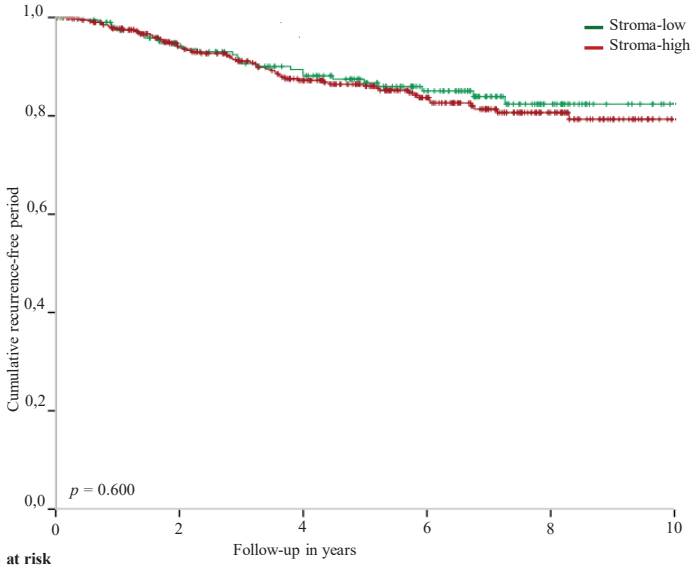
Most of the 619 tumors were categorized as stroma-high (67%). Eighty-five patients developed a tumor recurrence. Among stroma-high tumors, a higher number of patients with positive lymph nodes (p = 0.004), an advanced T-stage (p = 0.014) and hormonal therapy (p = 0.038) was observed. Older age was associated 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

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.

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.

ACKNOWLEDGEMENTS

We would like to thank dr. G.J. Liefers and dr. P.J.K. Kuppen for their valuable feedback and suggestions.
Funding information: This work was supported by Genootschap Keukenhof voor de Vroege Opsporing van Kanker, Lisse, The Netherlands. No grant number applicable.
Conflict of interest: The authors declare that there is no conflict of interest.

REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. Eur J Cancer. 2018.
2. DePinho RA. The age of cancer. Nature. 2000;408(6809):248-54.
3. van Pelt GW, Sandberg TP, Morreau H, Gelderblom H, van Krieken J, Tollenaar R, et al. The tumour-stroma ratio in colon cancer: the biological role and its prognostic impact. Histopathology. 2018;73(2):197-206.
4. Pierga JY, Girre V, Laurence V, Asselain B, Dieras V, Jouve M, et al. Characteristics and outcome of 1755 operable breast cancers in women over 70 years of age. Breast. 2004;13(5):369-75.
5. Benz CC. Impact of aging on the biology of breast cancer. Crit Rev Oncol Hematol. 2008;66(1):65-74.
6. Wildiers H, Kunkler I, Biganzoli L, Fracheboud J, Vlastos G, Bernard-Marty C, et al. Management of breast cancer in elderly individuals: recommendations of the International Society of Geriatric Oncology. Lancet Oncol. 2007;8(12):1101-15.
7. Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. J Natl Cancer Inst. 2000;92(7):550-6.
8. Lodi M, Scheer L, Reix N, Heitz D, Carin AJ, Thiebaut N, et al. Breast cancer in elderly women and altered clinico-pathological characteristics: a systematic review. Breast Cancer Res Treat. 2017;166(3):657-68.
9. Brouwers B, Fumagalli D, Brohee S, Hatse S, Govaere O, Floris G, et al. The footprint of the ageing stroma in older patients with breast cancer. Breast Cancer Res. 2017;19(1):78.
10. de Kruijf EM, van Nes JG, van de Velde CJ, Putter H, Smit VT, Liefers GJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat. 2011;125(3):687-96.
11. Dekker TJA, van de Velde CJH, van Pelt GW, Kroep JR, Julien JP, Smit VTHBM, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Tr. 2013;139(2):371-9.
12. Downey CL, Thygesen HH, Sharma N, Shaaban AM. Prognostic significance of tumour stroma ratio in inflammatory breast cancer. Springerplus. 2015;4:68.
13. Downey CL, Simpkins SA, White J, Holliday DL, Jones JL, Jordan LB, et al. The prognostic significance of tumour-stroma ratio in oestrogen receptor-positive breast cancer. Br J Cancer. 2014;110(7):1744-7.
14. Gujam FJ, Edwards J, Mohammed ZM, Going JJ, McMillan DC. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. Br J Cancer. 2014;111(1):157-65.

15. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. Ejso. 2012;38(4):307-13.
16. Roeke T, Sobral-Leite M, Dekker TJA, Wesseling J, Smit V, Tollenaar R, et al. The prognostic value of the tumour-stroma ratio in primary operable invasive cancer of the breast: a validation study. Breast Cancer Res Treat. 2017;166(2):435-45.
17. Vangangelt KMH, van Pelt GW, Engels CC, Putter H, Liefers GJ, Smit V, et al. Prognostic value of tumor-stroma ratio combined with the immune status of tumors in invasive breast carcinoma. Breast Cancer Res Treat. 2017.
18. Kramer CJH, Vangangelt KMH, van Pelt GW, Dekker TJA, Tollenaar R, Mesker WE. The prognostic value of tumour-stroma ratio in primary breast cancer with special attention to triple-negative tumours: a review. Breast Cancer Res Treat. 2018.
19. Biganzoli L, Wildiers H, Oakman C, Marotti L, Loibl S, Kunkler I, et al. Management of elderly patients with breast cancer: updated recommendations of the International Society of Geriatric Oncology (SIOG) and European Society of Breast Cancer Specialists (EUSOMA). Lancet Oncol. 2012;13(4):e148-60.
20. Smith BD, Jiang J, McLaughlin SS, Hurria A, Smith GL, Giordano SH, et al. Improvement in breast cancer outcomes over time: are older women missing out? J Clin Oncol. 2011;29(35):4647-53.
21. Holleczek B, Brenner H. Trends of population-based breast cancer survival in Germany and the US: decreasing discrepancies, but persistent survival gap of elderly patients in Germany. Bmc Cancer. 2012;12:317.
22. Jensen JD, Cold S, Nielsen MH, Jylling AM, Soe KL, Larsen LB, et al. Trends in breast cancer in the elderly in Denmark, 1980-2012. Acta Oncol. 2016;55 Suppl 1:59-64.
23. Bastiaannet E, Portielje JE, van de Velde CJ, de Craen AJ, van der Velde S, Kuppen PJ, et al. Lack of survival gain for elderly women with breast cancer. Oncologist. 2011;16(4):415-23.
24. Derks MGM, Bastiaannet E, Kiderlen M, Hilling DE, Boelens PG, Walsh PM, et al. Variation in treatment and survival of older patients with non-metastatic breast cancer in five European countries: a population-based cohort study from the EURECCA Breast Cancer Group. Br J Cancer. 2018;119(1):121-9.
25. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
26. van Pelt GW, Kjaer-Frifeldt S, van Krieken J, Al Dieri R, Morreau H, Tollenaar R, et al. Scoring the tumor-stroma ratio in colon cancer: procedure and recommendations. Virchows Arch. 2018.
27. LaBarge MA, Mora-Blanco EL, Samson S, Miyano M. Breast Cancer beyond the Age of Mutation. Gerontology. 2016;62(4):434-42.
28. Jackaman C, Tomay F, Duong L, Abdol Razak NB, Pixley FJ, Metharom P, et al. Aging and cancer: The role of macrophages and neutrophils. Ageing Res Rev. 2017;36:105-16.

29. Provinciali M, Argentati K, Tibaldi A. Efficacy of cancer gene therapy in aging: adenocarcinoma cells engineered to release IL-2 are rejected but do not induce tumor specific immune memory in old mice. Gene Ther. 2000;7(7):624-32.
30. Hankinson SE, Manson JE, Spiegelman D, Willett WC, Longcope C, Speizer FE. Reproducibility of plasma hormone levels in postmenopausal women over a 2-3-year period. Cancer Epidemiol Biomarkers Prev. 1995;4(6):649-54.
31. Key T, Appleby P, Barnes I, Reeves G, Endogenous H, Breast Cancer Collaborative G. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst. 2002;94(8):606-16.
32. Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev. 1993;15(1):17-35.
33. Cohen SM, Ellwein LB. Cell proliferation in carcinogenesis. Science. 1990;249(4972):1007-11.
34. Gennari R, Curigliano G, Rotmensz N, Robertson C, Colleoni M, Zurrida S, et al. Breast carcinoma in elderly women: features of disease presentation, choice of local and systemic treatments compared with younger postmenopasual patients. Cancer. 2004;101(6):1302-10.
35. van de Water W, Markopoulos C, van de Velde CJ, Seynaeve C, Hasenburg A, Rea D, et al. Association between age at diagnosis and disease-specific mortality among postmenopausal women with hormone receptor-positive breast cancer. JAMA. 2012;307(6):590-7.
36. de Glas NA, Bastiaannet E, Engels CC, de Craen AJ, Putter H, van de Velde CJ, et al. Validity of the online PREDICT tool in older patients with breast cancer: a population-based study. Br J Cancer. 2016;114(4):395-400.
37. van Maaren MC, van Steenbeek CD, Pharoah PDP, Witteveen A, Sonke GS, Strobbe LJA, et al. Validation of the online prediction tool PREDICT v. 2.0 in the Dutch breast cancer population. Eur J Cancer. 2017;86:364-72.

4

5

The prognostic value of the tumor-stroma ratio in tumor-positive axillary lymph nodes of breast cancer patients

K.M.H. Vangangelt
L.S.A. Tollenaar
G.W. van Pelt
E.M. de Kruijf
T.J.A. Dekker
P.J.K. Kuppen
R.A.E.M. Tollenaar
W.E. Mesker

International Journal of Cancer, 2018; 143:3194-3200

ABSTRACT

Purpose

The tumor-stroma ratio (TSR) has previously been found to be a strong prognostic parameter in primary breast cancer tumors. Since the presence of tumor cells in lymph nodes is important for clinical decision making, the influence of the TSR in the primary breast tumor combined with the TSR in tumor-positive lymph nodes on prognosis was evaluated.

Methods

Women with invasive breast cancer without distant metastasis who underwent an axillary lymph node dissection between 1985 and 1994 at the Leiden University Medical Center were analyzed retrospectively. TSR assessment was performed on hematoxylin and eosin stained tissue slides.

Results

In total, 87 (45.5%) primary tumors were scored as stroma-low and 104 (54.5%) as stroma-high. Patients with a high stromal percentage in the primary tumors had a statistically significant worse relapse-free period (RFP) compared to stroma-low tumors (HR 1.97, 95% CI 1.37-2.82, $p < 0.001$). A total number of 915 lymph nodes were assessed for the TSR. In 101 (52.9%) patients, heterogeneity was observed between stroma percentage category in the primary tumor and lymph nodes. The combination of the TSR of the primary tumor and the TSR of tumor-positive lymph nodes strengthened each other as an independent prognostic parameter for RFP ($p = 0.019$). Patients with primary tumor stroma-low/lymph nodes stroma-low tumors showed strongly improved RFP rates compared to patients with primary tumor stroma-high/lymph node stroma-high tumors with 10-year percentages of 58% versus 8%, respectively.

Conclusions

Assessing the TSR on tumor-positive lymph nodes can provide additional prognostic information. Stromal activation strongly differs between primary tumors and lymph node metastases.

INTRODUCTION

In patients with invasive breast cancer, the presence of a regional lymph node (LN) metastasis is one of the most important prognostic parameters for long-term prognosis (1). Careful evaluation of LN status is crucial to decide whether patients should undergo an axillary lymph node dissection (ALND) or axillary radiotherapy and also plays a large role in deciding on adjuvant chemotherapy. As breast cancer is a heterogeneous disease (2), distinguishing patients who need more aggressive therapy from patients who would benefit from a more conservative approach remains a difficult challenge. Prognostic parameters derived from the stromal compartment might provide an important tool. The interaction between tumor cells and cells in the tumor microenvironment has gained significant interest in the last two decades. The tumor stroma consists of inflammatory cells, capillaries, fibroblasts and extracellular matrix (3). Fibroblasts that surround and infiltrate the primary tumor (PT), the so-called cancer-associated fibroblasts (CAFs), are believed to play a key role in tumor progression by secreting chemokines and growth factors. This may lead to increased cancer cell proliferation, promoting motility and invasiveness, enhanced angiogenesis and tumor-promoting inflammation (4, 5). Based on the analysis of hematoxylin and eosin (H&E) stained histologic slides, our research group developed an internationally validated prognostic tool, the tumor-stroma ratio (TSR). This tool assesses the amount of stromal proliferation within the borders of the PT. This parameter has shown to be of high prognostic value in several types of epithelial neoplasms, including breast cancer (6-10), colon cancer (11-14), gastric cancer (15) and esophageal cancer (16). These studies have invariably shown a worse prognosis in patients with so-called stroma-high tumors compared to patients with stroma-low tumors.

The additional prognostic value of TSR assessment in metastatic LNs for disease-free survival (DFS) in patients with stage III colorectal cancer was published by Van Pelt et al. (17). By our knowledge, the influence of stromal growth in LNs affected by breast cancer has not yet been investigated. The objective of this current study was to evaluate the prognostic value of the TSR in the primary tumor combined with the TSR in tumor-positive LNs in primary breast tumors compared to the TSR in primary breast tumors alone.

MATERIAL AND METHODS

Study population

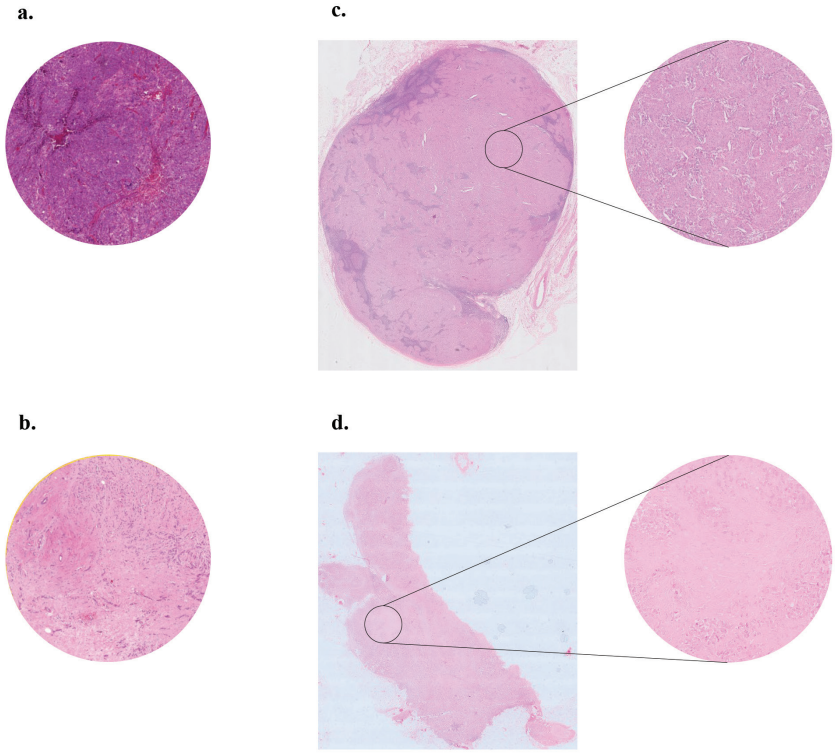
The patients included in this study were selected from a database consisting of patients with invasive breast cancer without distant metastasis, who were primarily treated with surgery between 1985 and 1994 at the Leiden University Medical Center. Patient data were assessed retrospectively (n = 677). Only patients who underwent an axillary lymph node dissection were included in this study. Patients with a history of cancer (other than basal cell carcinoma or cervical carcinoma in situ), bilateral breast cancer or absence of resected tissue slides were excluded, leaving 193 patients for analysis. The resected tumors were graded by an experienced breast cancer pathologist using the current pathological standards. TSR assessment of the primary breast tumors was described earlier (9). All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

TSR assessment

The TSR was visually assessed by conventional light microscopy on 5 µm routine H&E stained slides. First, the PT and LNs were evaluated with a 5x objective to identify the most stroma-rich tissue area(s). The most stroma-abundant area was selected and assessed with a 10x objective. Only tumor fields with tumor cells present at all borders of the image field were eligible. The stroma percentage was scored by increments of 10%. A stroma percentage ≤50% was categorized as stroma-low and a stroma percentage >50% was considered stroma-high (Figure 1). Positive LNs were identified as stroma-high if at least one of the LNs had a stroma percentage of >50% (figure 1). Lymph node metastases of >0.2 mm but ≤2 mm were defined as micrometastases. In the case of micrometastases, the TSR was evaluated in a smaller image field as long as tumor cells were present at all borders.

FIGURE 1. Examples of the tumor-stroma ratio in breast cancer. Lymph nodes were scanned with an automated scanning system (Philips Ultra Fast Scanner 1.6 RA) at 20x magnification.

a. Primary tumor stroma-low **b.** Primary tumor stroma-high **c.** Stroma-low tumor-positive lymph node **d.** Stroma-high tumor-positive lymph node.



Statistical analyses

SPSS software version 23.0 (SPSS Inc., IBM Company Chicago, IL, USA) was used to perform the statistical analyses. Cohen's kappa value was used to assess the inter-observer agreement. A value above 0.6 was considered as valid. The χ^2 test was used for the evaluation of statistically significant differences for categorical variables between patients with stroma-high or stroma-low tumors. For numerical variables (lymph node yield), distribution was tested for normality using the Shapiro-Wilk

test. Statistically significant differences of non-parametric variables were analyzed using the Mann-Whitney U test. The primary endpoint was the relapse-free period (RFP), which was defined as the time from date of surgery until local, regional or distant recurrence of breast cancer. Patients who died or were lost to follow-up were censored at the last date on which they were known to be recurrence-free and/or alive. The definition of secondary endpoint overall survival (OS) was the time from date of surgery until death from any cause. Kaplan-Meier curves were compared with log-rank tests to assess differences in RFP. Univariate and multivariate Cox regression analyses were calculated for RFP and OS. Parameters with a *p*-value of less than 0.10 in univariate analysis were entered in multivariate analysis. For all analyses, a *p*-value of less than 0.05 was considered statistically significant. Effect modification was evaluated by adding interaction in the Cox regression analysis.

RESULTS

Patients

In total, H&E slides derived from 193 breast cancer patients could be evaluated for the TSR. Two patients were excluded due to poor quality of LN tissue slides, leaving 191 patients for analysis. The study group consisted of women with a median age at time of diagnosis of 57.4 years (range 27.5-87.6 years). The median follow-up period was 7.3 years (range 0.2-23.0 years). Table 1 provides a detailed overview of patient characteristics.

TABLE 1. Patient characteristics and statistically significant differences between stroma-low and stroma-high primary tumors calculated with the χ^2 test.

		Stroma-low		**Stroma-high**		
	n	n = 87	*%*	n = 104	*%*	*p*-value
Age (in years)						
<40	15	9	10.3	6	5.8	0.364
>40-60	94	39	44.8	55	52.9	
>60	82	39	44.8	43	41.3	
Grade						
I	18	5	5.7	13	12.5	0.170
II	85	37	42.5	48	46.2	
III	88	45	51.7	43	41.3	

TABLE 1. Continued.

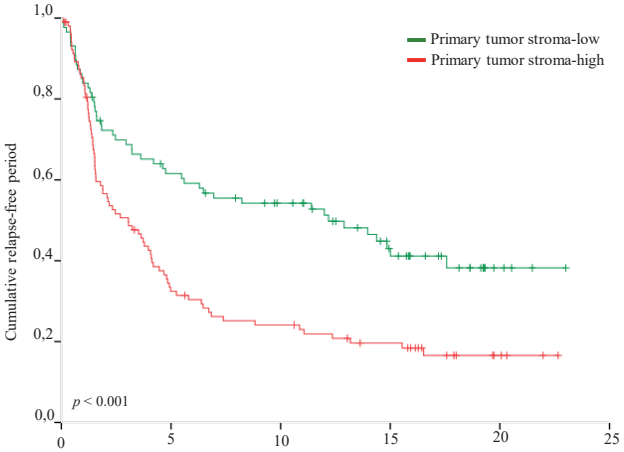
		Stroma-low		Stroma-high		
	n	n = 87	%	n = 104	%	*p*-value
Histological type						
Ductal carcinoma	171	83	96.5	88	85.4	0.010
Lobular carcinoma	18	3	3.5	15	14.6	
Tumor stage						
pT1	42	16	18.6	26	26.3	0.449
pT2	109	54	62.8	55	55.6	
pT3/4	34	16	18.6	18	18.2	
Nodal stage						
pN1	148	75	86.2	73	70.2	0.011
pN2	11	1	1.1	10	9.6	
pN3	32	11	12.6	21	20.2	
ER status						
Negative	83	40	47.1	43	44.8	0.760
Positive	98	45	52.9	53	55.2	
PR status						
Negative	86	36	42.4	50	51.0	0.241
Positive	97	49	57.6	48	49.0	
HER2 status						
Negative	118	57	82.6	61	82.4	0.978
Positive	25	12	17.4	13	17.6	
Surgery with or without radiotherapy						
MST without RT	62	30	34.5	32	30.8	0.860
MST with RT	63	28	32.2	35	33.7	
BCS without RT	0	0	0	0	0	
BCS with RT	76	29	33.3	37	35.6	
Chemotherapy						
No	127	52	59.8	75	72.1	0.072
Yes	64	35	40.2	29	27.9	
Hormonal therapy						
No	136	61	70.1	75	72.1	0.761
Yes	55	26	29.9	29	27.9	

Abbreviations: BCS = breast conserving therapy, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, MST = mastectomy, PR = progesterone receptor, RT = radiotherapy

Prognostic value of the TSR in the primary tumor

In total, 87 (45.5%) PTs were determined to be stroma-low and 104 (54.5%) as stroma-high. Patients with stroma-high PTs had a statistically significant worse RFP compared to stroma-low tumors (HR 1.97, 95% CI 1.37-2.82, $p < 0.001$) (figure 2). After 10 years of follow-up, 75% of patients with stroma-high tumors developed a recurrence compared to 46% of patients with stroma-low tumors. The multivariate analysis showed that the TSR in the PT is a statistically significant independent prognostic factor for RFP (HR 1.70, 95% CI 1.16-2.49, $p = 0.006$) (table 2) and OS (HR 1.49, 95% CI 1.04-2.14, $p = 0.029$) (supplementary table 1). In the stroma-high group, statistically significant more patients had a tumor of lobular type and a higher nodal stage (table 1). The TSR assessment of the PTs in the total group of patients was previously published by our group (9). The tissue slides were scored in a blinded fashion by a second observer with a Cohen's kappa of 0.85 (almost perfect agreement).

FIGURE 2. Kaplan-Meier analysis for relapse-free period of patients with stroma-low primary tumors and stroma-high primary tumors.



Numbers at risk				
Primary tumor stroma-low	86	49	35	14
Primary tumor stroma-high	102	32	22	11

The TSR in tumor-positive lymph nodes

In total, 915 LNs were analyzed (range 1-18 per patient). LNs were categorized as stroma-high if at least one of the LNs had a stroma percentage of >50%. The LNs of 160 (83.8%) patients were scored as stroma-low and 31 as stroma-high (16.2%). Stroma-low PTs and stroma-low LNs were seen in 73 patients (38.2%). Stroma-high PTs and stroma-high LNs were seen in 17 patients (8.9%). In 101 (52.9%) patients, heterogeneity was observed between the stroma percentage category in the primary tumor and in the lymph nodes. No interaction between the TSR in the PTs and LNs was found, as well as between the TSR in LNs and nodal status. The Mann-Whitney U test did not show a statistically significant difference between lymph node yield (not normally distributed) and the TSR category of LNs. In 10 patients, only micrometastases were observed. These small tumor fields consisted of tumor cells for more than 90%. Thirty percent of the LNs were scored in a blinded fashion by a second observer with a Cohen's kappa of 0.79.

Prognostic value of the TSR in primary tumor combined with tumor-positive lymph nodes

The TSRs of the PT and positive LNs were combined to evaluate the possibility of an additional prognostic effect. The four different combinations of the TSR (PT stroma-low/LNs stroma-low, PT stroma-low/LNs stroma-high, PT stroma-high/LNs stroma-low and PT stroma-high/LNs stroma-high) were plotted for the RFP with an overall *p*-value of 0.001 (figure 3). The patient characteristics of these four groups were described in supplementary table 2. Patients with PT stroma-low/LNs stroma-low showed better 10-year RFP rates compared to patients with PT stroma-high/LNs stroma-high with percentages of 58% versus 8%, respectively. These analyses showed a strong prognostic impact of high amounts of stroma in the PT as well as LNs with regard to RFP. Multivariate analysis showed that the combination of the TSR in PT and LNs is an independent prognostic factor for RFP ($p = 0.019$) (table 2). A non-statistically significant trend was seen in favor of stroma-low PT/stroma-low LNs for OS ($p = 0.084$) (supplementary table 1)

TABLE 2. Univariate and multivariate analyses for the relapse-free period calculated by Cox regression analysis.

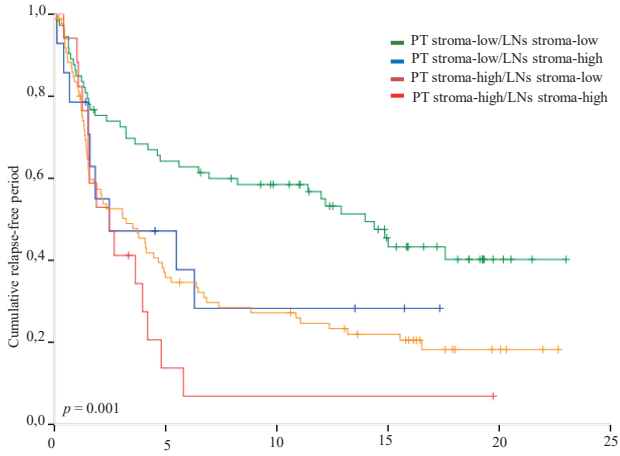
	Relapse-free period									
	Univariate analysis				*Multivariate analysis: TSR in PT*			*Multivariate analysis: TSR in PT and LNs*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age (in years)										
<40	15			0.868						
>40-60	94	1.17	0.62-2.22							
>60	82	1.10	0.57-2.12							
Grade										
I	18			0.745						
II	85	0.99	0.53-1.85							
III	88	1.14	0.61-2.11							
Histological type										
Ductal carcinoma	171			0.131						
Lobular carcinoma	18	1.52	0.88-2.60							
Tumor stage										
pT1	42			0.472						
pT2	109	0.88	0.58-1.34							
pT3/4	34	1.17	0.69- 1.98							
Nodal stage										
pN1	148			0.001			0.610			0.674
pN2	11	2.46	1.27-4.77		1.42	0.71-2.84		1.35	0.67-2.71	
pN3	32	1.90	1.23-2.93		1.11	0.68-1.82		1.13	0.69-1.84	
ER status										
Negative	83			0.311						
Positive	98	1.21	0.84-1.73							
PR status										
Negative	86			0.311						
Positive	97	0.83	0.59-1.19							

TABLE 2. Continued.

	Relapse-free period									
		Univariate analysis			*Multivariate analysis: TSR in PT*			*Multivariate analysis: TSR in PT and LNs*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
HER2 status										
Negative	118			0.331						
Positive	25	0.76	0.43-1.33							
Surgery with or without radiotherapy										
MST without RT	62			0.017			0.039			0.050
MST with RT	63	1.62	1.05-2.48		1.65	1.04-2.63		1.64	1.03-2.62	
BCS without RT	0									
BCS with RT	66	0.94	0.61-1.47		0.99	0.63-1.55		1.02	0.64-1.61	
Chemotherapy										
No	127			<0.001			0.004			0.004
Yes	64	0.47	0.32-0.70		0.53	0.35-0.82		0.53	0.35-0.82	
Hormonal therapy										
No	136			0.488						
Yes	55	0.87	0.59-1.29							
TSR										
Stroma-low	87			<0.001			0.006			
Stroma-high	104	1.97	1.37-2.82		1.70	1.16-2.49				
TSR PT combined with LNs										
PT low/LN low	73			0.001						0.019
PT low/LN high	14	1.78	0.86-3.68	0.120				1.58	0.76-3.30	0.223
PT high/LN low	87	2.04	1.37-3.04	<0.001				1.75	1.15-2.65	0.009
PT high/LN high	17	2.86	1.56-5.24	0.001				2.41	1.29-4.49	0.006

Abbreviations: BCS = breast conserving therapy, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, LN = lymph nodes, MST = mastectomy, PR = progesterone receptor, PT = primary tumor, RT = radiotherapy, TSR = tumor-stroma ratio

FIGURE 3. Kaplan-Meier analysis for relapse-free period of patients with PT stroma-low/LNs stroma-low, PT stroma-low/LNs stroma-high, PT stroma-high/LNs stroma-low, PT stroma-high/LNs stroma-high.



Numbers at risk				
PT stroma-low/LNs stroma-low	73	44	33	14
PT stroma-low/LNs stroma-high	13	5	3	1
PT stroma- high/LNs stroma-low	89	30	21	11
PT stroma-high/LNs stroma-high	17	2	1	1

Abbreviations: LN = lymph node, PT = primary tumor

DISCUSSION

This is the first study investigating the TSR in tumor-positive LNs in patients with invasive breast cancer. Patients with LN metastases were previously considered to be immediately eligible for adjuvant chemotherapy, irrespective of other clinic-pathological parameters. As studies have shown that patients with 1-3 positive LNs do not necessarily have a worse prognosis compared to node-negative tumors, subsequent guidelines have since stated that LN involvement in itself is not a reason for adjuvant chemotherapy (18). However, further research is needed to refine the

prognosis of lymph node-positive patients further, both to omit chemotherapy in some cases or possibly to escalate chemotherapy for others.

Analogous to our work regarding the prognostic implication of stromal proliferation in PTs, we investigated the added significance of assessing stroma in breast cancer positive LNs. We found that incorporating the TSR of LNs combined with the TSR of the corresponding PT provided a superior prediction of RFP compared to the TSR of the PT alone. When the TSR is solely evaluated in the PT, the disease recurrence rate after 10 years is 75% in primary stroma-high tumors, whereas the number is 46% in primary stroma-low tumors. When the TSR of the LNs is added to these two groups, a group of patients with high risk can be identified, namely PT stroma-high/LNs stroma-high. Considering that this patient group has a recurrence rate of 92% after 10 years, this method seems capable of identifying a group of patients with a worse prognosis.

An interesting result is a strong discrepancy between the TSR in the PT and the LNs of the same patients. In 101 (52.9%) patients, heterogeneity was observed between the stroma percentage category in the PT and LNs. Only a small proportion of patients was scored as stroma-high when evaluating the LNs (n = 31), which is in stark contrast with the fairly large amount of stroma-high PTs (n = 104). Consequently, a high number of patients with stroma-high tumors presented with stroma-low LN metastases. This finding might be reflective of differential activity of signaling processes across primary and metastatic tumors. The formation of genetically and transcriptionally distinct subclones of tumor cells that arise during tumor evolution might influence the activation of tumor-associated stroma as well as tumor cell dissemination. In the current study, we found that at least one LN with a high amount of stroma was predictive for a statistically significant decreased RFP. A previously published study by Van Pelt et al. also showed the additional value of the TSR in lymph nodes. The authors concluded that the assessment of the TSR in the PT combined with the TSR in metastatic LNs has an additional value with regards to the prediction of DFS in patients treated with adjuvant therapy for stage III colon cancer (17). Incorporating the TSR in clinical practice has certain advantages compared to other potential biomarkers. TSR scoring can be carried out on standard H&E slides and is performed by visually eyeballing the tissue area during the standard pathological assessment. TSR scoring takes less than a

minute and requires no additional costs. Implementation of this method in daily practice is, therefore, an easy and non-expensive option. The concordance of the inter-observer variability has been high between researchers from our group, which is also confirmed in the current study (6, 10, 14).

The patients for this study were primarily treated with surgery between 1985 and 1994 and are part of a well-characterized treatment cohort with long-term follow-up. However, this obviously means that modern-day adjuvant chemotherapy and hormonal regimens and selection of these treatment modalities according to current guidelines were not applied to this dataset. This is reflected by the relatively poor prognosis of the included patients compared to currently treated patient groups. Therefore, before definitive conclusions can be drawn regarding the prognostic and therapeutic implication of tumoral LN fibrosis, validation of the current results in modern-day cohorts should be undertaken.

Lastly, according to treatment guidelines, breast cancer patients first undergo a sentinel lymph node biopsy (SLNB) in case of no suspicion of positive lymph nodes by ultrasound or clinical examination (1). Depending on the presence of LN metastasis, an ALND will be performed. Evaluation of the TSR in a tumor-positive LN dissected during sentinel node procedure is interesting. A recent publication from Giuliano et al. showed that a less invasive SLNB alone was non-inferior to predicting overall survival compared to ALND in women with T1 or T2 tumors, no palpable axillary lymphadenopathy and 1 or 2 positive sentinel LNs (19). Evaluation of the TSR in sentinel nodes could be an important next step to evaluate if this clinical prognostic marker can select patients who will benefit from ALND or axillary radiotherapy.

CONCLUSIONS

The TSR is a simple, fast and cheap method. Assessing the TSR on tumor-positive LNs can provide further prognostic stratification in breast cancer patients. Stromal activation strongly differs between PTs and LN metastases, likely reflecting heterogeneity of the tumor stroma metastatic process.

ACKNOWLEDGEMENTS

We would like to thank prof. dr. H. Putter for his valuable statistical advice.

Funding information: This work was supported by Genootschap Keukenhof voor de Vroege Opsporing van Kanker, Lisse, The Netherlands. No grant number applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

REFERENCES

1. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2015;26 Suppl 5:v8-30.
2. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70.
3. Ronnov-Jessen L, Petersen OW, Bissell MJ. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev. 1996;76(1):69-125.
4. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6(5):392-401.
5. Marsh T, Pietras K, McAllister SS. Fibroblasts as architects of cancer pathogenesis. Biochim Biophys Acta. 2013;1832(7):1070-8.
6. Dekker TJA, van de Velde CJH, van Pelt GW, Kroep JR, Julien JP, Smit VTHBM, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Tr. 2013;139(2):371-9.
7. Gujam FJ, Edwards J, Mohammed ZM, Going JJ, McMillan DC. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. Br J Cancer. 2014;111(1):157-65.
8. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. Ejso. 2012;38(4):307-13.
9. de Kruijf EM, van Nes JG, van de Velde CJ, Putter H, Smit VT, Liefers GJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat. 2011;125(3):687-96.
10. Roeke T, Sobral-Leite M, Dekker TJA, Wesseling J, Smit V, Tollenaar R, et al. The prognostic value of the tumour-stroma ratio in primary operable invasive cancer of the breast: a validation study. Breast Cancer Res Treat. 2017;166(2):435-45.
11. Mesker WE, Junggeburt JMC, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
12. West NP, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, et al. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. Br J Cancer. 2010;102(10):1519-23.
13. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Ann Oncol. 2014;25(3):644-51.
14. Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013;24(1):179-85.

15. Aurello P, Berardi G, Giulitti D, Palumbo A, Tierno SM, Nigri G, et al. Tumor-Stroma Ratio is an independent predictor for overall survival and disease free survival in gastric cancer patients. Surgeon. 2017;15(6):329-35.
16. Wang K, Ma W, Wang JB, Yu L, Zhang XM, Wang ZB, et al. Tumor-Stroma Ratio Is an Independent Predictor for Survival in Esophageal Squamous Cell Carcinoma. J Thorac Oncol. 2012;7(9):1457-61.
17. van Pelt GW, Hansen T.F, Bastiaannet E., Kjær-Frifeldt S., Han J., van Krieken J.M., Tollenaar R.A.E.M., Sørensen F.B., Mesker W.E. . Stroma-High Lymph Node Involvement Predicts Poor Survival More Accurately for Patients with Stage III Colon Cancer. Journal of Medical & Surgical Pathology 2016.
18. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013;24(9):2206-23.
19. Giuliano AE, Ballman KV, McCall L, Beitsch PD, Brennan MB, Kelemen PR, et al. Effect of Axillary Dissection vs No Axillary Dissection on 10-Year Overall Survival Among Women With Invasive Breast Cancer and Sentinel Node Metastasis: The ACOSOG Z0011 (Alliance) Randomized Clinical Trial. JAMA. 2017;318(10):918-26.

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE 1. Univariate and multivariate analyses for overall survival calculated by Cox regression analysis.

	Overall Survival									
	Univariate analysis				*Multivariate analysis: TSR in PT*			*Multivariate analysis: TSR PT and LNs*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age (in years)										
<40	15			<0.001			0.296			0.305
>40-60	94	1.25	0.62-2.51		0.70	0.33-1.48		0.73	0.34-1.55	
>60	82	2.40	1.20-4.80		0.91	0.41-2.02		0.95	0.42-2.15	
Grade										
I	18			0.835						
II	85	1.06	0.59-1.88							
III	88	1.15	0.65-2.05							
Histological type										
Ductal carcinoma	171			0.274						
Lobular carcinoma	18	1.34	0.79-2.25							
Tumor stage										
pT1	42			0.384						
pT2	109	1.17	0.78-1.77							
pT3/4	34	1.44	0.86- 2.42							
Nodal stage										
pN1	148			<0.001			0.269			0.280
pN2	11	2.74	1.46-5.16		1.69	0.88-3.27		1.67	0.86-3.22	
pN3	32	1.94	1.29-2.92		1.20	0.75-1.91		1.21	0.76-1.93	
ER status										
Negative	83			0.809						
Positive	98	1.04	0.75-1.46							
PR status										
Negative	86			0.006			0.504			0.523
Positive	97	0.63	0.45-0.88		0.89	0.62-1.26		0.89	0.62-1.27	
HER2 status										
Negative	118			0.736						
Positive	25	0.92	0.55-1.52							

SUPPLEMENTARY TABLE 1. Continued.

	Overall Survival								
	Univariate analysis				*Multivariate analysis: TSR in PT*			*Multivariate analysis: TSR PT and LNs*	
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Surgery with or without radiotherapy										
MST without RT	62			0.001			0.021			0.033
MST with RT	63	1.04	0.71-1.53		1.02	0.66-1.58		1.02	0.66-1.59	
BCS without RT	0									
BCS with RT	66	0.51	0.34-0.77		0.58	0.37-0.91		0.60	0.38-0.94	
Chemotherapy										
No	127			<0.001			<0.001			<0.001
Yes	64	0.35	0.23-0.52		0.41	0.26-0.66		0.42	0.26-0.68	
Hormonal therapy										
No	136			0.126						
Yes	55	1.31	0.93-1.86							
TSR										
Stroma-low	87			0.003			0.029			
Stroma-high	104	1.65	1.86-2.29		1.49	1.04-2.14				
TSR PT combined with LNs										
PT low/LN low	73			0.002						0.084
PT low/LN high	14	2.14	1.11-4.14	0.023				1.56	0.78-3.14	0.209
PT high/LN low	87	1.73	1.20-2.49	0.003				1.55	1.05-2.29	0.029
PT high/LN high	17	2.50	1.41-4.42	0.002				1.91	1.03-3.52	0.039

Abbreviations: BCS = breast conserving therapy, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, LN = lymph nodes, MST = mastectomy, PR = progesterone receptor, PT = primary tumor, RT = radiotherapy, TSR = tumor-stroma ratio

SUPPLEMENTARY TABLE 2. Patient characteristics categorized in patients with stroma-low PTs/stroma-low LNs, stroma-low PTs/stroma-high LNs, stroma-high PTs/stroma-low LNs and stroma-high PTs/stroma-high LNs.

	Stroma-low PT/ stroma-low LNs		**Stroma-low PT/ stroma-high LNs**		**Stroma-high PT/ stroma-low LNs**		**Stroma-high PT/ stroma-high LNs**		
	n = 73	%	n = 14	%	n = 87	%	n = 17	%	*p*-value
Age (in years)									
<40	8	11.0	1	7.1	4	4.6	2	11.8	0.281
>40-60	35	47.9	4	28.6	49	56.3	6	35.3	
>60	30	41.1	9	64.3	34	39.1	9	52.9	
Grade									
I	5	6.8	0	0	10	11.5	3	17.6	0.475
II	32	43.8	5	35.7	41	47.1	7	41.2	
III	36	49.3	9	64.3	36	41.4	7	41.2	
Histological type									
Ductal carcinoma	69	95.8	14	100	72	83.7	16	94.1	0.034
Lobular carcinoma	3	4.2	0	0	14	16.3	1	5.9	
Tumor stage									
pT1	15	20.8	1	7.1	22	26.8	4	23.5	0.248
pT2	46	63.9	8	57.1	43	52.4	12	70.6	
pT3/4	11	15.3	5	35.7	17	20.7	1	5.9	
Nodal stage									
pN1	63	86.3	12	85.7	62	71.3	11	64.7	0.095
pN2	0	0	1	7.1	8	9.2	2	11.8	
pN3	10	13.7	1	7.1	17	19.5	4	23.5	
ER status									
Negative	33	45.8	7	53.8	36	45.0	7	43.8	0.943
Positive	39	54.2	6	46.2	44	55.0	9	56.3	
PR status									
Negative	28	38.9	8	61.5	41	50.0	9	56.3	0.278
Positive	44	61.1	5	38.5	41	50.0	7	43.8	
HER2 status									
Negative	49	83.1	8	80.0	52	83.9	9	75.0	0.895
Positive	10	16.9	2	20.0	10	16.1	3	25.0	
Surgery with or without radiotherapy									
MST without RT	23	31.5	7	50.0	29	33.3	3	17.6	0.268
MST with RT	22	30.1	6	42.9	27	31.0	8	47.1	
BCS without RT	0	0	0	0	0	0	0	0	
BCS with RT	28	38.4		7.1	31	35.6	6	35.3	

SUPPLEMENTARY TABLE 2. Continued.

	Stroma-low PT/ stroma-low LNs		**Stroma-low PT/ stroma-high LNs**		**Stroma-high PT/ stroma-low LNs**		**Stroma-high PT/ stroma-high LNs**		
	n = 73	%	n = 14	%	n = 87	%	n = 17	%	*p*-value
Chemotherapy									
No	42	57.5	10	71.4	63	72.4	12	7.06	0.233
Yes	31	42.5	4	28.6	24	27.6	5	29.4	
Hormonal therapy									
No	53	72.6	8	57.1	63	72.4	12	70.6	0.686
Yes	20	27.4	6	42.9	24	27.6	5	29.4	

Abbreviations: BCS = breast conserving therapy, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, LNs = lymph nodes, MST = mastectomy, PR = progesterone receptor, PT = primary tumor, RT = radiotherapy

6

Prognostic value of the tumor-stroma ratio combined with the immune status of tumors in invasive breast carcinoma

K.M.H. Vangangelt
G.W. van Pelt
C.C. Engels
H. Putter
G.J. Liefers
V.T.H.B.M. Smit
R.A.E.M. Tollenaar
P.J.K. Kuppen
W.E. Mesker

Breast Cancer Research and Treatment, 2018; 168:601-612

ABSTRACT

Purpose

The tumor microenvironment coexists of complex interactions between cancer cells and cells in the microenvironment. In this study, the prognostic value of the interplay between the tumor-stroma ratio (TSR) and the immune status of tumors in breast cancer patients was evaluated.

Methods

A cohort of 574 breast cancer patients was analyzed. The percentage of tumor stroma was visually estimated on hematoxylin and eosin (H&E) stained histological tumor tissue sections. Immunohistochemical staining was performed for classical human leukocyte antigen (HLA) class I, HLA-E, HLA-G, markers for regulatory T (Treg) cells, natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).

Results

The TSR ($p < 0.001$) and the immune status of tumors ($p < 0.001$) were both statistically significant for the recurrence-free period (RFP) and both independent prognosticators ($p < 0.001$) in which tumors with a high stromal content behave more aggressively as well as tumors with a low immune status. The 10-year RFP for patients with a stroma-low tumor and high immune status profile was 87% compared to 17% of patients with a stroma-high tumor combined with a low immune status profile ($p < 0.001$). The classical HLA class I was the most prominent immune marker in the immune status profiles.

Conclusions

Determination of the TSR is a simple, fast and cheap method. The effect on RFP of the TSR when combined with the immune status of tumors or expression of classical HLA class I is even stronger. Both are promising for further prediction and achievement of tailored treatment for breast cancer patients.

INTRODUCTION

Survival for patients with invasive breast cancer has increased in the last decade due to new and improved therapeutic options as well as new insights in molecular biology. Methods to select patients based on tumor phenotype are important to reduce over- and undertreatment. For example, gene expression profiles that identify subtypes (1, 2) associated with higher risk of metastasis. Although these techniques result in prognostic and predictive valuable information for specific patient groups, optimization of risk assessment might benefit from further improvement.

Despite an important update on the role of the microenvironment on cancer development by Hanahan et al. (3, 4), the classification system for predicting metastasis and disease-specific survival is still based on traditional tumor staging criteria (AJCC/UICC-TNM Classification) (5-7), which focusses largely on the tumor cell-autonomous processes and not on the microenvironment.

The tumor microenvironment coexists of complex interactions between cancer cells and cells in the microenvironment, such as immune and stromal cells. High stromal content is associated with worse prognosis in different solid cancer types, including breast cancer and especially triple-negative breast cancer (8-14). Together with the development of malignant tumor stroma, the connective tissue framework of the tumor becomes active. The collagen bundles degrade, the number of inflammatory cells increases, fibroblasts differentiate into myofibroblasts and proliferate, and angiogenesis increases (15). Also, the cellular immune response has a fundamental role in cancer development. An example of the prognostic value of the activity of the immune system is represented by the Immunoscore, which analyzes the distribution of $CD3^+$ lymphocytes and $CD8^+$ cytotoxic T cells (16). In breast cancer, especially in triple-negative tumors, the increased presence of tumor- infiltrating lymphocytes has been associated with good prognosis (17, 18). De Kruijf et al. showed that the immune status of tumors, which is based on six cellular immune markers, has a statistically significant effect on prognosis in favor of tumors with a high immune status (19). These six cellular immune markers (HLA-E, HLA-G, classical HLA class I (HLA-A, HLA-B and HLA-C), natural killer (NK) cells, cytotoxic T lymphocytes (CTLs) and regulatory T (Treg) cells) were selected based on biological rationale and the balance between their various interactions.

It is suggested that the tumor stroma influences the suppression of the immune response (9, 20-23). In this present study, the prognostic value of the interplay between the tumor-stroma ratio (TSR) and the immune status of tumors in breast cancer patients was evaluated. We hypothesize that stroma-high tumors, in combination with low immune status, behave more aggressively, resulting in a high risk of disease progression.

MATERIALS AND METHODS

Study population

The study population was assessed retrospectively and consisted of primary non-metastasized breast cancer patients. The patients were primarily treated with surgery between 1985 and 1994 at Leiden University Medical Center (n = 584). Exclusion criteria were bilateral breast tumors and a history of cancer (other than basal cell carcinoma or cervical carcinoma in situ). The resected breast tumors were graded by experienced breast cancer pathologists using current pathological standards. All samples were handled in a coded fashion, according to the national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). Approval of the study was obtained from the LUMC Medical Ethics Committee. The recommendations for reporting on tumor markers (the REMARK criteria) in prognostic studies were respected (24).

Tumor-stroma ratio

The TSR was visually estimated on routine hematoxylin and eosin (H&E) stained slides from formalin-fixed and paraffin-embedded (FFPE) blocks of the primary tumor (n = 584) as previously described by our group (25). Thirty-two percent of the tissues were scored in a blinded fashion by a second observer, with a concordance of classification of 94% (Cohen's kappa = 0.85). Ten tissues were not eligible for TSR scoring due to poor quality. Evaluation of TSR started with microscopical orientation using a 5x objective. Subsequently, a 10x objective was used in the most stroma-abundant area. The field of highest stromal percentage was selected and scored per tenfold increments. Tumor cells must be present on all sides (north,

east, south and west). Stroma percentage ≤50% was categorized as stroma-low and stroma percentage >50% as stroma-high (supplementary figure 1) (8, 12).

Immunohistochemistry

Tissue sections from intra-operatively derived FFPE tissue micro-array (TMA) material and immunohistochemistry analysis were used as previously described (19, 26, 27). Whole FFPE sections were immunohistochemically stained with mouse antibodies against $CD8^+$ and PEN5 recognizing CTLs and NK cells, respectively. TMA tissue sections were immunohistochemical stained for the expression of classical HLA class I (anti-HLA-A and anti-HLAB/C), non-classical HLA-E, HLA-G and Treg cell infiltration as previously described in the literature (26, 27). Quantification of $CD8^+$ cells and PEN5 cells was performed in a blinded set-up by two independent observers. Tumor infiltration of $CD8^+$ was divided into low CTL infiltration (0-100 $CD8^+$ tumor-infiltrating cells/mm^2) and high CTL infiltration (100-3.000 $CD8^+$ tumor-infiltrating cells/mm^2). Tumor infiltration of NK cells was divided into the presence or absence of NK cells. Classical HLA class I was categorized into loss versus expression and HLA-E was divided into no expression versus expression. HLA-G and Treg infiltration were categorized in absent versus present (supplementary figure 2).

These six immune markers were classified into three immune status profile groups (figure 1) as previously described by De Kruijf et al. for this cohort (19).

FIGURE 1. Evaluation of the immune status and classification.

Immune status	Evaluation of immune status and classification		Classification based on three categories
High	1a	Tumors with expression of classical HLA class I, high infiltration of CTL, and absence of infiltration of Treg	1 High immune status
	1b	Tumors with loss of expression of classical HLA class I, no expression of HLA-EG, present infiltration of NK cells, and absent infiltration of Treg	
	2a	Tumors with expression of classical HLA class I but low CTL infiltration	2 Intermediate immune status
	2b	Tumors with expression of classical HLA class I, high infiltration of CTL, but also present infiltration of Treg	
	3a	Tumors with loss of expression of classical HLA class I and absent NK-cell infiltration	3 Low immune status
	3b	Tumors with loss of expression of classical HLA class I, present NK-cell infiltration, but also present Treg infiltration	
Low	3c	Tumor with loss of expression of classical HLA class I and expression of HLA-E and G	

Abbreviations: CTL = cytotoxic T lymphocytes, HLA = human leukocyte antigen, Treg = regulatory T cells, NK = natural killer

Statistical analyses

Statistical analyses were performed using IBM SPSS statistics (version 23.0 for Windows). The inter-observer agreement for the assessment of TSR, CTLs and PEN5 was calculated with Cohen's kappa. A value above 0.6 was valid. The χ^2 test was used for the evaluation of statistically significant differences between included and excluded patients, distribution of the separate immune markers between stroma-high and stroma-low cases and three immune status categories. A p-value <0.05 was considered statistically significant. The Kaplan-Meier method was performed to analyze the overall survival (OS) and recurrence-free period (RFP). The log-rank test was applied for comparison between these curves. A p-value <0.05 was considered statistically significant. The time from date of surgery until any recurrence of breast cancer was defined as RFP. The OS was defined as the time from date of surgery until death from any cause. Univariate and multivariate analyses for RFP and OS were calculated by Cox regression analysis. Variables with p-value <0.10 in the univariate analysis were entered in multivariate analysis.

Effect modification was evaluated by adding interaction in Cox regression analysis. Stepwise regression analyses (backward and forward) of the different immune cells were evaluated. Missing values were not included.

RESULTS

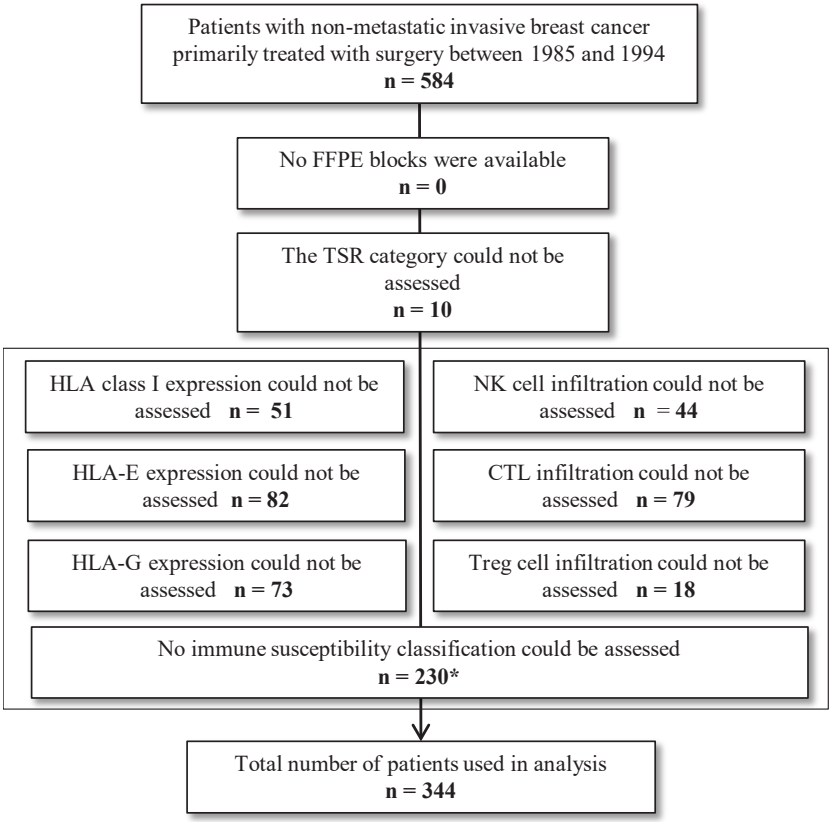
Patients

The FFPE blocks of all patients (n = 584) were available. TSR could be evaluated in 98% of the cases (n = 574). In 43% of the cases, no classification of the immune status could be made due to the low quality of tissues or TMAs. The loss or damage of TMA cores is a known problem associated with the preparation, staining and mounting of TMA slides.

Moreover, the cores we used were rather small. Since several markers were combined in the profiles, the patient was excluded from further analysis when data of one or more markers were missing. Figure 2 provides a flowchart of the subjects included. By comparison of prognostic parameters, no differences were found between included (n = 344) and excluded cases (n = 230), except for the treatment with hormonal therapy ($p < 0.001$). This can be explained by the fact that this therapy was only given sporadically between 1985 and 1988. No statistically significant differences were found for age, grade, tumor stage, tumor type, nodal stage, histological type, estrogen receptor, progesterone receptor, HER2 expression, TSR, chemotherapy and radiotherapy in these two groups.

The median follow-up of the 344 included patients was 10.2 years (0.2–22.4 years). The mean age at presentation was 58.0 years (27.5–90.2 years). There is no statistically significant difference in the distribution of the separate markers between stroma-high and stroma-low cases, nor in the three immune status categories ($p = 0.30$). Table 1 provides a detailed overview of the immune markers stratified by TSR and Table 2 shows the clinicopathological and treatment characteristics.

FIGURE 2. Flowchart of subject inclusion.



* For categorizing in one of the three immune status categories, not all six groups need to be known.
Abbreviations: CTL = cytotoxic T lymphocyte, FFPE = formalin-fixed and paraffin-embedded, NK = natural killer, Treg = regulatory T, TSR = tumor-stroma ratio.

TABLE 1. Distribution of the separate elements of the three immune status profiles.

	Stroma-low		**Stroma-high**		
	n = 177	%	n = 167	%	*p*-value
HLA class I					
Loss or downregulation	98	55.4	103	61.7	0.24
Expression	79	44.6	64	38.3	
HLA-E					
Negative	97	54.8	93	55.7	0.87
Positive	80	45.2	74	44.3	
HLA-G					
Negative	108	61.0	105	62.9	0.72
Positive	69	39.0	62	37.1	
NK cells					
Negative	78	44.1	79	47.3	0.47
Positive	95	53.7	82	49.1	
Missing	4	2.2	6	3.6	
CTL					
Low infiltration	115	65.0	121	72.5	0.19
High infiltration	55	31.0	42	25.1	
Missing	7	4.0	4	2.4	
Treg cells					
Absence	97	54.8	98	58.7	0.62
Presence	74	41.8	67	40.1	
Missing	6	3.4	2	1.2	
Immune status profiles					
High IS	39	22.0	26	15.5	0.30
Intermediate IS	108	61.0	109	65.3	
Low IS	30	17.0	32	19.2	

The subtypes were constructed according to the criteria shown in table 1. Only the cases for which both stromal content and immune subtyping could be performed, were included in the analysis.
Abbreviations: CTL = cytotoxic T lymphocyte, HLA = human leukocyte antigen, IS = immune status, NK = natural killer, Treg = regulatory T cells

TABLE 2. Patient characteristics.

	n = 344	%
Age (in years)		
<40	27	7.9
>40-60	168	48.8
>60	149	43.3
Grade		
I	52	15.1
II	171	49.7
III	118	34.3
Missing	3	0.9
Histological type		
Ductal carcinoma	309	89.8
Lobular carcinoma	32	9.2
Missing	3	0.9
Tumor stage		
pT1	121	35.2
pT2	170	49.4
pT3/4	43	12.5
Missing	10	2.9
Nodal stage		
Negative	189	55.0
Positive	147	42.7
Missing	8	2.3
ER status		
Negative	134	39.0
Positive	206	59.9
Missing	4	1.1
PR status		
Negative	139	40.4
Positive	200	58.1
Missing	5	1.5
HER2 status		
Negative	254	73.8
Positive	25	7.3
Missing	65	18.9
Breast cancer subtypes		
Luminal A	192	55.8
Luminal B	10	2.9

TABLE 2. Continued.

	n = 344	%
HER2-like	15	4.4
Triple-negative	62	18.0
Missing	65	18.9
Surgery and radiotherapy		
MST without RT	143	41.6
MST with RT	64	18.6
BCS without RT	1	0.3
BCS with RT	136	39.5
Chemotherapy		
No	265	77.0
Yes	79	23.0
Hormonal therapy		
No	273	79.4
Yes	71	20.6
TSR		
Stroma-low	177	51.5
Stroma-high	167	48.5
Immune status of the tumor		
High	65	18.9
Intermediate	217	63.1
Low	62	18.0
Combination TSR and immune status		
Stroma-low/high IS	39	11.3
Stroma-low/intermediate IS	108	31.4
Stroma-low/low IS	30	8.7
Stroma-high/high IS	26	7.6
Stroma-high/intermediate IS	109	31.7
Stroma-high/low IS	32	9.3

Abbreviations: BCS = breast conserving therapy, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, IS = immune status, MST = mastectomy, PR = progesterone receptor, RT = radiotherapy, TSR = tumor-stroma ratio

Prognostic value of the TSR

Tumors with low and high stromal contents were observed in 51.5% and 48.5% of the cases (n = 574), respectively. Patients with stroma-high tumors had a worse RFP (HR 1.75, 95% CI 1.37-2.25, $p < 0.001$) and OS (HR 1.28, 95% CI 1.04-1.58, $p = 0.02$) compared to patients with stroma-low tumors (not shown). After 10 years, 32% of the patients with a stroma-low tumor had developed a recurrence of disease compared to 50% of patients with a stroma-high tumor. These results for RFP in favor of stroma-low tumors were also seen in the group of patients (n = 344) in which the immune status could be assessed (HR 1.76, 95% CI 1.28- 2.42, $p < 0.001$) (figure 3a) with a 10-year RFP of 67% for patients in the stroma-low group compared to 49% in the stroma-high group. OS showed no significant difference between both stroma groups (HR 1.25, 95% CI 0.95-1.64, $p = 0.114$). The analyses of breast cancer subgroups showed that patients with a triple-negative tumor had a high hazard ratio of 2.41 (95% CI 1.32-4.40, $p = 0.003$) for RFP in both the total group (known TSR), as well as in the selected group (known TSR and immune status). Furthermore, within the luminal A subgroup, the TSR showed a significant difference in RFP (HR 1.57, 95% CI 1.13-2.19, $p = 0.008$), but not for OS. For the other subgroups (Luminal B and HER2-like tumors) no prognostic value of the TSR was found (supplementary table 1a and 1b).

Prognostic value of the immune status of tumors

The immune status of tumors was classified as high in 18.9%, intermediate in 63.1% and low in 18.0% of the breast cancer cases. The RFP (figure 3b) and OS curves (not shown) of the three immune status categories were statistically significant ($p < 0.001$) in which patients with a high immune status profile had a better outcome compared to patients with a low immune status profile. After 10 years of follow-up, 79% of the patients in the high immune status category did not develop a recurrence of disease compared to 58% in the intermediate immune status category and 36% in low immune status category. The analyses of breast cancer subgroups showed that patients with a luminal A or triple-negative tumor have a worse prognosis for both RFP and OS (supplementary table 2).

Prognostic value of the TSR and the immune status of tumors combined

The RFP data of the TSR and the immune status subtypes were combined and plotted in figure 3c. The overall *p*-value between the subgroups was statistically significant ($p < 0.001$) (table 3). A trend was observed for stroma-high tumors compared to stroma-low tumors with a high immune status profile ($p = 0.15$) and intermediate immune status profile ($p = 0.08$). However, only for the low immune status profile, the difference between stroma-high and stroma-low tumors showed significance ($p = 0.002$). The 10-year RFP for stroma-low and high immune status showed a recurrence rate of 87% versus 17% of patients with stroma-high and low immune status tumors.

Table 3 shows the results of the univariate and multivariate Cox regression analyses. The TSR remained statistically significant for RFP ($p < 0.001$) in multivariate Cox regression analysis and the immune status for RFP ($p < 0.001$) and OS ($p = 0.001$). Effect modification of stroma and immune status was not statistically significant. As expected, the TSR combined with immune status showed additional prognostic value in the analyzed patient cohort.

TABLE 3. Univariate and multivariate Cox regression analyses for recurrence-free period and overall survival.

	Recurrence-free period							Overall survival					
		Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*- value	HR	95% CI	*p*-value
Age (in years)													
<40	27			0.551						<0.001			
>40-60	168	1.08	0.61-1.94					1.62	0.81-3.24				
>60	149	0.90	0.49-1.64					3.45	1.74-6.83				
Grade													
I	52			0.004						0.009			
II	171	1.50	0.89-2.55					1.42	0.91- 2.22				
III	118	2.25	1.32-3.85					1.94	1.23-3.07				
Histological type													
Ductal carcinoma	309			0.199						0.163			
Lobular carcinoma	32	1.39	0.84-2.30					1.37	0.88-2.14				
Tumor stage													
pT1	121			<0.001						<0.001			
pT2	170	1.90	1.31-2.75					1.89	1.36-2.63				
pT3/4	43	2.89	1.76- 4.77					3.36	2.19-5.15				
Nodal stage													
Negative	189			<0.001						<0.001			
Positive	147	3.02	2.18-4.18					2.06	1.56-2.72				
ER status													
Negative	134			0.580						0.264			
Positive	206	0.91	0.66-1.26					0.85	0.65-1.13				
PR status													
Negative	139			0.290						0.211			
Positive	200	0.84	0.61-1.16					0.84	0.64-1.11				

TABLE 3. Continued.

	Recurrence-free period							Overall survival					
		Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*- value	HR	95% CI	*p*-value
HER2 status													
Negative	254			0.840						0.174			
Positive	25	1.07	0.58-1.98					1.40	0.86-2.29				
Breast cancer subtypes													
Luminal A	192			0.823						0.189			
Luminal B	10	1.06	0.39-2.89					1.17	0.51-2.66				
HER2-like	15	1.15	0.53-2.48					1.74	0.96-3.15				
Triple-negative	62	1.22	0.80-1.84					1.31	0.92-1.87				
Surgery and radiotherapy													
MST without RT	143			<0.001						<0.001			
MST with RT	64	1.99	1.34-2.95					1.31	0.93-1.85				
BCS without RT	1												
BCS with RT	136	0.76	0.52-1.10					0.47	0.34-0.65				
Chemotherapy													
No	265			0.976						0.019			
Yes	79	1.01	0.70-1.45					0.65	0.46-0.93				
Hormonal therapy ER positive													
No	161			0.242						0.023			
Yes	45	1.32	0.83-2.10					1.61	1.07-2.43				
Hormonal Therapy HER2 positive													
No	17			0.600									
Yes	8	1.39	0.41-4.76					1.71	0.66-4.23	0.270			

TABLE 3. Continued.

	Recurrence-free period							Overall survival					
		Univariate analysis			*Multivariate analysis*			*Univariate analysis*			*Multivariate analysis*		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*- value	HR	95% CI	*p*-value
TSR													
Stroma-low	177			<0.001			<0.001			0.114			0.048
Stroma-high	167	1.76	1.28-2.42		2.10	1.50-2.93		1.25	0.95-1.64		1.34	1.00-1.80	
Immune status of tumor													
High	65			<0.001			<0.001			<0.001			0.001
Intermediate	217	1.87	1.13-3.10		2.10	1.22-3.61		1.85	1.22-2.82		1.87	1.19-2.94	
Low	62	4.19	2.43-7.24		4.32	2.40-7.76		2.74	1.70-4.42		2.67	1.60-4.46	
Combination TSR and Immune Status													
Stroma-low/ high IS	39			<0.001			<0.001			<0.001			0.003
Stroma-low/ intermediate IS	108	2.11	0.99-4.51		2.70	1.13-6.46		1.83	1.04-3.20		1.74	0.94-3.23	
Stroma-low/low IS	30	3.53	1.52-8.18		4.75	1.84-12.27		2.00	1.02-3.90		2.39	1.16-4.93	
Stroma-high/ high IS	26	1.95	0.77-4.94		2.86	1.03-7.94		1.06	0.49-2.30		1.15	0.50-2.61	
Stroma-high/ intermediate IS	109	3.09	1.47-6.49		5.00	2.11-11.85		1.97	1.13-3.45		2.29	1.24-4.23	
Stroma-high/ low IS	32	9.25	4.21-20.31		11.54	4.63-28.79		3.97	2.12-7.46		3.27	1.64-6.52	

Abbreviations: BCS = breast conserving therapy, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, IS = immune status, MST = mastectomy, PR = progesterone receptor, RT = radiotherapy, TSR = tumor-stroma ratio

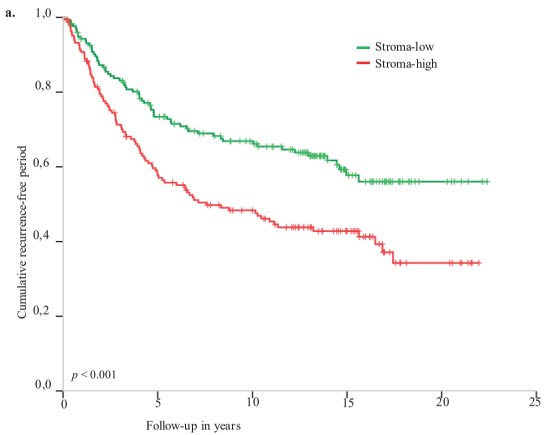
Prognostic value of the TSR combined with classical HLA class I

A stepwise regression analysis was performed to evaluate whether one or more of the six cellular immune cells were decisive in the immune status categories. In this analysis classical HLA class I showed to be statistically significant in the immune status categories for RFP ($p = 0.007$), but not for OS ($p = 0.06$), whereas the other immune cells were not significant for both RFP and OS. These results indicate that classical HLA class I is the most determinant factor in the three immune status profiles. In 523 of the 574 cases (91%) classical HLA class I could be assessed. Tumors expressing classical HLA class I had significantly fewer recurrences ($p = 0.001$), with a 10-year RFP of 66% versus 55%. In the same group, the TSR showed RFP of 67% versus 49% in benefit for stroma-low tumors ($p < 0.001$).

Figure 3d shows a statistically significant difference ($p < 0.001$) for RFP for the combination of the TSR and classical HLA class I. This indicates that patients with a stroma-low tumor and expression of classical HLA class I have a better prognosis compared to patients with a stroma-high tumor and loss of expression or downregulation of classical HLA class I with a 10-year RFP 72% versus 46%, respectively.

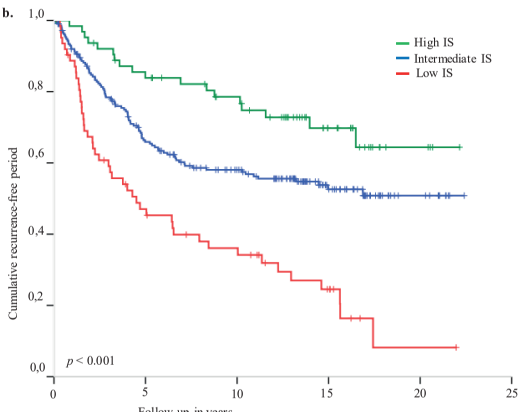
In triple-negative tumors classical HLA class I (n = 92) was also of prognostic value (HR 0.28, 95% CI 0.15-0.55, $p < 0.001$). Patients with loss of expression or downregulation of classical HLA class I showed a 10-year RFP of 35% compared to 73% of the patients in which HLA class I is expressed. The TSR and classical HLA class I combined showed a statistically significant difference in RFP ($p = 0.001$). Patients with stroma-low tumors and expression of classical HLA class I showed fewer recurrences compared to patients with stroma-high tumors and loss of expression or downregulation of classical HLA class with a 10-year RFP of 75% versus 26%, respectively.

FIGURE 3. Kaplan-Meier analysis for recurrence-free period of the tumor-stroma ratio, the immune status profiles and classical HLA class I. **a.** RFP for stroma-low and stroma-high tumors, **b.** RFP for the three IS profiles.



Numbers at risk

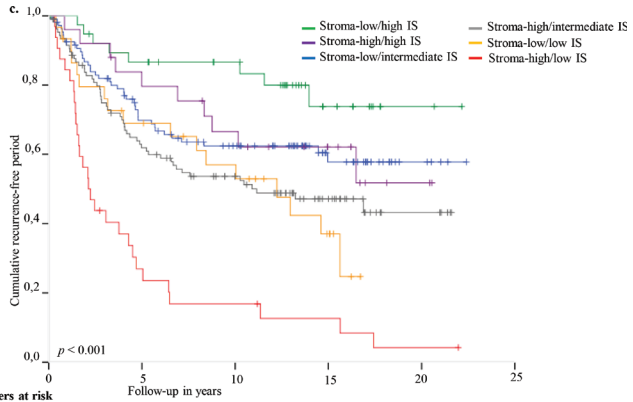
Stroma-low	171	111	70	23	4
Stroma-high	162	84	53	23	4



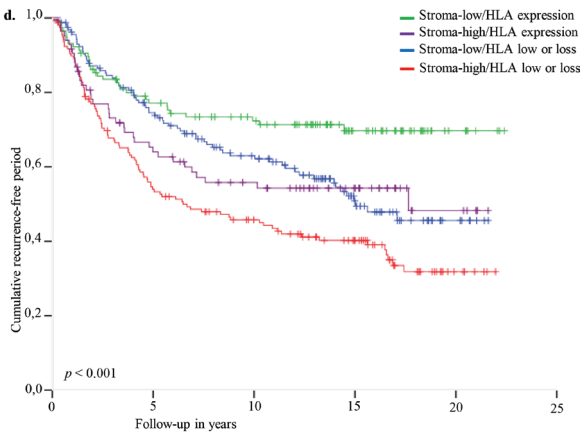
Numbers at risk

High IS	65	63	48	33	12
Intermediate IS	217	209	121	74	28
Low IS	62	61	26	17	7

FIGURE 3 Continued. Kaplan-Meier analysis for recurrence-free period of the tumor-stroma ratio, the immune status profiles and classical HLA class I. **c.** RFP for the TSR combined with the IS profiles, **d.** RFP for the TSR combined with classical HLA class I.



Numbers at risk					
Stroma-low/ high IS	38	30	20	6	1
Stroma-low/ intermediate IS	104	64	38	14	3
Stroma-low/ low IS	29	18	12	4	
Stroma-high/ high IS	25	19	13	6	1
Stroma-high/ intermediate IS	105	58	36	14	3
Stroma-high/ low IS	32	8	5	3	1



Numbers at risk					
Stroma-low/ HLA low or loss	155	102	64	24	4
Stroma-low/ HLA expression	113	80	54	21	4
Stroma-high/ HLA low or loss	156	77	53	26	4
Stroma-high/ HLA expression	82	46	30	14	3

Abbreviations: HLA = human leukocyte antigen, IS = immune status

DISCUSSION

There is a growing body of evidence that the TSR and immune cell response in cancer development might be important factors in patient stratification for treatment decision making. The relation of the stromal involvement and immune response for the determination of patients for adjuvant treatment has merely been investigated. Gujam et al. described the relationship between the TSR and clinicopathological parameters as tumor inflammatory infiltrate, $CD68^+$ macrophage infiltrate and $CD4^+$ and $CD8^+$ T- lymphocyte infiltrate in ductal breast cancer. They concluded that a high TSR was consistently associated with low tumor inflammatory infiltrate (9). Hynes et al. also published on the combination of the TSR with peritumoral diffuse lymphoid inflammation and Crohn's disease-like reaction in stage II/III colon cancer. A combination of these three parameters showed a significant association with survival outcomes (23).

Our study showed that the TSR and the combination of six cellular immune cells, categorized into three immune status subgroups, are both independent prognostic factors. A combination of both parameters even strengthens each other's' effect.

The six cellular immune cells were selected based on biological rationale and the balance between their various interactions. Classical HLA class I presents tumor-associated antigens on the cell surface. CTLs are capable of recognizing the presence of these antigens by HLA-A, HLA-B or HLA-C (28). Tumor cells can escape recognition by CTLs by losing classical HLA class I expression. This makes the tumor cells more prone to recognition by NK cells (29). On the other hand, HLA-E and HLA-G, also known as non-classical HLA class I, play a crucial role in the immune surveillance by NK cells. Expression of non-classical HLA I has an inhibitory effect on the function of NK cells (29-31). Other cells which are important in tumor development are Tregs. Tumor cells can escape immune-surveillance by attraction and induction of Tregs (32).

In this study, the prognostic value of the TSR in addition to classical HLA class I was also shown. The effect was smaller than the combination with the three immune status subgroups, but better applicable in daily routine pathology practice. Patients with stroma-low tumors also expressing classical HLA class I have a better prognosis than patients with stroma-high tumors with loss of expression or downregulation of classical HLA class I.

The estimation of the TSR is simple, inexpensive and takes only a few minutes. It can be done on regular H&E slides during routine pathology investigation of the resected tissue. Since the introduction of pre-operative chemotherapy, it might be of interest to score the TSR on tumor biopsies. Pre-operative chemotherapy can lead to the formation of non-desmoplastic stroma, which makes the resection material unsuitable for TSR scoring. In esophageal adenocarcinoma biopsies, the reproducibility of TSR scoring on biopsies was good (33), and it is plausible that this is even better in breast cancer due to the lack of the muscular area (34). Promising is the current interest in the automation of the TSR parameter (13). Assessment of the six cellular immune markers is relatively time-consuming, however the assessment of only classical HLA class I takes less effort and may help to optimize risk stratification in combination with the TSR.

Patients with early-stage breast cancer are often treated with adjuvant systemic therapy (endocrine therapy, chemotherapy or agents against HER2) based on tumor characteristics such as HER2 status, tumor size and lymph node status. A substantial number of women with breast cancer is overtreated. These patients do not benefit from adjuvant therapy, but they are exposed to the risk of toxic effects. The TSR, the immune status or a combination of these prognostic markers might be used to select patients with more confidence regarding adjuvant treatment or to select patients who need more intensive monitoring. Especially patients with stroma-high tumors and low immune status could benefit from more aggressive treatment, whereas for patients with stroma-low tumors and high immune status, less aggressive treatment could be discussed. The method described in this paper could give valuable additional pathology-based information for patients with invasive breast cancer.

CONCLUSIONS

Simple H&E stained sections contain more information than previously fathomed. The TSR is a simple, fast and cheap method for the identification of patients with more aggressive disease. Tumor immune status profiling is promising for further prognostication and the achievement of tailored treatment for breast cancer

patients. The combination of the TSR and the immune status of tumors is a strong prognosticator, applicable for daily routine use.

ACKNOWLEDGEMENT

Funding information: This work was supported by Genootschap Keukenhof voor de Vroege Opsporing van Kanker, Lisse, The Netherlands. No grant number applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

REFERENCES

1. Anderson WF, Rosenberg PS, Prat A, Perou CM, Sherman ME. How many epidemiological types of breast cancer: Two, three, four, or more. Cancer Res. 2014;74(19).
2. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. New Engl J Med. 2004;351(27):2817-26.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
5. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2009. Ann Oncol. 2009;20(8):1319-29.
6. Galea MH, Blamey RW, Elston CE, Ellis IO. The Nottingham Prognostic Index in Primary Breast-Cancer. Breast Cancer Res Tr. 1992;22(3):207-19.
7. Olivotto IA, Bajdik CD, Ravdin PM, Speers CH, Coldman AJ, Norris BD, et al. Population-based validation of the prognostic model ADJUVANT! for early breast cancer. J Clin Oncol. 2005;23(12):2716-25.
8. Dekker TJA, van de Velde CJH, van Pelt GW, Kroep JR, Julien JP, Smit VTHBM, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Tr. 2013;139(2):371-9.
9. Gujam FJ, Edwards J, Mohammed ZM, Going JJ, McMillan DC. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer. Br J Cancer. 2014;111(1):157-65.
10. Wang K, Ma W, Wang JB, Yu L, Zhang XM, Wang ZB, et al. Tumor-Stroma Ratio Is an Independent Predictor for Survival in Esophageal Squamous Cell Carcinoma. J Thorac Oncol. 2012;7(9):1457-61.
11. Moorman AM, Vink R, Heijmans HJ, van der Palen J, Kouwenhoven EA. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. Ejso. 2012;38(4):307-13.
12. Mesker WE, Junggeburt JMC, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
13. West NP, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, et al. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. Br J Cancer. 2010;102(10):1519-23.
14. Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CS. The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer. Ann Oncol. 2014;25(3):644-51.

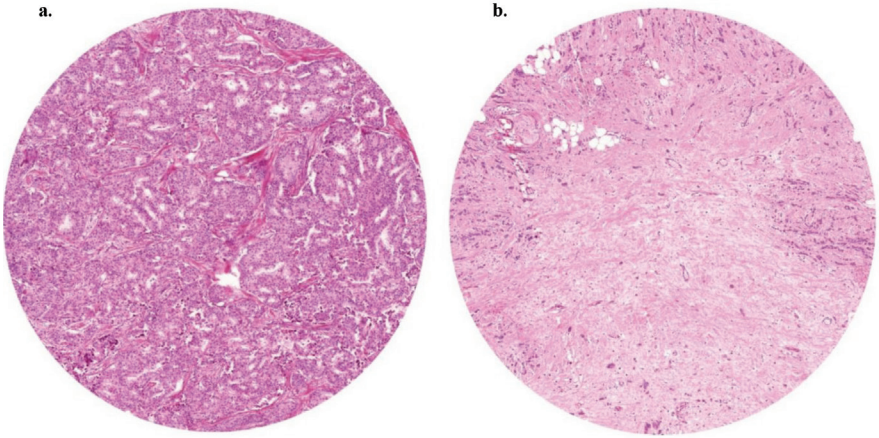
15. Mueller MM, Fusenig NE. Friends or foes - Bipolar effects of the tumour stroma in cancer. Nat Rev Cancer. 2004;4(11):839-49.
16. Kirilovsky A, Marliot F, El Sissy C, Haicheur N, Galon J, Pages F. Rational bases for the use of the Immunoscore in routine clinical settings as a prognostic and predictive biomarker in cancer patients. Int Immunol. 2016;28(8):373-82.
17. Pruneri G, Vingiani A, Bagnardi V, Rotmensz N, De Rose A, Palazzo A, et al. Clinical validity of tumor-infiltrating lymphocytes analysis in patients with triple-negative breast cancer. Ann Oncol. 2016;27(2):249-56.
18. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol. 2013;31(7):860-7.
19. de Kruijf EM, Engels CC, van de Water W, Bastiaannet E, Smit VT, van de Velde CJ, et al. Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients. Breast Cancer Res Treat. 2013;142(2):355-64.
20. Cirri P, Chiarugi P. Cancer-associated-fibroblasts and tumour cells: a diabolic liaison driving cancer progression. Cancer Metastasis Rev. 2012;31(1-2):195-208.
21. Hu M, Polyak K. Microenvironmental regulation of cancer development. Curr Opin Genet Dev. 2008;18(1):27-34.
22. Kim JB, Stein R, O'Hare MJ. Tumour-stromal interactions in breast cancer: the role of stroma in tumourigenesis. Tumour Biol. 2005;26(4):173-85.
23. Hynes SO, Coleman HG, Kelly PJ, Irwin S, O'Neill RF, Gray RT, et al. Back to the future: routine morphological assessment of the tumour microenvironment is prognostic in stage II/III colon cancer in a large population-based study. Histopathology. 2017.
24. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Tr. 2006;100(2):229-35.
25. de Kruijf EM, van Nes JG, van de Velde CJ, Putter H, Smit VT, Liefers GJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. Breast Cancer Res Treat. 2011;125(3):687-96.
26. de Kruijf EM, van Nes JG, Sajet A, Tummers QR, Putter H, Osanto S, et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res. 2010;16(4):1272-80.
27. de Kruijf EM, Sajet A, van Nes JG, Natanov R, Putter H, Smit VT, et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. J Immunol. 2010;185(12):7452-9.

28. Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother. 2004;53(10):904-10.
29. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol. 2007;17(6):459-68.
30. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol. 2002;3(11):999-1005.
31. Marin R, Ruiz-Cabello F, Pedrinaci S, Mendez R, Jimenez P, Geraghty DE, et al. Analysis of HLA-E expression in human tumors. Immunogenetics. 2003;54(11):767-75.
32. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A. 2001;98(20):11521-6.
33. Courrech Staal EF, Smit VT, van Velthuysen ML, Spitzer-Naaykens JM, Wouters MW, Mesker WE, et al. Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies. Eur J Cancer. 2011;47(3):375-82.
34. Dekker TJ, Charehbili A, Smit VT, ten Dijke P, Kranenbarg EM, van de Velde CJ, et al. Disorganised stroma determined on pre-treatment breast cancer biopsies is associated with poor response to neoadjuvant chemotherapy: Results from the NEOZOTAC trial. Mol Oncol. 2015;9(6):1120-8.

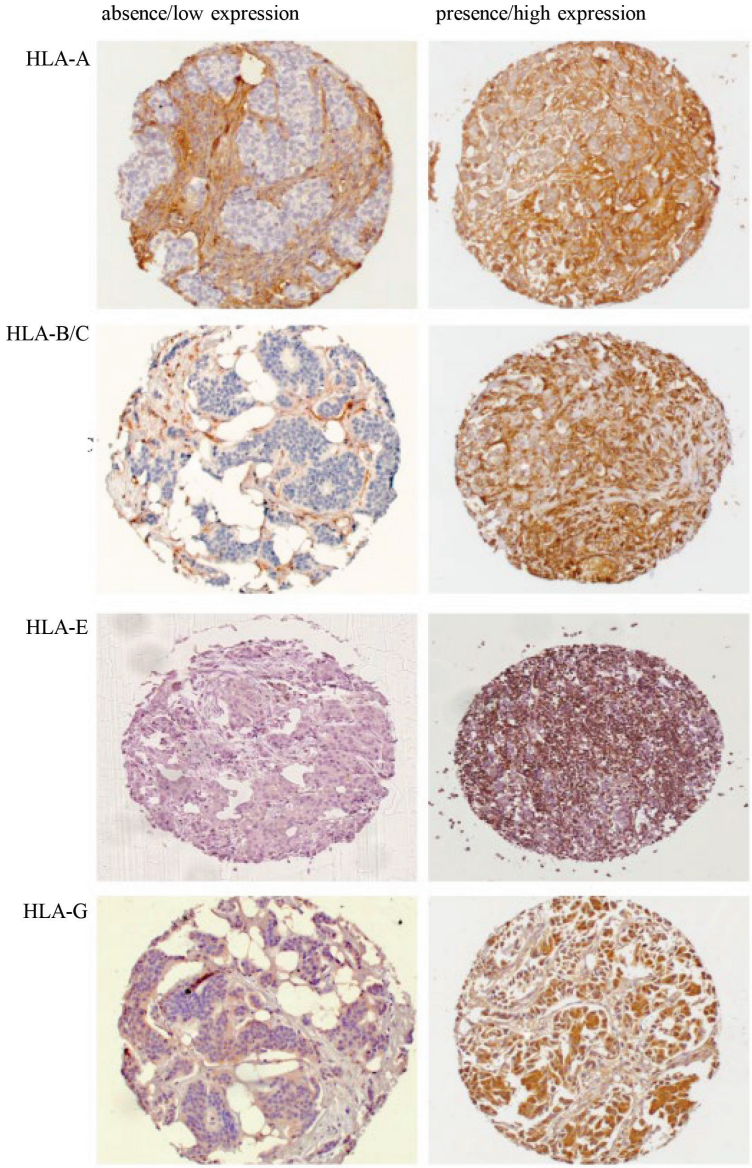
SUPPLEMENTARY DATA

SUPPLEMENTARY FIGURE 1. The tumor-stroma ratio.

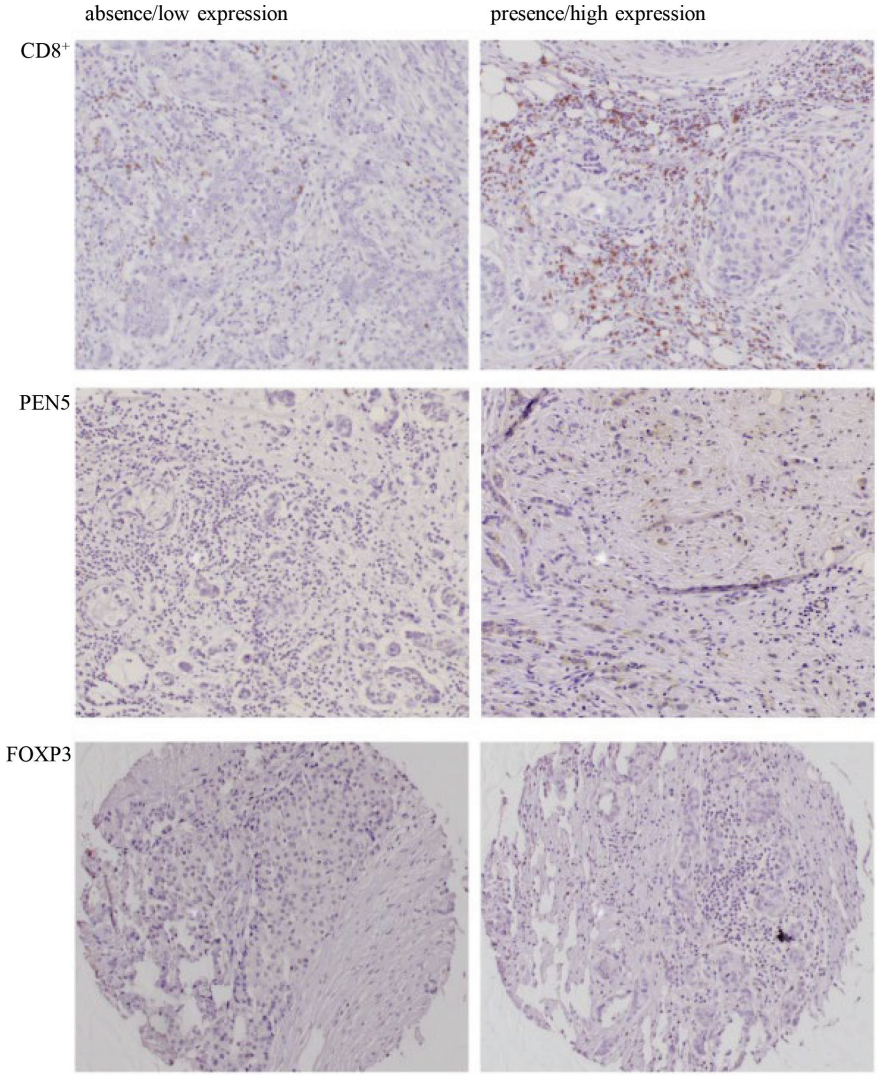
a. Stroma-low tumor **b.** Stroma-high tumor.



SUPPLEMENTARY FIGURE 2. Staining results of the immune markers.



SUPPLEMENTARY FIGURE 2. Continued.



Abbreviation: HLA = human leukocyte antigen

SUPPLEMENTARY TABLE 1a. Prognostic value of the tumor-stroma ratio stratified for breast cancer subtypes in total group (n = 420).

		Recurrence-free period			Overall survival		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Luminal A							
Stroma-low	164			0.008			0.139
Stroma-high	144	1.57	1.13-2.19		1.24	0.93-1.65	
Luminal B							
Stroma-low	10			0.394			0.242
Stroma-high	5	1.78	0.47-6.71		2.05	0.62-6.82	
HER2-like							
Stroma-low	14			0.396			0.183
Stroma-high	21	1.57	0.55-4.46		1.77	0.77-4.09	
Triple-negative							
Stroma-low	36			0.004			0.231
Stroma-high	26	2.41	1.32-4.40		1.46	0.78-2.73	

Abbreviations: HER2 = human epidermal growth factor receptor 2

SUPPLEMENTARY TABLE 1b. Prognostic value of the tumor-stroma ratio stratified for breast cancer subtypes within the known immune status group (n = 279).

		Recurrence-free period			Overall survival		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Luminal A							
Stroma-low	99			0.100			0.508
Stroma-high	93	1.42	0.94-2.15		1.13	0.79-1.62	
Luminal B							
Stroma-low	6			0.133			0.111
Stroma-high	4	5.69	0.59-55.19		4.07	0.73-22.88	
HER2-like							
Stroma-low	5			0.953			0.891
Stroma-high	10	1.05	0.20-5.45		1.10	0.29-4.20	
Triple-negative							
Stroma-low	36			0.010			0.231
Stroma-high	26	2.60	1.26-5.39		1.46	0.78-2.73	

Abbreviations: HER2 = human epidermal growth factor receptor

SUPPLEMENTARY TABLE 2. Prognostic value of the immune status stratified for breast cancer subtypes (n = 279).

		Recurrence-free period			Overall survival		
	n	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Luminal A							
High IS	33			<0.001			0.010
Intermediate IS	123	1.53	0.80-2.94		1.72	0.97-3.05	
Low IS	36	3.53	1.75-7.14		2.65	1.40-5.02	
Luminal B							
High IS	3			0.651			0.979
Intermediate IS	7	1.69	0.17-16.40		0.98	0.18-5.44	
Low IS	0						
HER2-like							
High IS	2			0.999			0.801
Intermediate IS	11				0.75	0.15-3.84	
Low IS	2				1.27	0.17-9.33	
Triple-negative							
High IS	12			0.003			0.011
Intermediate IS	36	2.25	0.65-7.78		3.07	1.06-8.87	
Low IS	14	6.39	1.79-22.84		5.54	1.79-17.18	

Abbreviations: HER2 = human epidermal growth factor receptor 2, IS = immune status

6

7

Summary, discussion and future perspectives

SUMMARY AND DISCUSSION

In the last decade, the tumor microenvironment has shown to play an important role in tumor progression. Still, no markers concerning the microenvironment have been implemented in clinical decision making. The research presented in this thesis emphasizes the prognostic value of the tumor-stroma ratio (TSR), a method focusing on the tumor microenvironment. The TSR assessment is performed by the scoring method developed by Mesker et al. on routine hematoxylin and eosin (H&E) stained tissue slides of the primary tumor (1). Various validation studies demonstrated that the TSR is a reliable, simple, quick and inexpensive parameter with a good to a very good inter-observer agreement, as described in the review in **chapter 2**. This review showed a significant association between a poor clinical outcome and tumors with a high amount of stroma in five out of seven studies. The two studies which were not in line with the previous results assessed the amount of stroma using semi-automated point counting. This method assessed the TSR in only two fields of 9 mm^2 selected at the leading and non-leading edge of the tumor. This semi-automated scoring method is in contrast to the other studies which performed the TSR scoring on the most stroma-abundant area using a 10x objective as described by Mesker and colleagues.

Breast cancer is a heterogeneous disease. Subgroup analyses are essential to evaluate the clinical value of the TSR as a prognostic parameter. The clinical value of TSR might differ for the various subgroups, for example, receptor status or histological type. A challenge is the relatively large amount of patients required for adequate statistical power. Previously published research represented not all subgroups adequately, although most analyses showed a worse clinical outcome in patients with stroma-high tumors. **Chapter 2** presented an overview of these results. To validate the prognostic value of the TSR in clinically important subgroups, a large UK cohort of 1794 primary breast cancer patients, primarily treated with surgery in the Nottingham City Hospital between 1993 and 2002, was analyzed. **Chapter 3** described this retrospective study. The results showed that the prognostic value of the TSR was more pronounced in patients with grade III tumors compared to patients with grade I and II tumors. Moreover, observations showed a more pronounced prognostic effect of the TSR in patients with triple-

negative tumors compared to nontriple-negative tumors. Comparable hazard ratios and confidence intervals for the TSR were observed in an independent Dutch cohort consisting of 737 early breast cancer patients diagnosed in the Netherlands Cancer Institute between 1990 and 1999. Age, tumor size, histology, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER2) status and lymph node status did not modify the prognostic value of the TSR.

The cohort from Nottingham City Hospital was scored using a digital version of the method developed by Mesker et al., showing the possibility of the TSR assessment on digital images. Digital pathology is becoming more important in current routine diagnostics. Advantages are, for example, availability of annotations and measurement tools, easier comparison between multiple slides, accessibility in sharing images for second opinions and/or extern research collaborations. A logical next step after the digitalization of images is the automation of the TSR, which is currently performed in collaboration with the University of Nijmegen. Visual TSR assessment is reliable, simple and quick, and accessible for all pathology laboratories. With the increased interest in automated pathology to handle the expanding numbers of analyses, automation of the TSR is desirable.

Chapter 4 illustrated the observation of a significant association between age and intra-tumoral stroma percentage assessed with the TSR. The results showed that the intra-tumoral amount of stroma increases with age. Different processes associated with the tumor and its microenvironment may play a role in the explanation of this observation, such as age-related alterations in the mamma, senescence of cells, changes in immune function and hormonal status. Additionally, **chapter 4** evaluated the prognostic value of the TSR in breast cancer patients of 70 years and older. Evaluation of prognostic markers in older patients is important because of a survival gap between younger and older patients with breast cancer, which may be caused partly by undertreatment. Currently, there are no specific guidelines concerning chemotherapy for older patients with breast cancer. Evidence-based treatment and accurate risk stratification are often not available since older patients are frequently excluded from studies. Comorbidities, which may strongly influence health status and clinical outcome, complicate research. Prognostic markers that

improve risk stratification in this specific group of patients are important for an accurate prognostic prediction regarding the additional benefit of adjuvant systemic therapy and can help patients and clinicians in shared-decision making. Unfortunately, so far, the TSR is likely to have no prognostic value in older patients with breast cancer.

Many efforts have been made to determine the best and the least invasive treatment of the axilla in breast cancer patients with positive axillary lymph nodes. There is a clinical need for an additional prognostic marker to improve risk stratification and personalized therapy.
In **chapter 5** the prognostic value of the TSR in tumor-positive lymph nodes in addition to the prognostic value of the TSR in the primary tumor in 191 patients was evaluated. The results showed a statistically significant difference between primary tumor stroma-low/lymph node stroma-low and primary tumor stroma-high/lymph node stroma-high for the relapse-free period, with recurrence rates of 42% versus 92%, respectively. The results in this study suggested that the assessment of the TSR in tumor-positive lymph nodes had additional value compared to the assessment of the primary tumor alone. Moreover, the strength of this study was the follow-up period of 15 years. The lymph nodes evaluated in this study were resected during axillary lymph node dissection, because the sentinel lymph node procedure was not part of standard clinical care. Compared to modern-day survival rates, the patients in this study had a relatively worse prognosis. Furthermore, a notable observation was the heterogeneity between the stroma category of the primary tumor and the lymph nodes in 52.9% of patients. A relatively high number of patients had stroma-high primary tumors and stroma-low lymph nodes. Literature shows that gene expression patterns differ between the primary tumor and the tumor disseminated to the lymph nodes. Downregulation of genes associated with cell-extracellular matrix (ECM) interaction, ECM remodeling, epithelial-mesenchymal transition (EMT) and loss of basement membrane are observed in the invaded lymph nodes compared to the primary tumor, which could confirm our observation of heterogeneity.

In **chapter 6**, the added value of immune markers to the TSR was evaluated in 344 patients with breast cancer without distant metastasis. Six markers involved in the immune response were chosen based on their interactions in tumor control and escape; HLA-E, HLA-G, classical HLA class I, natural killer cells, cytotoxic T lymphocytes (CTLs) and regulatory T cells (Tregs). Based on the interaction of these cells, the immune status of the tumors was divided into three categories: high, intermediate and low. For example, tumors with a high immune status showed expression of classical HLA class I, high infiltration of cytotoxic T cells and no infiltration of regulatory T cells. On the other hand, tumors with a low immune status showed no expression of classical HLA class I and no natural killer cell and regulatory T cell infiltration. As hypothesized at the start of this study, the results confirmed that breast cancer patients with a stroma-low tumor combined with a high immune status had a far more favorable prognosis compared to patients with a stroma-high tumor and a low immune status. The classical HLA class I was the most important prognostic determinant of the analyzed set of immune markers.

FUTURE PERSPECTIVES

Based on the published literature and the research presented in this thesis, the TSR is likely to be an independent prognostic parameter. The future perspectives of the TSR are two-sided, namely (1) clinical implementation of the TSR as a prognostic parameter and (2) research into the biological mechanism of stroma-low and stroma-high tumors in the search for new stroma derived markers for diagnostics, prognosis, disease monitoring and targeted therapy.

A next step toward clinical implementation is adding the TSR to the frequently used online prediction tool PREDICT. There is a clinical need to improve risk stratification to help clinicians and patients in shared decision-making toward personalized therapy. Implementation of the TSR in daily clinical practice needs further international validation in a very large retrospective assembled cohort consisting of patients with 10 years of follow-up or in a large prospective study. For clinical implementation of the TSR in colon cancer patients, the UNITED study has been started. This is an ongoing international prospective multicenter

study to validate the TSR in colon cancer patients. This study also includes training of pathologists for the assessment of the TSR to evaluate the inter-observer and intra-observer variation. Advantages of a prospective study for breast cancer are data collection and more up-to-date treatment regimes, but disadvantages are the long follow-up time of 10 years and the logistic challenge of including patients in many hospitals across the globe.

The PREDICT tool is used in patients primarily treated with surgery. The group of breast cancer patients treated with neoadjuvant chemotherapy is increasing. Therefore, it is of interest to evaluate the prognostic and predictive value of the TSR on core-needle biopsies of primary breast tumors instead of H&E slides originating from the primary tumor. Dekker et al. observed no association between the TSR assessed on H&E stained slides from tumor biopsies and complete pathological response to chemotherapy in 175 tumors of patients included in the NEOZOTAC trial (2). Besides, the authors evaluated the predictive value of stromal organization on tumor biopsies for the response to neoadjuvant chemotherapy and concluded that stromal organization was related to pathological response to chemotherapy (2). This study is the only publication on the TSR evaluation performed on core biopsies of breast tumors so far. For a more decisive conclusion, evaluation of a larger cohort is desirable. It would be of additional value if the assessment of the TSR on tumor biopsies could help to discriminate which patients are likely to respond to pre-operative chemotherapy in addition to standard pathological parameters and the relation to clinical outcome. This might result in an improved selection of patients for preoperative chemotherapy.

In this thesis, the additional prognostic value of the TSR in tumor positive lymph nodes resected during axillary lymph node dissection (ALND) to the TSR in the primary tumor was evaluated. In the last years, the intention of axillary management is the de-escalation of treatment and the reduction of morbidity associated with ALND. The omission of ALND is widely discussed in patients with 1 or 2 tumor-positive sentinel nodes in clinically node-negative disease (3-6). Evaluation of the TSR in tumor-positive sentinel nodes may add to better stratification of low or high-risk patients and finally to improve treatment decision making. In case a patient receives pre-operative chemotherapy, a core needle biopsy, instead of fine needle aspiration for cytology, of the lymph node suspicious for tumor involvement

might be performed to define the TSR. However, this may not be possible if the sentinel node is small.

Further research on the influence of the tumor stroma and the immune response regarding prognostication and interaction would be beneficial. Immune cells are an important component of the tumor microenvironment. Much research is performed on the prognostic role of tumor-infiltrating lymphocytes (TILs). The main components of TILs in breast cancer are $CD4^+$ and $CD8^+$ cells. Scoring of TILs are, like the TSR, assessed on standard H&E slides. High infiltration of TILs is associated with a better outcome (7-11). Especially stromal TILs have shown prognostic and predictive value (for example pathological complete response to neoadjuvant chemotherapy) in breast cancer patients, in particular in patients with triple-negative breast cancer and human epidermal receptor 2 (HER2) positive cancer (11). In this thesis, the TSR was combined with the immune status of tumors whereby the immunohistochemical evaluation of cytotoxic T lymphocytes (CTLs) and regulatory T cells (Tregs) was included as part of six immune markers (12, 13). As $CD8^+$ T cells are generally CTLs and $CD4^+$ T cells are helper T cells and Tregs, there is overlap with the immune status presented in this thesis. On the other hand, the inflammatory cells in the stroma are part of the stroma percentage score. Therefore, combining the TSR and TILs might strengthen each other.

The programmed death-ligand 1/programmed death-1 (PD-L1/PD-1) signaling pathway has become an important research topic in recent years. Inhibition and activation of T cells as a result of targeting this signaling pathway can influence the tumor microenvironment by preventing tumor immune evasion. Jiang et al. suggests in a review that inflammatory factors in the tumor microenvironment may induce PD-L1 and thereby influence the therapeutic efficiency of blocking PD-L1/PD-1 (14). It would be valuable to evaluate if the TSR could help in predicting therapeutic efficiency.

Research into the biological mechanism of stroma-low and stroma-high tumors in the search for new stroma derived markers may lead to new diagnostic, prognostic, monitoring and therapeutic opportunities. Stroma-high tumors likely reflect an activated stroma, supporting tumor aggressiveness. However, the underlying biological process in stroma formation of tumors is highly complex and largely

unknown. In-depth research is required to understand the biological differences in the tumor stroma of patients with stroma-low and stroma-high tumors, for example by evaluating gene expression profiles. Cancer-associated fibroblasts (CAFs), vascular endothelial growth factor, stromal cell-derived factor 1, platelet-derived growth factor, and transforming growth factor-β are thought to be strongly involved in cancer progression. CAFs are one of the most important components of the tumor microenvironment and play a role in remodeling the tumor microenvironment. Through the secretion of growth factors, cytokines and chemokines, CAFs enable tumor cells to invade the tumor microenvironment.

Regarding the improvement of therapeutic agents based on the stromal compartment, CAFs are promising. However, specific markers on CAFs are still lacking and therefore restrain direct depletion. Another way of influencing CAFs is via indirect routes, such as targeting processes influencing CAF activation or effectors. In a preclinical trial based on a triple-negative breast cancer model, doxorubicin combined with an antifibrotic agent pirfenidone inhibited tumor growth and metastasis. This agent has an anti-TGF-beta activity and may reduce collagen and hyaluronan levels (15). More knowledge about the role of the tumor microenvironment in chemo-resistance is also crucial to improve the success of chemotherapy.

Stromal markers can also be used for optical imaging techniques in oncological breast surgery, which could help to optimize the surgical procedure. Current preoperative imaging techniques do not provide enough information about the tumor borders resulting in surgical reintervention in approximately 25% of patients undergoing breast-conserving surgery. Intra-operative visualization techniques are therefore desirable, and tumor stroma might be a valuable source.

Furthermore, specific reliable markers originated from the stromal compartment can be used as tumor tracers, for example in positron emission tomography scan (PET-scan). These markers can be useful in monitoring disease progression, detecting cancer, determining disease aggressiveness and/or drug effectiveness before histology is available. For example, diagnostic dosages of drugs can be applied to patients to evaluate if the drug reaches the tumor and could, later on, be used in developing new therapeutics. Additionally, recently, the TSR was correlated with the images of a breast MRI. The authors concluded that short-tau

inversion-recovery (STIR) T2 weighted imaging and dynamic sequence of breast MRI reflected the stromal compartment of invasive breast tumors (16). Finally, molecules in the blood released by the stromal compartment also have potential in the early detection of breast cancer. Tumor stroma specific molecules in the 'liquid biopsy' could be identified by, for example, a proteomic approach.

CONCLUSIONS

The new insights presented in this thesis contribute to a better understanding of the role of the TSR on predicting clinical outcome in subgroups of breast cancer patients and in combination with other prognostic parameters. Furthermore, the described research is important for further research toward clinical implementation of the TSR and might finally be useful for decision-making regarding therapy. Moreover, molecular research of the stromal compartment in the near future is desirable for the development of new diagnostic, prognostic, monitoring and therapeutic markers.

REFERENCES

1. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
2. Dekker TJ, Charehbili A, Smit VT, ten Dijke P, Kranenbarg EM, van de Velde CJ, et al. Disorganised stroma determined on pre-treatment breast cancer biopsies is associated with poor response to neoadjuvant chemotherapy: Results from the NEOZOTAC trial. Mol Oncol. 2015;9(6):1120-8.
3. Morrow M, Van Zee KJ, Patil S, Petruolo O, Mamtani A, Barrio AV, et al. Axillary Dissection and Nodal Irradiation Can Be Avoided for Most Node-positive Z0011-eligible Breast Cancers: A Prospective Validation Study of 793 Patients. Ann Surg. 2017;266(3):457-62.
4. Giuliano AE, Hunt KK, Ballman KV, Beitsch PD, Whitworth PW, Blumencranz PW, et al. Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. JAMA. 2011;305(6):569-75.
5. Morrow M. Management of the Node-Positive Axilla in Breast Cancer in 2017: Selecting the Right Option. JAMA Oncol. 2018;4(2):250-1.
6. Giuliano AE, Ballman KV, McCall L, Beitsch PD, Brennan MB, Kelemen PR, et al. Effect of Axillary Dissection vs No Axillary Dissection on 10-Year Overall Survival Among Women With Invasive Breast Cancer and Sentinel Node Metastasis: The ACOSOG Z0011 (Alliance) Randomized Clinical Trial. JAMA. 2017;318(10):918-26.
7. Dieci MV, Conte P, Bisagni G, Brandes AA, Frassoldati A, Cavanna L, et al. Association of tumor-infiltrating lymphocytes with distant disease-free survival in the ShortHER randomized adjuvant trial for patients with early HER2+ breast cancer. Ann Oncol. 2019;30(3):418-23.
8. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26(2):259-71.
9. Loi S, Drubay D, Adams S, Pruneri G, Francis PA, Lacroix-Triki M, et al. Tumor-Infiltrating Lymphocytes and Prognosis: A Pooled Individual Patient Analysis of Early-Stage Triple-Negative Breast Cancers. J Clin Oncol. 2019;37(7):559-69.
10. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple-negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol. 2014;25(8):1544-50.
11. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40-50.

12. de Kruijf EM, van Nes JG, Sajet A, Tummers QR, Putter H, Osanto S, et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res. 2010;16(4):1272-80.
13. de Kruijf EM, Engels CC, van de Water W, Bastiaannet E, Smit VT, van de Velde CJ, et al. Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients. Breast Cancer Res Treat. 2013;142(2):355-64.
14. Jiang X, Wang J, Deng X, Xiong F, Ge J, Xiang B, et al. Role of the tumor microenvironment in PD-L1/PD-1-mediated tumor immune escape. Mol Cancer. 2019;18(1):10.
15. Dykes SS, Hughes VS, Wiggins JM, Fasanya HO, Tanaka M, Siemann D. Stromal cells in breast cancer as a potential therapeutic target. Oncotarget. 2018;9(34):23761-79.
16. Yamaguchi K, Hara Y, Kitano I, Hamamoto T, Kiyomatsu K, Yamasaki F, et al. Tumor-stromal ratio (TSR) of invasive breast cancer: correlation with multi-parametric breast MRI findings. Br J Radiol. 2019;92(1097):20181032.

8

Nederlandse samenvatting

Borstkanker is de meest voorkomende oorzaak van kanker gerelateerde sterfte bij vrouwen. De overleving van vrouwen met borstkanker is de laatste jaren toegenomen door enerzijds het bevolkingsonderzoek en anderzijds verbeterde behandelingen. Om de overleving verder te verbeteren is het nodig dat het risico op terugkeer of overlijden ten gevolge van borstkanker beter wordt ingeschat. Door een betere risicoschatting kunnen vrouwen op maat worden behandeld, waardoor onder- en overbehandeling zoveel mogelijk kan worden voorkomen. Dit houdt in dat er een betere afweging gemaakt kan worden tussen het risico op terugkeer van ziekte en bijwerkingen van chemotherapie, zoals hartfalen en cognitieve disfunctie. De voorspelling over het verloop van een ziekte wordt ook wel prognose genoemd. Het inschatten van de agressiviteit van de ziekte gebeurt in de klinische praktijk op basis van kenmerken van de tumorcellen, zoals hormoongevoeligheid, gradering en de grootte van de tumor. In de afgelopen jaren is het duidelijk geworden dat de omgeving waarin de tumorcellen zich bevinden, ook wel het tumorstroma genoemd, van belangrijke invloed is op de ontwikkeling van kanker. Het tumorstroma bestaat uit verschillende componenten, zoals bindweefsel, met daarin fibroblasten, cellen van de bloedvaten en cellen van het afweersysteem. De interactie tussen kankercellen en stromacellen is complex en verandert bij het voortschrijden van de ziekte. Tot op heden zijn er echter nog geen specifieke tumorstroma markers beschikbaar die klinisch toepasbaar zijn.

In dit proefschrift wordt de tumor-stroma ratio bestudeerd als marker om de mate van agressiviteit van borstkanker beter in te schatten. Het grote voordeel van de tumor-stroma ratio is dat de beoordeling van deze parameter simpel, snel en relatief goedkoop is. Het scoren vindt namelijk plaats door middel van het beoordelen van het tumorweefsel met een conventionele lichtmicroscoop. Tumorweefsel wordt in de huidige routine diagnostiek al uitvoerig door de patholoog beoordeeld om bijvoorbeeld het soort kanker en de uitgebreidheid van het proces te bepalen. De tumor-stroma ratio is een maat om de proportie van tumorcellen versus stromacellen uit te drukken in het meest stroma bevattende deel van een tumor. Deze scoringsmethode is als eerste beschreven door Mesker en collega's in darmkankerweefsel en daarna verder onderzocht in andere kankersoorten. De meeste studies laten zien dat patiënten met veel stroma in de tumor (stroma-hoog)

eerder kans hebben op terugkeer van ziekte en overlijden ten gevolge van kanker ten opzichte van patiënten met een tumor die weinig stroma (stroma-laag) bevat.

Het onderzoek beschreven in dit proefschrift levert een bijdrage aan het beter begrijpen van (1) de rol van de tumor-stroma ratio als prognostische factor in verschillende subgroepen borstkankerpatiënten en (2) de voorspellende waarde van de tumor-stroma ratio in combinatie met andere tumor gerelateerde parameters.
Hoofdstuk 2 begint met een overzicht van artikelen die gepubliceerd zijn waarin de prognostische waarde van de tumor-stroma ratio in vrouwen met borstkanker wordt onderzocht. Dit review laat zien dat in vijf van de zeven studies patiënten met een stroma-hoog tumor een slechtere klinische uitkomst hebben ten opzichte van patiënten met een stroma-laag tumor. In de twee studies waarin dit effect niet wordt gezien, is een andere methode gebruikt om de tumor-stroma ratio te bepalen, namelijk een semi-automatische punttelling. Een belangrijk verschil is dat bij deze methode niet het meest stromarijke deel wordt geselecteerd, zoals bij de methode van Mesker en collega's. Het review in **hoofdstuk 2** laat tevens zien dat de tumor-stroma ratio niet alleen in de algemene borstkanker populatie een prognostische waarde heeft, maar ook in een aantal klinisch relevante subgroepen.
Hoofdstuk 3 onderzoekt de waarde van de tumor-stroma ratio in een grote groep vrouwen met borstkanker en kijkt tevens naar de prognostische waarde van de tumor-stroma ratio in de meest klinisch relevante subgroepen. Hiervoor worden de studiegegevens van 1794 patiënten afkomstig van de University of Nottingham (Engeland) gebruikt, alsmede de gegevens van 737 patiënten afkomstig van het Nederlandse Kanker Instituut-Antoni van Leeuwenhoek (Nederland). De studie illustreert dat in de totale patiëntengroep, een stroma-hoog tumor geassocieerd is met een klinisch slechtere uitkomst. Deze studie laat tevens zien dat de voorspellende waarde van de tumor-stroma ratio in de meeste klinisch relevante subgroepen niet verschilt ten opzichte van het effect in de totale patiëntengroep. Een opvallende bevinding is dat in patiënten met een graad III tumor een beter onderscheidend vermogen van de tumor-stroma ratio wordt gezien, alsmede in patiënten met triple-negatieve borstkanker. De mate van gradering geeft aan in hoeverre de kankercellen lijken op gezond weefsel, onderverdeeld in graad I tot en met graad III. Kankercellen in graad III tumoren lijken vrijwel niet meer op

gezonde cellen van het borstweefsel. Triple-negatieve borstkanker is niet gevoelig voor de hormonen progesteron en oestrogeen en daarnaast ontbreekt het eiwit humane epidermale groeifactor receptor 2. Deze vorm van borstkanker is vaak agressiever, waarbij de ziekte vaker terugkomt in vergelijking met hormoongevoelige borstkanker.

In **hoofdstuk 4** is onderzocht hoe de hoeveelheid stroma, bepaald met de tumor-stroma ratio, verandert met de leeftijd. De resultaten van dit onderzoek laten zien dat het stroma toeneemt met de leeftijd van de patiënt. Dit is een nieuwe bevinding die nog niet eerder is gepubliceerd en draagt bij aan de opvatting dat tumoren bij oudere vrouwen verschillen van tumoren bij jongere vrouwen. Het immuunsysteem functioneert namelijk anders naarmate men verouderd, maar ook de hormonale status verandert door de jaren heen. De resultaten in dit hoofdstuk tonen aan dat er geen voorspellende waarde is van de tumor-stroma ratio voor het voorspellen van de overleving bij vrouwen van 70 jaar en ouder.

In het volgende hoofdstuk, **hoofdstuk 5**, wordt de toegevoegde waarde van de tumor-stroma ratio in tumor-positieve lymfeklieren in de oksel onderzocht. De resultaten laten zien dat het bepalen van de tumor-stroma ratio in positieve lymfeklieren toegevoegde waarde heeft ten opzichte van de bepaling van de tumor-stroma ratio in de primaire tumor alleen. Een andere belangrijke bevinding is het verschil ten aanzien van de hoeveelheid stroma in de primaire tumoren en de tumor bevattende oksellymfeklieren.

Het laatste onderzoek dat gepresenteerd wordt in **hoofdstuk 6** gaat over de toegevoegde waarde van immuunmarkers op de prognostische waarde van de tumor-stroma ratio. Er zijn zes verschillende immuunmarkers onderzocht die gekozen zijn op basis van hun interactie en rol in tumorontwikkeling: humaan leukocytenantigenen (HLA)-E, HLA-G, klassieke HLA klasse I, cytotoxische T-cellen en regulatoire T-cellen. De resultaten laten zien dat patiënten met een stroma-hoog tumor met een lage immuunstatus de slechtste overleving hebben en patiënten met een stroma-laag tumor en een hoge immuunstatus de beste overleving. Daarnaast is aangetoond dat klassieke HLA klasse I de meest bepalende factor van de zes immuunmarkers is.

Vervolgonderzoek naar de prognostische waarde van de tumor-stroma ratio zal zich richten op de klinische implementatie van deze parameter, bijvoorbeeld door toevoeging van de tumor-stroma ratio aan het online predictiemodel PREDICT. Dit model wordt in Nederland door oncologen gebruikt om de overlevingskansen en de toegevoegde waarde van aanvullende chemotherapie, hormonale therapie en immunotherapie te voorspellen. Indien door middel van de toevoeging van de tumor-stroma ratio aan de PREDICT een nauwkeurigere inschatting kan worden gemaakt over de agressiviteit van de tumor, kan dit mogelijk leiden tot nog gerichtere therapie. Verder is het zeer interessant om meer inzicht te krijgen in biologische processen in het tumorstroma, met name in het biologische verschil tussen stroma-laag en stroma-hoog tumoren. Dergelijk onderzoek zou potentieel nieuwe stromale markers kunnen opleveren, welke van invloed kunnen zijn op het nauwkeuriger bepalen van de prognose van een patiënt met borstkanker. Tevens kunnen nieuwe stromale markers bijdragen aan het optimaliseren van de diagnostiek, monitoring en ontwikkeling van gerichte therapeutische strategieën. Hierbij kan worden gedacht aan *image guided surgery*, betere visualisatie van de tumor op scans en specifieke therapie die aangrijpt op stromacellen die van belang zijn voor tumorontwikkeling.

Samengevat laat het onderzoek gepresenteerd in dit proefschrift zien dat de tumor-stroma ratio een mogelijk veelbelovende prognostische marker is. De nieuwe inzichten beschreven in dit proefschrift dragen bij aan de kennis voor verder onderzoek richting de klinische implementatie van de tumor-stroma ratio.

9

List of publications, curriculum vitae and acknowledgments

LIST OF PUBLICATIONS

Vreeker G.C.M., **Vangangelt K.M.H.**, Nicolardi S., Mesker W.E., Tollenaar R.A.E.M., van der Burgt Y.E.M., Wuhrer M.
Serum N-glycome in breast cancer
Submitted

Vangangelt K.M.H., Green A.R., Heemskerk M.F., Cohen D., van Pelt G.W., Sobral-Leite M., Schmidt M.K., Putter H., Rakha E.A., Tollenaar R.A.E.M., Mesker W.E.
The prognostic value of the tumor-stroma ratio is most discriminative in patients with grade III or triple-negative breast cancer
Int J Cancer. 2020 Apr 15;146(8):2296-2304

Vangangelt K.M.H., Kramer C.J.H., Bastiaannet E., Putter H., Cohen D., van Pelt G.W., Rakha E.A., Green A.R., Tollenaar R.A.E.M., Mesker W.E.
The intra-tumoral stroma in patients with breast cancer increases with age
Breast Cancer Res Treat. 2020 Jan;179(1):37-45

Kramer C.J.H., **Vangangelt K.M.H.**, van Pelt G.W., Dekker T.J.A., Tollenaar R.A.E.M., Mesker W.E.
The prognostic value of the tumor-stroma ratio in primary breast cancer with special attention to triple-negative tumors: a review
Breast Cancer Res Treat. 2019 Jan;173(1):55-64

Vangangelt K.M.H., Tollenaar L.S.A., van Pelt G.W., de Kruijf E.M., Dekker T.J.A., Kuppen P.J.K., Tollenaar R.A.E.M., Mesker W.E.
The prognostic value of tumor-stroma ratio in tumor-positive axillary lymph nodes of breast cancer patients
Int J Cancer. 2018 Dec 15;143(12):3194-3200

Vangangelt K.M.H., van Pelt G.W., Engels C.C., Putter H., Liefers G.J., Smit V.T.H.B.M., Tollenaar R.A.E.M., Kuppen P.J.K., Mesker W.E.
Prognostic value of tumor-stroma ratio combined with the immune status of tumors in invasive breast carcinoma
Breast Cancer Res Treat. 2018 Apr;168(3):601-612

CURRICULUM VITAE

Kiki Vangangelt is geboren op 29 augustus 1989 te Maastricht. Na het behalen van haar VWO diploma aan het Sint-Maartenscollege te Maastricht in 2007 is zij geneeskunde gaan studeren. In 2014 heeft zij haar master geneeskunde behaald aan Maastricht University. Haar semi-arts stage heeft zij volbracht bij de afdeling heelkunde en plastische chirurgie in het Maastricht University Medical Center. Vervolgens is zij gestart als ANIOS heelkunde in het Ziekenhuis Amstelland. Haar interesse voor wetenschappelijk onderzoek is gedurende deze periode gegroeid, waarna zij in november 2015 is aangesteld als promovenda bij de onderzoeksgroep van dr. W.E. Mesker en prof. dr. R.A.E.M. Tollenaar in het Leids Universitair Medisch Centrum.

DANKWOORD

Graag wil ik iedereen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift. Met speciale dank aan mijn copromotor dr. Wilma Mesker en promotor prof. dr. Rob Tollenaar.

Prof. dr. Tollenaar, beste Rob, dank dat jij mij de mogelijkheid hebt gegeven om bij jouw groep te mogen promoveren.
Dr. Mesker, beste Wilma, tijdens mijn sollicitatiegesprek werd ik ontzettend nieuwsgierig naar jullie onderzoek, mede vanwege jouw tomeloze energie, positiviteit en enthousiasme. Dank voor het vertrouwen dat je in mij hebt gehad ten aanzien van het behalen van het doctoraat.
Beste collega's en co-auteurs, bedankt voor de prettige samenwerking de afgelopen jaren.
Dr. Green, Andy, it has been a great pleasure to work with you. The clinical data from your institute were of great value. Many thanks!
Geachte leden van het genootschap landgoed keukenhof, jullie financiële ondersteuning is van onschatbare waarde voor het onderzoek naar de vroege opsporing van kanker en het onderzoek naar de klinische waarde van de tumor-stroma ratio.
Stéphanie en Randa, jullie waren mijn steun en toeverlaat gedurende de afgelopen jaren. Ik ben ontzettend blij dat ik jullie heb mogen leren kennen.
Gabi, dank voor jouw geduld om al mijn vragen te beantwoorden. Jij was onmisbaar gedurende mijn PhD.
Vrienden, ondanks dat we elkaar niet meer zo vaak zien, wil ik jullie bedanken voor jullie vriendschap.
Omake en Dré, dank voor de tijd, aandacht en liefde die jullie mij hebben gegeven. Jullie inzet heeft er mede voor gezorgd dat ik de kans heb gekregen om te gaan studeren.
Jos en Maria, dank voor jullie steun en vertrouwen. Ik ben dankbaar dat ik onderdeel van jullie familie mag uitmaken.
Pap en mam, dank voor jullie eindeloze vertrouwen en mogelijkheden die jullie mij hebben gegeven om te studeren, maar bovenal dank voor alle liefde. Ik kan me geen betere ouders wensen.

Zoë, mijn zusje, ik kan me geen liever, spontaner en mooier zusje wensen. Op het moment van schrijven is jullie zoontje nog niet geboren, maar één ding dat ik zeker weet is dat jullie fantastische ouders worden! Ik kijk er naar uit om hem te mogen ontmoeten.
Floris, dank voor jouw onvoorwaardelijke liefde en steun. Ik ben ontzettend gelukkig met ons gezin.
Lily Rose, jij laat ons iedere dag weer ervaren waar het echt om gaat in het leven. Dank dat je er bent!