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

Vangangelt, K.M.H.

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.