



Universiteit
Leiden
The Netherlands

Optimizing breast cancer survival models based on conventional biomarkers and stromal parameters

Dekker, T.J.A.; Dekker T.J.A.

Citation

Dekker, T. J. A. (2017, September 26). *Optimizing breast cancer survival models based on conventional biomarkers and stromal parameters*. Retrieved from <https://hdl.handle.net/1887/55957>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/55957>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/55957> holds various files of this Leiden University dissertation

Author: Dekker T.J.A.

Title: Optimizing breast cancer survival models based on conventional biomarkers and stromal parameters

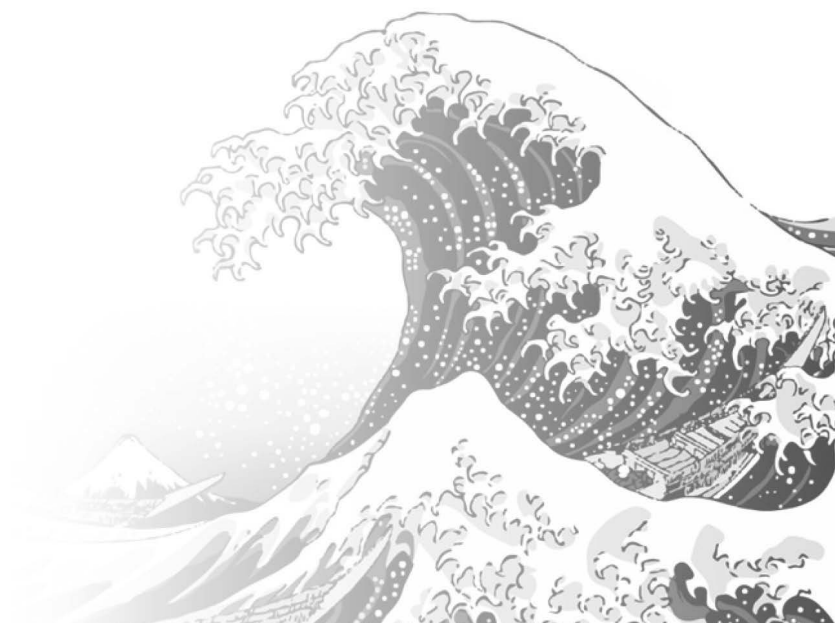
Date: 2017-09-26

14

Stromal alignment determined on pre-treatment breast cancer biopsies is related to response to neoadjuvant chemotherapy: results from the NEOZOTAC trial

Molecular Oncology 2015; 9(6): 1120-1128

T.J.A. Dekker, A. Charehbili, V.T.H.B.M. Smit, E.M. Meershoek-Klein
Kranenbarg, C.J.H. van de Velde, G.J. Liefers, J.W.R. Nortier, R.A.E.M. Tollenaar,
W.E. Mesker, J.R. Kroep.



Introduction

Neoadjuvant chemotherapy is an increasingly used treatment modality for locally advanced breast cancers. The goal of neoadjuvant therapy in this setting is to reduce the size of the tumor enabling the use of breast-conserving therapy. Neoadjuvant chemotherapy also provides the opportunity to evaluate tumor response to chemotherapy via histopathological evaluation (e.g. Miller-Payne score (Ogston et al., 2003)). Published patient series indicate that neoadjuvant chemotherapy pathological complete responses are achieved in 15-20% of all patients treated with neoadjuvant hormonal- or chemotherapy (i.e. no detectable invasive tumor cells) (Cortazar et al., 2014). Triple-negative breast cancer (estrogen receptor [ER]-negative, progesterone receptor [PR]-negative, human epidermal growth factor 2 [HER2]-negative, TNBCs) and HER2-positive, hormone receptor-negative tumors who received trastuzumab are most likely to undergo complete pathological response (Cortazar et al., 2014 and Kuerer et al., 1999). The paradox between the high likelihood of pCR and generally worse prognosis of aggressive tumor subtypes, might in part be explained by the distinct biology that underlies these tumors. Additional markers for predicting response to neoadjuvant chemotherapy and patient survival in TNBCs as well as ER-positive breast tumors are essential in order to improve patient quality of life and survival.

Both tumor-associated and physiologic stroma consist of cross-linked collagen fibers of which the organization is influenced by tumor cells (Provenzano et al., 2006). The activation of stroma has been shown to activate several different mechanisms that influence tumor progression and patient prognosis (Hawinkels et al., 2014, Trimboli et al., 2009 and Witkiewicz et al., 2011) and has also been implicated in resistance to chemotherapy (Liu et al., 2012). Previous preclinical studies have investigated the influence of the collagen matrix organization on the perfusion of drugs towards the intended target cells (Stylianopoulos et al., 2010). The organization of the stromal extracellular matrix can increase the effective path of molecules towards the target cells (Ramanujan et al., 2002), possibly reducing drug diffusion and treatment efficacy. This organization of the tumor-associated stroma can be observed under the microscope, and was therefore quantified with a simple image analysis method and correlated to response to chemotherapy. To our knowledge, this principle has never been investigated in clinical tumor samples treated with neoadjuvant chemotherapy. In this study we hypothesize that the stromal organisation parameter can discriminate between pathological responders and non-responders. The predictive influence of the organisation of the tumor-associated stroma was investigated in HER2-negative,

stage II/III breast cancer patients treated with neoadjuvant chemotherapy in the NEOZOTAC trial (Charehbili et al., 2014).

Methods

Patient population

The NEOZOTAC trial is a national, multicenter, phase III, randomized trial that investigated the efficacy of addition of zoledronic acid to the TAC (docetaxel, doxorubicin, cyclophosphamide) chemotherapy regimen. A number of 250 patients were entered in this trial between 2010 and 2012. These patients presented with large resectable or locally advanced (stages T2, T3, T4, every N, M0) breast cancers. All tumors were confirmed to be negative for HER2 amplification. Central pathology revision, was performed according to the Miller-Payne (MP) criteria (Ogston et al., 2003). All clinical samples were obtained according to Dutch ethical rules and guidelines. Briefly, the tumor cell cellularity was assessed in pre-operative biopsies and resection specimens. The decrease in tumor cellularity was estimated by two observers (TD and AC) and grouped in one of five categories (1- no decrease in cellularity, 2- < 30% decrease, 3- between 30 and 90% decrease, 4- > 90% decrease and 5- complete pathological response). Radiological response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria (Therasse et al., 2000) via MRI imaging modalities at local participating centers.

Stromal organisation

Stromal organisation was determined by capturing three separate images of haematoxylin and eosin (H&E)-stained intra-tumoral stroma tissues from all available pre-treatment tumor biopsies. When insufficient material was available for three separate images, two images were acquired. A single image was considered insufficient. The images were of randomly selected intratumoral stromal regions. Only stromal tissues that were surrounded by tumor cells in all corners were eligible, as previously done in studies investigating the prognostic effect of the tumor-stroma ratio (de Kruijf et al., 2011, Dekker et al., 2013 and Courrech Staal et al., 2011). These images were then loaded into ImageJ (Image Processing and Analysis, WS Rasband, <http://rsb.info.nih.gov/ij/>). Straight lines were drawn onto the image alongside the orientation of the stromal fibers. A number of 12-15 lines were drawn until the overall stromal orientation of the tumor was captured alongside these lines (Figure 1A, B) according to the observer. The mean orientation of these vectors and the standard deviation of this

orientation was determined as a measure for the organization of the fiber network and noted for each tumor. A high value for this standard deviation indicates broad distribution of the drawn vectors and thus signifies disorganised stromal orientation, whereas a low value for this parameter indicates that the stroma was radially organised.

AZAN trichrome staining protocol

AZAN trichrome staining was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections to evaluate whether detection of the stromal organisation parameter might be improved by using stains to highlight the collagen component of the tissue specifically. FFPE slides were deparaffinized using demineralized water, and then incubated in azocarmine solution at 56 °C. Slides were then allowed to cool and were rinsed with tap water. Subsequently, slides were incubated in 5% phosphotungstic acid solution for 1.5 h and again washed with tap water, prior to incubation with Aniline Blue – Orange G solution for 10 min. Slides were again washed with tap water and subsequently rinsed with 50% and 70% alcohol solutions and dehydrated with a 100% xylene solution.

Stromal organisation was assessed on the AZAN-stained slides and compared with the organisation score that was determined on H&E-stained slides. To avoid discordance caused by possible regional heterogeneity regarding stromal organisation, this comparison was performed on the same stromal regions within the tumor as much as possible (Figure 2). This was done by selecting a suitable region on the AZAN-stained slides and then highlighting the corresponding region on the H&E-stained slide. Images were captured, loaded into ImageJ and the analyses were performed as described above.

Immunohistochemistry protocol

Phospo-Smad2 (pS2; activated form of Smad2) immunohistochemical stainings were performed on FFPE tissues. Slides were deparaffinized by dipping the sections three times in xylene and in decreasing concentrations of ethanol. Slides were subsequently peroxidase blocked for 20 min in H₂O₂ solution (0.3%). Antigen retrieval was performed by heat treatment in citrate buffer (pH 6.0) for 10 min. After cooling down, slides were washed with phosphate buffered saline (PBS) for three times and incubated overnight with a pS2 antibody (Cell signaling, US) with a concentration of 1:100. After PBS wash, the secondary antibody was applied (Envision, HRP, anti-rabbit, DAKO, Denmark). Reaction was developed by adding 3,3'-Diaminobenzidine (DAB) for

a 10 min period and subsequently washed and counterstained with Mayer's hematoxylin. The pS2-immunostained slides were analyzed via conventional light microscopy. The amount of positive stromal cells were estimated in two categories: 1- < 50% pS2 positive stromal cells and 2- more than 50% positive stromal cells.

Statistical analysis

All statistical analyses were performed in Statistical Package for the Social Sciences (SPSS) statistics version 20 (IBM, US). As this study was not prospectively designed and was not a part of the study protocol of the NEOZOTAC trial, this was a post-hoc exploratory analysis. The standard deviation of the mean direction vector was determined on all available images and the average of these values was used as the final score for the tumor in question. The association of stromal organisation between ER status, cN-status and cT-status was investigated by performing independent sample t-tests. Association of stromal organisation and pS2 with response to chemotherapy was performed by stratifying chemotherapy response to two categories (MP 1-2 vs MP 3-5) and was compared in univariate analyses by comparing independent sample t-tests. Stromal organisation was converted to an ordinal variable for multivariate analyses by calculating tertile cut-offs. These were used to stratify the entire dataset into three groups based on the stromal organisation groups (1- highly aligned, 2- intermediate category, 3 – highly disorganized stroma). The relationship between ER, cN-status, cT-status and stromal organisation with response to chemotherapy was investigated in univariate logistic regression analyses. Parameters that were associated ($P \leq 0.05$) with significant pathological response (MP3-5) were entered into a multivariate model. Intra- and interobserver correlation and AZAN-staining and H&E-staining correlations were both calculated as Pearson correlation coefficient.

Results

Study population

175 H&E slides were centrally available and included for analysis. Stromal organisation evaluation was achieved for 162 tumors from the entire cohort. For 13 additional tumors, only two images were captured, due to limited presence of intratumoral stroma and these were also included in the analysis. Clinicopathological characteristics from these patients can be found in table 1.

Stromal organisation

After determining the organisation score for these 175 tumors (as the mean standard deviation from the mean direction vector) for the individual slides, the mean of these values was used as the final score. High scores indicate that the mean standard deviation was high, thus reflecting disorganized extracellular matrix (ECM). This stroma organisation parameter was positively correlated to ER status ($P = 0.003$) and cT-status ($P = 0.041$, table 2). Interestingly, this parameter was negatively correlated to cN-status ($P = 0.029$), indicating that tumors with lymph node metastases were more likely to display aligned collagen. Stromal organisation was also correlated to pathological response to neoadjuvant chemotherapy ($P < 0.001$; table 3). In order to test the clinical applicability of this parameter, stromal organisation was stratified into three categories of equal size and tested in multivariate analyses as an ordinal categorical variable. The stromal organisation parameter could discriminate between responders and non-responders. Tumors with disorganized stroma displayed a reduced Odds Ratio (OR) for showing significant pathological response (MP 3-5) to chemotherapy compared to tumors that were highly aligned (OR 0.276, 95% CI 0.124-0.614, $P = 0.002$, table 2) independent of ER-status. In an exploratory analysis, the association between previously mentioned tumor-stroma ratio and pCR was not significant in the current trial.

Correlation with AZAN trichrome stain

In order to verify whether standard H&E-staining was sufficiently reliable for determining this stromal organisation parameter, AZAN trichrome stainings were performed on the pre-operative biopsies and compared to the organisation determined on H&E-stained slides. The comparison between these methods was performed on 51 images captured randomly from 19 different tumors. Correlation between the analysis of H&E stain and AZAN stain was 0.806. The mean absolute difference between these methods was 4.91 (range 0.17-16.44). However, no statistically significant difference was found between the average mean scores between H&E and AZAN (difference between means -0.02173 , 95% CI $-1.85-1.81$, $P = 0.981$), indicating that H&E-stained slides can be used for adequate evaluation without over- or underestimation of stromal organisation. This observation in combination with the high correlation, led us to conclude that the stromal organisation can be determined on H&E-stained slides.

Reproducibility (intra/interobserver variation)

To test the reproducibility of the stromal organisation assessment, all captured images from approximately one third of the entire patient set (63 cases, 36%) were again analyzed by the same observer. Correlation between the first and second observation by the same observer for these 188 images was 0.815 (figure 2A). These images were also measured by a second independent observer which resulted in a correlation of 0.689 (figure 2B). After this initial assessment, images that were most discordant (absolute difference more than 10, $N = 68$) between the two observers were again observed by two observers together. The correlation between these scores increased to 0.732 (from 0.426) and the mean difference from with the original score decreased from 15.17 to 5.1425.

Relation with TGF- β /Smad2 signaling

Because TGF- β has previously been shown to be a central mediator of stromal activation, presence of active TGF- β signaling in the stromal compartment was evaluated for relations with stromal organisation. Smad2 is an intracellular effector of TGF- β , which becomes phosphorylated upon TGF- β receptor activation. Analysis of TGF- β /Smad2 signaling activity in pretreatment biopsied was performed by staining with an antibody directed at carboxy terminal phosphorylated Smad2 (pS2). The expression of pS2 was scored in two categories (< 50% positive stromal cells, Figure 3A and > 50% positive stromal cells, Figure 3B). Stromal pS2 status was finally assessed in 142 tumors, of which 31 tumors had high stromal pS2 expression, whereas 111 tumors had low pS2 expression. Although no relationship was found between stromal pS2 expression and response to chemotherapy ($P = 0.624$), stromal organisation was negatively related to pS2 expression ($P = 0.025$, figure 3C). This suggests that stromal compartments of tumors with active signaling in the TGF- β pathway are likely to have more organised stroma by altered synthesis of the collagen matrix, hypothetically influencing both tumor cell mobility and vulnerability to chemotherapy.

Discussion

Neoadjuvant chemotherapy is an increasingly used treatment modality. Yet easily-applicable, reliable biomarkers for predicting response to this treatment are currently insufficiently available. Features of the tumor-associated stroma, such as for example the tumor-stroma ratio, have been frequently related to patient prognosis and clinical outcome (de Kruijf et al., 2011 and Dekker et al., 2013). However, fewer studies have described clinical correlations between stromal features and tumor response

to chemotherapy. Gene-expression studies have previously shown the influence of tumor-associated stroma on response to therapy (Farmer et al., 2009). In the current study, tumors whose stroma consisted of organised collagen showed a higher benefit from neoadjuvant chemotherapy compared to tumors with disorganized tumor-stroma, independent from other conventional variables. To our knowledge, this principle has never been shown to be relevant in a clinical cohort of tumors treated with neoadjuvant chemotherapy.

Interestingly, tumors with disorganised stroma were not only associated with poor response to chemotherapy, but also showed an inverse relation with lymph node metastases. Studies have shown that tumor-surrounding stromal tissue is characterized by stromal changes with possible mechanical implications, such as increased linearisation and stiffness, due to increased collagen crosslinking (Egeblad et al., 2010 and Levental et al., 2009). Alignment of the collagen matrix at the tumor-stromal border may be a process induced by tumor cells, which facilitates migration of tumor cells alongside these collagen bundles (Provenzano et al., 2006). Results from our study suggest that this mechanism both promotes tumor cell dissemination while at the same time also sensitizing tumors to chemotherapy.

The possible prognostic role of the stromal organisation parameter was also identified by Conklin et al, who found that presence of straightened and aligned collagen is prognostic for poor disease-free survival (Conklin et al., 2011). Conklin et al. described the use of multiphoton second harmonic generation imaging to enhance visibility collagen fibers (Conklin et al., 2011), followed by visual estimation of collagen alignment by three observers. Other methods used to increase visibility of collagen fibers are AZAN trichrome stains, which we also applied in this study. However, conventional light microscopy visualization of common H&E-stained images from the NEOZOTAC cohort were used for determining stromal organization. The H&E method used in this current study is arguably the most feasible method for determining this parameter in clinical practice. Regarding the determination of the organisation of collagen, this was aided by drawing vectors in freely available image analysis software whereas previous studies have relied on visual estimation. The use of this software provides a numerical estimation of stromal organisation. In order to implement such a parameter, cut-off values are of course necessary. For the present study only the upper tertile (the most highly disorganized tumors) were found to have an independently worse response to neoadjuvant chemotherapy. Aside from the use of the image analysis software, these are all common tools for practicing pathologists and thus make this parameter fairly easily implementable.

TGF- β signaling has previously been shown to lead to activation of stromal fibroblasts (Hawinkels et al., 2014, Kojima et al., 2010 and Lohr et al., 2001) and results in our study have demonstrated an association between stromal TGF- β signaling and stromal organisation. This indicates that TGF- β signaling in the stromal compartment might contribute to chemotherapy sensitivity while at the same time leading to a poor prognosis. This observed relationship between TGF- β signaling and collagen organisation is concordant with a study performed by Bowes et al. which demonstrated that wounds treated with TGF- β display more aligned collagen (Bowes et al., 1999). Another study in collagen models showed that TGF- β 1 lead to α -smooth muscle actin (SMA) upregulation and alignment of the collagen matrix (Ng et al., 2005). Regarding matrix stiffness, Leight et al. observed that increased rigidity is accompanied by increased epithelial-mesenchymal transition (Leight et al., 2012). Intriguingly, Liu et al. (2012) found that blockade of TGF- β actually improves doxorubicin distribution in the tissue by decreasing the amount of collagen in a breast cancer model. Stromal organisation was not assessed in the latter study. The beneficial effect of TGF- β inhibition in that model might be explained by the effect that this pathway has on pericytes. Selective inhibition of TGF- β type 1 receptor kinase activity has been shown to decrease pericyte-coverage and increase vascular permeability in tumor models (Kano et al., 2007 and Kano et al., 2009). TGF- β thus seems to have dual effects on chemotherapy sensitivity and this might explain why although a positive association was found between TGF- β signaling and stromal organisation, no direct relationship was found between stromal pS2 expression and response to neoadjuvant chemotherapy. Furthermore, while the correlation between stromal pS2 expression and organisation was statistically significant, organised stromal features were also found in tumors that displayed no pS2 signal within the stromal compartment. Other signaling pathways are likely to be involved as well in this organization, and these might prove to be modulators of chemotherapy sensitivity as well.

Stromal organisation was correlated to ER status in this study as ER negative tumors were found to have more organised stroma than ER-positive counterparts. Besides the recognized more aggressive tumor cell behavior and therefore increased sensitivity to chemotherapy, stromal organization might in part explain the tendency for ER-negative breast carcinomas to respond favorably to neoadjuvant chemotherapy. Interaction between basal like (ER-negative) tumor cells with fibroblasts has been shown to lead to increased production of TGF- β 1 (among others) compared to luminal (ER-positive) breast cancer cell lines (Camp et al., 2011). This can in turn lead to the stromal organisation shown in this study and other, as well as leading to the TGF- β -induced promotion of cell motility (Muraoka et al., 2002). This might lead to the relatively poor prognosis of ER-negative tumors while also explaining the relative

chemotherapy-sensitivity of this tumor group. Apart from the correlation with ER status, stromal organisation was independently related with response to chemotherapy in multivariate analyses. We hypothesize that this effect is a direct representative of decreased diffusion of drugs towards the tumor cells when the stromal matrix is composed of haphazardly organized matrix fibers. This effect might also be related to a possible association of stromal organisation with both structural and molecular stromal features such as collagen density and stromal caveolin1 expression (Goetz et al., 2011), which might also be related to response to chemotherapy. In addition, it has previously been shown that quantification of the amount of stroma using the tumor-stroma ratio is of prognostic value (Dekker et al., 2013). Although, not confirmed in the current trial, we aim to further evaluate this feature as well as other features of the tumor-surrounding stroma in future studies. Alternatively, the differential chemo-sensitivity on basis of stroma organization may be the result of qualitative aspects of cells in the stromal tumor microenvironment, such as for example the extent of tumor infiltrating lymphocytes and the differentiation state of macrophages (M1 vs. M2) (Chanmee et al., 2014 and Salgado et al., 2015). Although our data show a clear significant relationship between stromal organization and pathological response, it cannot be excluded that stromal organization only correlates with response, instead of being causative for differences in pathological response patterns.

These proposed mechanisms for the association between stromal organisation and chemotherapy efficacy warrant further study.

The stromal organization tool we present is limited by the fact that it is an on observer dependent method. We found relatively good intra- and inter-observer correlation values for estimating the stromal organization variable ($r = 0.689$). In practice, a pathology workflow might aid for this (supplemental file). Our study suggests that stromal organization measured by human observers may be an easy and adequate predictor for chemo-sensitivity. However, automated measurement methods may provide a more reproducible method. Recently, approaches have described with machine-based visualization of collagen fibers (Bredfeldt et al., 2014a and Bredfeldt et al., 2014b). For example, Cui et al. measured fiber organization in aorta and skin samples using two-photon excitation fluorescence and second-harmonic generation signals (Cui et al., 2014). Such methods may prove useful for assessment of stromal organization. However, as H&E and observer assessment is still the gold standard for pathologists, who have to evaluate large amounts of samples in limited timeframes, we present a method which can be easily and directly applied in clinical practice.

Of note, it must be acknowledged that the data described in our manuscript are based on a post-hoc exploratory analysis. Therefore, further (prospectively planned) studies are necessary to validate our findings.

In conclusion, stromal organisation was related to response to neoadjuvant chemotherapy and also confirmed a previously shown relationship between stromal organisation and prognosis. This parameter might prove a useful tool for determining the treatment plan for breast cancer patients. Additionally, this might provide insight into additional pathways that govern sensitivity to chemotherapy.

Author contributions

TD and AC designed the experiments. TD, AC and VS performed experiments and data-analysis. VS, EM, JN, JK and CV provided clinical and archival specimens. RT and WE provided intellectual input and critical evaluation of the results. TD, AC and JK prepared the manuscript. All authors discussed the results and provided comment on the manuscript.

Financial support

The NEOZOTAC trial was funded with research grants from Amgen, Novartis, Sanofi and the Dutch Cancer Foundation 2010-4682.

Acknowledgments

We want to thank the Datacenter of the Department of Surgery of the Leiden University Medical Center for their assistance with collecting the clinical data, especially Linda Verhoeff.

References

1. Bowes LE et al.: Collagen fiber orientation as quantified by small angle light scattering in wounds treated with transforming growth factor-beta2 and its neutralizing antibody. *Wound Repair Regen.*, 7 (3) (1999 May), pp. 179–186
2. Bredfeldt JS et al.: Automated quantification of aligned collagen for human breast carcinoma prognosis. *J. Pathol. Inform.*, 5 (2014), p. 28
3. Bredfeldt JS et al.: Computational segmentation of collagen fibers from second-harmonic generation images of breast cancer. *J. Biomed. Opt.*, 19 (1) (2014 Jan), p. 16007
4. Camp JT et al.: Interactions with fibroblasts are distinct in Basal-like and luminal breast cancers. *Mol. Cancer Res.*, 9 (1) (2011 Jan), pp. 3–13
5. Chanmee T et al.: Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel)*, 6 (3) (2014), pp. 1670–1690
6. Charehbili A et al.: Addition of zoledronic acid to neoadjuvant chemotherapy does not enhance tumor response in patients with HER2-negative stage II/III breast cancer: the NEOZOTAC trial (BOOG 2010-01). *Ann. Oncol.*, 25 (5) (2014 May), pp. 998–1004
7. Conklin MW et al.: Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am. J. Pathol.*, 178 (3) (2011 Mar), pp. 1221–1232
8. Cortazar P et al.: Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet*, 384 (9938) (2014 Jul 12), pp. 164–172
9. Courrech Staal EF et al.: Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies. *Eur. J. Cancer*, 47 (3) (2011 Feb), pp. 375–382
10. Cui JZ et al.: Quantification of aortic and cutaneous elastin and collagen morphology in Marfan syndrome by multiphoton microscopy. *J. Struct. Biol.*, 187 (3) (2014 Sep), pp. 242–253
11. De Kruijf EM 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.*, 125 (3) (2011 Feb), pp. 687–696
12. Dekker TJA 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.*, 139 (2) (2013 Jun), pp. 371–379
13. Egeblad M et al.: Dynamic interplay between the collagen scaffold and tumor evolution. *Curr. Opin. Cell Biol.*, 22 (5) (2010 Oct), pp. 697–706
14. Farmer P et al.: A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat. Med.*, 15 (1) (2009 Jan), pp. 68–74
15. Goetz JG et al.: Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell*, 146 (1) (2011 Jul 8), pp. 148–163
16. Hawinkels LJ et al.: Interaction with colon cancer cells hyperactivates TGF-beta signaling in cancer-associated fibroblasts. *Oncogene*, 33 (1) (2014 Jan 2), pp. 97–107

17. Kano MR et al.: Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-beta signaling. *Proc. Natl. Acad. Sci. USA*, 104 (9) (2007 Feb 27), pp. 3460–3465
18. Kano MR et al.: Comparison of the effects of the kinase inhibitors imatinib, sorafenib, and transforming growth factor-beta receptor inhibitor on extravasation of nanoparticles from neovasculature. *Cancer Sci.*, 100 (1) (2009 Jan), pp. 173–180
19. Kojima Y et al.: Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc. Natl. Acad. Sci. USA*, 107 (46) (2010 Nov 16), pp. 20009–20014
20. Kuerer HM et al.: Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. *J. Clin. Oncol.*, 17 (2) (1999 Feb), pp. 460–469
21. Leight JL et al.: Matrix rigidity regulates a switch between TGF-beta1-induced apoptosis and epithelial-mesenchymal transition. *Mol. Biol. Cell*, 23 (5) (2012 Mar), pp. 781–791
22. Levental KR et al.: Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*, 139 (5) (2009 Nov 25), pp. 891–906
23. Liu J et al.: TGF-beta blockade improves the distribution and efficacy of therapeutics in breast carcinoma by normalizing the tumor stroma. *Proc. Natl. Acad. Sci. USA*, 109 (41) (2012 Oct 9), pp. 16618–16623
24. Lohr M et al.: Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res.*, 61 (2) (2001 Jan 15), pp. 550–555
25. Muraoka RS et al.: Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J. Clin. Invest.*, 109 (12) (2002 Jun), pp. 1551–1559
26. Ng CP et al.: Interstitial fluid flow induces myofibroblast differentiation and collagen alignment in vitro. *J. Cell Sci.*, 118 (Pt 20) (2005 Oct 15), pp. 4731–4739
27. Ogston KN et al.: A new histological grading system to assess response of breast cancers to primary chemotherapy: prognostic significance and survival. *Breast*, 12 (5) (2003 Oct), pp. 320–327
28. Provenzano PP et al.: Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med.*, 4 (1) (2006), p. 38
29. Ramanujan S et al.: Diffusion and convection in collagen gels: implications for transport in the tumor interstitium. *Biophys. J.*, 83 (3) (2002 Sep), pp. 1650–1660
30. Salgado R et al.: The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann. Oncol.*, 26 (2) (2015 Feb), pp. 259–271
31. Stylianopoulos T, al: Diffusion anisotropy in collagen gels and tumors: the effect of fiber network orientation. *Biophys. J.*, 99 (10) (2010 Nov 17), pp. 3119–3128

32. Therasse P et al.: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J. Natl. Cancer Inst.*, 92 (3) (2000 Feb 2), pp. 205–216
33. Trimboli AJ et al.: Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature*, 461 (7267) (2009 Oct 22), pp. 1084–1091
34. Witkiewicz AK et al.: Molecular profiling of a lethal tumor microenvironment, as defined by stromal caveolin-1 status in breast cancers. *Cell Cycle*, 10 (11) (2011 Jun 1), pp. 1794–1809

Table 1. Characteristics of the two study populations described in this study.

ER = Estrogen Receptor, MP = Miller&Payne, PR = Partial Response, CR = Complete Response, SD = Stable Disease, PD = Progressive Disease.

Parameter	Stromal organisation known	Stromal organisation unknown
	N (%)	N (%)
cT2	110 (62.9)	34 (45.3)
cT3 + cT4	63 (36.0)	39 (52.0)
Unknown	2 (1.1)	2 (2.7)
cN0	76 (43.4)	35 (46.7)
cN+	98 (56.0)	38 (50.7)
Unknown	1 (0.6)	2 (2.7)
ER+	141 (80.6)	63 (84.0)
ER-	33 (18.9)	10 (13.3)
Unknown	1 (0.6)	2 (2.7)
MP response (categories 3–5)	98 (56.0)	42 (56.0)
MP response (categories 1–2)	72 (41.1)	31 (41.3)
Unknown	5 (2.9)	2 (2.7)
Radiological response (PR + CR)	114 (65.1)	48 (64.0)
Radiological response (SD + PD)	31 (17.7)	12 (16.0)
Unknown	30 (17.1)	15 (20.0)

Table 2. Relationship between prognostic factors and stromal organisation.

MP = Miller&Payne. Higher values represent disorganized stroma.

Parameter	Stromal organisation	P-value
cT2	37.73	0.041
cT3 + cT4	40.25	
cN0	40.07	0.029
cN+	37.47	
ER+	39.45	0.003
ER-	34.98	
MP response (categories 3–5)	36.85	< 0.001
MP response (categories 1–2)	41.26	

Table 3. Factors predicting significant pathological response (MP3-5) to neoadjuvant chemotherapy.

Parameter	Univariate analyses			Multivariate analyses		
	OR	95% CI	P-value	OR	95% CI	P-value
cT2	1.000	Ref	0.051	1.000	Ref	
cT3 + cT4	0.596	0.355–1.001		0.838	0.430–1.631	0.602
cN–	1.000	Ref	0.639	N.I.		
cN+	1.139	0.678–1.885				
ER+	1.000	Ref		1.000	Ref	
ER–	2.379	1.133–4.995	0.022	2.096	0.852–5.159	0.107
Highly organised stroma	1.000	Ref		1.000	Ref	
Intermediate category	0.497	0.224–1.102	0.085	0.498	0.220–1.128	0.095
Highly disorganized stroma	0.252	0.115–0.554	0.001	0.263	0.117–0.593	0.001

N.I. = Not Included. Lymph node status was not included in multivariate analysis as there was no association between lymph node status and pathological response in univariate analysis

Figure 1. (A) example of breast tumor biopsy with linearly aligned stroma. (B) example of breast tumor biopsy with disorganised stroma.

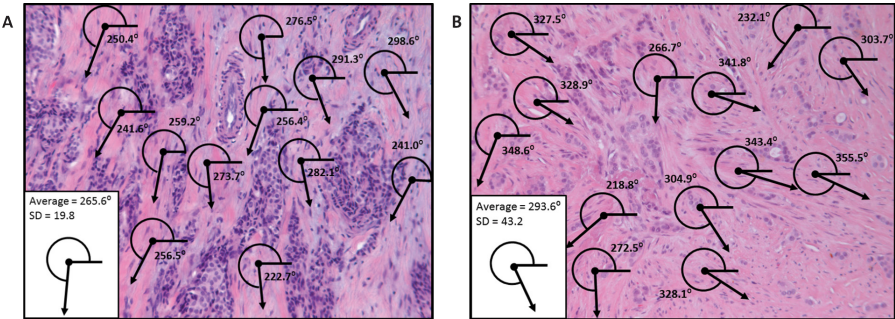


Figure 2. Example of the trichrome AZAN-staining for collagen fibers.

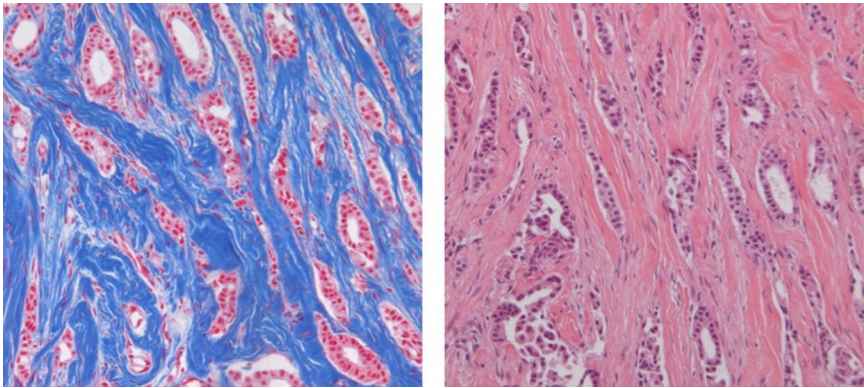


Figure 3. Correlation between pS2 immunostaining and stromal alignment.

