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

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Gathering evidence for prognostic and predictive factors from the breast cancer stroma

Background

The tumor-associated stroma has received increased attention in the past decade due to its significant effect on breast cancer growth and progression [1]. Morphological changes in the stromal compartment of breast tumors include fibroblast proliferation, dense fibrosis (or desmoplasia) and altered alignment of collagen fibers. Compared to their normal counterparts, cancer-associated fibroblasts (CAFs) are both morphologically and functionally different, including increased cell size, spindle-cell shaped appearance and increased secretion of cytokines and hormones that stimulate cellular division, angiogenesis [2] and the epithelial-to-mesenchymal transition [3]. Due to these and other functionalities, CAFs have been shown to promote tumor progression in murine models [2]. Transforming growth factor-β (TGF-β) is regarded as a major factor in the activation of the tumor-associated stroma as it has been found to exert a wide range of tumor-promoting effects in the tumor microenvironment. These effects include differentiation of physiologic fibroblasts into CAFs (thereby increasing collagen synthesis), enhanced angiogenesis [4, 5] and generating a tumor-promoting inflammatory response [6]. Murine breast cancer models with high TGF-β signaling have been shown to be associated to tumor ER-negativity, stromal enrichment and a pro-inflammatory immune response [7].

Arguably the most frequently reported marker associated with CAFs and stromal activation is α-smooth muscle actin (α-SMA). α-SMA is also expressed in other cell types, which hinders the use of this protein as a specific marker for activated fibroblasts. α-SMA also lacks sufficient sensitivity to identify all CAFs [8]. Although other biomarkers have been reported that are thought to identify CAFs, the overlap between these markers concerning the identification of CAFs remains ill-defined [9]. The predominant origin of CAFs is also unknown for the most part. CAFs are commonly thought to be derived from resident fibroblasts, but may also be the result of migration of mesenchymal stem cells [10], differentiation of adipocytes [11] and the epithelial-to-mesenchymal transition of epithelial cells. In order to fully understand mechanisms of stromal activation and CAF functionality, novel markers for tumor-associated stromal activation are needed to resolve these issues and reach agreement on both the origin and functionality of these cells.

Prognostic stromal biomarkers

Although the ability to predict breast cancer progression and patient survival has benefited many patients, there is still room for improvement. Treatment guidelines have increasingly broadened the inclusion of patients in the high-risk,

chemotherapy-eligible group [12], yet low risk patients also experience disease [13]. In order to reduce under- and overtreatment of breast cancer patients, novel strategies for risk stratification are needed. No stromal markers are currently incorporated as standard markers for patient prognostication. This is despite a study that showed that the use of a digital pathologist system was capable of retrieving a significant amount of prognostic information from solely the morphology of the tumor-associated stroma [14]. Furthermore, these imaging features from the tumor-associated stroma actually had a higher prognostic power than morphological information derived from the tumor epithelium [14]. Gene-expression profiling of the stromal compartment of breast tumors has also resulted in prognostic parameters in multiple studies [15-17]. In this section, results from both retrospective and prospective studies that have investigated specific individual components of the microenvironment for prognostic information will be discussed (figure 1). Additionally, levels of evidence for prognostic markers identified in these studies are summarized in table 1.

Fibroblasts

In accordance with its supposed importance in stromal activation, the stromal expression of the TGF-β receptor type II has been linked to poor prognosis in breast cancer [18]. However, this finding has been contradicted by another study, which showed a relatively favorable outcome when TGF-β receptors were expressed in the stroma [19]. These studies show that stromal TGF-β functionality likely depends on more than one factor and might be strongly context-dependent. Therefore, additional biomarkers are needed to further characterize breast cancer stroma into low-risk and high-risk categories.

An example of such a biomarker is **caveolin-1**, as caveolin-1 down-regulation in stromal cells has been shown to be predictive of both a worse disease-specific- and overall survival in invasive breast cancer [20]. Interestingly, differential caveolin-1 expression has not shown to be of any prognostic value in epithelial cells [21]. Loss of caveolin-1 in stromal cells is induced by tumor cells via induction of oxidative stress [22], leading to mitochondrial autophagy and citric acid cycle dysfunction [23]. This leads to production of energy-rich substrates (e.g. lactate) which are then shuttled to and utilized by tumor cells [24]. The expression of the **platelet-derived growth factor receptor (PDGFR)** by fibroblasts has been shown in preclinical models to contribute to the recruitment and activation of tumor-associated stroma. Although epithelial cancers do not often show expression of this marker, presence of the PDGF-β receptor in the stromal compartment of breast tumors has been linked to an adverse effect on the disease-free survival period, especially in premenopausal patients [25].

The p53 gene is one of the most described tumor suppressors and has been linked to a multitude of malignancies. While traditionally studied for its role in the tumor epithelium, loss of p53 expression in the stromal compartment or p53 mutant fibroblasts have been shown to promote metastases [26]. Mutations in p53 have been identified in breast tumor-associated stromal cells [27], although this seems to conflict with the previously described genetic stability of these cells [28,29] and has led to some discussion [30]. Regardless, p53 protein accumulation in fibroblasts has been linked to prognosis in one study by Hasebe et al. [31]. Another study by Patocs et al. discovered an association between stromal p53 mutations and lymph-node metastases in sporadic breast cancer [32].

Additional prognostic markers identified in cancer-associated fibroblasts include **podoplanin** [33, 34], β-arrestin-1 [35], **CD105** [36] and **CD10** [37, 38], which have all shown prognostic value in relatively large retrospective patient series. Although these will not be discussed in further detail, further information on these studies can be found in the provided references.

Extracellular matrix (ECM)

The ECM consists of a complex network of collagens, proteoglycans and glycoproteins which functions as a scaffold for epithelial cells. The ECM is produced by fibroblasts and is subject to remodeling by several factors after secretion. ECM molecules are important in cell-to-matrix and cell-to-cell adhesions, and as such, can influence cellular movement (reviewed elsewhere [39]). While the fibroblast is the most predominant cell-type, the extracellular matrix (ECM) takes up the largest volume of all components of the tumor-microenvironment. De Kruijf et al. showed that tumors that contained areas (assessed under 10X magnification field) with more stromal than tumor epithelium (tumors with a low **tumor-stroma ratio**) were significantly associated with an adverse prognosis, which was especially pronounced in triple-negative tumors [40-42]. Similarly, **the fibrotic focus**, which is a relatively acellular fibrosclerotic core within the primary tumor, is related to breast cancer disease relapse and decreased overall survival following surgery [43, 44]. Changes in the directionality of the collagen bundles that compose the ECM have been shown to promote tumor cell dissemination [45]. Under influence of the tumor cells, the stromal tissue is reorganized into straight, aligned bundles (referred to as **TACS3**), which can be used to predict breast cancer disease-specific survival [46].

Besides these parameters based on the morphology and organization of the ECM, the molecular content of the ECM has also been investigated for prognostic parameters.

Certain glycoproteins like **tenascin and fibronectin** are not present in the physiologic breast ECM, yet expression of these markers is found to occur in the majority of breast tumors and is related to a poor patient outcome [47-51]. Similarly, **hyaluronan** is an ECM polysaccharide that is normally seen in healing wounds [52] and is associated with increased cell migration and poor prognosis in breast cancer [53]. Matrix metalloproteinases (most notably **mmP-2** and **mmP-9)** are a family of related proteinases that are capable of cleaving ECM proteins. Although these proteins are generally not expressed in normal breast cells, these are strongly expressed in both breast cancer cells and stromal cells [54]. Interestingly, contrasting prognostic effects of these markers dependent on cell type have been published. For instance, for mmP-9, stromal expression seems to be associated with a poor prognosis and tumor cell expression seems to indicate a relatively favorable prognosis [55]. Further studies have shown that the prognostic effect of mmPs might not only be cell type- but also disease stage specific [56], possibly accounting for some of the contrasting results that have been published so far, regarding mmP-2 in particular [57-60]. This data suggests that the current understanding of the complex interactions between mmPs and their inhibitors potentially limit the translation of these parameters into reliable and reproducible prognostic biomarkers [54].

Inflammatory cells

The presence and extent of the inflammatory infiltrate in breast tumors has been shown to be reproducible source of prognostic information. For instance, immunohistochemical assessment of the amount of CD8+, CD4+ FOXP3+ cells [61-63], γδ-T-cells [64], macrophages [65] within the stromal compartment have been found to result in prognostic information. Stromal presence of tumor-infiltrating lymphocytes (TILs) quantified by means of H&E stains have recently been related to clinical outcome in several large clinical trial cohorts [66]. Tumors whose stromal tissues show massive influx of immune cells (more than 50-60% of the stromal tissues) have been termed lymphocyte predominant breast cancer (LPBC)[66] and constitute 5.4% of all breast carcinomas [67]. The LPBC phenotype and the presence of intratumoral- and stromal lymphocytes were not associated with prognosis in the overall population of BIG 02-98 adjuvant phase III trial [67]. However, when stratifying for ER-negative/HER2-negative carcinomas, tumors with the LPBC phenotype were associated with a very favorable prognosis (5-year survival was 92% for this phenotype compared to 71% of the non-LPBC ER-negative/HER2-negative tumors). Similarly, increasing amounts of TILs in non-LPBC, ER-negative tumors have been shown to be related to favorable patient prognosis. The favorable effect of TILs in this subgroup was later verified in an independent study that included 506 triple-negative breast cancer patients [68].

Vascular parameters

Angiogenesis is the formation of novel blood vessels induced by the presence of tumor epithelial cells. Vascular endothelial growth factor (VEGF) is considered as the major contributor to this process and is expressed by both tumor epithelial and stromal cells [69]. The density of microvessels (or **microvessel density, MVD**) has been assessed in a multitude of prognostic studies, with contradictory results [70-72]. These contrasting results might be explained by the difference in methodology employed by various studies regarding the visualization and detection of these blood vessels, and was never incorporated into routine diagnostics. A meta-analysis published in 2004 concluded that MVD is a prognostic factor but is only weakly predictive of overall survival [73]. While these studies investigated all vessels present within the tumor border, other studies have focused on vessels that were newly created by tumor cells. Arnes et al. published the results of a study investigating **endothelial proliferation** within the tumor border with the combination of stains for CD31 and Ki-67 and showed strong prognostic power in three datasets especially among high grade and ER-negative breast carcinomas [72]. An alternative marker for proliferating endothelial cells is **endoglin**, which might be prognostic in breast cancer [74, 75].

Predictive stromal biomarkers

Although chemotherapy can greatly impact the survival of breast cancer patients, a significant number of tumors show no response to cytotoxic agents [76], leaving the patient exposed to the harmful side-effects without a significant benefit. Although some epithelial features have been associated with non-response to chemotherapy [76], this phenomenon remains inadequately understood. Preclinical data shows that the stroma at least in part governs response to chemotherapy [77, 78]. The tumor-associated stroma might prove valuable for the discovery of new markers not only for assessing patient prognosis but response to therapy as well.

Few studies have directly investigated the response of chemotherapy with regards to expression of stromal markers. Farmer et al. demonstrated that high expression of a **stromal metagene** signature was related to resistance to chemotherapy [79]. This gene signature was also related to the amount of reactive stroma present in the tumor biopsy. The composition of this gene signature did reveal that several factors (e.g. PDGF-βR and mmP2) that were found to be prognostic stromal biomarkers were also factors involved in response to chemotherapy, suggesting that significant overlap might exist between these two mechanisms.

The stromal presence of **tumor-infiltrating lymphocytes (TILs)** has been shown to increase the likelihood of achieving pCR [66, 80-82]. Data describing the assessment of intratumoral and stromal lymphocytes in 1058 breast cancers that were treated with anthracycline/taxane-based chemotherapy showed that high numbers of tumor-infiltrating lymphocytes predict relative higher benefit from these chemotherapy regimens [66, 67, 80]. The predictive effect of TILs in HER2-positive and triple-negative tumors that were treated with neoadjuvant therapies has been investigated in a subanalysis of the GeparSixto trial [66]. Interestingly, another study performing these analyses via the same method has shown that HER2-positive tumors with the LPBC phenotype have an improved survival compared to non-LPBC HER2-positive tumors when both are treated with trastuzumab [83], suggesting that this marker might predict response to this agent. This might be explained by increased induction of antibody dependent cytotoxicity (one of the mechanisms of action of trastuzumab [84]) in the presence of a high amount of immune cells. Although the reproducibility regarding the pathologic evaluation of these criteria remains to be seen, these parameters provide an interesting low-cost application for the tumor microenvironment regarding therapy selection.

Lastly, the **matrix fiber organization** might also hypothetically affect response to chemotherapy. Increased interstitial flow within tumors with aligned collagen bundles might benefit both the movement of tumor cells and also the flow of chemotherapeutic drugs towards the intended targets. The orientation of stromal fibroblasts has been mainly studied in vitro system and although imperfectly understood, the relationship between interstitial flow and fibroblast orientation has been established, and fits the hypothesis above [85, 86]. Additionally, the alignment of collagen has been related to TGF-β signaling [87,88], possibly providing more insight on how this pathway regulates response to chemotherapy. In this thesis, matrix fiber organization is investigated in a series of breast cancer patients treated with neoadjuvant chemotherapy and related to response to therapy. To our knowledge, the relationship between stromal organization and response to chemotherapy has not been investigated in previous publications.

Conclusion

An increasing amount of both prognostic and predictive data has emerged from the tumor-associated stroma in recent years. These data vary from parameters derived from morphological information to individual biomarkers assessed via H&E-staining and IHC-stains to gene-expression profiles. The applicability of these markers depends on several factors, including their prognostic and predictive power,

the reliability of their assessment, the availability of such techniques in the everyday practice and the levels of evidence for individual parameters. The question still remains which patient groups benefit most from these markers, depending on the strength of the prognostic and predictive power of the markers involved and the relative paucity of other markers for some patient groups (thus increasing the urgency for the discovery of novel prognosticators). Regarding this last issue, the triple-negative population seems an ideal candidate. Relatively little prognostic data is available regarding the biology of these tumors compared to hormone receptor-positive and HER2-positive tumors. Multiple studies concerning stromal biomarkers have shown a differential prognostic power in the triple-negative population. The highest levels of evidence are derived from randomized clinical trials which have the benefit of including relatively uniform patient populations, central review of pathology and relatively uniform treatments (aside from a randomized intervention). The validation of these stromal markers should be performed in different clinical trial populations as much as possible in order to achieve level I evidence.

Outline of part II of this thesis

The second part of this thesis discusses several issues regarding the tumor-associated stroma, namely stromal-derived prognostic parameters, investigation into the molecular content of the tumor-associated stroma and the use of stromal parameters and pathways for predicting breast cancer disease progression and response to therapies.

In **chapter 10,** a validation study of the tumor-stroma ratio (TSR) is presented. This parameter is determined on the amount of tumor-associated stroma (desmoplastic response) in breast cancers. The study presented in this chapter assesses this parameter in a series of patients treated as part of EORTC trial 10854. The prognostic role of the TSR in triple-negative patient was also investigated as well as the implementation of this parameter alongside other clinico-pathological parameters.

Due to poor understanding of the origination and functionality of the tumor-associated stroma, novel stromal markers are needed to further investigate these tissues. **Chapter 11** discusses a study utilizing a technique called matrix assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI) for providing proteomic signatures distinguishing cancer-activated stromal tissues from quiescent stromal tissues. This study was performed by analyzing breast cancer tissues in two MALDI-MSI centers, namely the Leiden University Medical Center and the Helmholtz Institute in Munich. Protein signatures identifying tumor-associated stromal tissues

were compared across these two centers and potential stromal markers were validated with the use of immunohistochemistry.

Stromal biomarkers reflective of adverse metabolic tumor-stromal interactions have recently been published in the literature and have shown promise regarding their prognostic power. As such, metabolic tumor-stromal interactions should be further investigated and stratified according to biomarker expression. The use of the Fourier transform ion cyclotron resonance (FTICR) mass spectrometer for detecting metabolic signatures in cancer tissues is described in **chapter 12.** These experiments were performed in order to set-up a method for registering metabolic signatures between the tumor and stromal compartments of breast cancers to be used in future studies on this subject.

TGF-β is a well-known mediator of stromal activation. The prognostic information of this pathway in the tumor component is unclear. **Chapter 13** describes the prognostic relevance of several components of the TGF-β pathway. This study was performed by investigating the interaction between several TGF-β-related biomarkers in a series of breast cancer patients.

The TGF-β pathway is also involved in the reorganization of the tumor-associated stroma. Previous studies have shown that this organization might partly determine the response to (neo)adjuvant chemotherapy, but this has never been investigated in clinical tissue samples. In **chapter 14,** a novel stromal-based parameter which incorporates the organization of the intratumoral extracellular matrix is described. This parameter was investigated for predictive power of response to neodjuvant chemotherapy in a set of patients treated in the NEOZOTAC trial. Also, the relationship between this stromal parameter and its relationship with TGF-β signaling was investigated.

The findings in this thesis are summarized in English and in Dutch in **chapters 15 and 16** respectively.

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Table 1. Overview of stromal biomarkers with a reported positive association with breast cancer prognosis, including the cut-off for marker expression, hazard for disease recurrence, level of evidence are assessed according to a publication on this matter [89].

Marker	Original study	Evaluation method	Original study cut-off
Caveolin1	Witkiewicz[20]	IHC: negative, < 30%, > 30%	Positive vs absent
PDGF-ßR	Paulsson[25]	IHC: negative, weak, moderate, strong	Strong vs other
p53	Hasebe[31]	IHC: modified Allred score	Allred score 4-8 vs Allred score 0-2
Podoplanin	Schoppmann[34]	IHC: < 10% positive stromal cells, > 10% positive stromal cells	Negative vs positive
ß-arrestin-1	Lundgren[35]	IHC, negative vs low vs moderate vs high	Low vs high
CD105	Martinez[36]	IHC, high vs low vs negative	High vs low or negative
CD10	Iwaya[37]	Positive (> 10%) versus negative (< 10%)	Positive vs negative
Tumor-stroma ratio	De Kruijff[40], Dekker[41]	H&E: stroma-low (≤ 50% stroma), stroma-high (> 50% stroma)	High vs low
Fibrotic focus	Hasebe[43]	H&E: presence of fibrotic focus or absence	Presence vs absence
TACS3	Conklin[46]	SHGI on TMA, analysed by observer	Score 1 vs score2 vs score 3
Tenascin	Ishihara[50]	IHC, strongly positive vs positive vs negative	Negative vs positive
Fibronectin	Fernandez-Garcia[48]	IHC, staining intensity and percentage	Negative vs positive
MMP profiles	Nakopoulou[57]	IHC, staining intensity and percentage	Negative vs positive
TILs	Loi[67]	Percentage of TIL in stroma, LPBC	Increments of 10%, LPBC
MVD	Multiple[73]	Various methods, IHC staining followed by counting (hotspots)	Various methods and cut-offs
Endoglin	Kumar[74]	IHC, counting vessels in hotspots	Lowest quartiles vs other quartiles

Second harmonic generation imaging (SHGI), platelet-derived growth factor receptor (PGDFR), immunohistochemistry (IHC), tumor-associated collagen signatures (TACS), estrogen receptor (ER), tissue mico-array (TMA), tumor-infiltrating lymphocytes (TILs), matrix metalloproteinases (MMP), microvessel density (MVD), tissue inhibitor of metalloproteinases 2 (TIMP2), lymphocyte-predominant breast cancer (LPBC)

Hazard ratio	Possible subgroup	Independent validation	Level of evidence
3.569 (not provided)	Triple-negative/basal-like(21)	Yes(90)	IIC
1.67 (1.08-2.58)	Premenopausal	No(90)	IIIC
3.8-32.2 (depending on subgroup)	Not investigated	No	IIIC
1.782 (1.374-3.862)	Not investigated	Yes(33)	IIC
2.72 (1.06-6.98)	Tamoxifen-treated patients	No	IIIC
6.44 (1.79-23.08)	Not investigated	No	IIIC
3.069 (1.408-6.693)	Not investigated	Yes(91;92)	IIC
2.92 (1.358-6.320)	Triple-negative	Yes, for triple negative(42)	IIB
2.8 (0.8-9.7)	Not investigated	Yes(93;94;94)	IIC
3.04 (1.19-7.76)	ER-positive breast cancer(46)	No	IIIC
Not provided	Not investigated	Yes(49)	IIC
Not provided	ER-positive, ductal carcinoma(47)	No(47)	IIIC
4.58 (1.12-18.79)	TIMP2-negative tumors	Contradictory(55-58;60)	IIIC
0.30 (0.11-0.81)	Triple-negative	Yes(68)	IB
1.99 (1.33-2.98), in meta-analysis	Node-negative	Contradictory results(73)	IIB
Not provided	Early-stage breast cancer[95]	Contradictory results(75;95)	IIIC

Figure 1. The intratumoral stromal compartment of a breast tumor. The tumor that is shown has a low tumor-stroma ratio and is thus stroma-high based on this field. Examples of fibroblasts are marked with black arrows. The dotted lines are drawn in parallel to the orientation of the extracellular matrix fibers. The red arrows indicate examples of inflammatory cells. The encircled structures are blood vessels.