Cyclooxygenase 2 Overexpression and The Collagen-dense Breast Tumor Microenvironment

By

Karla Esbona

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Clinical Investigation)

at the

UNIVERSITY OF WISCONSIN-MADISON

2015

Date of final oral examination: 12/04/2015

The dissertation is approved by the following members of the Final Oral Committee:

Patricia J. Keely, Ph.D., Professor, Cell and Regenerative Biology
Lee G. Wilke, M.D., Professor, Surgery
Mark Burkard, M.D.-Ph.D., Associate Professor, Medicine
Andreas Friedl, M.D.-Ph.D., Professor, Pathology and Laboratory Medicine
Amy Trentham-Dietz, Ph.D., Professor, Population Health Sciences

ACKNOWLEDGEMENTS

Over the past five years I have received tremendous support and encouragement from a great number of people. I am very grateful to Dr. Patricia Keely, who has been a mentor, colleague, and friend. Her guidance has made this a thoughtful and rewarding journey. I will always value her passion for science, her optimism which always motivated me to move forward and her activism to make our community a better place. Likewise, I would also like to thank Dr. Lee Wilke, who has been a wonderful co-mentor and friend, always keeping me motivated and challenging me to think very critically from the clinical perspective of my research. I will always appreciate the fact that both of you were there when I most needed you, inspiring me to be the best I can.

I would like to thank my dissertation committee for their support and valuable insight over the past years as I moved from a project proposal to a completed study. I have to emphasize that I have been very fortunate to work with Dr. Mark Burkard and Dr. Wilke in pursuing my dream of translating my preclinical research in to a human clinical trial and his mentorship towards this project and the next steps in my career will be priceless. Similarly, I am very thankful to Suzanne Ponik, who has been there for me in the tougher and cheerful times as a mentor and most importantly as a friend. In addition, I would like to thank Matt Conklin, David Inman, Brian Burkel and Pepper Schedin for their friendly work and life guidance, thought-provoking questions and making science so much fun.

In addition, through this journey I met wonderful people who helped me with their different expertise. I thank Sandeep Saha, Yanyao Yi, Zhanhai Li and Menggang Yu for their valuable statistical advice and collaborations. Also, I would like to thank Beth Gray at RARC for countless hours of processing mice histology samples and being such a great teacher and friend and Ruth Sullivan from RARC for teaching me how mammary tumors are supposed to look in the mouse and her insight about life and career paths. None of the Vectra imaging would have been possible without the help of Sally Drew from the TRIP lab and her endless advice on histology experimental design and staining. Similarly, I am thankful to Dr. Wei Huang who always was available to teach me and troubleshoot the image analysis software Nuance and InForm. PET imaging and help with analysis would have not been possible without Justin Jeffery from UW Small Animal Imaging Facility. Laura Hogan was very instrumental in giving me insights on my manuscripts as the science editor from ICTR. I will always be thankful to all of these people who were kind enough to offer me their assistance and enthusiasm to collaborate and move this project forward. I would like to recognize the past and present members of the Keely Lab, who are friends and colleagues. I am very thankful for their support and insight in all aspects of work and life and most importantly to make the lab such a fun place to work. It will always be a pleasure to work with you all.

I thank all the individuals, the ones who are still here and the ones who are not here with me anymore, who have influenced my life and kept me strong and motivated to reach all my goals. I thank my parents, family and friends, who have been there for me from day one. I am infinitely thankful to my brilliant husband, Doug Graham, who has been very patient and supportive of my career, for his words of courage, very insightful conversations and helping me write code for

more efficient experimental analysis. Thank you for all of the love, support, encouragement and dedication. Finally, this is a tribute to all the women who have to battle breast cancer, especially the women who donated their tissue for research and made my TMA study possible. Your courage to defeat this disease is my motivation to be involved in breast cancer research.

This work was possible by funding of multiple parties**.** The projects described in this Thesis were supported by the Clinical and Translational Science Award (CTSA) program, through the NIH National Center for Advancing Translational Sciences (NCATS), grants 9U54TR000021 and UL1TR000427. In addition, this work was supported by several National Institutes of Health grants: R01CA114462-02, R01 CA142833, U01 CA143069, and R01 CA179556 (PJK)**.** I am thankful to the University of Wisconsin Translational Research Initiatives in Pathology laboratory, in part supported by the UW Department of Pathology and Laboratory Medicine and UW Carbone Cancer Center grant P30 CA014520, for use of its facilities and services. This work was not funded by an industry sponsor.

TABLE OF CONTENTS

Page

LIST OF FIGURES AND TABLES

Page

ABSTRACT

Cyclooxygenase 2 Overexpression and The Collagen-dense Breast Tumor Microenvironment

Karla Esbona

under the supervision of Dr. Patricia J. Keely and Dr. Lee G. Wilke

at the University of Wisconsin-Madison

Breast cancer is the most common type of cancer in American women, excluding skin cancers, with 200,000 diagnosed cases and causing 40,000 deaths yearly. There are many risks that are concomitant with breast cancer, and increased breast density is associated with 4-6 times higher risk of breast cancer. Collagen I is the most abundant protein in breast stroma and it has been correlated to elevated breast density. In addition, in a rodent model of breast cancer, high levels of collagen present in the tumor microenvironment leads to a more aggressive mammary tumor formation and progression to metastasis. Furthermore, *in vitro* experiments show that elevated collagen density increases expression of PTGS2 (prostaglandin-endoperoxide synthase 2), the gene for cyclooxygenase-2 (COX-2), which is an enzyme involved in the inflammation process. This thesis investigates the role of COX-2 and its impact in tumor progression within a collagen-dense microenvironment. A collagen-dense (HD) mammary cancer mouse model was utilized in two different ways: therapeutically and preventively. In the therapeutic model, tumors in the HD animals were larger, more proliferative, expressed higher levels of COX-2, had more

inflammatory cells and higher levels of cytokines than tumors in the wild-type (wt) mice. Conversely, after treatment with the COX-2 inhibitor, celecoxib, all these effects were reversed. Next, when celecoxib was used as a preventive agent, tumors in HD animals were fewer and smaller than untreated HD animals. Furthermore, the associations between COX-2 overexpression, collagen deposition and high infiltration of tumor-associated macrophages (TAMs) were examined utilizing a human invasive carcinoma tissue microarray containing 371 cases. COX-2 overexpression in the tumor-associated stroma (TS) was associated with increased collagen deposition. Additionally, localization of COX-2 and TAMs within the tumor microenvironment significantly affected patient outcome. For instance, COX-2 overexpression in the tumor nest (TN) was associated with worse patient outcome and $CD68^{+}$, $CD163^{+}$ TAMs and their co-expression with COX-2 in the TS led to poor patient prognosis. Findings of this work suggest that invasive carcinoma patients with collagen-dense tumors, overexpressing COX-2 and/ or have high infiltration of TAM's will benefit from treatment with a COX-2 selective inhibitor, such as celecoxib.

CHAPTER 1

Introduction

The role of mammographic breast density and collagen alignment in breast cancer

Every year over 200,000 women are diagnosed with breast cancer in the Unites States. Advances in early detection techniques and improvement in systemic treatment of early stage breast cancer have led to a small decline in overall breast cancer mortality in the last 20 years. Despite the decline in overall mortality, approximately 40,000 women will succumb to invasive breast carcinoma [1]. Considering these high statistics, it is of great importance to find effective prognostic measures that are present as early as breast carcinoma is detected in the patient, in order for oncologists to take proper action on treating this disease.

There are several risks factors associated with breast cancer including advanced age [2], obesity [3], early age at menarche and advance age at menopause [4], and environmental factors such as smoking at early age [5]. Likewise, high breast density is correlated with a four- to six-fold increased risk of developing breast neoplasia, making it one of the strongest risk factors for breast cancer [6, 7]. Breast density is determined by measuring fat and fibroglandular tissue content in the breast. Increased mammographic density in 50% or more of the breast accounts for a third of breast neoplasia cases [8]. There are several studies that associate increased breast

density with various factors and these vary depending on women's age and menopause status. For instance, increased breast density is associated with longer menopause [9], high levels of estrogen [10], progesterone [9] and the use of hormone replacement therapy (HRT) [11]. In the later study, the effects of HRT used on breast collagen density were dynamic; where the use of HRT was associated with high breast density but discontinuation of HRT promoted the opposite effect. These findings suggest that there are underlying mechanisms where hormones may modulate collagen deposition.

Moreover, several types of breast cancer are associated with high breast density. In ductal carcinoma *in situ* (DCIS), cancer lesions were found in denser regions of the breast [12] and women with higher breast density have a higher risk of getting DCIS [13]. Furthermore, increased breast density has been associated with invasive breast carcinoma [13, 14] and more aggressive tumors that are larger in size, higher lymphatic and vascular invasion and positive node status [15]. In addition, increased breast density has been associated with breast cancer recurrence. Women with DCIS and highly dense breasts, had three times higher risk of getting a later breast cancer, including: DCIS, invasive or ipsilateral cancer [14]. A study by Cil et al. reported that women who had invasive breast carcinoma, increased breast density and did not received radiotherapy had a higher risk of recurrence [16]. Even though a causal relationship for breast density as a breast carcinoma prognostic or recurrence factor has not been established, the association between breast cancer and increased breast density had evoked several new studies that involved possible mechanisms in tumor growth and progression to metastasis.

An important aspect of breast density is the presence of excessive and altered structure of collagen. As mammographic density increases, deposition of extracellular matrix (ECM) components, mainly collagen, increases [17–19]. Several rodent studies have demonstrated evidence on the effects of collagen deposition and alignment in cancer invasion and progression. In a mouse model of mammary carcinoma, changes in stromal collagen organization were characterized. In this study, Tumor Associated Collagen Signatures (TACS) were identified and describe collagen attributes such as morphology and alignment [20]. TACS are categorized into 3 groups with TACS-3 being associated with local tumor invasion. TACS-1 is accumulation of collagen around the mammary tumor lesion. TACS-2 is defined as aligned collagen fibers tangentially around a tumor boundary. TACS-3 is defined as the reorientation of bundles of straightened and aligned collagen fibers perpendicular to the tumor boundary [20]. This breakthrough study demonstrates that stromal collagen remodeling has impact in the tumor microenvironment, promoting a more invasive cellular phenotype.

Until recently, a causal link between collagen deposition and tumor formation was not established, in part because suitable models did not exist. Thus, a mouse model of mammary carcinoma and increased collagen deposition, MMTV-PyVT x Col1a1^{tm1jae} (Figure 1.1), was created to evaluate the effects of highly dense collagen on the mammary tumor microenvironment [21]. To produce mice with high levels of collagen, the collagenase cleavage site in the collagen alpha-1 chain was removed [22]. This prevented collagen to be degraded by collagenases, yielding increased levels of stromal collagen in the mice. By utilizing the MMTV-PyVT mice bearing Col1a1^{tm1ja} transgene, Provenzano et al. found that these mice had higher

incidence of TACS-3 when compared to wildtype mice, indicating that collagen alignment is associated with density in the mouse mammary tumor [21]. In addition, tumor incidence, invasion, and metastasis increased three-fold [21], suggesting that increased levels of collagen and increased alignment are part of a mechanism that promote more aggressive mammary tumors. Moreover, Conklin et al. tested over 200 breast carcinoma patient samples using second harmonic generation (SHG) microscopy to qualitatively analyze collagen alignment [23]. They found that aligned collagen, specifically TACS-3, is a predictor of poor survival in human breast neoplasia. Together, these studies suggest that an increase in collagen density and alignment is part of the mechanism by which high breast density is correlated with increased risk in humans.

Furthermore, a link between pregnancy-associated breast cancer and breast density has been established. Women diagnosed with breast cancer within a period of five years after postpartum have a worse prognosis for this disease [24–26]. Involution of the mammary gland is a multi-step process that requires extensive cellular apoptosis and tissue remodeling in order to regress the lactating mammary gland to a pre-pregnant state [27]. Throughout pregnancy, the epithelium of the breast follows an extraordinary process of proliferation to produce milk ducts and milk secretion. After lactation is complete, the mammary gland epithelium and stroma reverts to resemble the pre-pregnant state [28]. However, in post-partum breast cancer, tissue remodeling and several cellular processes that are involved in mammary involution are altered, causing a tumor promoting environment rather than regression to a pre-parous state [29]. A recent study found that mammary gland involution promotes tumor progression in a mouse model of post-partum involution [30]. Notably, increased stromal collagen deposition, higher levels of COX-2

and tumor promotion to a more invasive phenotype were characteristics associated with tumors that arose from involuting mice when compared to nulliparous mice [30]. Together, these findings suggest that the involuting mammary gland microenvironment, promotes high levels of collagen deposition and inflammation, leading to tumorigenesis and a more invasive phenotype which may explain the high risk in mortality for the post-partum breast cancer population.

The role of the inflammatory stroma in breast cancer

The inflammatory response is a cascade of many chemical signals that elicit host response to aid tissue injury. This process starts first with the activation and migration of neutrophils to the site of injury, followed by monocytes which differentiate into macrophages. Then activated macrophages and mast cells will secrete several growth factors, cytokines and matrix remodeling proteins that promote ECM deposition by fibroblasts and migration and proliferation of endothelial, epithelial and mesenchymal cells to repair the normal tissue microenvironment [31]. Besides monocytes, dendritic cells, which play an important role in adaptive immunity, process and present foreign antigens to $CD4^+$, $CD8^+$ T lymphocytes and B lymphocytes to start clonal expansion leading to adaptive immunity [32]. Several cytokines elicit attraction and recruitment of leukocytes which in turn control other cells and dictate the fate of the inflammatory process. For instance, tumour necrosis factor-α (TNF-α) and tumor growth factor-β1 (TGF-β1) control several aspects of inflammatory cell populations including cell growth, proliferation,

differentiation and apoptosis [33]. The persistence of cytokines secreted at the injury site, lead to chronic inflammation and eventually the alteration of any of these factors could promote disease.

Many reports have shown a relationship between cancer development and the progression and activation of the chronic immune system [31, 34, 35]. It is believed that after tumor formation, immune response processes will convert the tumor microenvironment to a tumor promoting milieu. For example, the activation of mesenchymal cells such as fibroblasts in normal inflammation is also observed in tumor stroma, which elicits a fibrosis response in the stroma [36, 37]. Besides mesenchymal cells, tumor epithelial cells are stimulated by the secretion of pro-inflammatory factors in the tumor microenvironment. For instance, chronic inflammation caused by pathogens or chemical irritants has been associated with multiple types of neoplasms, including gastric, liver and pulmonary cancers. Infection with the bacterium *Helicobacter pylori* has been associated with gastric cancer; human papillomavirus (HPV) infection is concomitant to the incidence of cervical cancer; and Chrohn's disease or chronic ulcerative colitis are associated with colorectal neoplasm [38]. In the normal inflammatory process, lymphocytes generate reactive oxygen and nitrogen species to fight infection [31]. However, there is evidence that persistence of inflammatory cells in the inflammatory tissue site, will cause DNA damage in epithelial cells undergoing proliferation through the production of reactive oxygen and nitrogen species, which in turn generate a mutagenic agent called peroxynitrite [39]. Thus, diseases that originate from multiple or persistent infections will cause chronic inflammatory responses, leading to permanent DNA alterations which will promote an environment for tumor development.

Furthermore, there is evidence that the immune response towards a tumor is dynamic and multifaceted and could dictate different outcomes depending on pro or anti-tumor behavior of inflammatory cells. Adaptive immunity mediated by T and B lymphocytes are thought to be protective against tumor development while innate immunity mediated by Th2, macrophages, neutrophils and mast cells results in tumor development and cancer progression [40, 41]. Tumor-associated macrophages (TAMs) form part of the inflammatory cascade in cancer. They differentiate from monocytes primarily recruited by the chemokine, monocyte chemotactic protein 1 (MCP-1) [31]. There are several reports indicating that TAMs have a dual role in breast cancer. They will elicit an anti-tumorigenic effect by activation of IL-2, interferon and IL-12 while also promoting a tumorigenic environment by secreting diverse cytokines, growth factors and proteases [31]. In addition, TAMs have been shown to aid in the processes of angiogenesis, degradation of the ECM, promotion of breast tumor epithelial cell migration and metastasis [42, 43]. Additionally, a study using a PyVT mouse mammary carcinoma model containing a mutation in the gene for colony-stimulating factor 1 (CSF-1), crucial for macrophage growth, reported that mammary cancer development was diminished and metastasis was abolished [44]. Furthermore, elevated neutrophil cell populations in blood circulation is commonly observed in mouse models of breast carcinoma, suggesting they play a role in the tumor inflammatory process [45]. Like TAMs, tumor-associated neutrophils (TANs) alter the ECM and promote cell proliferation, migration, and metastasis [46–48]. A recent study, reports that increased neutrophil-to-lymphocyte ratio in solid tumors, including breast solid tumors, has been associated with worse prognostic outcome for cancer patients [49]. These observations suggest that lymphocyte and leukocyte recruitment to the tumor microenvironment is essential in the

growth and spread of mammary tumors. However, further studies are needed to define mechanisms of intervention to target cancer-specific inflammation.

Inflammatory cells are not the only players in the mammary tumor inflammation cascade. Multiple secretion of cytokines and growth factors can stimulate cell proliferation in tumor cells and stromal fibroblasts [50]. For instance, leukocytes, monocytes and macrophages can secrete several interleukins, TNF-α, TGF-β, as well as proteolytic enzymes that remodel the ECM and enhances proliferation of fibroblasts, as well as the survival of tumor cells by promoting angiogenesis, cell migration and metastasis [32, 51]. Fibroblasts are stromal cells that secrete ECM proteins such as fibronectin and collagen. Normal fibroblasts are part of the stromal cell population and they tend to express higher levels of vimentin [50]. In contrast to normal fibroblasts, cancer-associated fibroblasts (CAFs) [52] have been associated with the stroma of breast cancer tumors and they enhance tumor growth and metastasis through the production of growth factors like TGF-β and ECM proteins [53]. CAFs are activated fibroblasts expressing α-smooth muscle actin (α-SMA) and exhibiting higher collagen contractility [37, 50]. Up to 80% of stromal fibroblast have the activated phenotype in breast cancer tumors and have been shown to produce growth factors and ECM proteins that promote proliferation and survival of tumor cells [36]. Extensive work by Orimo et al. demonstrated that in invasive carcinoma, CAFs secrete elevated levels of SDF-1 (stromal cell-derived factor-1) which promotes the recruitment of endothelial progenitor cells into the tumor epithelium, enhancing tumor vascularization [37]. In addition, SDF-1, MCP-1 and vascular endothelial growth factor (VEGF) secretion by CAFs promote tumor growth via paracrine signaling to breast tumor cells and activation of

inflammatory cells [37, 38]. Overall, these studies provide compelling evidence that chronic inflammation contributes to the initiation and progression of mammary carcinoma. In addition, the secretion of growth factors, cytokines and ECM remodeling proteins by stromal cell populations indicate that changes in the stroma of the tumor microenvironment are as significant as DNA alterations in tumor epithelial cells, and they both contribute to the process of tumor formation and progression.

Significance of COX-2 inhibition in breast cancer

The most important clinical evidence that explains a causal link between chronic inflammation and progression to cancer, comes from epidemiological research describing the used of non-steroidal anti-inflammatory drugs (NSAIDs) and their protective effect towards neoplasms in patients that have predisposition towards cancer [55-64]. These studies found that long-term use of NSAIDs like aspirin or ibuprofen and selective COX-2 inhibitors, significantly reduced the risk of colorectal, gastric, esophageal, breast, among other carcinomas [54, 57]. NSAIDs block the body's production of fever, pain and inflammation by inhibiting cyclooxygenases. Non-selective NSAIDs like aspirin or ibuprofen irreversibly inhibit both COX-1 and COX-2 enzymes by acetylation and blocks platelet synthesis of prostaglandins and thromboxane A2 [31]. Celecoxib, also called Celebrex® by Pfizer Inc., is the only selective COX-2 inhibitor [66] currently approved by the FDA to be used in the United States. Celecoxib with its anti-inflammatory, analgesic, and antipyretic therapeutic effects, has less risk for endoscopic mucosal

injury [67] and decreased incidence of cardio-renal toxicity [68] when compared with ibuprofen. Moreover, there is evidence that NSAIDs may use other mechanisms of action besides cyclooxygenases, since NS-398, a COX-2 selective inhibitor was able to induce apoptosis on colorectal carcinoma cell lines containing or lacking expression of COX-2 [69]. Alternative mechanisms include apoptosis by activation of caspace-3 and -9, cell cycle progression, reduction of carcinogen activation and promotion of the immune surveillance [70]. These findings support the association between cancer formation and chronic immune response, however, there are no clearly defined mechanisms by which NSAIDs inhibit tumor development in breast cancer.

Furthermore, COX-1 is a constitutively expressed cyclooxygenase enzyme and plays a biological role in platelet formation, protection of gastrointestinal mucosa and renal hemodynamics [66]. COX-2 is an inducible enzyme that is activated at sites of injury as part of the inflammation response that generates prostaglandins from arachidonic acid, which trigger inflammation [71]. Cyclooxygenases, with their peroxidase activity, convert arachidonic acid into prostaglandin H2 (PGH_2), which in turn activates prostaglandin E synthase (PGES) to initiate prostaglandin E2 (PGE2) production [71]. PGE2 is a lipid mediator that is produced enzymatically to achieve rapidly higher levels of this molecule in response to inflammation. There are four G-coupling receptors that regulates PGE2 activity and stimulate downstream signaling transduction processes: EP_1, EP_2, EP_3 and EP_4 [72]. Besides its role in inflammation, PGE2 activity influences gastric mucosal integrity, cellular proliferation, promotes bone formation, inhibits sleep, among others [73]. On the other hand, COX-2 through biosynthesis of PGE2 is a contributor to many neoplasms. Expression levels of COX-2 are elevated in breast, colorectal

and other carcinomas in comparison to normal tissue and increased levels of COX-2 lead to overproduction of PGE2 [74]. COX-2 can also be stimulated by cytokines, interleukins, hormones and growth factors such as VEGF, TNF-α and TGF-β [75]. There are reports that specific mutations in *Wnt*, *Ras* and *HER2/neu* may cause COX-2 overexpression [76, 77]. In addition, PGE2 signaling also up-regulates COX-2 mainly through PGE2's EP_2 and EP_4 receptors, creating a positive feedback loop where it increases its own biosynthesis [78]. EP_2 and EP_4 receptors elevate cAMP levels, promoting activation of the transcription factor CREB, which in turn alters gene expression level of COX-2 and VEGF [78]. Besides, there is evidence that increased levels of the urinary PGE2 metabolite, PGE-M, are associated with an increased risk of tumor development and progression to metastasis in breast cancer patients [79–81]. Additionally, these studies found that levels of urinary PGE-M were diminished when both healthy humans and breast cancer patients had treatment with NSAIDs, including COX-2 selective inhibitors. This indicates that the majority of PGE2 biosynthesis in these subjects were derived from COX-2. Taken together, these evidence suggest that COX-2 through PGE2 has a major contribution towards cancer and not only COX-2 but also PGE2 receptors may be an important therapeutic target for breast cancer.

There have been extensive evidence on the effect of COX-2 inhibition in rodent models of mammary carcinoma. Celecoxib suppressed the incidence, burden, and volume of malignant tumors in breast cancer rat models. In a chemoprevention study, rats were pretreated 7 days with celecoxib before 7,12-dimethyl-benz(a)anthracene (DMBA) was administered, then after celecoxib therapy and DMBA continued for 105 days, fewer tumors and diminished tumor growth of up to 68% was observed [82]. Additionally, a dose-dependent relationship was

established between celecoxib and mammary tumor incidence. As rats were fed a diet of celecoxib of 500, 1,000, or 1,500 ppm, tumor incidence resulted in 55%, 45%, and 25%, respectively [82]. To evaluate the chemotherapeutic activity of celecoxib, DMBA-treated rats were randomized to control or celecoxib following 6 weeks of DMBA treatment. Tumor growth increased 5 times in comparison to baseline in the control group whereas in the celecoxib group, tumor regression resulted in 90% of animals [83]. In addition, in a transgenic mouse model, COX-2 overexpression was sufficient to promote mammary carcinoma in the normal mammary gland [84]. Moreover, Harris et al, found that COX-2-derived PGE2 can also stimulate aromatase transcription leading to the production of estrogens [85, 86]. Based on the association between PGE2 and aromatase gene expression, data demonstrates that the combination of exemestane, an aromatase inhibitor, and celecoxib is more effective than either agent alone in reducing tumor burden, volume and incidence in the DMBA animal model [87]. Data reveals that while celecoxib restores the overexpression of aromatase to physiologic production, the addition of exemestane is required to block the activity of aromatase enzyme below basal levels [87, 88]. Thus, these pre-clinical data, indicate that COX-2 inhibition with celecoxib may be a great way to prevent and treat different types of human breast cancer.

Furthermore, several lines of evidence connect increased COX-2, with the effects of increased matrix density. Increased levels of COX-2 are found in the post-partum involuting mammary gland, DCIS and invasive breast cancer and high COX-2 levels are associated with increased mammographic density [89] and increased collagen deposition [30, 90]. COX-2 contributes to the increased levels of aligned collagen that are found during involution, and remodeling of the breast ECM promotes mammary cell metastasis and are associated with progression of DCIS

[91]. In addition, mammary epithelial cells in high density collagen matrices upregulate *PTGS2* (prostaglandin-endoperoxide synthase 2), the gene for COX-2, by 4-fold [92]. Even though pre-clinical data demonstrate that celecoxib is an effective chemopreventive agent and significantly delays tumor formation, the role of COX-2 and collagen remodeling in invasive breast cancer is still unclear.

COX-2 over-expression is observed in 40% of human invasive breast carcinoma cases, it is implicated in DCIS recurrence and correlates with poor prognosis [93]. Epidemiological studies found that COX-2 inhibition by NSAIDs is associated to decreased breast cancer recurrence and increased survival [94–96]. Based in these findings, some clinical trials have evaluated the use of celecoxib alone or in combination with chemotherapy regimens in different breast cancer settings [97]. Overall, when combination therapy of celecoxib and exemestane was used, it had similar or better efficacy compared with exemestane monotherapy alone using the end points of progression-free survival, time to progression, overall response and clinical benefit rates. Most clinical trials lasted about two years and were well tolerated except for some cardiovascular adverse events at longer time frames and at the higher doses [64, 65, 98–101]. Additionally, there are reports of three clinical trial studies with biological endpoints. The first study by Brandao et al., shows significant anti-tumor transcriptional response after a two to three-week treatment with celecoxib, in invasive carcinoma patients [102]. The other two trials show no difference with celecoxib alone [103] or in combination with exemestane [104] compared to placebo. However, the second study conclusions cannot be taken fully into consideration, since the trial ever reached statistical power for lack of enrollment and was terminated early. In this

study celecoxib did not significantly affect apoptosis, COX-2, estrogen receptor (ER) or progesterone receptor (PR) expression, and there was only modest evidence for a biological effect of celecoxib in primary breast cancer. The third study demonstrated that in ER-positive DCIS, celecoxib treatment had no effect on proliferation or apoptosis alone, or in combination with exemestane. It could be possible that celecoxib could work best in invasive carcinoma cases of patients with dense breasts and positive for ER or PR. To date, there is no clinical study addressing COX-2 inhibition with celecoxib and its biological response in relation to matrix density in a specific patient population of ER and PR positive invasive breast carcinoma.

Mammary carcinoma model for pre-clinical study of COX-2 inhibition in the collagen-dense tumor microenvironment

In order to study the effects of COX-2 and its inhibition in the collagen-dense tumor microenvironment, a mammary carcinoma mouse model containing elevated levels of stromal collagen resembling high breast density in humans is desirable. The work of Provenzano et al. in creating such rodent mammary carcinoma model [21], helped explained the association between elevated breast density and the initiation and progression of mammary carcinoma.

While there are numerous mouse models of mammary carcinoma, mice carrying the mouse mammary tumor virus (MMTV), is one of the most established and widely used in experiments.

Mice containing MMTV have a transforming retrovirus that causes mammary tumors and this virus is inherited as an integrated retrovirus through the germline or as an exogenous retrovirus via transmission through lactation [105]. There are several MMTV models, however, MMTV-PyVT (polyomavirus middle T antigen) is a murine signal transduction breast cancer mouse model [106] and it is the most broadly utilized mouse model for its prompt development of tumors and progression to metastasis. Premalignant adenocarcinomas can be detected within the first month of age [107]. Furthermore, MMTV-PyVT mice carrying the Col1a1^{tm1ja} transgene were created to produce mice with high levels of collagen deposition. In this mouse model the cleavage site for collagenase in the collagen alpha-1 chain was removed, preventing collagen to be degraded [22]. MMTV-PyVT mice carrying the Col1a1^{tm1ja} transgene usually develop palpable tumors after 9 weeks of age, measuring around 2mm in diameter. The MMTV-PyVT x Col1a1^{tm1jae} mouse model typically develops multiple mammary gland tumors and progression to metastasis in a more rapid manner. Therefore, by utilizing the MMTV-PyVT mice carrying the Col1a1^{tm1ja} transgene, it is possible to carry pre-clinical studies that examine the role of COX-2, inflammation and breast density.

Motivation for studying increased COX-2 expression levels in the collagen-dense tumor microenvironment

Yearly, in the United States, 200,000 women are diagnosed with breast carcinoma and 40,000 will die from this disease. New advances in breast cancer research will require understanding of

breast cancer and the tumor microenvironment at the molecular level. Inhibition of COX-2 and analysis of its effect in the breast cancer tumor microenvironment provide one such fruitful therapeutic target. There is emerging evidence in post-partum breast cancer that there may be a link between COX-2 expression levels and collagen deposition in the breast [30], however, there are no studies evaluating the role of COX-2 and dense collagen stroma in invasive breast carcinoma.

Moreover, there is evidence for the role of COX-2 and PGE2 in breast cancer inflammation. Several studies describe the effects of cytokines, interleukins and growth factors on several inflammatory cells and on COX-2 creating a tumor-promoting environment. However, no clear links between the tumor inflammatory response and high levels of collagen deposition or altered collagen structure have been established in breast cancer. If high COX-2 levels in association with altered inflammatory response and increased collagen density are connected in invasive breast carcinoma, then this relationship could serve as a prognostic tool for patient disease outcome and election of more suitable therapies depending on the disease prognosis.

Hypothesis

From all the evidence described in this chapter, it was hypothesized that high breast density stimulates increased COX-2 expression, which promotes tumor initiation and progression. The

goal of this study was to describe the role of COX-2 and inflammation in response to collagen density in the breast tumor microenvironment. Here we utilize both, a preventive and a therapeutic collagen dense mouse mammary carcinoma model (MMTV-PyVT x Col1a1^{tm1jae}) to establish whether COX-2 inhibition with celecoxib in response to collagen density is effective as chemopreventive and therapeutic breast cancer treatment. Additionally, we describe changes in the inflammatory response with respect to collagen density in mammary tumors. Finally, findings from the pre-clinical animal models were translated into a human invasive breast carcinoma study to measure associations between high COX-2 levels, inflammatory cells and collagen deposition with patient outcome. The chapters that follow in this thesis describe the experiments and results that were designed to address our hypothesis and to contribute further understanding to the role of high breast density and inflammation in the initiation and progression of invasive breast cancer.

Figure 1.1

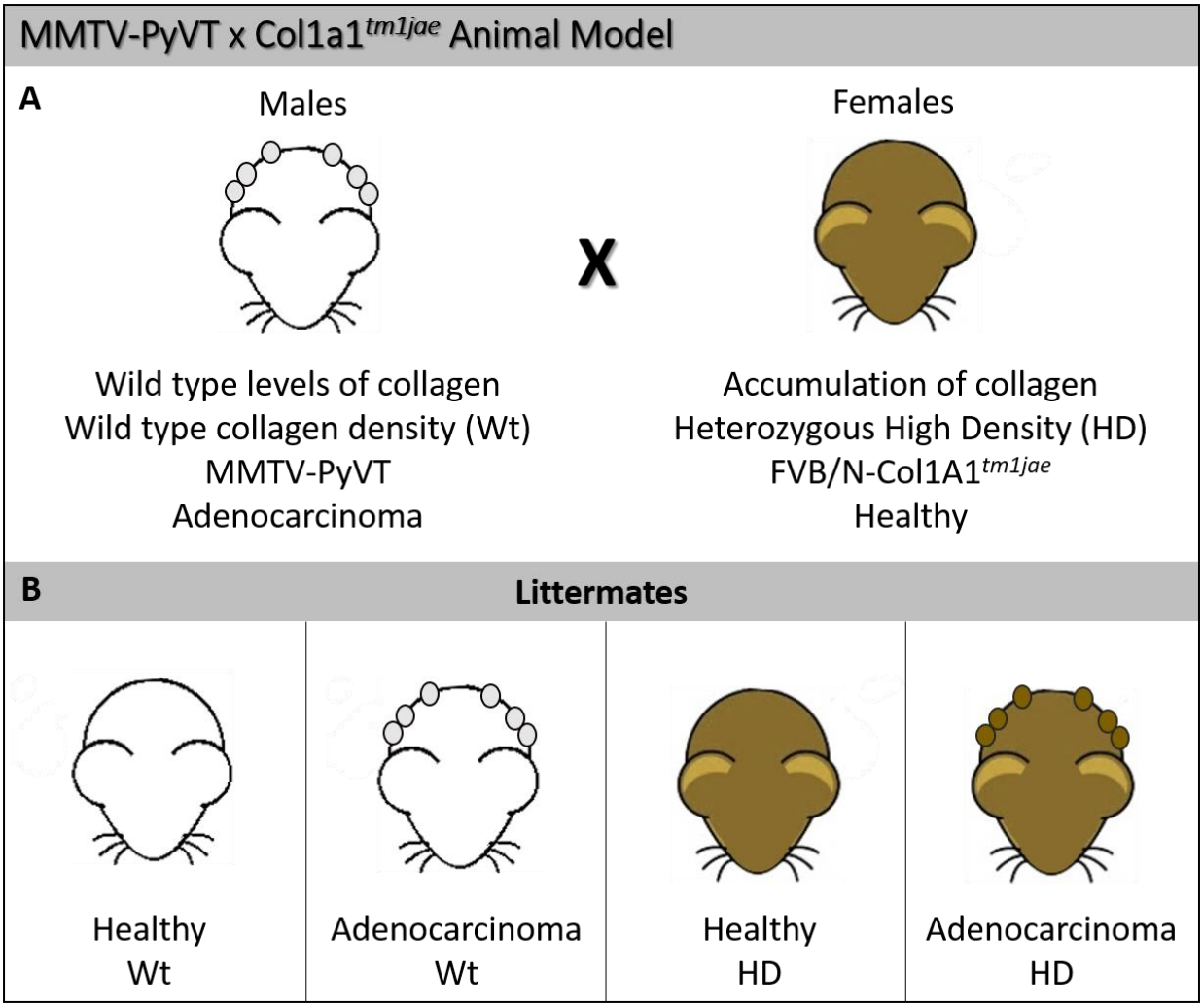

Figure 1.1. MMTV-PyVT x Col1a1^{tm1jae} mouse model of mammary carcinoma. A. MMTV-PyVT male mice were crossed with female FVB/N mice carrying the Col1a1^{tm1jae} transgene **B.** Nulliparous female littermates from the crosses in **A** were used for experiments in Chapter 2. Mammary glands were extracted from "Healthy Wt and HD" and tumors were extracted from "Adenocarcinoma Wt and HD" to make study comparisons.

Chapter 2

COX-2 modulates mammary tumor progression in response to collagen density

Karla Esbona, David Inman, Sandeep Saha, Justin Jeffery, Pepper Schedin, Lee Wilke, and Patricia Keely

Manuscript submitted to Breast Cancer Research.

Abstract

Introduction: High breast density correlates with increased collagen in breast tissue, and high levels of collagen in the MMTV-PyVT x Col1a1^{tm1jae} mouse promote accelerated mammary tumor formation and progression. Previous gene expression analysis *in vitro* suggests that increased collagen density elevates expression of PTGS2 (prostaglandin-endoperoxide synthase 2), the gene for cyclooxygenase-2 (COX-2).

Methods: To understand the role of COX-2 in tumor progression within a collagen-dense microenvironment, we utilized the MMTV-PyVT x Col1a1^{tm1jae} collagen-dense mammary cancer mouse model both preventively and therapeutically. Animals received treatment of celecoxib, a specific COX-2 inhibitor, or placebo. Mammary tumors were examined for COX-2, inflammatory and stromal cell components and collagen deposition through immunohistochemistry, immunofluorescence, multiplex cytokine ELISA and tissue imaging techniques.

Results: Tumors in the Col1a1^{tm1jae} (high density, HD) mice were larger, more proliferative, and expressed higher levels of COX-2 and PGE2 than tumors in wild-type (wt) mice. Treatment with celecoxib decreased expression levels of COX-2, PGE2, and Ki-67. Several cytokines were over-expressed in HD tumors compared to wt, and celecoxib treatment prevented their over-expression. Furthermore, macrophage and neutrophil recruitment were enhanced in HD tumors, and this effect was inhibited by celecoxib. Notably, COX-2 inhibition reduced collagen deposition. Finally, when celecoxib was used as a preventive agent, tumors in HD animals were fewer and smaller than untreated HD animals.

Conclusion: These findings suggest that COX-2 has a direct role in modulating tumor progression in tumors arising within collagen-dense microenvironments, and suggest that COX-2

may be an effective therapeutic target for women with dense breast tissue and early stage breast cancer.

Introduction

Breast cancer is the most common invasive cancer in women with upwards of 40,000 deaths annually in the United States [1]. Women who have over 75% mammographic breast density have a more than four-fold increased risk for developing breast cancer, making it one of the most significant risk factors for this disease [6, 7, 109, 110]. High breast density correlates to higher amounts of collagen fibers in the breast tissue [12] and a key feature of this density/collagen association is the presence of excessive and altered collagen structure and distribution. Our group has defined changes in collagen structure that manifest as bundles of straightened and aligned collagen fibers oriented perpendicular to a tumor boundary, which we term Tumor Associated Collagen Signature-3 (TACS-3) [20]. We showed increased TACS-3 corresponds to decreased survival for breast cancer patients [23]. In addition a transgenic mouse model with increased collagen deposition (MMTV-PyVT x Col1a1^{tm1jae}) has three-fold increased rates of tumor incidence, invasion, and metastasis [20, 21]. Finally, mammary epithelial cells cultured in a collagen-dense matrix have a four-fold higher expression of PTGS2 (prostaglandin-endoperoxide synthase 2), the gene for cyclooxygenase-2 (COX-2), compared to the same cells in a non-dense matrix [30, 92]. Despite the accumulation of data suggesting a role for increased collagen in mammary tumor progression, the molecular mechanisms for the increased risk and subsequent cancer development are unknown.

COX-2 over-expression is observed in 40% of invasive breast carcinoma cases and correlates with poor prognosis [97, 111, 112]. Unlike the constitutive activity of COX-1, COX-2 is an inducible enzyme that synthesizes prostaglandins and is activated at sites of injury as part of the inflammation response [71]. In rodent models of mammary neoplasms, COX-2 over-expression promotes tumor formation and progression to metastasis, in addition to increased angiogenesis, cell migration, and invasion [30, 84, 113–115]. Recent findings suggest that the increase in collagen density in the breast cancer tumor micro-environment may relate to increased levels of COX-2. In a preclinical study of women with increased mammographic density, samples from adjacent normal breast had elevated COX-2 expression compared to samples from women with low mammographic density [116]. The Schedin lab reported that high COX-2 expression and increased levels of aligned collagen are the driving force for the development of ductal carcinoma *in situ* in a postpartum mammary gland involution mouse model. Moreover, treatment with non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenases, reversed this effect [30]. Celecoxib is a selective NSAID that specifically inhibits COX and is the only COX-2 inhibitor currently approved by the FDA for use in the U.S. [66]. Several studies have demonstrated that NSAIDs decrease the risk of cancer development [58, 62–64, 117–120]. Specifically, celecoxib prevents sporadic colorectal adenomas [121] and several clinical trials have evaluated the use of celecoxib alone or in combination with chemotherapy regimens in breast cancer settings [97]. Despite these associations, the role of COX-2, collagen remodeling and development of an invasive breast cancer is still unclear.

Inflammation is emerging as an important mediator of tumor progression. Many reports have shown a relationship between cancer progression and activation of the chronic immune system

[40, 122, 123]. It is believed that after tumor formation, immune response processes change the tumor microenvironment to a tumor promoting milieu. Tumor-associated macrophages (TAM) stop the activated immune system by secreting anti-inflammatory cytokines [124], and play important roles in many aspects of tumor growth and progression [46]. Macrophages can polarize into M1 and M2 phenotypes, although the distinction becomes less clear in TAMs, which often demonstrate both M1 and M2 features [46]. Likewise, tumor-associated neutrophils (TAN) can polarize in response to chronic inflammation signaling in the tumor microenvironment [125]. Both TAMs and TANs can promote tumor progression by remodeling the extracellular matrix (ECM) and by increasing tumor cell proliferation, migration, and metastasis [46–48]. Moreover, cytokines released by both tumor cells and TAMs can activate stromal fibroblasts, the main depositors of ECM and regulators of inflammation [126, 127]. The resulting activated cancer-associated fibroblasts (CAFs) enhance tumor growth and metastasis through the production of growth factors and ECM proteins that further promote proliferation and survival of tumor cells [36]. To date, there is no clarity regarding the role of these inflammatory and stromal cell populations in a dense breast tumor microenvironment and how they relate to high expression of COX-2.

In this report we tested the hypothesis that breast density promotes high COX-2 levels, which support tumor growth and progression. The goal of this study was to describe the role of COX-2, inflammation, and density in the breast tumor microenvironment using a collagen dense mouse mammary carcinoma model. We found that COX-2 and prostaglandin E2 (PGE2) levels are elevated in the collagen dense (HD) tumors, and COX-2 inhibition with celecoxib decreases these expression levels. Treatment with celecoxib significantly diminished tumor growth and

proliferation in the collagen dense tumors. Many cytokines were over-expressed in HD tumors, and COX-2 inhibition reversed their over-expression. Results from this cytokine panel led us to look closer at different immune and stromal cell populations and their response to COX-2 inhibition in HD and wild-type (wt) tumor microenvironments. We found that macrophage and neutrophil recruitment are enhanced in HD tumors and enhancement was blocked by COX-2 inhibition. In addition, celecoxib decreased α-SMA^{+} fibroblast numbers in HD tumors. Collagen deposition in both wt and HD tumor microenvironments was diminished with celecoxib; however, normal mammary glands were not affected by COX-2 inhibition. Together, these findings suggest that COX-2 has a direct role in modulating tumor progression in dense matrices, which promote a more invasive cancer effect. COX-2 may be an effective therapeutic target for women with dense breast tissue-associated breast cancer.

Results:

COX-2 Expression Levels are Elevated in Collagen Dense Tumors

To assess whether COX-2 is involved in tumor growth and enhanced in a collagen-dense tumor microenvironment, we utilized our previously characterized transgenic mouse model of increased stromal collagen based on the Col1a1^{tm1jae} mouse. This transgenic line has a mutation in the collagenase cleavage site of the α1 chain of collagen I, leading to increased collagen accumulation and tumor progression [21]. For this study, MMTV-PyVT mammary gland mice were introduced into a wt or MMTV-PyVT x Col1a1^{tm1jae} (HD = high density collagen) background.

Tissue from 14-week-old nulliparous female mice was used for quantitative immunofluorescence (IF) to detect COX-2 and its product PGE2 to assess expression levels with respect to mammary tumor collagen density. We measured both COX-2 and PGE2 levels in the stroma adjacent to tumors and epithelium from tumors to see if their expression is predominant within a particular tissue compartment. Col1a1^{tm1jae} mouse mammary tumors contained higher stromal COX-2 expression levels compared to the wt tumors (Figure 2.1A and C). Similarly, collagen-dense tumors had a small increase in stromal PGE2 expression levels (Figure 2.1B and D).

COX-2 Inhibition with Celecoxib Diminishes Tumor Growth, Proliferation and Progression to Metastasis

To test whether COX-2 inhibition reverses COX-2 and PGE2 expression levels, we treated wt and HD tumor mice with 0.2mg celecoxib or vehicle (5% methyl cellulose) for 21 days (Figure 2.2A). This dose was selected because it is comparable to the human dose of 600mg a day (when using a liner scale, 10mg/ kgbw) and no serious adverse side effects in humans have been reported at this dose for this short period. Mice were started on a daily treatment at 11 weeks of age and tissues were collected at 14 weeks of age. At the 11 week time point, tumors are established and uniformly palpable among all experimental animals. Four treatment arms were created for this study: wt vehicle, wt celecoxib, HD vehicle, and HD celecoxib. We found HD mice had larger tumors compared to wt mice and that COX-2 inhibition with celecoxib diminished tumor growth only in collagen dense tumors (Figure 2.2 B-C). Next, we collected lung tissue to quantify lung metastasis; despite a trend toward increased metastasis in HD mice compared to wt mice, there was no statistically significant difference (Figure S2.1). Since there

was a significant different in tumor growth, we measured cell proliferation in these mouse mammary tumors. Immunohistochemistry (IHC) to detect the proliferation marker Ki-67 demonstrated that celecoxib diminished proliferation both in wt and HD mammary tumors (Figure 2.2 D-E). While COX-2 levels are significantly increased in HD tumors compared to wt, expression levels of COX-2 were significantly decreased in wt and high density collagen mouse tumors when treated with celecoxib (Figure S2.2 A-B). Moreover, PGE2 expression levels were significantly elevated in collagen-dense tumors and inhibition with celecoxib reversed this effect in both wt and HD tumors. These findings suggest that COX-2 has a role in tumor growth and progression and that its inhibition has differential effects in mammary tumors arising in dense collagen compared to the wt milieu.

Inflammatory Cytokines Regulated by Density and COX-2 Inhibition

Cancer, inflammatory and stromal cells can secrete and respond to cytokines that stimulate growth, diminish apoptosis and enable invasion and metastasis in the tumor microenvironment. Having demonstrated that dense collagen tumors have increased expression of COX-2, and its over-expression enhanced tumor growth, cell proliferation and metastasis, we investigated the role of COX-2 in inflammation within collagen dense tumor microenvironments using a quantitative chemiluminescence assay to detect the expression of 23 cytokines. Samples from three mouse tumors per each of the study arms were pooled and cytokines were monitored simultaneously for their expression level relative to collagen deposition and COX-2 inhibition with celecoxib. Most cytokines were increased by 2-fold or more in HD tumors compared to wt tumors. Cytokines with substantially increased expression included Interlukin-2 (19-fold), β-

Nerve Growth Factor (β-NGF) (17-fold), Interlukin-4 (13-fold), Platelet Derive Growth Factor (8.7-fold) and Interlukin-17A (IL-17A) (5-fold) (Figure 2.3). COX-2 inhibition with celecoxib diminished overall cytokine expression levels in both wt and HD animals (Figure 2.3). These results indicate that there is an effect of high collagen density in altering cytokine expression levels, and that these high cytokine expression levels are decreased by COX-2 inhibition with celecoxib.

To validate the cytokine expression in the epithelium and stroma of the tumors, we performed quantitative IF for two highly expressed cytokines. We studied overall β-NGF distribution in the tumor microenvironment. β-NGF is secreted by epithelial cells, macrophages, neutrophils, and neurons. It is expressed in 80% of breast cancers and it activates the survival and proliferation of tumor cells [128, 129]. Overall, we found that β-NGF expression was higher in the tumor epithelial compartment than the stroma, and that there was more stromal β-NGF in HD tumors compared to wt tumors. Treatment with celecoxib reversed β-NGF in HD tumors, within both the epithelial and stromal compartments (Figure 2.4 A and C).

Because we observed high levels of IL17A in the cytokine array data, we characterized the expression of IL-17A receptor (IL-17A-R) as a measure of cell populations that may be recruited by this cytokine. We observed that IL-17A-R is mostly expressed by cell populations in the stromal compartment, and was significantly elevated in HD tumors compared to wt tumors (Figure 2.4 B and D). Celecoxib treatment inhibited IL-17A-R-expressing cell populations within the stroma of the collagen dense tumor microenvironment (Figure 2.4 B and D). Together,

these findings indicate that tumor density and COX-2 expression levels play a role in cytokine expression, perhaps via inflammatory pathways.

Collagen Dense Tumors and COX-2 Regulate Macrophage and Neutrophil Populations

To characterize cytokine-mediated recruitment of inflammatory and stromal cell populations to the tumor microenvironment, we performed quantitative IF to identify populations of mature macrophages (F4/80 positive) and neutrophil granulocytes (Ly6g positive). Stromal F4/80 macrophages were significantly increased in HD tumors compared to wt tumors. Treatment with celecoxib diminished F4/80+ macrophage numbers in collagen dense tumors (Figure 2.5 A and C). Moreover, epithelial $Ly6g^{+}$ neutrophils were also increased in the collagen dense tumors compared to wt tumors and celecoxib diminished $Ly6g^{+}$ neutrophil populations only in HD tumors, as well (Figure 2.5 B and D). These results reinforce the data from our cytokine array study and suggest that increased tumor collagen density leads to increased COX-2 function and recruits macrophages and neutrophils into the collagen-dense microenvironment.

Collagen Dense Tumors and COX-2 Regulate Fibroblast Populations

Our cytokine array data revealed elevation of several cytokines and growth factors associated with tumor cell proliferation and inflammatory response modulation in the HD tumor microenvironment (Figure 2.3). To determine whether the collagen-dense microenvironment and high COX-2 levels regulate different fibroblast populations known to secrete such factors, we performed quantitative IF with vimentin as a general fibroblast marker and α-SMA as a marker of cancer associated fibroblasts (CAF). We observed that stromal $vimentin^{+}$ fibroblast populations were similar in all treatment arms of the study. However, there is a trend in

decreased vimentin^{+} fibroblast numbers only in the HD tumors with celecoxib treatment (Figure 2.6 A and C). Notably, celecoxib decreased α-SMA^{+} fibroblasts within the HD tumor microenvironment (Figure2. 6 B and D).

Since CAFs induce higher collagen deposition to alter the extracellular matrix, and COX-2 inhibition diminishes α-SMA^{+} fibroblasts in HD tumors, we characterized collagen levels in mammary tumors with Masson's trichrome staining. We found there was more collagen deposited in HD tumors and COX-2 inhibition by celecoxib reversed collagen levels in both wt and HD tumors (Figure 2.7 A and C). To discriminate effects related to tumor formation vs COX-2 treatment, we stained mammary glands of nulliparous mice treated with celecoxib or vehicle in wt and HD backgrounds. COX-2 inhibition by celecoxib did not affect collagen deposition in either wt or HD mammary glands (Figure 2.7 B and D). These results indicate that inhibition of COX-2 by celecoxib specifically affects the tumors of HD mice by reducing CAF populations, and by diminishing the tumor-associated collagen deposition prevalent in HD tumors.

Celecoxib diminishes tumor growth and number in a preventive mouse mammary carcinoma model

To study whether celecoxib inhibition of COX-2 expression in response to collagen density is effective as a preventive breast cancer therapy, we treated early postnatal animals before palpable tumors arose. Female MMTV-PyVT x Col1a1^{tm1jae} (HD) and their wild type counterparts at 10 days of age were randomly assigned to treatment with celecoxib or vehicle (Figure 2.8A). Mice were treated until they were 9 weeks of age and their tissues were collected

for analysis. PET scans were used to determine mammary gland tumor weight, number, growth rate, and volume in wt and HD mice with and without celecoxib. Mice were imaged at 6 weeks and 9 weeks. At 9 weeks of age, mice bearing collagen-dense tumors were larger when compared to wt tumors. Consistent with the above treatment regimen, celecoxib diminished tumor weight only in HD mice (Figure 2.8B). Tumor growth features were measured and compared longitudinally over time using PET scans, and the number of tumors counted at 6 and 9 weeks of age. Collagen-dense animals developed more tumors than wt mice over time. Celecoxib reduced tumor numbers only in HD mice (Figure 2.8C and F). In addition, the rate of tumor volume was enhanced over time in animals bearing HD tumors compared to wt tumors. Again, celecoxib reduced tumor volume in HD mice (Figure 2.8D). Surprisingly, we did not find differences when we measured mean glucose uptake with ^{18}Fluorodeoxyglucose-PET tracer, suggesting that neither density nor treatment with celecoxib altered glucose metabolism (Figure 2.8E).

To further understand the underlying mechanisms of COX-2 inhibition and its response to the collagen dense tumor microenvironment in a preventive treatment setting, we measured levels of different cytokines using the multiplex ELISA cytokine array described above. The relative levels of cytokines followed the same trends as in the therapeutic study (Figure 2.3). Most cytokines had 2-fold increased expression; Epithelial Growth Factor (3.4-fold), Leptin (3.2-fold), Interleukin 1α (3.3-fold), Granulocyte Macrophage Colony Stimulating Factor (3.7-fold), Monocyte chemotactic protein 1 (3.1-fold) and β-Nerve Growth Factor (3.8-fold) had the highest increases in expression (Figure 2.8G). Collagen-dense tumors tended to express significantly higher levels of cytokines compared to wt. Additionally, when wt and HD mice were treated

with celecoxib, relative levels of cytokines declined (Figure 2.8G). Similar to the therapeutic study, these results suggest that collagen density affects cytokine expression levels in a manner reversed by celecoxib. Together, these data indicate that COX-2 is a significant driver of tumor formation and growth in collagen-dense tumors. Celecoxib is able to reverse the increased tumor progression of the collagen-dense microenvironment, although it cannot completely prevent tumor incidence in this genetically-driven PyVT mouse model.

Discussion:

COX-2 over-expression in breast cancer is associated with poor patient prognosis. Here, we tested the hypothesis that COX-2 over-expression plays a role in regulating tumor- associated inflammation in the collagen-dense tumor microenvironment. We find that collagen dense breast tissue is associated with a more inflammatory tumor microenvironment, which promotes larger and more aggressive tumors. Over-expression of COX-2 is a major contributor to the inflammatory milieu of collagen-dense tumors, and leads to recruitment of tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs). Subsequently, we demonstrated that we can reverse recruitment of inflammatory cell populations in the dense tumor microenvironment by selectively inhibiting COX-2 with celecoxib. In addition, COX-2 inhibition with celecoxib reduced collagen deposition and decreased αSMA^+ fibroblasts mainly in the HD tumors. These data suggest there may be a therapeutic opportunity to treat tumors arising in dense breast tissue with celecoxib.

Cytokines are cell signaling molecules that have critical biological roles in processes such as cell growth, differentiation, gene expression, migration, immunity and inflammation [91]. Consistent with the enhanced inflammatory environment of the collagen-dense tumors, we found a dramatic increase in several cytokines in the Col1a1^{tm1jae} background. Several granulocyte and macrophage-recruiting cytokines, including GM-CSF, G-CSF, MCP-1, MIP-1α and RANTES (CCL5), were increased in collagen-dense tumors. Moreover, there was a strong increase in IL-17A, which contributes to macrophage and neutrophil recruitment [130]. IL-17A is a pro-inflammatory cytokine that is secreted by Th17 cells and induces the production of other cytokines, growth factors and prostaglandins from other cells including fibroblasts [130]. Also, IL-17A promotes angiogenesis, cell proliferation and chemoresistance and it is associated with poor patient prognosis [131, 132]. We find increased levels of IL-17R-A in the stroma of collagen-dense tumors, which decreased after celecoxib treatment. Accordingly, decline of IL17A and its receptor expression levels following treatment with celecoxib was accompanied by declining populations of F4/80$^+$ macrophages and Ly6g$^+$ neutrophils.

Platelet derived growth factor ββ (PDGF-ββ) is produced by epithelial and endothelial cells and stimulates nearby mesenchymal cells including fibroblasts in a paracrine fashion [133]. Consistent with this, we found a significant over-expression of PDGF-ββ in HD tumors compared to wt, and COX-2 inhibition by celecoxib diminished this effect. We did not find an increase in VIM$^+$ or αSMA$^+$ fibroblasts in the stroma surrounding collagen-dense tumors when compared to wt; yet, COX-2 inhibition with celecoxib decreased αSMA$^+$ fibroblasts in only the HD tumors. Besides, there was a trend in decreased vimentin$^+$ fibroblasts only in the HD tumors with celecoxib treatment. Possibly, some of the VIM+ or αSMA$^+$ cells may also be tumor cells

that have undergone epithelial-mesenchymal transition (EMT). Therefore, the observation that αSMA^{+} cells are decreased following celecoxib treatment may be due to effects on both fibroblasts and tumor cells undergoing EMT.

CAFs secrete elevated levels of many growth factors, including β-NGF. The continuous secretion of β-NGF activates an autocrine loop where more fibroblasts are recruited [36]. Additionally, β-NGF can be secreted by epithelial cells, macrophages, and neutrophils. Interestingly, we found that stromal β-NGF is elevated only in HD tumors and COX-2 inhibition with celecoxib decreases β-NGF over-expression in both wt and collagen-dense tumors.

There is a supporting effect of COX-2 on increased collagen deposition. Treatment with celecoxib leads to a reversal of increased collagen deposition that occurs around tumors, even in the $Col1a1^{tm1jae}$ background. This finding adds important insight into the mechanism of desmoplasia often observed around breast tumors. Moreover, these data are consistent with our observation that celecoxib diminishes matrix deposition. Interestingly, Lyons et al. also demonstrated that COX-2 inhibition reduces the collagen deposition associated with tumor growth and progression to metastasis in the involuting mammary gland [30]. Our results suggest that higher collagen deposition and elevated levels of COX-2 do not promote more VIM+ fibroblast recruitment. Nevertheless, high levels of cytokines promote activation of more αSMA^{+} fibroblasts, which in turn elicit higher deposition of collagenous stroma. Moreover, it has been demonstrated that macrophages are associated with local regions of collagen deposition in the postpartum involuting mammary gland [134]. Increased recruitment of macrophages could also contribute to elevated collagen deposition in HD tumors. Consistent with this, we observed

reduced numbers of CAFs and F4/80^{+} macrophages when collagen-dense tumors are treated with celecoxib.

Celecoxib, with its anti-inflammatory effects, is associated with less risk for endoscopic mucosal injury [67] and decreased incidence of cardio-renal toxicity [121] compared with ibuprofen. There is evidence that COX-2 inhibitors can decrease breast cancer risk by 16% [135]. Thus, we tested whether celecoxib could be used as a chemopreventive agent with respect to collagen density. Using no more than the maximum celecoxib dosage recommended by the FDA, we show that COX-2 inhibition with celecoxib produces smaller and fewer tumors than treatment with vehicle alone in collagen-dense animals. In addition, in HD mice, treatment with celecoxib decreases expression of all 23 cytokines tested in our ELISA array. This indicates that COX-2 inhibition modulates several immune and stromal cell populations and delays tumor formation and progression. A limitation of our preventive mouse model is that it is a very aggressive genetically-driven model of mammary carcinoma. Thus, it would have been impossible to observe complete abolishment of mammary tumors in this context.

Together, these findings support a mechanism in which COX-2 modulates tumor progression in collagen-dense matrices and produces a more aggressive tumor microenvironment. In addition, in dense mammary tumors, COX-2 inhibition with celecoxib reduces tumor growth; the amount of tumors developed, collagen deposition, and significantly decreases expression of several cytokines in either therapeutic or preventive settings. There is evidence of the effect of celecoxib as a therapeutic agent for primary breast cancer. A randomized controlled phase II clinical trial demonstrated that pre-operative treatment with celecoxib changes expression of several genes at

the transcription level in patients with invasive breast cancer when compared to placebo [102]. Additionally, Fabi et al. found that treatment with celecoxib facilitates the tolerability of capecitabine, a drug that aids in the delivery of the anti-cancer agent 5-fluorouracil (5-FU) in metastatic breast cancer patients. Results from this phase II clinical trial showed that patients with COX-2 overexpressing tumors had significantly longer time to progression and median overall survival [136]. Moreover, the increased gene expression of both COX-2 and collagen I is associated with decreased survival and shorter time to metastasis [30]. Added to these findings, our results here suggest that COX-2 may be an effective preventive or therapeutic molecular target that will preferentially benefit women with dense breast tissue. A clinical trial that studies the impact of celecoxib on women with dense breast and high COX-2 expression will be of great clinical significance.

Methods:

Mice and trial design

Mice were maintained and bred at the University of Wisconsin under the oversight of the University of Wisconsin Animal Use and Care Committee. To evaluate the effects of high COX-2 expression in an advanced stage of mammary cancer, a therapeutic mouse model was used (Figure 2.2A). Nulliparous female MMTV-PyVT x Col1a1^{tm1jae} (HD), their wt counterparts bearing mammary tumors (wt), Col1a1^{tm1jae} (no tumor), and their wt littermates were randomly assigned to a daily treatment of 0.2 mg (linear scale from 600 mg human dose or 10mg/kgbw) celecoxib (Pfeizer Inc.) suspended in 5% methyl cellulose or 5% methyl cellulose alone (vehicle)

at 11 weeks of age for a duration of 21 days. Dosage calculations were made for a 20g mouse. At 14 weeks of age, tissues were collected for study.

To evaluate the effects of COX-2 inhibition with celecoxib in response to collagen density as a preventive breast cancer therapy, a preventive mouse mammary model was used (Figure 2.8A). Female MMTV-PyVT x Col1a1^{tm1jae}, their wild type counterparts bearing mammary tumors, Col1a1^{tm1jae} (no tumor), and their wild type littermates at 10 days of age were randomly assigned to treatment with celecoxib suspension or vehicle. Ten days of age is as early as neonate mice can be handled for oral administration of their assigned treatment, and is a developmental stage that precedes tumor formation in this model. First, neonate mice were orally fed with celecoxib 3.3mg/kgbw (linear scale from 200mg human dose) or vehicle every other day until they were weaned at 3 weeks of age. Dosage calculations were made for a 10g mouse. At this low dose, celecoxib is not thought to interfere with development or cause other physiological complications in the pediatric population [137, 138]. At weaning, the dose was increased to 6.7mg/kgbw (linear scale from 400mg human dose) every other day until mice reached 9 weeks of age. Dosage calculations were made for a 20g mouse. At 9 weeks of age, tumors were clearly palpable, and animals were sacrificed for tissue analysis.

Antibodies:

The following antibodies were used for IHC and/ or IF: COX-2 (Cayman 160126), PGE2 (Abcam ab2318), Ki-67 (Abcam ab15580), β-NGF (Abcam ab6199), IL-17A-R (LSBio LS-B6706), F4/80 (AbD Serotec MCA497R), Ly6g (Biolegend 127601), Vimentin (Abcam ab92547) and a-SMA (Abcam ab5694).

Histology, Immunohistochemistry and Immunofluorescence:

For histology, tissues were fixed in 10% formalin for 48 hours followed by paraffin-embedding (FFPE). Tissue sections were stained with hematoxylin and eosin (H&E). For IHC, FFPE tissues were subject to standard deparaffinization, followed by dehydration and antigen retrieval with Citra Plus (Biogenex HK080-5K) for 15 minutes, blocking with BLOXALL, avidin/ biotin (Vector SP-6000 and SP-2001, respectively), and normal serum. Primary antibodies were incubated either overnight at 4˚C (anti-COX-2 or -PGE2 1:500) or for 1 hour at room temperature (anti-Ki-67 1:200). Tissue sections were incubated with biotinylated rabbit IgG (Vector, BA-1100) for 10 minutes following 30 minute incubation with R.T.U. Vectastain kit Elite ABC (Vector PK-7100). For IF, tissue sections were treated as described above for 20 minutes to retrieve antigens and then subjected to the TSA Plus kit for tissue labeling following manufacturers' protocols (Perkin Elmer, fluorescein NEL741E001KT, Cy 3.5 NEL744E001KT and Cy 5 NEL745E001KT). Briefly, primary antibodies were incubated as following: COX-2 (1:1000, O/N); PGE2 (1:5000, 1hr); β-NGF (1:6000, O/N); IL17RA (1:10000, 1hr); F4/80 (1:1000, 1hr); Ly6g (1:1000, 1hr); Vimentin (1:1000, 1hr); α-SMA (1:1000, 1hr). HRP-conjugated anti-rabbit (Abcam, ab7090) or anti-rat (Abcam, ab7097) was added for 10 minutes following 10 minutes incubation with TSA Plus kit working solution including desired fluorophore. Tissues underwent the antigen retrieval step for 20 minutes if the same tissue would be subjected to multiple labelings before counterstaining with DAPI for 2 minutes 1:10000 (Life Technologies, D21490).

Nuance and InForm Software

IF and IHC image experiments were acquired using a Nuance microscope with 20X objective and software version 3.0.12 (Perkin Elmer) with analysis done as previously described [139]. Briefly, a spectral library was created using image cubes to define distinctive spectral curves for each fluorophore, chromogen, and counterstain to adjust for background effects and accurately quantify positive staining of biomarkers using InForm version 1.4.0 software (Perkin Elmer). This software analysis allows objective counting of cell populations and biomarkers and increases the accuracy of the statistical analysis. Algorithms for tissue and subcellular compartment separation were created by machine learning and all algorithms were above 95% for precision (Figure S2.3). Algorithms were created for separating tissue compartments into stroma and epithelium and to identify nuclei to accurately assign associations for positive staining to a specific compartment in the tumor microenvironment. To create each algorithm, 10% of the image data set was used for each experiment.

Masson's Trichrome and Color Segmentation Software

To assess collagen deposition in the tumor tissue and mammary glands, Masson trichrome staining (Cancer Diagnostics Inc., SS1026-MAB-250) was used on paraffin embedded sections. Color images were analyzed with FIJI software and the Color Segmentation plugin (Daniel Sage, 2008 http://bigwww.epfl.ch/sage/soft/colorsegmentation/) using the K-means algorithm clustering method. All images had the same pixel size so the total area of collagen could be quantitated as blue pixels over total number of pixels per image.

Cytokine Array

To describe a cell signaling mechanism for collagen density changes in response to high COX-2 levels and to COX-2 inhibition, a mouse cytokine ELISA plate array (Signosis, Sunnyvale, CA) was utilized. In this quantitative chemiluminescence plate array, 23 mouse cytokines were monitored simultaneously for their expression level in relation to collagen deposition and COX-2 inhibition with celecoxib. Cytokine signal was measured with a fluorometer (Fluoroskan, Ascent, FL) and Ascent software version 2.6 (Thermo Scientific). To compare fold-change differences, data was normalized to a blank and graphically represented by normalization to wt vehicle cytokine data levels.

Positron Emission Tomography (PET) Imaging and Analysis:

Highly sensitive and quantitative PET imaging was used to study potential preventative effects of COX-2 inhibition. All mice were fasted 8 hours prior to intravenous injection of approximately 5 MBq of 2′-deoxy-2′-[^{18}F]fluoro-D-glucose (FDG) 1 hour before imaging. Mice were anesthetized with inhalation gas (2% isoflurane gas mixed with 1L/min of pure oxygen) and kept under a heat lamp during injection until imaging. Mice were imaged in a prone position on a Siemens Inveon Hybrid micro-PET/CT (Siemens Medical Solutions, Knoxville, TN). A 10 minute PET scan was acquired and data was displayed as a histogram in one static frame; data was subsequently reconstructed using ordered- subset expectation maximization (OSEM) of three dimensions followed by the maximum *a posteriori* algorithm (Matrix size = [128,128,159], Pixel size = [0.776, 0.776, 0.796]mm, iterations = 18, subsets = 16, and beta smoothing factor = 0.004). Data were not corrected for attenuation or scatter. PET analysis used Siemens Inveon

software (Siemens Medical Solutions, Knoxville, TN).The data was normalized to animal weight, amount of injected PET tracer, and tracer decay. A sphere was drawn and positioned over identified tumors and tumor volume and mean FDG uptake were calculated by the software.

Statistical Analysis:

The analyses were performed with the goal of studying the effect of Cox-2 in mammary tumor progression in response to cell matrix density. Mixed linear models were used to assess differences between the various factors. The data were tested for normality and log transformations were made as necessary. Every statistical test was two-sided, and a p-value less than 0.05 was considered statistically significant. All analyses were performed using the procedure PROC MIXED from the SAS/STAT® software (version 9.4).

Figure 2.1:

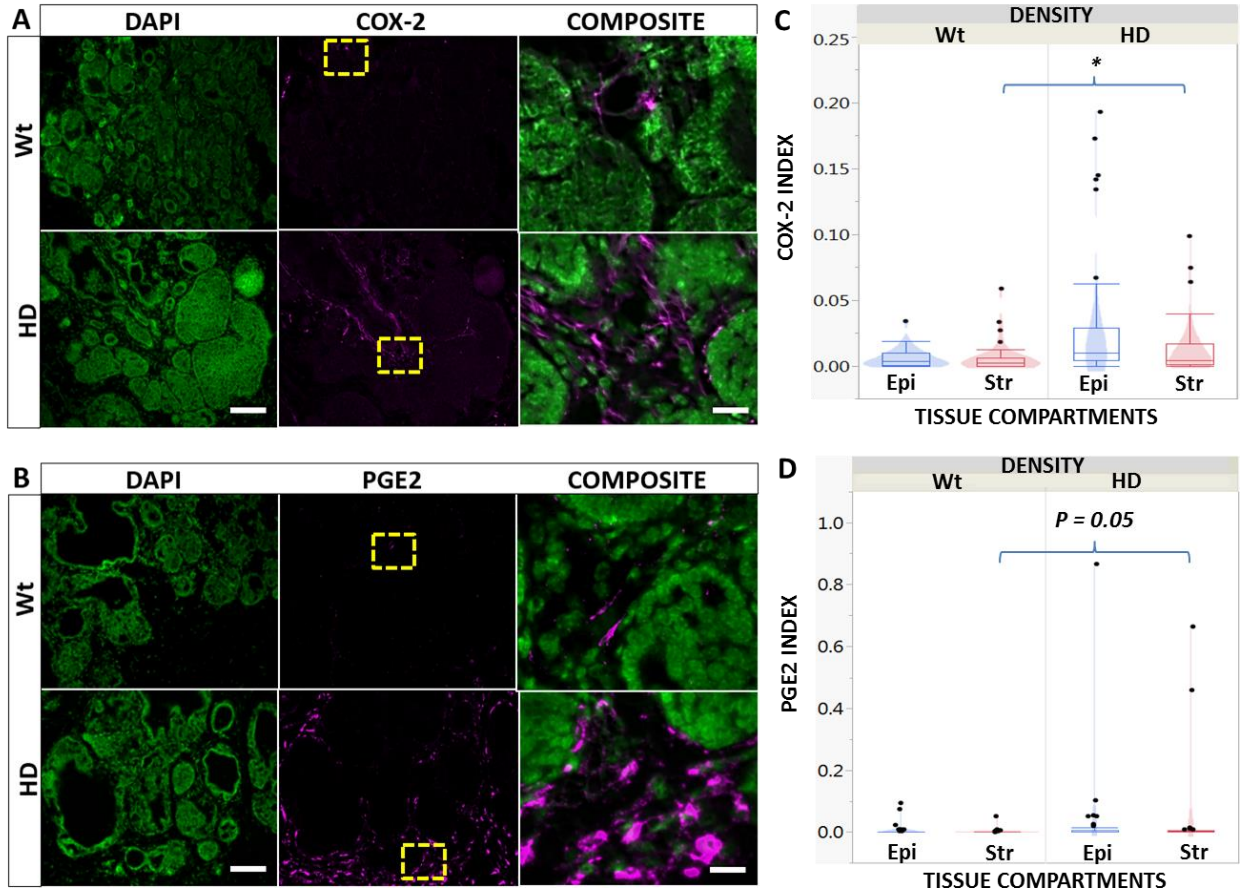

Figure 2.1. COX-2 and PGE2 levels are elevated in collagen-dense tumors. A, B. Immunofluorescence images of COX-2 (A) or PGE2 (B) (magenta) counterstained with DAPI (green). 20x objective, scale bar = 100um. Composite is enlarged image of area marked by the yellow window, scale bar = 15um. **C, D.** Quantitation of several images as shown in A and B. Graphs are box plots overlaid with violin plots to indicate differences in data point density distribution. Graphs depict raw data. Epi = epithelium, Str = stroma. Quantitative values represent the number of positive stained cells divided by the total number of cells in that compartment. **C.** COX-2 levels are elevated in tumor and stromal cells from collagen-dense (high density, HD) compared to tumors arising in wt mice. **D.** PGE2 levels are moderately elevated in HD tumors of cells in the stroma compartment. *$P < 0.05$. n=5 mice per arm, at least 8 image fields per tumor analyzed per 2-3 tumors per animal, mixed linear model.

Figure 2.2:

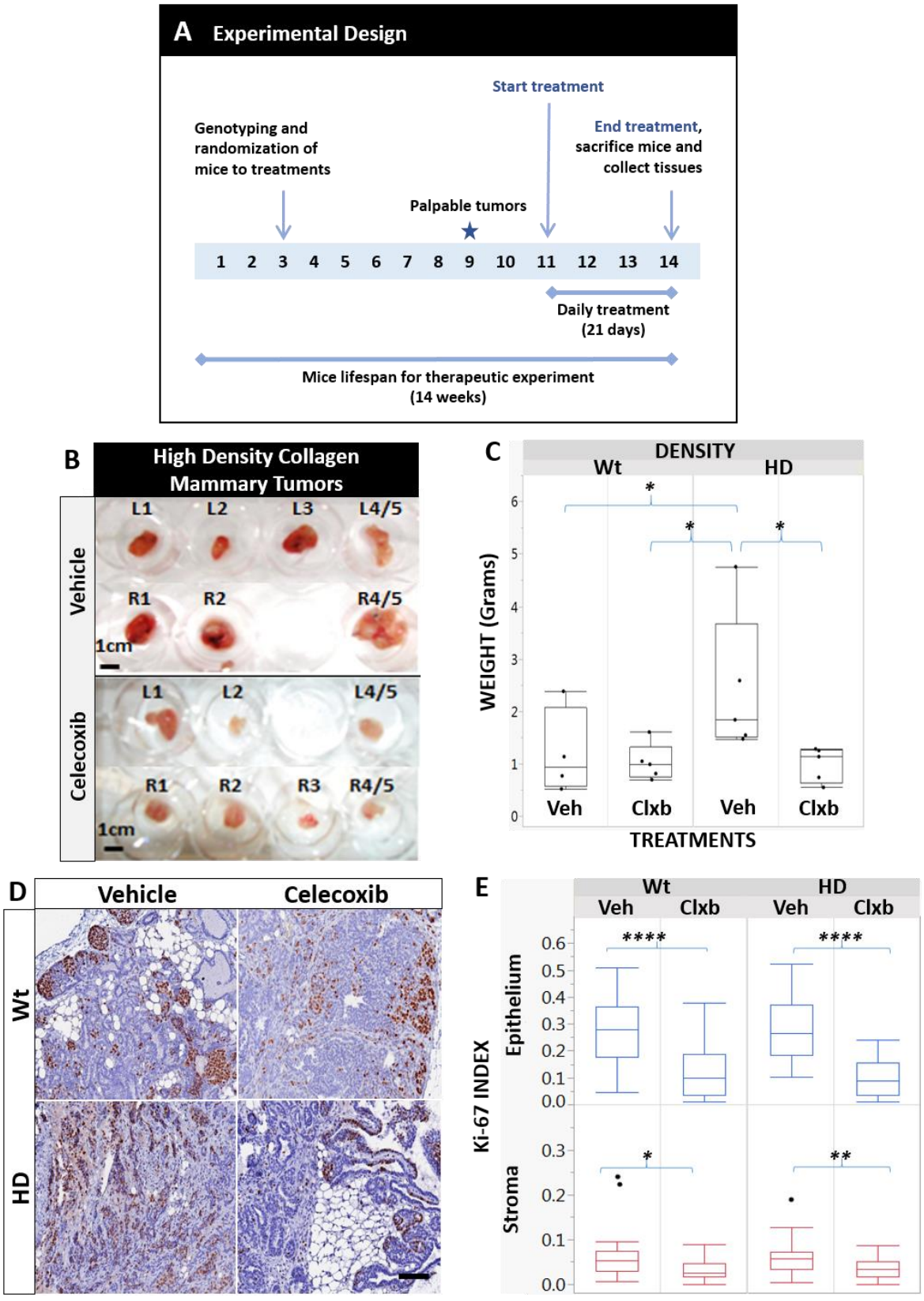
A Experimental Design
Start treatment
Genotyping and randomization of mice to treatments
End treatment, sacrifice mice and collect tissues
Palpable tumors
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Daily treatment (21 days)
Mice lifespan for therapeutic experiment (14 weeks)
B
High Density Collagen Mammary Tumors
Vehicle
Celecoxib
L1 L2 L3 L4/5
R1 R2 R4/5
R1 R2 R3 R4/5
1cm
C
DENSITY
Wt
HD
WEIGHT (Grams)
Veh Clxb Veh Clxb
TREATMENTS
D
Vehicle
Celecoxib
Wt
HD
E
Wt
HD
Veh Clxb Veh Clxb
Ki-67 INDEX
Epithelium
Stroma
****
*
**

Figure 2.2. Celecoxib diminishes collagen-dense tumor growth. A. Timeline for celecoxib therapeutic study. See Methods for more details. **B.** Representative example of tumors arising in collagen-dense mice treated with vehicle or celecoxib. **C.** Tumor weights from wt and HD animals, treated with vehicle or celecoxib. Tumor weights are higher in HD tumors compared to wt tumors and celecoxib delays tumor growth in HD mice. **D.** IHC of representative tumor sections stained with anti-Ki-67 antibody (visualized with DAB, brown) and counterstained with hematoxylin. 20x objective, scale bar = 100um. **E.** Quantitation Ki67 positive cells normalized to the total number of cells. Celecoxib treatment diminishes proliferation levels as measured by Ki67 detection in both wt and HD tumors. Veh = vehicle, Clxb = celecoxib. Graphs depict raw data. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. n=5 mice per arm, at least 8 image fields analyzed per 2-3 tumors per animal, mixed linear model.

Figure 2.3:

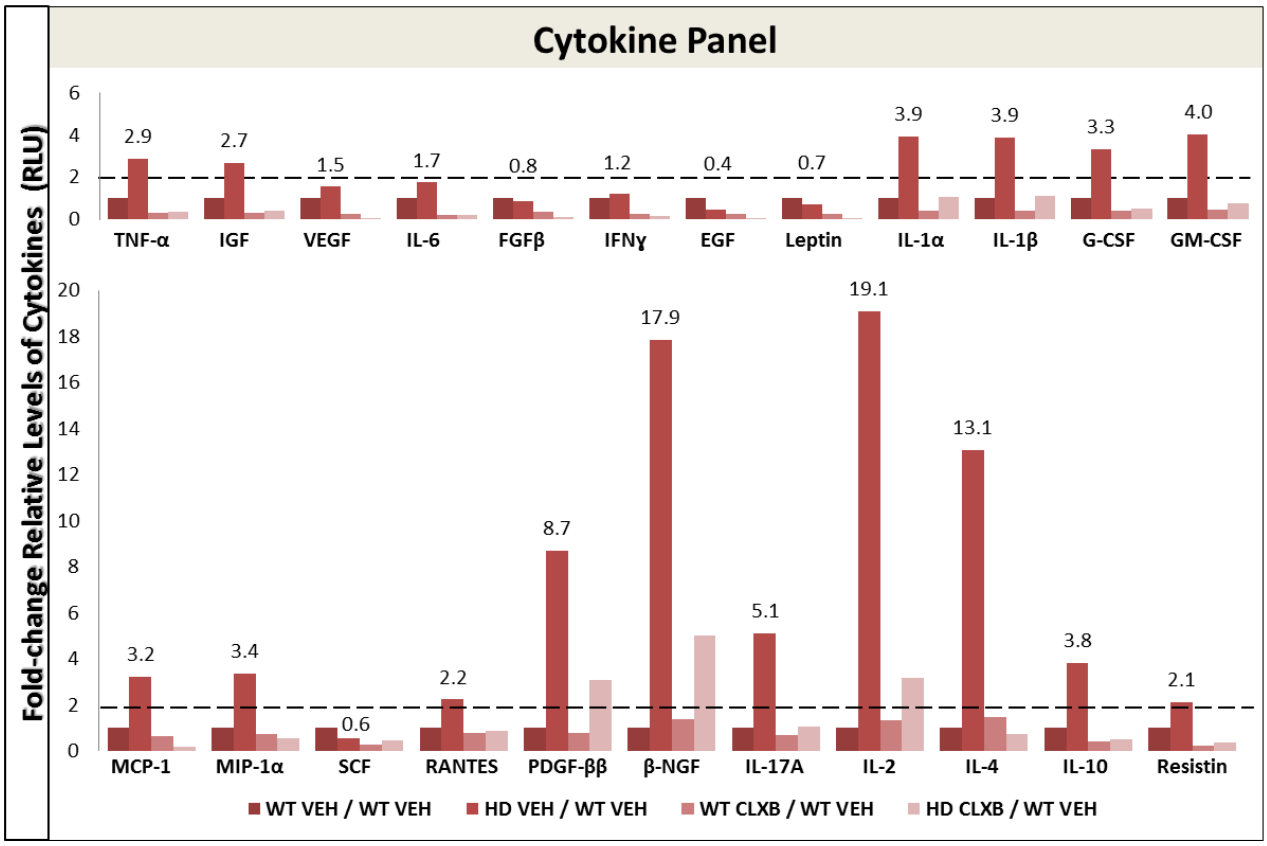

Figure 2.3. Regulation of cytokines by density and celecoxib. Several cytokines are upregulated in HD mammary tumors compared to wt tumors. Treatment with celecoxib (clxb) diminishes cytokine levels in wt and HD mice. Relative levels of cytokines are represented as the fold-change normalized to wt vehicle. Cytokines having a 2-fold or greater expression change are considered significantly upregulated. Three tumors, each from a single animal, were pooled per treatment arm to perform the multiplex cytokine ELISA array. Tumor necrosis factor alpha (TNF□), IGF = Insulin growth factor, VEGF = Vascular endothelial growth factor, IL = Interleukin, FGFβ = Fibroblast growth factor beta, IFNγ = Interferon gamma, EGF = Epithelial growth factor, G-CSF = Granulocyte-colony stimulating factor (CSF-2), GM-CSF = Granulocyte-macrophage colony-stimulating factor (CSF-3), MCP-1 = Monocyte chemotactic protein 1 (CCL2), MIP-1α = Macrophage Inflammatory Protein 1 alpha (CCL3), SCF = Stem cell factor, RANTES = Regulated on activation, normal T cell expressed and secreted (CCL5), PDGF-ββ = Platelet-derived growth factor beta-beta and β-NGF = Nerve growth factor beta.

Figure 2.4:

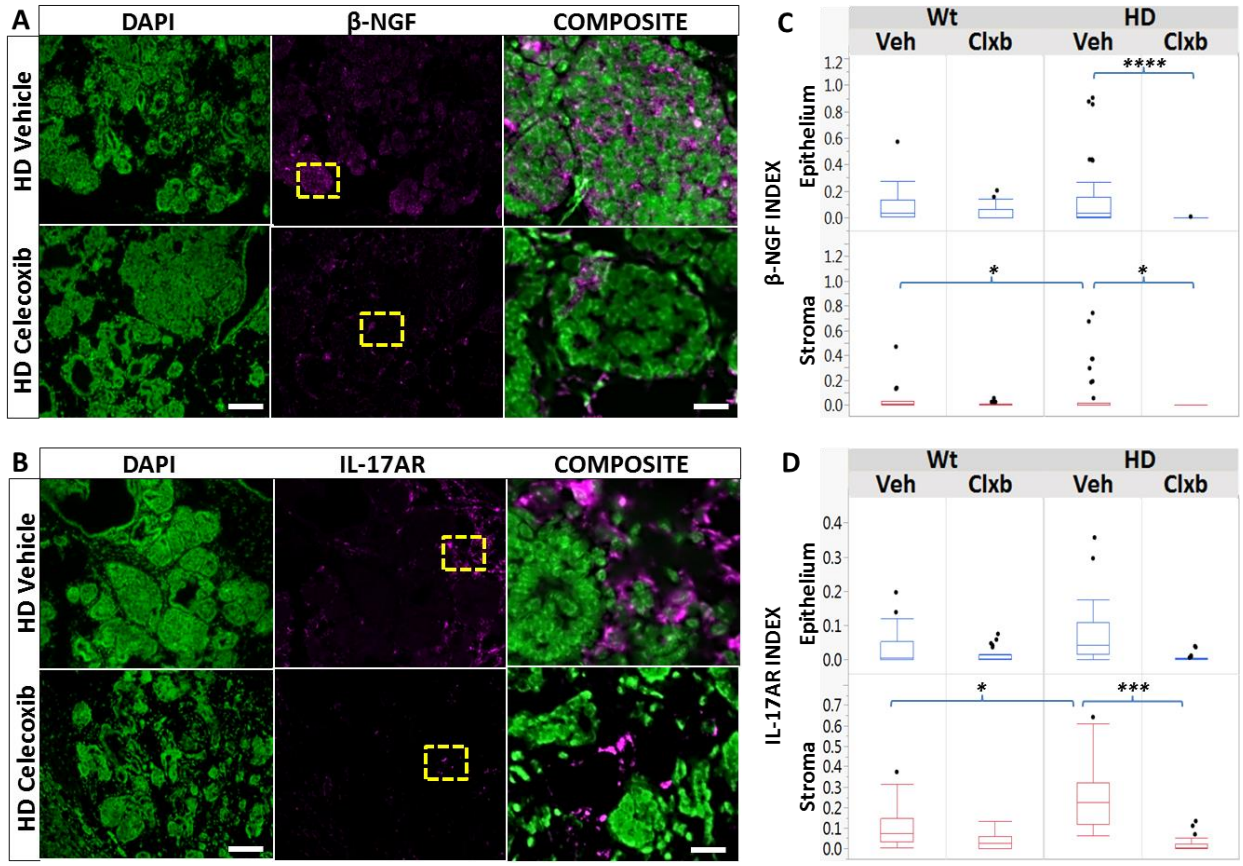

Figure 2.4. Validation of cytokine results in tumor and stroma. A, B. Representative IF images of β-NGF or IL-17A Receptor (IL-17A-R) (magenta) counterstained with DAPI (green). 20x objective, scale bar = 100um. Composite is enlarged image of area demarked by the yellow window, scale bar = 15um. **C.** Quantitation of β-NGF levels normalized to the total number of cells. β-NGF levels are elevated in the stroma of HD tumors. Celecoxib decreases β-NGF levels in both epithelium and stroma of HD tumors. β-NGF levels are higher in the tumor/epithelium consistent with its known role in promoting survival and proliferation of epithelial breast cancer cells. **D.** Quantitation of IL-17A-R levels normalized to the total number of cells. IL-17AR levels are elevated in the stroma of HD tumors, consistent with its role in macrophage/ neutrophil recruitment. Celecoxib treatment decreases IL-17A-R levels only in the stroma of HD tumors. Graphs depict raw data. * = $P < 0.05$, *** = $P < 0.001$, **** = $P < 0.0001$. n=5 mice per arm, at least 8 image fields analyzed per 2-3 tumors per animal, mixed linear model.

Figure 2.5:

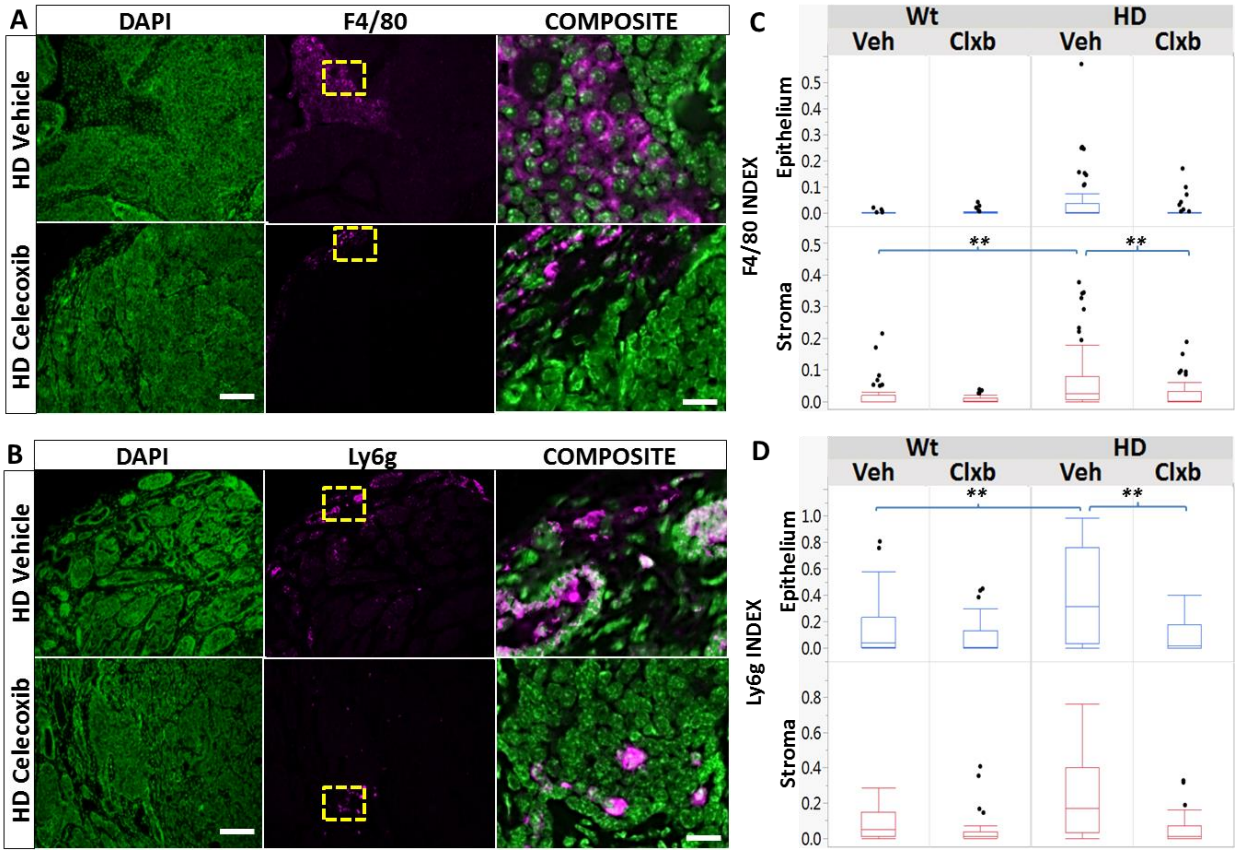

Figure 2.5. Regulation of macrophages and neutrophils by collagen density and celecoxib. **A, B.** IF images of F4/80$^+$ macrophages or Ly6g$^+$ neutrophils (magenta), respectively, counterstained with DAPI (green). 20x objective, scale bar = 100um. **C, D.** Index was calculated by dividing positive stained cells by total numbers of cells. Graphs depict raw data. **C.** Quantitation of total F4/80$^+$ cells normalized to the total number of cells. F4/80$^+$ macrophage numbers are elevated in the stroma of HD tumors and decreased by treatment with celecoxib. **D.** Total number of Ly6g$^+$ neutrophils normalized to total number of cells. Ly6g$^+$ neutrophil numbers are elevated in the epithelium of HD tumors and decreased by treatment with celecoxib. ** = P <0.01. n=5 mice per arm, at least 8 image fields analyzed per 2-3 tumors per animal, mixed linear model.

Figure 2.6:

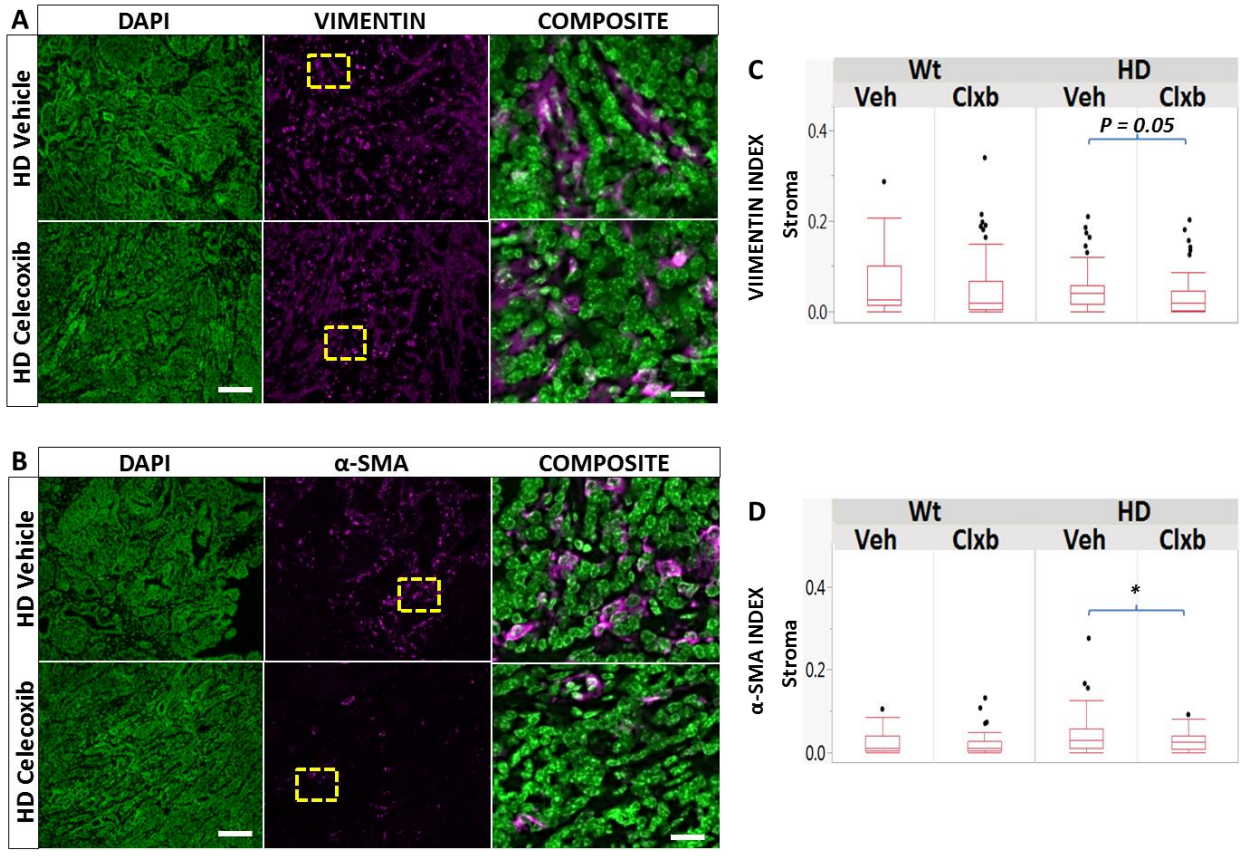

Figure 2.6. Regulation of fibroblasts by density and celecoxib. A, B. IF images of vimentin$^+$ fibroblasts or α-SMA$^+$ fibroblasts (magenta) counterstained with DAPI (green). 20x objective, scale bar = 100um. **C.** The number of vimentin$^+$ fibroblasts remains the same regardless of collagen density. Values represent vimentin$^+$ cells normalized to total cell number. **D.** Celecoxib decreases the number of α-SMA$^+$ fibroblasts in the stroma of HD tumors. Values represent the number of α-SMA$^+$ cells normalized to the total number of cells. Graphs depict raw data. * = P <0.05. n=5 mice per arm, at least 8 image fields analyzed per 2-3 tumors per animal, mixed linear model.

Figure 2.7:

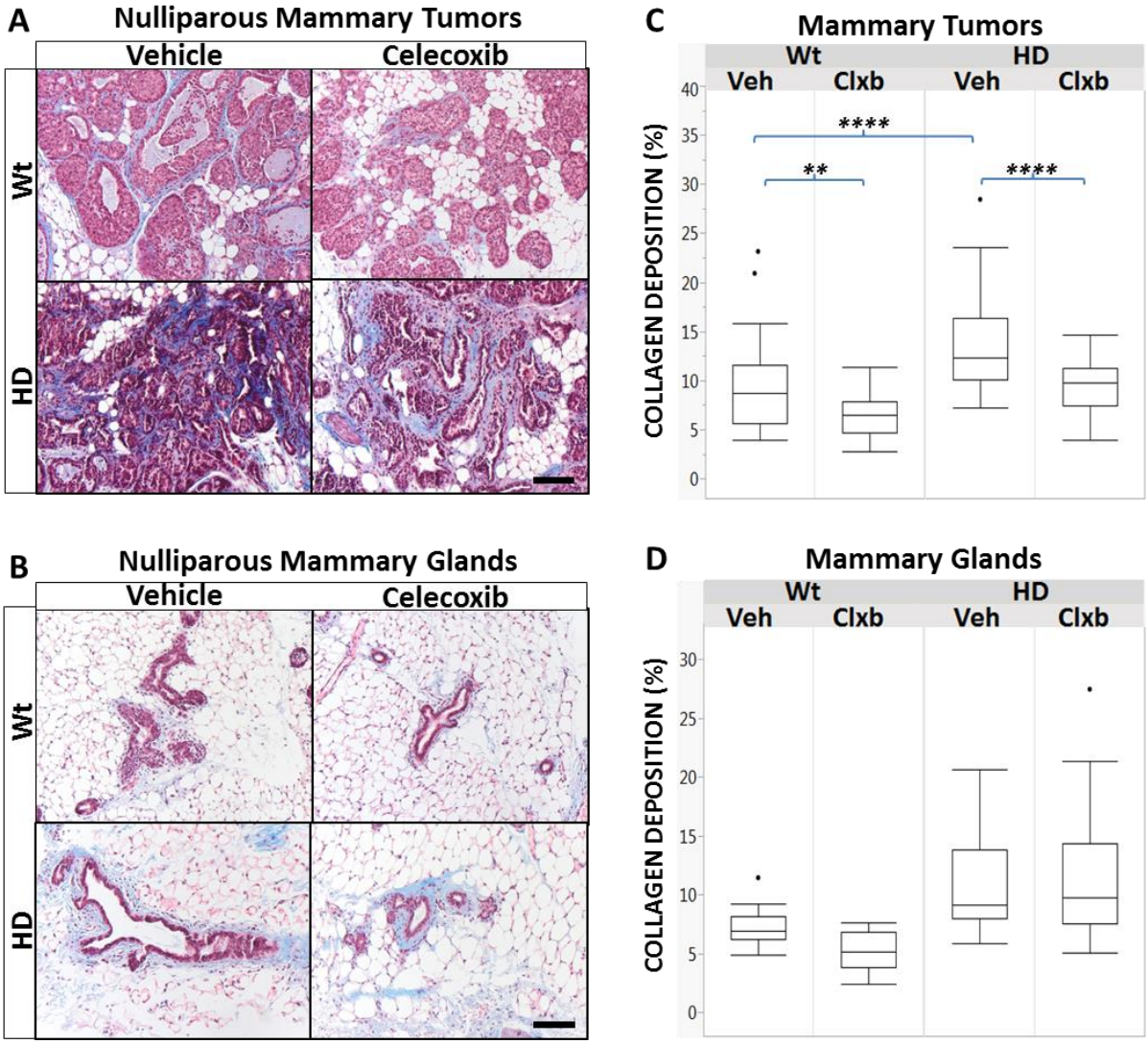

Figure 2.7. Celecoxib diminishes collagen deposition in mammary tumors but not tumor-free mammary glands. A, B. Masson's trichrome stain of mammary tumors or mammary glands of nulliparous mice treated with vehicle or celecoxib. Collagen fibers are in blue, cell nuclei are black and the cell cytoplasm, muscle tissue and erythrocytes are stained red. 20x objective, scale bar = 100um. **C, D.** Quantitation of Masson's trichrome, performed as described in Methods. **A, C.** In tumors, there is a significant increase in collagen in HD compared to wt. Celecoxib diminishes collagen deposition in both wt and HD tumors. **B, D.** In normal, tumor-free mammary glands, there is no significant effect of treatment with celecoxib. Glands in HD animals tend to have higher collagen accumulation. Graphs depict raw data. ** = P <0.01, **** = P <0.0001. n=5 mice per arm, separate true normal study, age is 14 weeks for both animal cohorts, at least 5 image fields analyzed per 2-3 tumors/glands per animal, mixed linear model.

Figure 2.8:

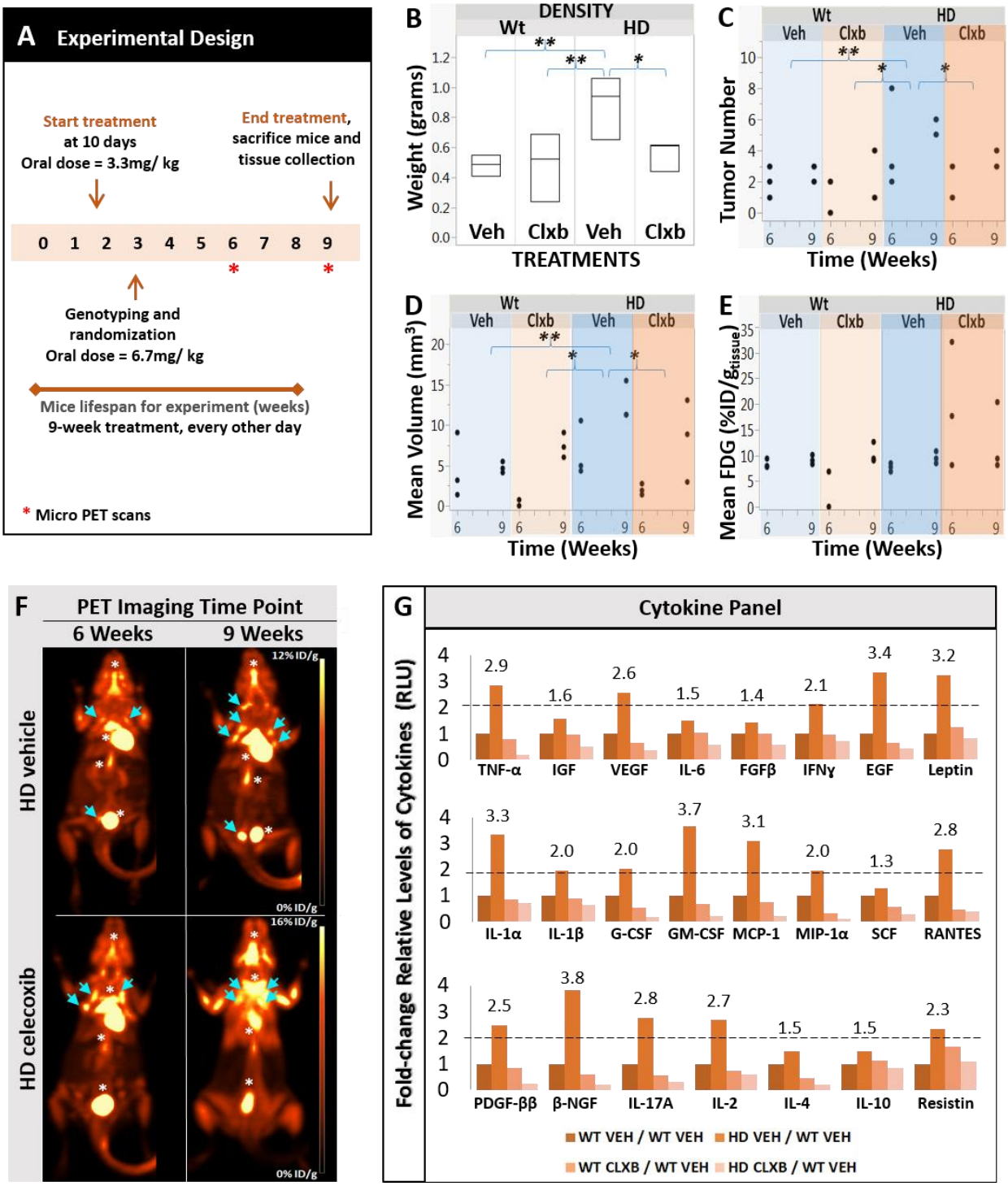
A Experimental Design
Start treatment at 10 days Oral dose = 3.3mg/ kg
End treatment, sacrifice mice and tissue collection
0 1 2 3 4 5 6 7 8 9
Genotyping and randomization Oral dose = 6.7mg/ kg
Mice lifespan for experiment (weeks)
9-week treatment, every other day
* Micro PET scans
B DENSITY
Wt HD
Weight (grams)
Veh Clxb Veh Clxb
TREATMENTS
C Wt HD
Veh Clxb Veh Clxb
Tumor Number
Time (Weeks)
D Mean Volume (mm3)
Time (Weeks)
E Mean FDG (%ID/gtissue)
Time (Weeks)
F PET Imaging Time Point
6 Weeks 9 Weeks
HD vehicle
HD celecoxib
12% ID/g
0% ID/g
16% ID/g
G Cytokine Panel
Fold-change Relative Levels of Cytokines (RLU)
TNF-α IGF VEGF IL-6 FGFβ IFNγ EGF Leptin
IL-1α IL-1β G-CSF GM-CSF MCP-1 MIP-1α SCF RANTES
PDGF-ββ β-NGF IL-17A IL-2 IL-4 IL-10 Resistin
WT VEH / WT VEH
HD VEH / WT VEH
WT CLXB / WT VEH
HD CLXB / WT VEH

Figure 2.8. A. Timeline for celecoxib preventive study. See Methods for more details. **B-E.** Quantitation of several tumors from wt and HD animals, treated with vehicle or celecoxib. **B.** Tumor weight is higher in HD tumors (n=5 per arm) when compared to wt tumors and celecoxib delays tumor growth in HD mice. **C.** HD animals developed more tumors than wt animals and celecoxib reduces tumor number in HD mice. **D.** Tumor volume is higher in HD tumors when compared to wt tumors and celecoxib reduces tumor volume in HD mice. **E.** The average amount of glucose uptake by the tumors remains the same regardless of collagen density or treatment with celecoxib. FDG (^{18}Fluorodeoxyglucose) PET tracer. %ID/g $_{(tissue)}$ is the percent injected dose of PET tracer per gram of tissue. **F.** Representative PET images of collagen-dense mice at 9 and 14 weeks of age either treated with celecoxib or vehicle. Images over time corresponds to same subject. Arrows indicate tumors and asterisks indicate tissue other than tumors that uptake the FDG tracer such as brain, carotid, brown fat, heart, kidneys, aorta, bladder or muscle tissue. **G.** Regulation of cytokines by density and celecoxib. Several cytokines are upregulated in HD mammary tumors compared to wt tumors. Treatment with celecoxib diminishes cytokine levels in wt and HD mice. Relative levels of cytokines are represented as the fold-change normalized to wt vehicle. Cytokines equal or greater than a 2-fold change are considered significantly upregulated. Three tumors, each from a single animal, were pooled per treatment arm to perform the multiplex cytokine ELISA array. **C-F.** For PET studies n=3 per arm. * = $P < 0.05$, ** = $P < 0.01$, mixed linear model.

Supplemental Figures:

Figure S2.1:

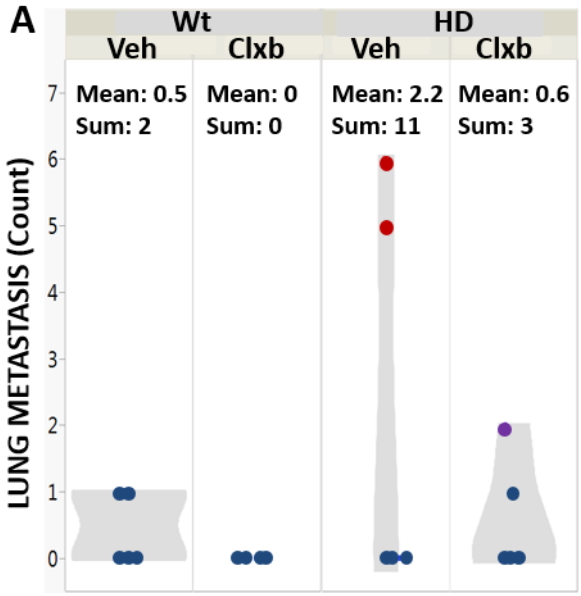

Figure S2.1. COX-2 inhibition and lung metastasis. A. Lung metastases tend to be increased in collagen-dense tumors and inhibited by celecoxib (n.s.). Gray shading depicts data density graph to better illustrate differences in data distribution. Veh = vehicle, Clxb = celecoxib. Graphs depict raw data. n=5 mice per arm.

Figure S2.2:

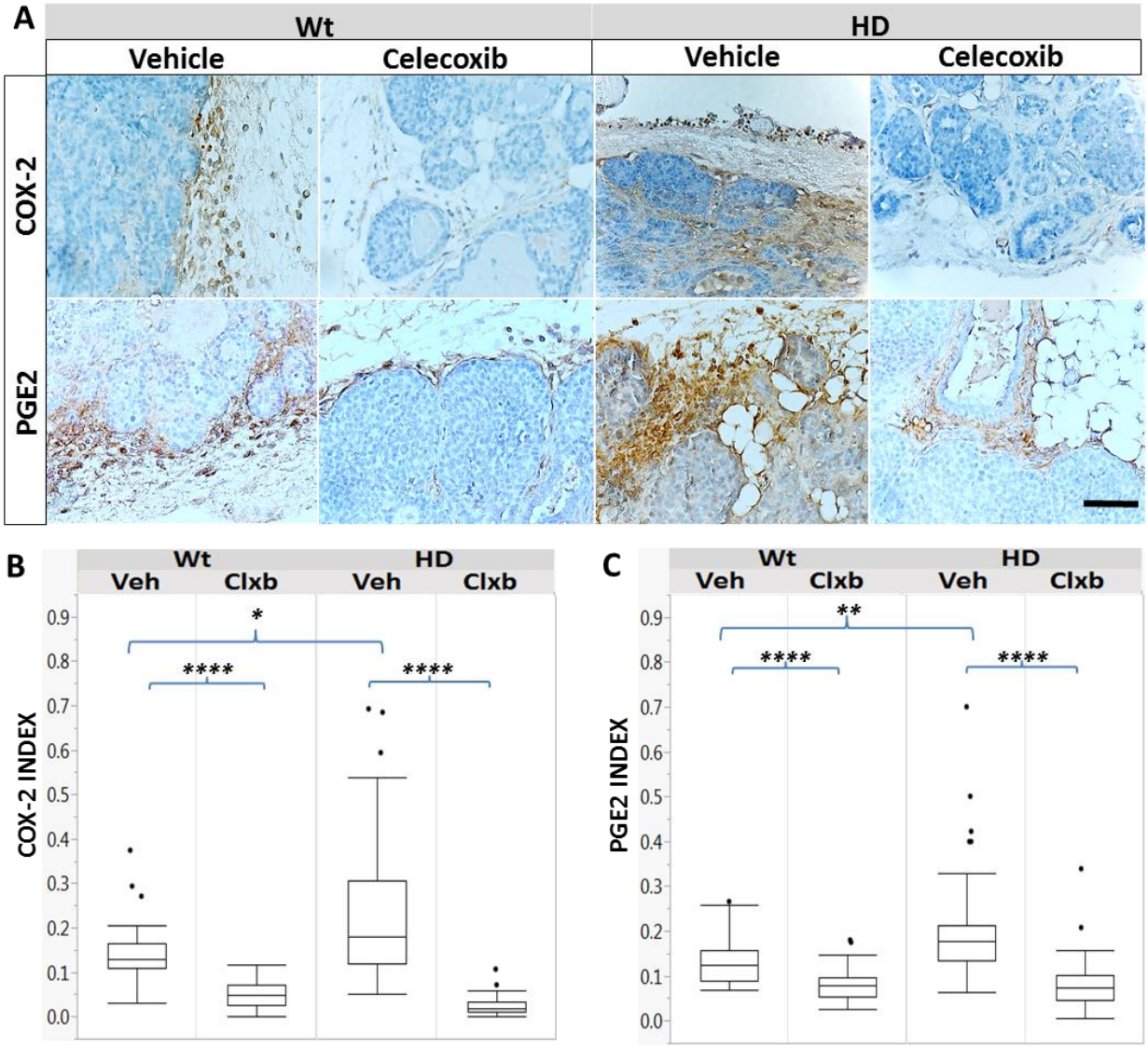

Figure S2.2. Celecoxib diminishes COX2 and PGE2. **A.** IHC images of COX-2 and PGE2 (DAB) counterstained with hematoxylin. 40x objective, scale bar = 50um. **B, C.** Index was calculated by dividing amount of positive stained cells over total amount of cells. Graphs depict raw data. **B.** COX-2 levels are elevated in HD tumors. Celecoxib diminishes COX-2 levels in wt and HD tumors. **C.** PGE2 levels are elevated in HD tumors and Celecoxib diminishes PGE2 levels in wt and HD tumors. * = $P < 0.05$, ** = $P < 0.01$, **** = $P < 0.0001$. n=5 mice per arm, at least 8 image fields analyzed per 2-3 tumors per animal, mixed linear model.

Figure S2.3:

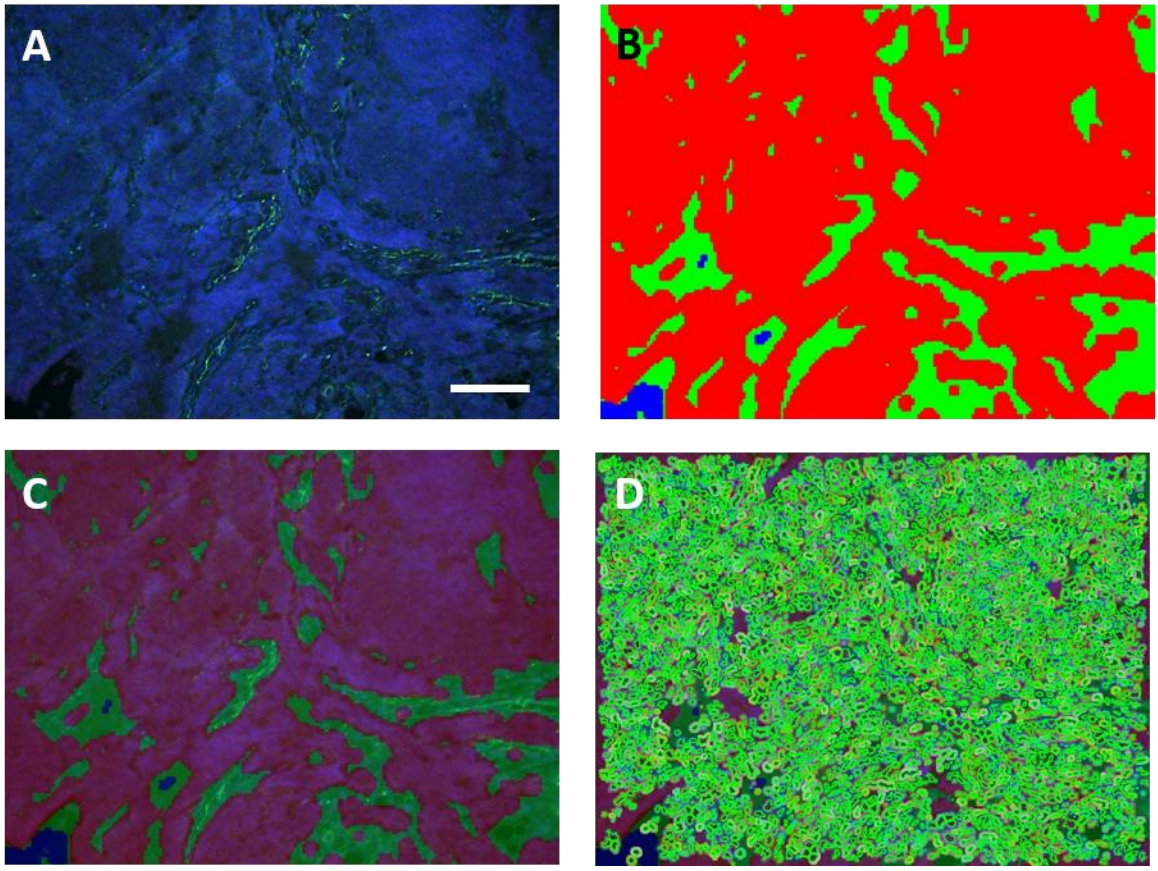

Figure S2.3. Tissue segmentation analysis. **A-D.** Images for the process of tissue segmentation. Algorithms for tissue segmentation, i.e. tumor epithelium versus tumor stroma, were created by machine learning (see methods). **A.** Sample IF image cube of mouse tumor stained with COX-2 (green) and counterstained with DAPI (blue). 20x objective, scale bar = 100um. **B.** Tissue segmentation mask after training the software. Red = epithelium, green = stroma, blue = other (empty space, debris/ artifacts) **C.** Tissue segmentation map (overlay of tissue segmentation mask and IF image). **D.** Overlay of object cell count map and tissue segmentation map. Each object (cell) circled in green was associated to its respective tissue compartment; tumor epithelium or stroma and debris was associated with the "other" category and not included in the statistical analysis.

CHAPTER 3

The presence of COX-2 and tumor associated macrophages as a prognostic marker for invasive breast carcinoma patients

Karla Esbona, Sandeep Saha, Yanyao Yi, Menggang, Yu, Lee Wilke, and Patricia Keely

Manuscript to be submitted to Breast Cancer Research.

Abstract

Introduction: The objective of this study was to assess whether the tissue localization of COX-2 and tumor-associated macrophages were associated with clinicopathological features of invasive carcinoma, including collagen deposition and patient survival outcome.

Methods: A tumor microarray (TMA) of 371 biopsy specimens from patients with invasive breast carcinoma was analyzed for expression of high levels of COX-2, CD68 and CD163 in either the tumor nest (TN) or the tumor-associated stroma (TS). The study population for this TMA was female patients; 26 to 94 years of age with a median follow up of 8.4 years. Survival curves were calculated according to the Kaplan-Meier method.

Results: We found that elevated collagen deposition was associated with stromal expression of high COX-2, however, collagen deposition was not a predictor for survival outcome. High COX-2 expression in the TN led to a worse patient outcome. In addition, high infiltration of $CD68^+$ in the TS and $CD163^+$ macrophages in both TN and TS was associated with worse overall survival (OS). Finally, dense infiltration of COX-2-expressing $CD68^+$ and $CD163^+$ macrophages in the TS led to poor patient OS and reduced progression-free survival (PFS).

Conclusion: These findings suggest that, in invasive carcinoma samples, the localization of inflammatory markers within the tumor play a role in patient outcome. The sole presence of dense infiltration of COX-2-expressing $CD68^+$ and $CD163^+$ macrophages in the TS is associated with poor patient survival. Suggesting, these patients may benefit from therapy with a selective COX-2 inhibitor such as celecoxib.

INTRODUCTION

Yearly, over 1.7 million women are diagnosed with breast cancer worldwide. Despite the improvement in early detection and treatment of breast cancer, 31% of women diagnosed with breast cancer will succumb to this disease [1]. Breast cancer is a complex disease caused by multiple factors including, genetic mutations, advanced age [2], obesity [3], reproductive factors and hormonal fluctuations [4, 5], environmental factors such as smoking at early age [6] and high breast density [7, 8]. Elevated mammographic density in 50% or more of the breast is associated with 30% of breast cancer cases [9]. For instance, high mammographic density was more prevalent in cancer lesions in ductal carcinoma in situ (DCIS) [10], and also has been associated with increased risk of DCIS [11], invasive breast carcinoma [11, 12] and more aggressive tumors that are larger in size, higher lymphatic and vascular invasion and positive node status [13]. However, no causal link has been established for increased mammographic density in cancer.

Moreover, breast cancer can be categorized into different histopathological types with invasive breast carcinoma being the most common and responsible for most deaths. About 80% of invasive cancers are invasive ductal carcinoma, with lobular, adenoid, papillary, and several others being less infrequent [14]. In addition, another way to categorize invasive carcinoma tumors is by molecular profiling which aids in targeted therapy for this disease. Assessment of estrogen receptor expression (ER^+), progesterone receptor expression (PR^+) and the overexpression of human epidermal growth factor receptor 2 ($HER2^+$) by immunohistochemistry, has demonstrated that these receptors are independently associated with patient outcome. For instance, patients with tumors that are ER^+ and/ or PR^+, tend to benefit

from hormonal therapy, whereas patients with $HER2^{+}$ tumors, have worse outcome, especially if they have cancer spread to the lymph nodes [15]. Other prognostic factors are tumor size, stage, grade and proliferation. Even though, all these prognostic factors are generally associated with patient outcome, these factors are not enough to deliver personalized breast cancer treatment nor to be predictive of positive disease outcome. Thus, it is imperative to find effective prognostic markers that are present in early breast carcinoma detection, in order to apply personalized proper treatment action that will benefit the outcome in breast cancer patients.

Furthermore, there is evidence that the immune response plays a critical role towards the development and progression of breast cancer. There are several reports that demonstrate that expression levels of cyclooxygenase 2 (COX-2) are elevated in breast, colorectal and other carcinomas in comparison to normal tissue [16]. Unlike COX-1, which is a constitutively expressed enzyme [17], COX-2 is an inducible enzyme that is activated at sites of injury as part of the inflammatory response [18]. Cyclooxygenases are responsible for the biosynthesis of prostaglandins, like prostaglandin E2 (PGE2), which it has been associated as a major contributor to many cancers [19]. COX-2 expression can be modulated by cytokines, interleukins, hormones, growth factors, genetic mutations and PGE2 itself, promoting its own biosynthesis [20–23]. COX-2 over-expression is observed in 40 - 75% of invasive breast carcinoma cases and correlates with more aggressive types of tumors and poor patient prognosis [24, 25]. In addition, several epidemiological studies demonstrated that COX-2 inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) is associated with decreased breast cancer recurrence and increased survival [26–28].

Additionally, tumor-associated macrophages (TAMs) play a dynamic and multifaceted role in breast cancer development that produces different outcomes depending on their pro- or anti-tumor behavior. TAMs have served a dual role in breast cancer. They can produce an anti-tumorigenic effect by activation interleukins and interferon while also promoting a tumorigenic environment by secreting diverse cytokines, growth factors and proteases [29]. Pro-tumoral TAMs, have been shown to aid in the processes of angiogenesis, proliferation, immunosuppression, degradation of the ECM, promotion of breast tumor epithelial cell migration and metastasis [30, 31]. There is evidence of macrophages expressing high COX-2 levels in the tumor microenvironment and PGE2 production, downstream of COX-2, is one of the key molecules that facilitates TAMs pro-tumoral capabilities [32, 33]. Through COX-2 enzymatic production of PGE-2, macrophages can be stimulated to produce cytokines and growth factors that will promote more pro-inflammatory cell recruitment and the development of colitis-associated tumorigenesis [33]. In addition, sera from obese breast cancer patients promoted higher expression levels of COX-2 and PGE2 production in macrophages, resulting in higher proliferation and migration of these cells when compared to average-weight patients and this effect was reversed by selectively inhibiting COX-2 with celecoxib [34]. Our previous work demonstrated that elevated expression of COX-2 and PGE2 were key elements in the inflammatory response of mouse collagen-dense mammary tumors, which led to increased recruitment of TAMs, elevated levels of several cytokines, enhanced proliferation and promoted tumor development [35]. Consequently, we validated these findings when tumor presence of TAMs was decreased in conjunction with diminished cytokine levels, smaller and less proliferative tumors and less collagenous stroma after COX-2 inhibition with celecoxib. These observations suggest that COX-2 through enzymatic production of PGE has an important role in

collagen deposition and macrophage recruitment to the tumor microenvironment and is essential in the growth and spread of mammary tumors. However, larger future studies are needed to determine the prognostic factor of high COX-2-expressing macrophages that will aid in therapies to target cancer-specific inflammation.

Furthermore, the tumor microenvironment is very complex and heterogeneous. It includes not only tumor epithelial cells but also tumor-associated stroma (TS) components such as stromal cells, immune cells, ECM and blood vessels, which each could serve as potential treatment target depending on the patient's tumor molecular expression characteristics [36]. Based on the tumor-promoting factors secreted by cells expressing COX-2 and by TAMs, the identification of the site of infiltration in the tumor microenvironment may lead to different treatment responses in breast cancer patients and may reveal unknown associations with patient survival. For instance, high stromal presence of TAMs is associated with poor OS in melanoma patients, however, in colorectal cancer, infiltration of TAMs in the tumor nest (TN) is associated with poor patient prognosis [37, 38]. Thus, it is imperative to study whether these markers of cancer-associated inflammation are concomitant to a specific compartment in the tumor microenvironment that may serve as a more relevant prognostic significance and treatment target in invasive breast cancer.

In this report we tested the hypothesis that localization of high COX-2 expression, dense infiltration of TAMs and increased stromal collagen deposition will lead to a worse patient outcome. The goal of this study was to assess whether localization of COX-2, macrophage markers CD68 and CD163 and their co-expression were associated with collagen deposition in

addition to examine their capability as independent prognostic makers in a larger U.S. cohort study of invasive carcinoma patients. Here we report that high collagen content is associated with high expression levels of COX-2 only in the TS. Compartment localization of COX-2 in the TN is associated with a worse patient prognosis. Moreover, dense infiltration of $CD68^+$ in the TS and $CD163^+$ macrophages in both TN and TS leads to worse overall survival (OS). Finally, high infiltration of COX-2-expressing $CD68^+$ and $CD163^+$ macrophages in the TS leads to poor patient OS and progression-free survival (PFS). Taken together, this data suggest that tumor localization of these inflammatory markers are associated with poor patient survival in invasive breast cancer. Most importantly, the mere presence of COX-2-expressing $CD68^+$ and $CD163^+$ macrophages leads to both poor overall survival and poor progression-free survival in only the TS. Invasive breast carcinoma patients with high COX-2 expression in the TN or dense TAMs also expressing COX-2 in the TS would benefit from therapy with a selective COX-2 inhibitor such as celecoxib.

RESULTS:

Characterization of the invasive carcinoma cohort

A total of 371 female confirmed invasive carcinoma cases were included in this study. Patients in this cohort were diagnosed from 1999 – 2009. At time of diagnosis, their age ranged from 26 to 94 years-old, with a median age of 54 (mean = 56). Most of the cohort were racially Caucasian (96%) and 1.9% were Black or African - American. Thirteen percent of the patients

had family history of breast cancer and 6% of the cohort had a clinical history of a prior breast cancer. Moreover, tumors were of ductal invasive carcinoma type (83%) and stage of I or II (41% or 47%, respectively). Positive receptor status for tumors were: 80% for ER, 72% for PR and 17% for HER2. Following the St. Gallen consensus 2013 [39], 39% of the tumors had a Ki-67 proliferation score of $\geq$14% and for molecular subtypes: 44% of the tumors were luminal A, 27% were luminal B and 12% were triple negative. For more detailed patient demographics and clinicopathological features, see Table 3.1. Furthermore, median duration of patient follow-up was 8.4 years, where 22% of patients experienced cancer recurrence within a period of 2.3 years and 26% were deceased (55% due to breast cancer) in a median timeframe of 3.5 years. For more detailed patient follow-up data, see Table S3.1.

Characterization and distribution of COX-2, $CD68^+$ and $CD163^+$ macrophages in invasive carcinoma

From the 371 invasive breast carcinoma cases, TMA cores from 352 cases (95%) were suitable for measuring COX-2 expression, and 313 cases (84%) were included to measure CD68, CD163. COX-2/ CD68 and COX-2/ CD163 macrophage infiltration. The exclusion of these cases were due to poor tissue quality, low tumor cell content or loss of cores after TMA processing. As demonstrated in Figure 3.1, COX-2, $CD68^+$ and $CD163^+$ cells were present in both TN and TS of invasive breast carcinoma cases. COX-2 expression was more common in TN and macrophage presence as labelled with CD68 and CD163 was more common in TS. Besides, we observed that cells could be double positive for CD68 and CD163 or single positive for either of these macrophage markers. This clearly suggests that there is more than 2 populations of tumor

associated macrophages. Overall COX-2 expression was associated with TN (P <0.0001), $CD68^+$ macrophages were associated with TS (P <0.0001) and $CD163^+$ macrophages were also associated with TS (P <0.0001) (Table S3.2). However, there was not a strong association between high expression of COX-2, high $CD68^+$ and high $CD163^+$ macrophage presence in TN or TS (Table S3.3).

Associations between COX-2, $CD68^+$ and $CD163^+$ macrophages and clinicopathological features in invasive carcinoma

To be able to assess the effect of COX-2, $CD68^+$ and $CD163^+$ macrophages in the invasive carcinoma cohort, and to examine whether localization of these cancer inflammatory biomarkers have a role in tumor initiation and/ or progression, we analyzed their association with several clinicopathologic features jointly in both TN and TS tissue compartments. Cases that had expression for COX-2, $CD68^+$ and $CD163^+$ above the 75^{th} percentile of the data, were consider as high expression or dense infiltration in the tumor microenvironment for each specific marker. It is important to note that in the majority of tumor samples CD68 and CD163 macrophage infiltration was absent or at very low levels, whereas most of the samples had some level of COX-2 expression. Interestingly, we observed that the distribution of high COX-2 differed in TN from TS. However, this account is not due to an increased in the amount of TN or TS content, since they are found to be not moderate or strongly correlated with high COX-2 expression (Table S3.2).

Furthermore, invasive carcinoma tumors that had high expression of COX-2 in the TN, were associated with ER^- status ($P = 0.026$) (Table 3.2). We found that higher COX-2 expression in the TS was associated with higher collagen deposition ($P < 0.0001$). Furthermore, there were several features that affected macrophage infiltration in invasive carcinoma tumors. Tumors that had high presence of $CD68^+$ macrophages in TN, were smaller ($P = 0.004$) and were associated with positive lymph node status ($P = 0.043$). Similarly, $CD68^+$ tumors in the TS were also smaller ($P = 0.011$) and were associated with lower collagen deposition ($P < 0.008$) (Table 3.2). In addition, invasive carcinoma tumors that had high infiltration of $CD163^+$ macrophages in the TN, were smaller ($P = 0.014$) and had positive lymph node status ($P = 0.012$). Likewise, tumors that had dense infiltration of $CD163^+$ macrophages in the TS, were smaller ($P = 0.004$) and had decreased collagen deposition ($P = 0.007$) (Table 3.2). Moreover, $CD68^+$ macrophages that also expressed COX-2 in the TS, were associated with smaller tumor size ($P = 0.013$) and decreased proliferation ($P = 0.015$). $CD163^+$ macrophages that also expressed COX-2 in both TN and TS were negatively correlated with collagen deposition ($P = 0.022$, $P = 0.047$, respectively). In addition, COX-2-espressing $CD163^+$ macrophages in the TN were associated with younger patients ($P = 0.026$) and in the TS with decreased proliferation ($P = 0.021$) (Table 3.2). Overall, these data indicates that increased collagen deposition is significantly associated with high expression of COX-2 in the TS. Besides, tumors with dense infiltration of macrophages in the TS, suggest a role in tumorigenesis where their increased infiltration is associated with smaller tumors, less proliferative cells and decreased collagen content.

Role of high COX-2 expression and high density of CD163+ and CD68+ macrophage infiltration in patient survival

The maximum duration of follow-up for the invasive carcinoma cohort was 13.6 years (median of 8.4 years). During this follow-up period, the cohort had 74% overall survival (95 deaths among 371 cases) and 22% disease recurrence (80 recurred among 371 cases) (Table S3.1). From the 80 cases that recurred, 76% (61 of 80 cases) passed away during follow-up. Furthermore, we found that high COX-2 expression in TN (P = 0.011), high infiltration of CD68 in TS (P = 0.015) and high infiltration of CD163 in both TN and TS (both, P = 0.004) were associated with poor patient survival (Figure 3.2 and Table 3.3). Additionally, the combined presence of high COX-2/ CD68 expression in both TN and TS (P = 0.02, P = 0.009, respectively) and the combined presence of high COX-2/ CD163 expression in both TN and TS (P = 0.005, P = 0.001, respectively) were associated with poor patient survival (Figure 3.2 and Table 3.3). Notably, COX-2 in the TS and CD68 in the TN were not associated with patient overall survival. Likewise, collagen deposition was not a significant marker for OS (P = 0.3685). Several clinicopathologic features, including age at diagnosis of invasive carcinoma, larger tumor size, higher grade and stage, lymph node positive status, ER^-, PR^-, triple receptor-negative status and higher Ki-67 index were associated with a worse disease prognosis (Figures S3.1, S3.2 and Table 3.3) and worse progression-free survival outcome (Figures S3.2, S3.3 and Table 3.3).

Moreover, we found that high expression of COX-2 in TN has a moderate association with progression-free survival (P = 0.051) (Figure 3.3 and Table 3.3). Also, the combined presence of high COX-2/ CD68 expression and high COX-2/ CD163 expression in TS (P = 0.016, P = 0.038,

respectively) were associated with poor survival due to breast cancer metastasis (Figure 3.3 and Table 3.3). Besides, high COX-2/ CD68 expression and high COX-2/ CD163 expression in TN had a borderline significance for poor patient survival due to metastatic breast cancer. Collagen deposition was not a significant marker for PFS ($P = 0.28$). Taken all together, this data suggest that high COX-2 expression in the TN leads to a worse patient prognosis. However, high presence of $CD68^+$ and $CD163^+$ macrophages in both TN and TS is associated with worse OS but not PFS. Finally, dense infiltration of $CD68^+$ and $CD163^+$ macrophages that also expressed high levels of COX-2 lead to poor patient overall survival and poor survival due to metastatic breast cancer.

To be able to describe how all significant clinicopathologic factors and experimental markers jointly impact patient survival, we performed a multivariate analysis. All covariates that had a p-value smaller than 0.05 were considered statistical significant. After adjusting for patient age at diagnosis and tumor stage, multivariate analysis for overall survival demonstrated that only the presence of high CD163 in the TN in addition to the proliferation marker Ki-67 affected negatively patient survival ($P = 0.000$ for both) (Table 3.4). In contrast, COX-2 and CD168 both in TN and TS, CD163 in the TS and macrophages expressing COX-2 did not provide prognostic information independently of other variables for OS and PFS. Moreover, high proliferation as measured by the marker Ki-67 was the only feature that had a negative effect on patient survival after they had metastatic breast cancer recurrence ($P = 0.001$) (Table 3.4). This analysis suggests that high density of $CD163^+$ macrophages in the TN and Ki-67 play an independent role in patient OS, however, Ki-67 alone is an independent prognostic factor for PFS.

DISCUSSION

COX-2 expression is often seen in 50-70% of breast cancers and dense macrophage infiltration is observed in a minority of cases. Here, we tested the hypothesis that the localization of COX-2 over-expression and dense TAMs infiltration have an association with high collagen content and poor invasive carcinoma patient outcome. We find that high collagen content in the TS is significantly associated with high expression of COX-2. In addition, tumors with high infiltration of macrophages in the TS compartment associate with smaller tumors, less proliferative cells and decreased collagen deposition, suggesting a role for macrophages in tumor progression. Interestingly, compartmentalization of high COX-2 expression in the TN is associated with decreased OS and PFS in invasive breast carcinoma patients. Dense infiltration of $CD68^{+}$ and $CD163^{+}$ macrophages in both TN and TS impairs OS. Additionally, high infiltration of $CD68^{+}$ and $CD163^{+}$ macrophages expressing COX-2 in the TS leads to poor patient OS and PFS. Finally, the multivariate analysis reveals that CD163 in the TN may be an independent prognostic marker for OS in invasive breast cancer. These data suggest that in invasive breast carcinoma patients, whose tumors express high COX-2 or have dense macrophage infiltration expressing COX-2 would benefit from a selective COX-2 inhibitor therapy, such as celecoxib.

Many studies have demonstrated the implication of COX-2 in the development and progression of many cancers, including breast and colon cancers [16]. Besides cytokines, interleukins, hormones and growth factors, COX-2 can also be stimulated by specific mutations in *Wnt*, *Ras* and *HER2* oncogenes [20–22]. The correlation between COX-2 and HER2 has been

controversial and could be due to the type and number of cases included in the cohort, such as varying patient demographics, tumor characteristics and disease stage, among others [40]. In this study, we observed an increase number of cases that were $HER2^+$ and also expressed high levels of COX-2 (Table S3.4). However, when we jointly compared all clinicopathological variables for their effect in COX-2 expression, HER2 receptor expression did not have an effect on COX-2 expression. Though, there was an association between COX-2 in the TN and ER^- status. In fact, other reports, found that high COX-2 expression was associated with decreased OS in ER^+ breast cancer tumors confirming our results [24, 40–42]. The proliferation marker Ki-67 has been previously associated with COX-2 in other studies [24, 41]. However, we did not find any association between these 2 markers.

To our knowledge, we are the first study to look at COX-2 and its expression in different tumor tissue compartments: Tumor nest and tumor-associated stroma. The univariate analysis revealed that only high COX-2 expression in the TN resulted in reduced OS and had a borderline negative association with PFS. Other studies containing patient cohorts from different regions of the world found a similar association for overall COX-2 overexpression (includes whole tissue) in breast cancer patients for OS, PFS and relapse-free survival [40–43]. However, Ristimaki et al, which utilized the largest patient cohort of 1984 cases of different types of breast cancer found that overall, COX-2 expression was only negatively associated with OS [24]. Clearly, our study demonstrates that compartment localization for COX-2 overexpression is an important factor for patient prognostic significance.

The tumor microenvironment contains a variety of stromal cells, such as tumor-associated macrophages. Recent evidence demonstrated that pro-tumoral TAMs, are involved in several tumor promoting processes including proliferation, promotion of breast tumor epithelial cell migration and metastasis [30, 31]. CD68 and CD163 are glycoproteins that are expressed in human monocytes and tissue macrophages and play a role in the immune response [44, 45]. CD163 is a scavenger receptor that is overexpressed by macrophages in an anti-inflammatory environment [46] and it is considered a highly specific monocyte/macrophage marker for polarized pro-tumoral macrophages [45, 47, 48]. On the other hand, CD68 is a pan-macrophage marker that recognizes both pro- and anti-tumoral macrophages [44]. In our study we find that CD68 and CD163 are associated with larger tumor size in both TN and TS and only CD163 is additionally associated with positive lymph node status in the TN and PR status in both TN and TS. Furthermore, in the univariate analysis, we find that high infiltration of CD68 in only TS and high infiltration of CD163 in both TN and TS are negatively associated with OS. Our results differ from Medrek et al, since they were able to find clinicopathological and survival associations with macrophage infiltration exclusively in the tumor-associated stroma [49]. Consequently, in the multivariate analysis, we find that dense infiltration of $CD163^{+}$ macrophages in the TN is an independent negative prognostic factor for overall survival. In contrast, Medrek et al, found that CD68 in the TS is an independent prognostic marker for breast cancer specific survival [49]. Possible differences in results could be due to very distinct patient cohorts: number of samples analyzed, ethnicity, variation in age, distribution of receptor status and molecular types and patient treatment.

Moreover, there is evidence of macrophages expressing high COX-2 levels in the tumor microenvironment. It has been suggested that COX-2-derived PGE2 can enhance TAMs pro-tumoral capabilities [32, 33, 50]. For instance, higher expression levels of COX-2 in TAMs has been suggested as a prognostic factor for melanoma progression and also dense infiltration of COX-2-expressing TAMs are observed after tumor irradiation, leading to early prostate cancer growth in mice [51, 52]. In addition, Cox-2 inhibition with etodolac, caused loss of pro-tumoral macrophage characteristics and diminished breast cancer metastasis [53]. We find in the univariate survival analysis that dense infiltration of $CD68^+$ and $CD163^+$ macrophages that also expressed high levels of COX-2 in both the TN and TS led to reduced patient OS and PFS. However, we did not find that dense infiltration of COX-2-expressing TAMs was an independent prognostic factor for invasive breast cancer. Interestingly, a study by Li et al., found in a Chinese cohort of patients that $COX\text{-}2^+$ TAMs, as measured with CD163 expression in the whole tissue, were associated with poor patient survival and may serve as an independent prognostic biomarker [50]. These data suggest that dense infiltration of TAMs that also express COX-2 may play an important role in breast cancer tumor development and play a negative role in patient survival.

Furthermore, it would be important to evaluate the role of high COX-2 expression and its association with tumor promoting TAMs in larger studies, which include more demographic data such as menopausal status, BMI and mammographic density. Such data is important to take into consideration since high BMI in post-menopausal women and mammographic density have been previously reported as breast cancer risks [54, 55]. Besides, it would be insightful to further evaluate the role of collagen matrix deposition and other related factors such as mammographic

density and collagen alignment to study plausible mechanisms in which the matrix is been remodeled in the tumor microenvironment due to both COX-2 over-expression and its inhibition. In this work we find that high collagen content in the tumor TS is associated with elevated levels of COX-2. However, our univariate analysis for patient survival did not revealed collagen deposition as a significant marker for OS or PFS (P = 0.3685 and P = 0.2803, respectively). Previously, we reported the association between high expression of COX-2 or PGE2 and increased collagen deposition in collagen-dense tumors [35]. In addition, other reports demonstrated that increased COX-2 and collagen I gene expression was correlated with reduced survival and shorter time to metastasis [56] and collagen alignment is negatively associated with patient survival outcome [57, 58]. Larger scale studies would be needed to conclude whether COX-2, pro-tumoral TAMs and collagen deposition may be independent prognostic markers or they are features involved in initiating and promoting breast cancer. COX-2 inhibitors are relatively inexpensive when compared to standard cancer treatments. For instance, celecoxib, a selective COX-2 inhibitor, have tolerable side effects and it has been previously demonstrated that it makes tumor cells more susceptible to radiotherapy [59]. Taken together, COX-2 overexpression in the tumor nest and the sole presence of dense COX-2-expressing TAMs play a role in decreased patient survival. Breast cancer patients with increased COX-2 expression and COX-2-expressing $CD163^{+}$ macrophages in the TN will benefit from COX-2 inhibitory therapy.

MATERIALS AND METHODS

Invasive breast carcinoma patient samples

The invasive breast carcinoma tissue microarray (TMA) used in this study was obtained from the University of Wisconsin Biobank. The protocol to build and use de-identified patient tissue and select clinical patient information was approved with a waiver of consent by the University of Wisconsin Comprehensive Cancer Center Health Sciences Institutional Review Board (UWCCC-HS IRB). Thus all research using this TMA was IRB exempt. The use of the TMA was the most efficient way to test hundreds of precious breast cancer tumor specimens and be subjected to the same experimental process at the same time. This invasive breast carcinoma TMA, consisted of 371 female cases diagnosed from 1999 to 2009 and 15 normal cases divided into 3 slides. For this study normal cases were excluded. The TMA sections were 4 microns thick and each case contained triplicate punch biopsies of 0.6mm in diameter and placed adjacent to each other. De-identified patients' demographics and clinical information corresponding to each case was entered into a data-base. The patient cohort was composed mainly by Caucasians (96%), with a median age at diagnosis of 54 years, a median duration of follow up of 100.7 months (8.39 years) and a median duration to recurrence of 27.5 months (2.29 years). Receptor status and Ki-67 data was obtained from manual chart review and registry database.

Histology and Multiplex Immunofluorescence:

For histology, tissues were formalin-fixed and prepared by standard methods for paraffin-embedding (FFPE) and sectioning as previously mentioned elsewhere [60]. One TMA set was stained with hematoxylin and eosin (H&E) to get a sense of tumor and stroma composition. For IF, the protocol was followed as described previously [35]. Briefly, FFPE tissues were subject to

standard deparaffinization, dehydration and antigen retrieval with Citra Plus (Biogenex HK080-5K) for 20 minutes, blocking with BLOXALL, avidin/ biotin (Vector SP-6000 and SP-2001, respectively), and normal serum. Then the TMA sections were subjected to the TSA Plus kit for tissue labeling following manufacturers' protocols (Perkin Elmer, fluorescein NEL741E001KT, Cy 3.5 NEL744E001KT and Cy 5 NEL745E001KT). Briefly, primary antibodies were incubated as following: Pan-cytokeratine (1:1000, 1hr); COX-2 (1:1000, O/N); CD163 (1:1000, 1hr) and CD68 (1:1000, 1hr). HRP-conjugated anti-rabbit (Abcam, ab7090) or anti-mouse (Abcam, ab97023) was added for 10 minutes following 10 minutes incubation with TSA Plus kit working solution including desired fluorophore. Tissues underwent the antigen retrieval step for 20 minutes if the same tissue would be subjected to multiple labelings before counterstaining with DAPI for 2 minutes 1:10000 (Life Technologies, D21490). TMAs were mounted with ProLong diamond (Molecular Probes-Life technologies, P36961).

Masson's Trichrome Staining

To assess collagen deposition in the TMA samples, Masson's trichrome staining kit (Cancer Diagnostics Inc., SS1026-MAB-250) was used on paraffin embedded sections and standard staining protocol provided by the manufacturer was applied. Briefly, FFPE TMAs were deparaffinized and rehydrated. Tissues were fixed in Bouin's solution for 1 hour at 56 C to improve staining quality and then immersed in Weigert's iron hematoxylin working solution and then Biebrich scarlet-acid fuchsin for 10 minutes each. Next, tissues were differentiated in phosphomolybdic-phosphotungstic acid solution for 10 minutes and transferred to aniline blue solution for 5 minutes. Staining of tissue was further differentiated by 1% acetic acid solution for 2 minutes, then tissues were dehydrated through a series of ethanol immersions, cleared in

xylene and mounted. Samples were subjected to washes with distilled water between staining and differentiation steps. Collagen fibers stained blue, cell nuclei stained black and cell cytoplasm, muscle tissue and keratin stained red.

Nuance and InForm Software

The Vectra system was used to acquire TMA core images with a 20X objective and Vectra software v2.0.8 (Perkin Elmer) with analysis done as previously described [35, 60]. A spectral library was generated using image cubes to delineate distinctive spectral curves for each of the fluorophores and DAPI counterstain to correct for background effects and subsequently, to accurately identify positive staining and quantitation of biomarkers using InForm version 2.1 software (Perkin Elmer). With the system's automated slide-handling and pattern recognition-based image analysis, samples were segmented, analyzed and quantified in an objective manner. Algorithms for tissue and subcellular compartment separation were created by machine learning and all algorithms were above 95% for precision (Figure S3.4). Algorithms using the guidance of epithelial cell marker, pan-cytokeratin, were created for separating tissue compartments into tumor nest (epithelium) and tumor-associated stroma. This process allowed us to identify nuclei, as stained with DAPI, to accurately assign associations for positive staining of a marker to a specific compartment in the biopsy sample. 10% of the image data set was used to create algorithms for each experimental analysis. For COX-2 expression, we used a semi-quantitative scoring (H-score), which reflects both percent of positive cells and intensity of the staining. For CD-68 and CD163, markers, we applied the percent positivity analysis which looked into the amount of cells that are positive for that particular marker normalized to the total number of cells present in the tissue sample. For COX-2/ CD68 and COX-2/ CD163 expression levels, we

utilized the percent double positivity analysis which looked into number of cells that were double positive for COX-2 and a macrophage marker at the same time and normalized to total number of cell present in the tissue sample. For Masson's trichrome, image analysis of spectral images were analyzed by the positive amount of blue pixels (collagen) over the total amount of pixels in the core area and multiplied by 100 to obtain percent area of collagen deposition. For all of the analyses, there were a maximum of three cores per case. The average of the cores was used as the final score for each case.

Statistical Analysis

The correlations between expression of COX-2, or CD68 or CD163 and amount of tumor or stroma content were calculated using Spearman's correlation test. A Spearman's p correlation value of 0.3 was considered an indication of weak to moderate correlation and a value over 0.7 was considered a strong correlation. The data was divided into quartiles to reduce the range of data points and confidence intervals. High COX-2 expression and high infiltration of TAMs included the cases that were in the upper 75^{th} percentile of the data for the specific marker. The associations between biomarker expression and clinicopathological parameters were assessed by a Wald test, were simultaneously all the clinical parameters were entered into a logistic model and compared for their effect contribution (predictors) to high COX-2, or CD68 or CD163 expression. For univariate analysis, survival curves for OS or PFS were calculated according to the Cox proportional hazards model. PFS was calculated from patient age at diagnosis to the occurrence of metastases outside the locoregional area or death from breast cancer, whichever occurred first. Different survival curves were compared with the Kaplan-Meier log-rank method. Multivariate survival analysis was performed using the Cox proportional hazards regression

model. In the multivariate analysis, all variables that were associated significantly with patient survival in the univariate analysis were included in the final analysis. Additionally, COX-2 TS and CD68 TN were included. Age at diagnosis and tumor stage are covariates that were forced into the model. Cox proportional hazards regression was done using forward, backward and stepwise selection of variables, and a *P* value of 0.1 was adopted as the limit for inclusion of a covariate. All selection models yielded the same variable inclusion results for OS and PFS. For all the statistical analysis, COX-2, CD68, CD163, COX-2/ CD68 and COX-2/ CD163 were converted into categorical variables divided into quartiles ranging from 0 (no expression) to 3 (high expression). For survival analyses, H-scores for COX-2 were converted into binary data and high COX-2 H-score included all data falling into the upper 75^{th} quartile. All statistical tests were two-sided. For all statistical tests, except when noted, a P value of < 0.05 was considered statistically significant. For statistical analysis and graphs preparation we used the following software packages: SAS version 9.4 (SAS Institute, NC), JMP version 11 Pro (SAS Institute, NC) and R software version 3.2.2 (R Core Team, Vienna, Austria).

Table 3.1. Patient characteristics for the study

Patient Demographics	**% (N)**
Total cases included in the study[#]	371
Date of diagnosis	1999 - 2009
Age at diagnosis (mean, SD)	56 ±14
Race	
Caucasian	96% (356)
Other*	4% (15)
Family history of breast cancer	13% (47)
Prior cancer site	
Breast	6% (24)
Multiple including breast	0.5% (2)
Non-breast	4% (15)
Clinicopathologic Features	**% (N)**
Histology type	
Ductal	83% (308)
Lobular	9% (34)
Mammary	7% (27)
Other**	1% (2)
Tumor stage	
I	41% (153)
II	47% (173)
III	12% (45)
Tumor grade	
1	24% (87)
2	42% (152)
3	34% (125)
Tumor size (Mean, range)	26.5 (2 - 150 mm)
Lymph node positive status	40% (150)
Receptor status	
ER+	80% (292)
PR+	72% (260)
HER2+	17% (44)
Ki67 ≥ 14%[≠]	39% (139)
Molecular subtypes[≠]	
Luminal A	44% (109)
$HER2^{+}$ Luminal B	11% (26)
$HER2^{-}$ Luminal B	16% (40)
Triple negative	12% (30)

352 cases were included for COX-2 data analysis and 313 cases were included for CD68, CD163, COX-2/ CD68 and COX-2/ CD163 data analyses. * Includes: Asian (3), Black (7), Hispanic (2) and other/unknown (2). ** Includes: Adenoid cystic (1) and phyllodes (1); ≠ Follows the St. Gallen consensus 2013. mm = millimeters; ( ) = number of cases.

Figure 3.1

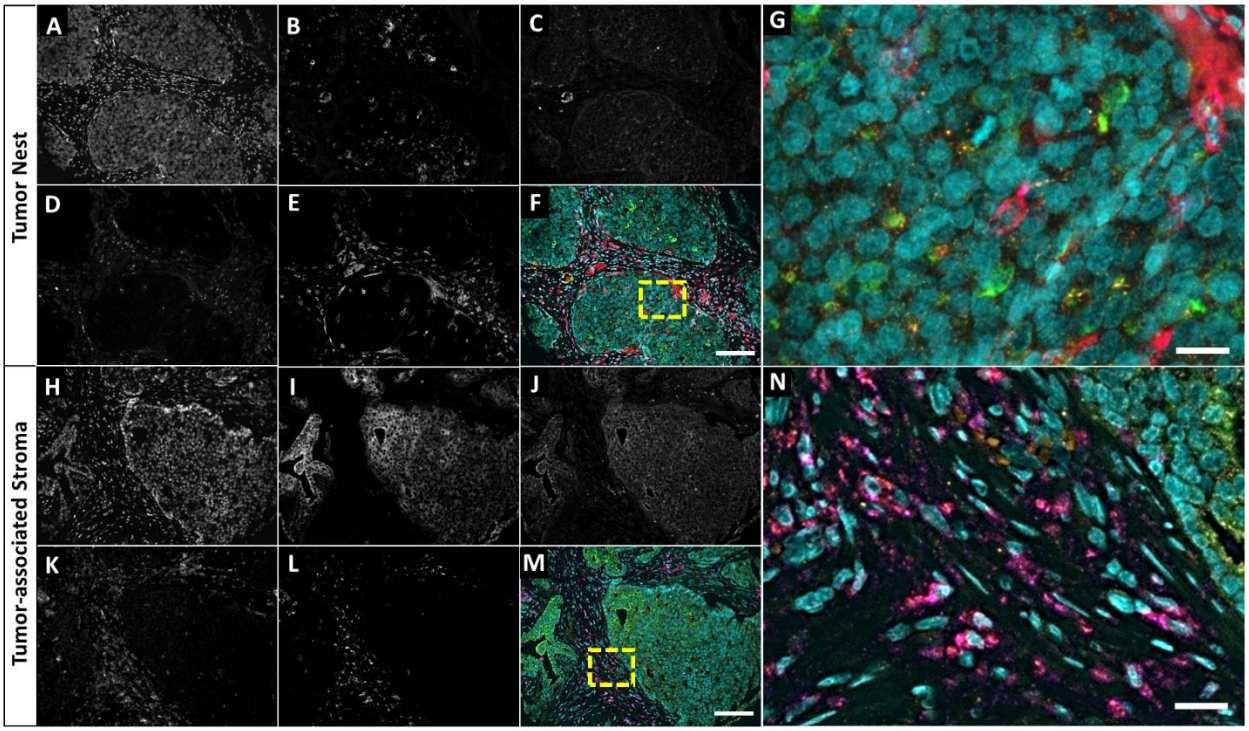

Figure 3.1. Immunofluorescence staining of invasive carcinoma tumors. A - N. Immunofluorescence images of COX-2 positive cells, $CD68^{+}$ and $CD163^{+}$ macrophages in tumor nest (A - G) or tumor-associated stroma (H - N). COX-2 positive cells, $CD68^{+}$ and $CD163^{+}$ macrophages are observed in both: tumor nest and tumor-associated stroma. **A, H.** Nuclear labeling with DAPI. **B, I.** Epithelial labeling with pan-cytokeratin. **C, J.** COX-2 staining. **D, K.** CD68 staining. **E, L.** CD163 staining. **F, M.** Composite image. Pan-cytokeratin = green, COX-2 = orange, CD68 = fuchsia, CD163 = red and DAPI = turquoise. 20x objective, scale bar = 100um. **G, N.** Zoomed-in image of area demarked by the yellow window of composites, scale bar = 15um.

Table 3.2. Individual clinicopathologic predictors for COX-2, CD68, CD163 and macrophages expressing COX-2 in tumor nest and tumor stroma in invasive breast cancer

Clinicopathologic features	COX-2				CD68				CD163				COX-2/ CD68				COX-2/ CD163			
	Tumor Nest		Tumor Stroma		Tumor Nest		Tumor Stroma		Tumor Nest		Tumor Stroma		Tumor Nest		Tumor Stroma		Tumor Nest		Tumor Stroma	
	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*
Age	1.55	0.213	0.90	0.342	1.77	0.183	0.00	0.997	0.56	0.454	1.25	0.263	2.55	0.110	0.52	0.469	4.95*	**0.026**	1.68	0.194
Tumor size	0.08	0.784	2.57	0.109	8.25**	**0.004**	10.20**	**0.001**	6.03*	**0.014**	5.55**	**0.004**	3.26	0.071	6.17*	**0.013**	2.47	0.116	1.4	0.237
Tumor grade	1.19	0.551	0.14	0.930	0.74	0.692	0.65	0.723	0.22	0.895	1.4	0.496	0.2	0.905	1.57	0.457	0.11	0.944	0.83	0.659
Tumor stage	1.42	0.493	0.01	0.997	5.23	0.073	3.22	0.200	5.96	0.051	1.7	0.428	1.66	0.437	3.82	0.148	1.15	0.564	0.3	0.861
Nodal stage	0.34	0.560	0.09	0.760	4.11*	**0.043**	2.04	0.153	6.37*	**0.012**	1.36	0.244	0.48	0.488	0.33	0.565	3.74	0.053	0.14	0.712
ER status	4.93*	**0.026**	0.59	0.444	0.05	0.817	0.93	0.335	0.05	0.827	0.1	0.757	0.41	0.521	0.09	0.763	2.6	0.107	0.03	0.863
PR status	0.23	0.634	0.60	0.438	0.04	0.845	0.10	0.753	0.52	0.469	0.41	0.524	0.21	0.650	0.01	0.918	0.34	0.563	0.23	0.633
HER2 status	0.07	0.793	0.21	0.647	0.95	0.329	0.04	0.848	0.47	0.495	0.17	0.681	2	0.157	0.73	0.395	2.49	0.115	1.35	0.246
Ki-67	1.83	0.177	2.19	0.139	3.54	0.060	4.19*	**0.041**	3.78	0.052	1.34	0.248	3.54	0.060	5.89*	**0.015**	0.16	0.693	5.35*	**0.021**
Collagen Deposition	0.72	0.397	13.8***	**0.000**	3.02	0.082	7.07**	**0.008**	6.41*	**0.011**	7.19**	**0.007**	3.19	0.074	0.9	0.342	5.28*	**0.022**	3.93*	**0.047**

* = $P < 0.05$; ** = $P < 0.01$ and *** = $P < 0.0001$;

Figure 3.2

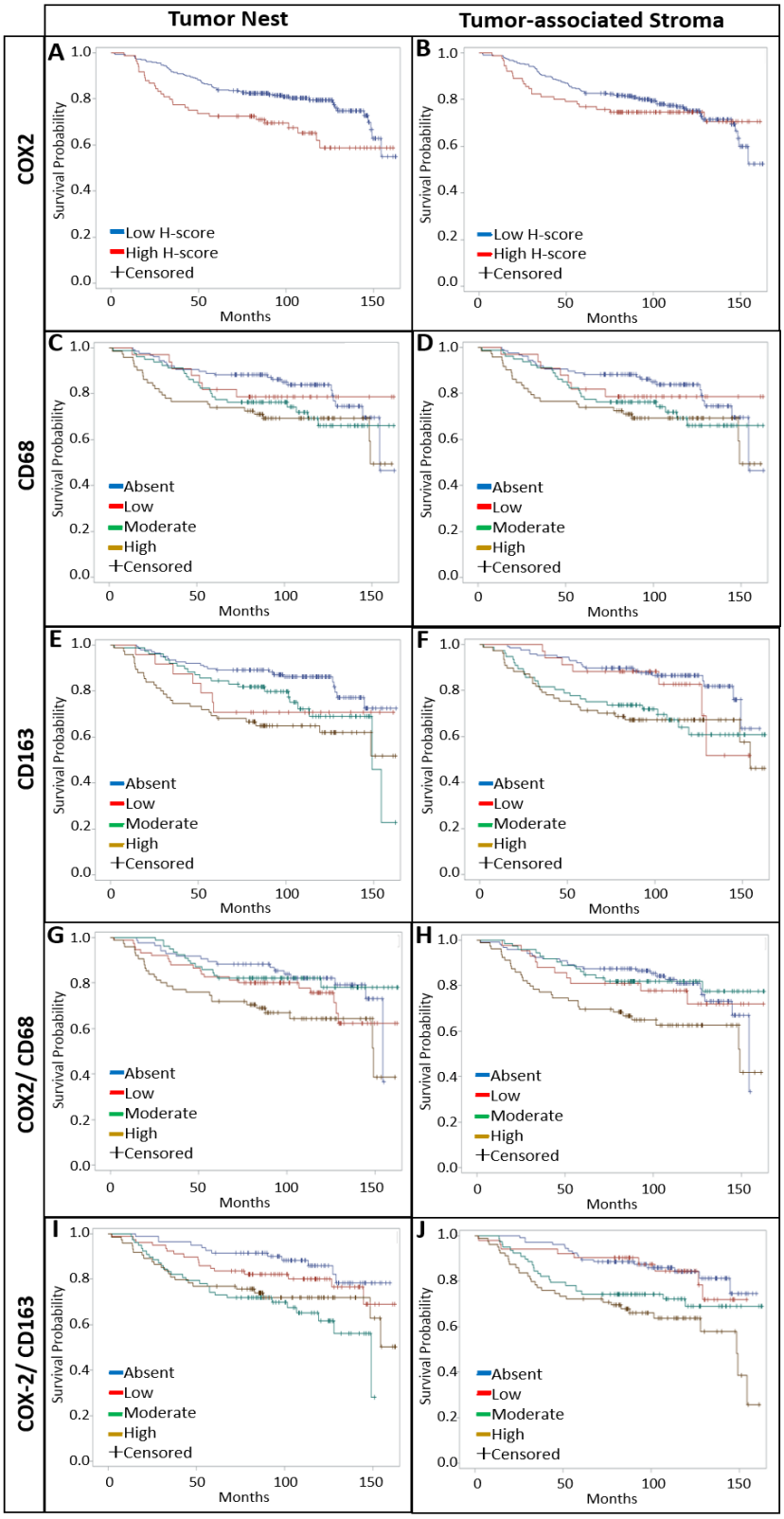
Tumor Nest
Tumor-associated Stroma
COX2
CD68
CD163
COX2/ CD68
COX-2/ CD163
A
B
C
D
E
F
G
H
I
J
Survival Probability
Months
Low H-score
High H-score
Censored
Absent
Low
Moderate
High

Figure 3.2. Overall survival with respect to COX-2 expression and macrophage infiltration in invasive carcinoma tumors. **A – F.** Overall survival curves according to COX-2 expression, $CD68^+$ and $CD163^+$ macrophages in the tumor nest or tumor-associated stroma of invasive carcinoma cases. **G - H**. Overall survival curves according to COX-2 expression in both $CD68^+$ and $CD163^+$ macrophages in the tumor nest or tumor-associated stroma of invasive carcinoma cases.

Figure 3.3

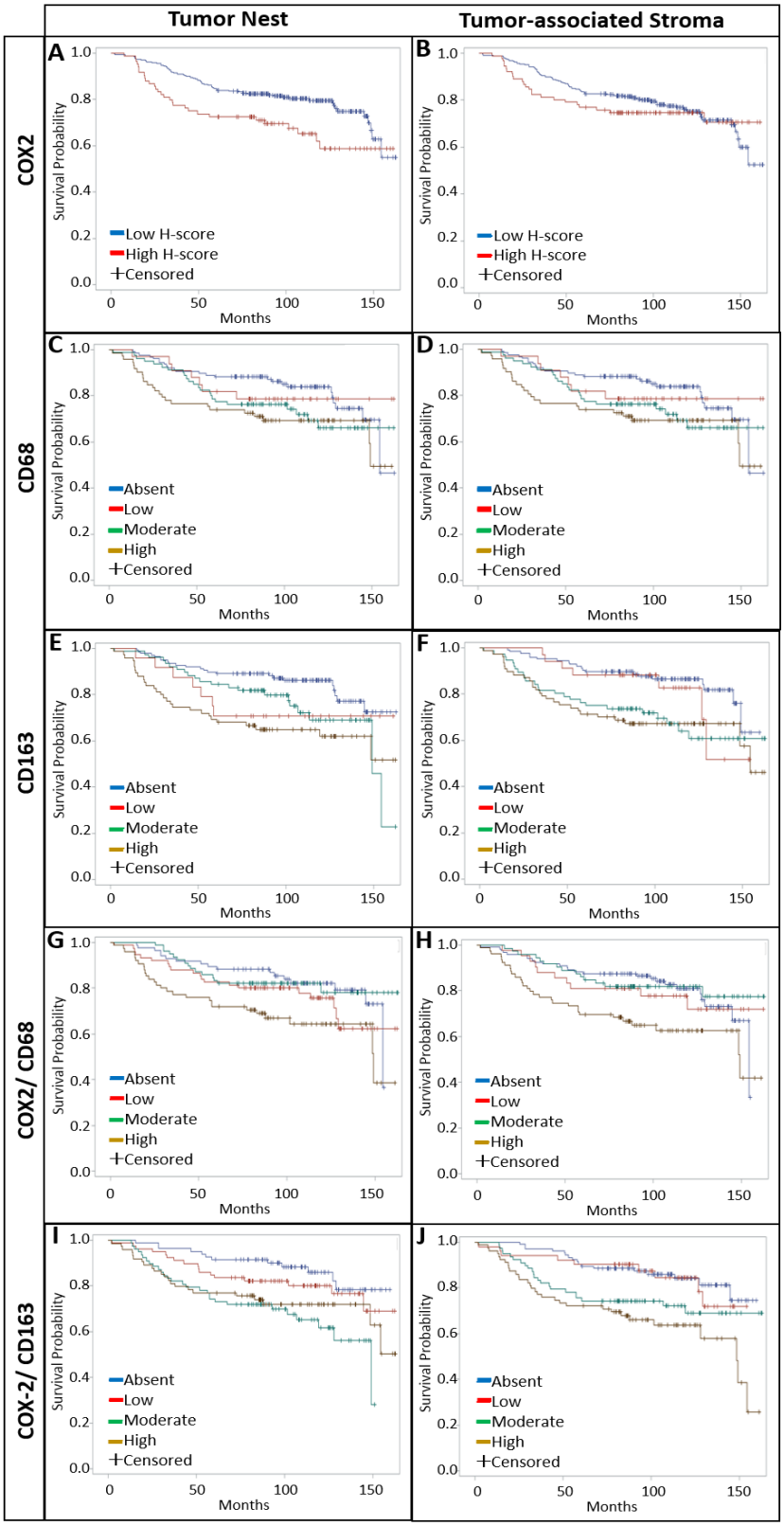
Tumor Nest
Tumor-associated Stroma
COX2
CD68
CD163
COX2/ CD68
COX-2/ CD163
A
B
C
D
E
F
G
H
I
J
Survival Probability
Months
Low H-score
High H-score
Censored
Absent
Low
Moderate
High
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150

Figure 3.3. Progression-free survival with respect to COX-2 expression and macrophage infiltration in invasive carcinoma tumors. A – F. Progression-free survival curves according to COX-2 expression, $CD68^+$ and $CD163^+$ macrophages in the tumor nest or tumor-associated stroma of invasive carcinoma cases. **G - H**. Progression-free survival curves according to COX-2 expression in both $CD68^+$ and $CD163^+$ macrophages in the tumor nest or tumor-associated stroma of invasive carcinoma cases.

Table 3.3. Univariate Cox regression analysis for OS and PFS

	OS	PFS
Variables	***P***	***P***
Age at diagnosis	0.000	0.013
Tumor size	0.000	0.000
Tumor grade	0.000	0.000
Tumor stage	0.000	0.000
Lymph node status	0.007	0.001
ER status	0.000	0.000
PR status	0.000	0.000
HER2 status	0.787	0.767
Ki67 (proliferation)	0.000	0.000
Molecular types	0.000	0.002
COX-2 TN	0.011	0.051
COX-2 TS	0.584	0.192
CD68 TN	0.107	0.157
CD68 TS	0.015	0.157
CD163 TN	0.004	0.108
CD163 TS	0.004	0.171
COX-2/ CD68 TN	0.020	0.069
COX-2/ CD68 TS	0.009	0.016
COX-2/ CD163 TN	0.005	0.054
COX-2/ CD163 TS	0.001	0.038

OS = Overall survival; PFS = Progression-free survival.

Table 3.4. Multivariate Cox regression analysis for OS and PFS

	OS			PFS		
Variables	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age at diagnosis	1.04	1.02 - 1.06	0.001	0.97	0.95 - 1.00	0.039
Tumor stage						
I	1	(Reference)		1	(Reference)	
II	3.53	0.76 - 16.27	0.106	1.42	0.18 - 11.19	0.742
IIA	1.96	0.90 - 4.25	0.090	1.84	0.76 - 4.50	0.179
IIB	1.95	0.76 - 5.04	0.167	2.62	1.07 - 6.43	0.036
III	0.00	0.00 - Inf	0.997	4.47	0.00 - Inf	0.999
IIIA	2.66	1.02 - 6.90	0.044	6.42	2.47 - 16.73	0.000
IIIB	1.99	0.44 - 9.00	0.371	0.00	0.00 - Inf	0.997
IIIC	0.16	4.14 - 63.87	0.000	3.05	0.38 - 24.58	0.296
Ki67 (proliferation)	1.02	1.01 - 1.04	0.000	1.02	1.01 - 1.03	0.001
CD163 TN						
Negative	1	(Reference)		Not retained in stepwise		
Low	0.93	0.28 - 3.10	0.909	model		
Moderate	1.03	0.40 - 2.63	0.955			
High	4.67	2.34 - 9.30	0.000			

OS = Overall survival; PFS = Progression-free survival; HR = hazard ratios; CI = Confidence intervals; Inf = infinite.

Supplemental Data:

Table S3.1. Patient follow-up characteristics

Patient Follow-up data	% (N)
Median duration of follow up (range)	100.7 (1.8 - 162.8 months)
Disease recurrence	
Overall	22% (80)
Local	4% (14)
Distant	20% (66)
Site of distant recurrence	
Bone	28% (18)
CNS	8% (5)
Lung	12% (8)
Lymph nodes	9% (6)
Multiple	32% (21)
Other*	11% (7)
Median duration to recurrence (range)	27.5 (1.4 – 159.7 months)
Vital status	
Alive	74% (276)
Dead	26% (95)
Median time to death (range)	42.4 (1.8 – 154.3 months)
Death due to breast cancer	55% (52)

*Includes: liver (2), peritoneum (1) and pleura (4).
( ) = number of cases.

Table S3.2. Correlation between the amount of tumor nest or stroma content and COX-2 expression, CD163$^+$ and CD68$^+$ TAMs in TN and TS.

Clinicopathologic features in tumor stroma	Tumor nest content			Tumor stroma content		
	Spearman's ρ	*P*	N	Spearman's ρ	*P*	N
COX-2	-0.299	<.0001	352	-0.029	0.585	352
CD68	-0.146	0.009	313	0.326	<.0001	313
CD163	-0.187	0.001	313	0.255	<.0001	313
COX-2/ CD68$^{\#}$	-0.111	0.05	313	0.196	0.0005	313
COX-2/ CD163$^{\#}$	-0.207	0.0002	313	0.054	0.344	313

$^{\#}$ Density of CD163$^+$ and CD68$^+$ TAMs that are also COX-2 positive. A Spearman's ρ correlation value of at least 0.3 was considered as an indication of a weak to moderate association.

Table S3.3. Correlation between the amount of tumor nest or stroma content and high COX-2 expression and high density of $CD163^+$ and $CD68^+$ TAMs in TN and TS

Clinicopathologic features in tumor stroma	Tumor nest content			Tumor stroma content		
	Spearman's ρ	*P*	N	Spearman's ρ	*P*	N
COX-2	-0.186	0.082	88	0.188	0.079	88
CD68	-0.264	0.019	79	0.386	0.0004	79
CD163	-0.224	0.047	79	0.271	0.016	79
COX-2/ $CD68^{\#}$	-0.13	0.253	79	0.013	0.912	79
COX-2/ $CD163^{\#}$	0.032	0.778	79	-0.155	0.174	79

$^{\#}$ Density of $CD163^+$ and $CD68^+$ TAMs that are also COX-2 positive. High COX-2 expression and high density of $CD163^+$ and $CD68^+$ TAMs only include all the cases that were over the 75^{th} percentile of the data for the specific marker. A Spearman's ρ correlation value of at least 0.4 was considered as an indication of a moderate but not strong association.

Table S3.4. Distribution of high COX-2 expression and dense infiltration of $CD68^+$ and $CD163^+$ macrophages in receptor-positive and triple-negative invasive breast cancer

Marker	High presence in tumor nest % (N)					High presence in tumor stroma % (N)				
	Whole cohort	ER	PR	HER2	Triple Negative	Whole cohort	ER	PR	HER2	Triple Negative
COX-2	25% (88)	19% (53)	19% (48)	25% (10)	27% (13)	25% (88)	21% (57)	20% (50)	30% (12)	20% (12)
CD68	25% (79)	21% (51)	21% (44)	37% (14)	19% (10)	25% (79)	20% (49)	19% (41)	34% (13)	20% (11)
CD163	25% (79)	22% (52)	21% (44)	32% (12)	19% (10)	25% (79)	22% (52)	21% (44)	29% (11)	20% (10)

High COX-2 expression and high density of $CD163^+$ and $CD68^+$ TAMs only include all the cases that were over the 75^{th} percentile of the data for the specific marker.

Figure S3.1

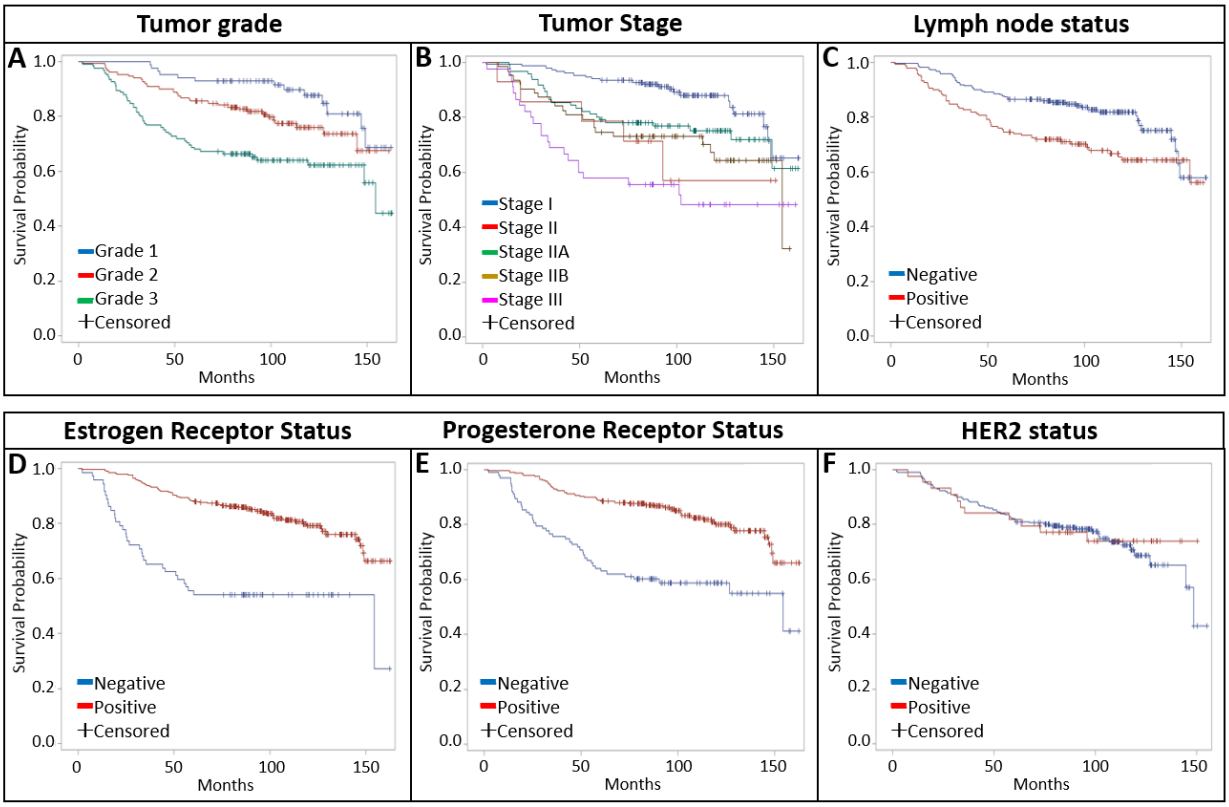

Figure S3.1. Overall survival (OS) according to several clinicopathological features in invasive carcinoma tumors. **A - F**. OS curves according to **A.** tumor grade, **B.** tumor stage, **C.** lymph node status, **D.** Estrogen receptor status, **E.** Progesterone receptor status and **F.** HER2 status of invasive carcinoma cases.

Figure S3.2

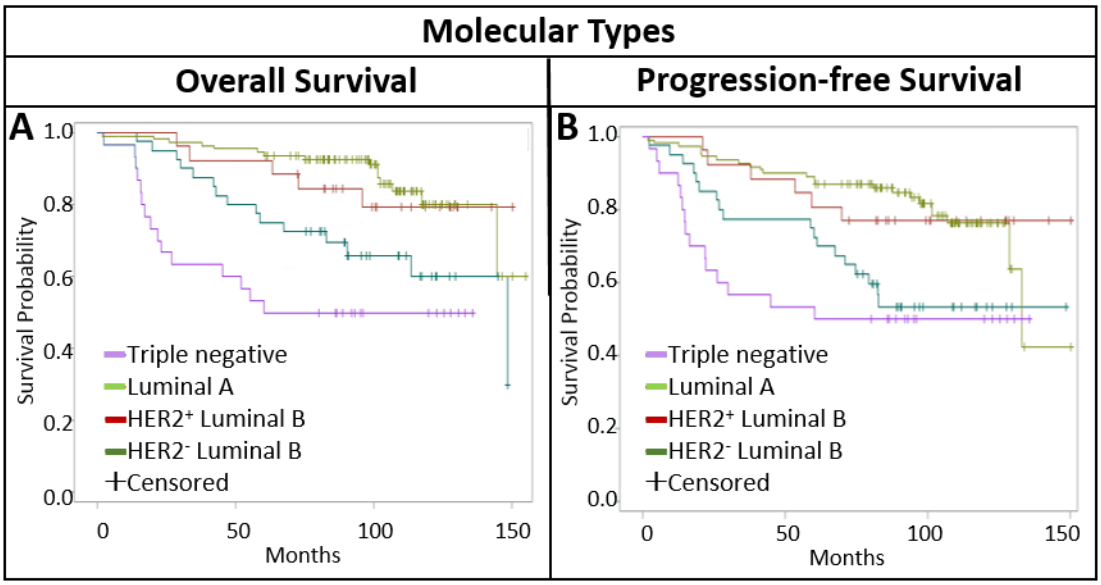

Figure S3.2. Overall survival (OS) and progression-free survival (PFS) according to several molecular types in invasive carcinoma tumors. **A**. OS curve according to molecular types. **B.** PFS curve according to molecular types in invasive carcinoma cases.

Figure S3.3

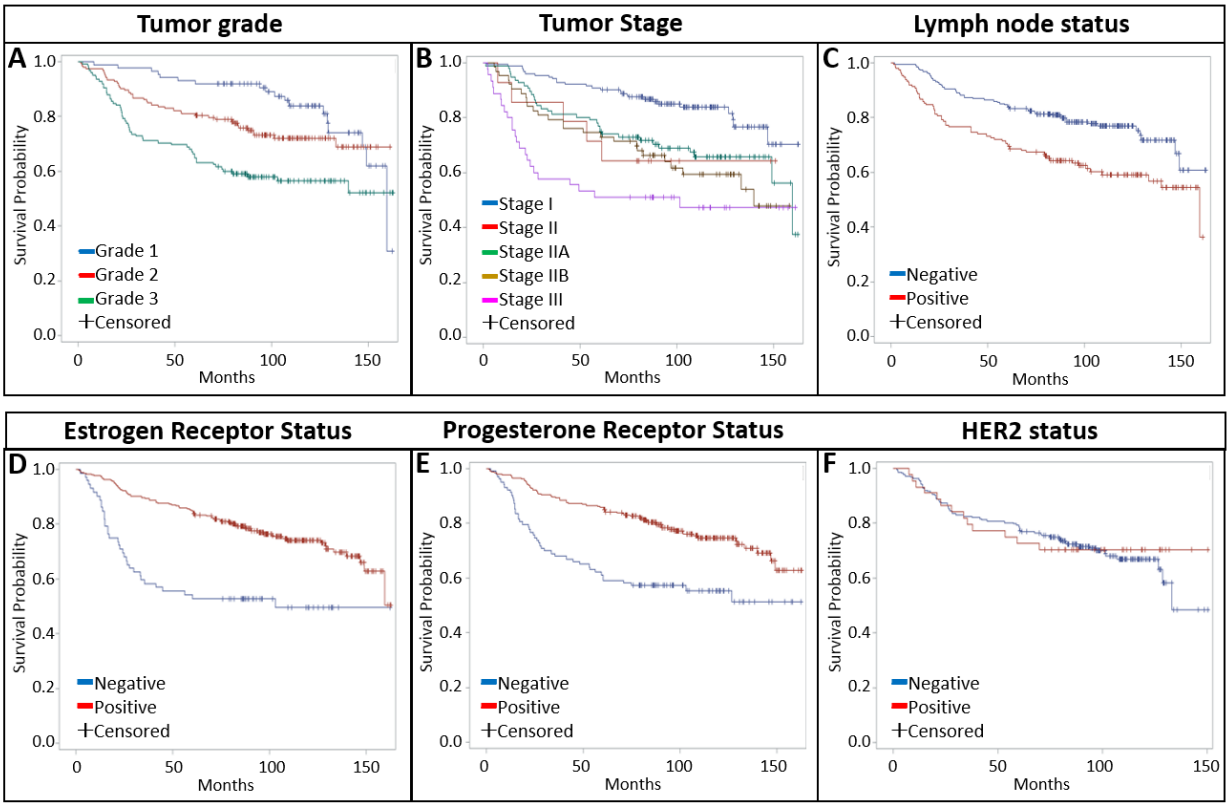

Figure S3.3. Progression-free survival (PFS) according to several clinicopathological features in invasive carcinoma tumors. **A - F**. PFS curves according to **A.** tumor grade, **B.** tumor stage, **C.** lymph node status, **D.** Estrogen receptor status, **E.** Progesterone receptor status and **F.** HER2 status of invasive carcinoma cases.

Figure S3.4

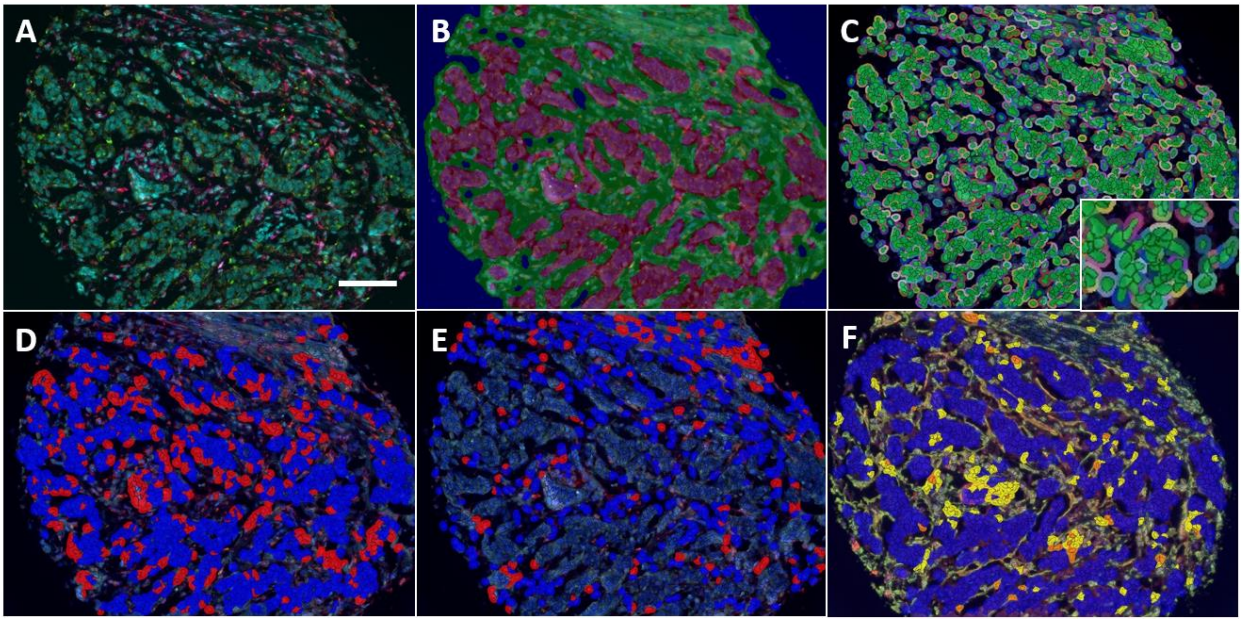

Figure S3.4. Tissue segmentation and marker analysis. A-F. Images for the process of tissue segmentation. Algorithms for tissue segmentation, i.e. tumor epithelium versus tumor stroma, were created by machine learning (see methods). **A.** Sample IF image cube of mouse tumor stained with pan-cytokeratin (green), COX-2 (orange), CD68 (purple), CD163 (red) and counterstained with DAPI (turquoise). 20x objective, scale bar = 100um. **B.** Tissue segmentation map after training the software. Red = epithelium, green = stroma, blue = other (empty space, debris/ artifacts) **C.** Overlay of total object cell count map. The insert zooms in a region to depict that for cell surface markers (colorful cell outlines), cells were further segmented into nuclei and membrane. **D - E.** Each object (cell) circled in green in **C** was associated to its respective tissue compartment; tumor nest **D** or tumor-associated stroma **E**. Blue = negative cells for a marker, red = positive cells for a marker. Debris was associated with the "other" category and not included in the statistics analysis. **F.** Cell count for COX-2 positivity. Blue = 0^+, yellow = 1^+, orange 2^+ and purple = 3^+. The sum of the percent of cells falling into each of the 0^+ to 3^+ categories were converted into H-score which ranges from 0 to 300.

CHAPTER 4

Conclusions and Future Directions

From the experiments described in this thesis, several important findings were discussed with respect to the role of COX2 and other inflammatory components of the tumor microenvironment, which negatively influenced mouse and human outcome in breast cancer. The main objective for this thesis was to have a better understanding of the role of COX-2 in breast cancer. To this end, the relationship between COX-2 and the ECM, specifically collagen dense matrices, and with other cancer-associated stromal and inflammatory cells, such as TAMs and CAFs, was investigated. Importantly, the impact of COX-2 overexpression overall or in specific stromal populations, such as pro-tumoral macrophages, on human survival was assessed.

Chapter 2 described numerous experiments in two preclinical rodent models of mammary carcinoma which describes the effect of COX-2 and its inhibition with celecoxib in response to collagen-dense tumors in a therapeutic and preventive setting. First, in the therapeutic pre-clinical mouse model, we found that collagen-dense tumors were larger, had higher proliferation rate, higher presence of macrophages and fibroblasts and expressed greater levels of COX-2, PGE2 and several cytokines when compared to tumors in wildtype mice. Consequently, treatment with the COX-2 inhibitor, celecoxib, resulted in smaller and less proliferative tumors, with decreased presence of stromal cell populations, such as macrophages and fibroblasts, and diminished levels of COX-2, PGE2, and several cytokines. It is important to emphasize that, COX-2 inhibition reduced collagen matrix deposition. It has been previously shown that

collagen alignment is negatively associated with patient survival outcome [23, 164]. Therefore, it would be insightful to study collagen alignment and structure to observe the matrix remodeling in the tumor microenvironment as a result of COX-2 over-expression and its inhibition. A better understanding at ECM regions of COX-2 cellular overexpression in these mouse model compare to intact mammary gland or wildtype animals would elucidate possible mechanisms for COX-2 overexpression and its effect in ECM modulation in the collagen-dense tumor microenvironment.

Finally, in Chapter 2, celecoxib was tested as a preventive agent, in a separate pre-clinical trial using the same dense collagen mammary carcinoma mouse model. Again tumors arising from collagen-dense animals were larger and more abundant and expressed increased levels of cytokines when compared to animals treated with celecoxib or their wild-type counterparts. To reinforce our results, there is overwhelming epidemiological evidence demonstrating that COX-2 inhibitors can significantly decrease breast cancer risk [117, 166]. Nevertheless, our preventive mouse model has the caveat of being genetically-driven and not a spontaneous model of mammary carcinoma. Consequently, it would have been unrealistic to accomplish complete elimination of mammary tumors in this context. An alternative experiment could take place in the 4T1- BALB/c x Col1a1^{tm1jae} syngeneic xenograft model of metastatic breast cancer. The 4T1- BALB/c model is well-characterized and based on luciferase-expressing 4T1 mammary tumor cell line of the 4T1 mouse [167, 168]. Taken into consideration the right controls, wildtype and collagen-dense experimental animals could be treated with celecoxib before and after orthotopic administration of 4T1 tumor cells to be able to confirm the outcomes described in Chapter 2. This study offers an ideal setting for studying the role of COX-2 and the immune system in tumor growth and metastasis in the context of collagen density in the breast.

Furthermore, by means of no more than the maximum celecoxib dose recommended by the FDA, we demonstrated in both animal models that the drug has the most effect only in subjects containing collagen-dense tumors. These findings could partly explain outcomes of human breast cancer clinical trials for Celebrex, which show no statistical significance and suggests that treatment with this drug should be catered to breast cancer patients with mammographically dense breasts and tumors with high COX-2 expression. Taken together, chapter 2 demonstrates that COX-2 modulates several components in the collagen-dense tumor microenvironment, including stromal and inflammatory cell populations, matrix deposition, tumor formation and progression. Conversely, inhibition of COX-2 with celecoxib, reduces tumor growth and development, collagen deposition, and reverses all the inflammatory and tumor promoting effects by significantly decreasing expression of several cytokines in either therapeutic or preventive settings. These key findings suggest that COX-2 may be an effective therapeutic target for women with dense breast tissue and early stage breast cancer.

Moreover, Chapter 3 described the findings of a TMA analysis of over 300 cases of invasive carcinoma and their association between high expression levels of COX-2, macrophage markers CD68 and CD163, high infiltration of COX-2-expressing macrophages and patient survival outcome. We concluded that tumor localization of these inflammatory markers were involved in patient survival outcome. For instance, high COX-2 expression in the TN, high infiltration of $CD68^+$ macrophages only in the TS and high infiltration of $CD163^+$ macrophages in both TN and TS were associated with poor OS. Overall, $CD68^+$ and $CD163^+$ macrophages in the TN and TS who also expressed COX-2, were associated with both OS and PFS. Additionally, the

multivariate analysis revealed that CD163 in the TN was an independent predictor for OS but not PFS in invasive breast cancer.

Even though the invasive breast carcinoma TMA allowed us to study a vast array of cases, it is important to note that the cores analyzed were a small snapshot of the patients' tumor microenvironment. It is known that the tumor microenvironment is a heterogeneous system, and for that reason, if the tissue sections were bigger, we would have had the opportunity to execute a more comprehensive and precise study. Accordingly, the effect of COX-2 in a multivariate analysis for patient survival may have to be observed in bigger sections and larger patient sample size. In fact, in a multivariate analysis which included close to 2000 breast cancer samples, Ristimaki et al., were able to find COX-2 as an independent predictor of breast cancer PFS [93]. Another difficulty with this TMA cohort was the limited ethnic and racial diversity, as our cohort was 96% Caucasian. It is necessary to include more racially and ethnically diverse cohorts for this type of studies. Several of the previous reports suggesting COX-2 as a negative predictor for patient survival and associations with diverse clinicopathological features are from Europe or Asia [93, 111, 150–152]. Additionally, this cohort included only 12% of triple-negative breast cancer cases. Triple-negative breast cancer is an aggressive subtype most often associated with women of African-American racial background, it leads to cancer recurrence within the first 2 years after diagnosis, has a tendency to form metastasis in the lungs and brain and it is associated to poor patient prognosis [169]. There is evidence that COX-2 over-expression is associated with poor survival in triple-negative breast cancer patients. Triple-negative breast cancer patients with high COX-2 expression had a 5-year DFS of 58.3%, compared to 83.9% of patients with no COX-2 expression [170]. Therefore, it will be critical to study these inflammatory

biomarkers in other minority groups with invasive breast cancer in the U.S. such as in people with Hispanic or African descent.

Our study cohort lacked information about patients' menopausal status and body mass index (BMI). Several lines of evidence demonstrated obesity, as measured by BMI, as a risk factor for post-menopausal breast cancer and a prognosis factor for reduced survival [3]. There is evidence that increased levels of the inflammatory marker PGE-M, a PGE2 metabolite found in the urine, is associated with obesity, greater risk of tumor growth and lung metastasis in breast cancer patients [80]. It would be fundamental to have a better understanding of the role of COX-2 and pro-tumoral macrophages in obese patients at risk of breast cancer or patients with this disease to prevent or treat this patient subpopulation accordingly. It may be plausible, that triple-negative breast cancer patients and obese patients could benefit from therapy with a COX-2 inhibitor such as celecoxib.

Likewise, it will be imperative to include additional mammographic density and collagen alignment data to our invasive breast cancer TMA database. This will allow the study of their association with COX-2 and other inflammatory stromal markers and their impact to patient outcome. A study found that increased gene expression of both COX-2 and collagen I was associated with decreased survival and shorter time to metastasis [30]. In Chapter 2, we found an association between high expression of COX-2 or PGE2 and increased collagen deposition in collagen-dense tumors. Moreover, cases from the breast cancer TMA described in Chapter 3 were additionally stained with Masson's trichrome to study collagen content in the samples. We found that increased collagen deposition was exclusively associated with COX-2 expression only

in the TS. However, in the univariate analysis for patient survival, collagen deposition was not a significant marker for OS or PFS (P = 0.3685 and P = 0.2803, respectively). Thus, collagen deposition was never included in the multivariate analysis. It would be necessary to do larger scale studies to conclude if collagen deposition could be a prognostic marker or is a factor largely involved in promoting breast cancer. It would also be clinically relevant to include analyses of collagen structure, such as alignment, since it has been previously demonstrated that TACS-3 aligned collagen fibers predict patient survival outcome [23, 164]. If associations are significant, they could be used as a prognostic tool to adequately choose therapies for breast cancer patients with higher risks of relapse and metastasis.

In breast cancer, there is emerging evidence of the effect of celecoxib as a therapeutic drug in human clinical studies and there are ongoing studies evaluating its effect alone or in combination with several regimens. For instance, in a randomized phase II clinical trial, the pre-operative treatment with celecoxib changed expression level of several genes, including genes related to cell cycle, proliferation, extracellular matrix and inflammatory immune response in invasive carcinoma patients when compared to placebo [102]. Moreover, combination therapy of celecoxib and exemestane, an aromatase inhibitor, had similar or better efficacy compared with exemestane alone and only cardiovascular adverse events were observed at longer time frames and at the higher doses [64, 65, 98–101]. Another phase II clinical study, combined treatment with celecoxib and capecitabine, a drug that aids in the delivery of the anti-cancer agent 5-fluorouracil (5-FU) in metastatic breast cancer patients and concluded patients with COX-2 overexpressing tumors had significantly longer time to progression and median overall survival [136]. However, there are two clinical trials with biological endpoints that show no difference

with celecoxib alone or in combination with exemestane. The first study never reached power because of enrollment difficulties and their results concluded that celecoxib did not significantly inhibit COX-2, apoptosis, ER or PR expression [103]. The second study showed that in DCIS patients with ER-positive status, celecoxib had no effect on proliferation or apoptosis alone, or in combination with exemestane [104]. Different results from this clinical trials, could be due to the patient population been tested and statistical power of the study.

As mentioned above, celecoxib may only benefit a selective patient population, such as women with mammographically dense breasts and tumors overexpressing COX-2. Thus a clinical trial that studies the impact of celecoxib on women with invasive breast carcinoma that have dense breast and high COX-2 expression will be of great clinical significance. It is important to note, that heavy intake of NSAIDs (above the standard recommended dose) has been associated with negative cardiovascular outcomes from two major colorectal cancer randomized clinical trials evaluating selective COX-2 inhibitors. The warning of possible severe cardiovascular effects caused the recall of the COX-2 inhibitor, Vioxx, and was the main reason for the delay in the evaluation of selective COX-2 inhibitors, such as celecoxib, in other cancers besides colorectal cancer [117]. Consequently, this has created a bad perception of the use of selective COX-2 inhibitors in treating breast cancer and misinformation about specific COX-2 inhibitors. For instance, there are some reports that indicate that celecoxib has no increased risks of severe cardiovascular effects when compared to other NSAIDs or placebo and the used of celecoxib at the standard dose of 400mg a day may have a slight protective cardiovascular effect [171, 172].

There is evidence in a rodent study of post-partum breast cancer, that xenographed tumors that arose from involuting mammary glands, were larger, with increased collagen deposition and higher COX-2 expression and this effect was reversed when animals were treated with celecoxib [30]. These data suggests that women at high risk for or diagnosed with postpartum breast cancer could also benefit from therapy with celecoxib. To our knowledge, we are the only group with evidence that COX-2 inhibition with celecoxib modulates collagen deposition and inflammatory cells in mammary adenocarcinoma.

Based on the compelling findings in Chapter 2 and Chapter 3, and associated evidence from the literature, we are in the process of initiating a clinical study addressing celecoxib's biological effect with respect to collagen matrix structure and the stromal inflammatory response in patients with invasive carcinoma. This study will have a window of opportunity design, where the whole trial will take two weeks duration comparing the effects of Celecoxib before and after treatment in the tumor microenvironment (Figure 4.1). The proposed study will take place at the University of Wisconsin Hospital and Clinics (UWHC) with the objective of assessing whether Celecoxib can reduce the change in collagen alignment and inflammatory response in the tumor tissue of primary breast cancer patients after two weeks of oral treatment intake.

It is hoped that the results of this trial will serve as a mechanism for intact characterization of the response of the tumor micro-environment in women with primary breast cancer. The end point of this trial is a biological end point comparing the anti-tumor and stromal properties of celecoxib, where collagen alignment before and after celecoxib treatment will be evaluated in the tumor microenvironment by multiphoton microscopy. As secondary endpoints, COX-2 levels

will be used as biomarker in response to therapy. In addition, CD68, CD163, Ki67, among others, will be analyzed as tumor and/or stromal response biomarkers. Finally, any adverse events associated with the 2-week intake of 200mg celecoxib twice a day will be evaluated. This trial will apply new cutting edge technology to advance in the proof of biologic principles. In the last 20 years, early detection and better systemic therapies for early stage breast cancer have led to a small decline in mortality. New advances will require understanding of breast cancer biology at the molecular level.

Inhibition of COX-2 and analysis of its effect in the breast cancer tumor microenvironment provide one such fruitful therapeutic target. Despite of new drugs being developed to treat breast cancer and tested in clinical trials, it is rarely possible to assess how the treatment is affecting breast cancer cells at the molecular level. This window trial provides a way to look at cancer and stromal cells before and after a new treatment to see if celecoxib as a therapeutic drug is actively working. If we can do this before and after a patient has surgery, and see how the tumor microenvironment responds, then the physician could pick a better suited adjuvant treatment for this patient after surgical intervention that would improve their overall survival rate. These findings could lead to novel chemopreventive care for breast cancer patients and further insight in drug effectiveness with respect to breast density.

The breast cancer tumor microenvironment is dynamic, complex and poorly understood. While more studies are required to have a better understanding of the complexity of the mechanisms, it is clear that several inflammatory components including COX-2 and pro-tumoral macrophages, play a role not only in tumor cell development but also, in extracellular matrix modulation,

which in turn, promote a more aggressive tumor microenvironment. The work described in this thesis provides additional support for the role of inflammation in breast cancer and suggests that women with high mammographic density and invasive carcinoma may effectively benefit from COX-2 inhibition therapy.

Figure 4.1

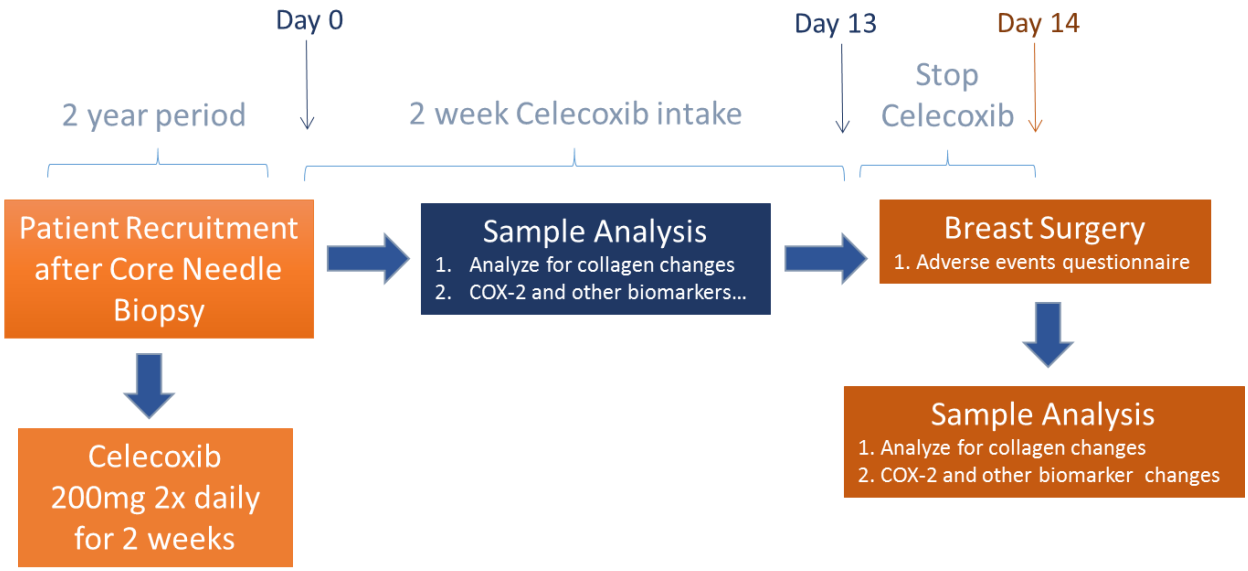

Figure 4.2. Schematic diagram of two-week pre-operative celecoxib window of opportunity clinical trial. Each eligible patient (ages 18 – 90) will have invasive breast carcinoma of stages T_1cN_0 to T_2N_0, with tumors greater than 1cm without lymph node spread. Each patient will intake 200mg of Celecoxib two times a day (400mg a day total) for 2 weeks after initial biopsy. Histologic tissue samples will be obtained for evaluation at time of biopsy of the tumor and at time of surgery removal of the tumor. Collagen alignment, COX-2, CD68, CD163, Ki67 and various cytokines expression levels will be measured as epithelium or stromal response biomarkers. At the end of the 2-week intake of celecoxib, an adverse events questionnaire will be offered to see if the patients had any side-effects due to the drug regimen they were taking.

APPENDIX I

Intraoperative Imprint Cytology and Frozen Section Pathology for Margin Assessment in Breast Conservation Surgery: A Systematic Review

This appendix is published as:

Karla Esbona, MS, Zhanhai Li, PhD, and Lee Wilke, MD, Ann Surg Oncol. 2012 Oct;19(10):3236-45.

PMID: 22847119

Abstract

Background. Achieving negative surgical margins is critical to minimizing the risk of local tumor recurrence in patients undergoing breast conserving surgery (BCS) for treatment of a breast malignancy. Our objective was to perform a systematic review comparing re-excision rates, sensitivity and specificity of the intraoperative use of the surgical margin assessment techniques of imprint cytology (IC) and frozen section analysis (FSA), against the gold standard of permanent section histology (PS) for breast cancer patients who underwent BCS.

Method. The databases PubMed, Web of Knowledge, Cochrane Library and CINAHL Plus were searched from January 1997 to July 1st 2011. Original investigations of patients who underwent BCS for breast cancer that evaluated margin assessment with permanent histology

and/or IC or FSA were included. Titles, abstracts, and articles were reviewed by a single author. Of 182 titles identified, 41 patient cohorts from 37 articles met the inclusion criteria: PS (n=19), IC (n=7) and FSA (n=15). Studies reporting re-excision rates after primary BCS using permanent section, IC and FSA as well as studies reporting specificity and sensitivity values for both intraoperative techniques were summarized qualitatively using the STROBE checklist for cohort studies and the SORT numerical scale for diagnostic studies. Statistical evaluation was done using SAS software.

Results. The final re-excision rates after primary BCS were 35% for permanent section, 11% for IC ($p=0.001$ *versus* PS) and 10% for FSA ($p<0.0001$ *versus* PS). For IC, re-excision rates decreased from 26% to 4% ($p=0.18$) and for FSA, re-excision rates decreased from 27% to 6% ($p<0.0001$). The pooled sensitivity of IC and FSA were 72% and 83%, correspondingly. The pooled specificity of IC and FSA were 97% and 95%, respectively. The average length of each technique during BCS was 13 minutes for IC and 27 minutes for FSA.

Conclusions. Patients who underwent BCS with intraoperative IC or FSA to assess negative surgical margins had significantly fewer secondary surgical procedures for excision of their breast malignancies.

Introduction

Breast conservation surgery (BCS) with radiation has become the preferred method of treatment for patients with Stage I and II breast cancer after the 1990 Consensus Conference on Breast Cancer from the National Institutes of Health (NIH)[1]. To achieve an oncologically and cosmetically successful BCS, performance by both the surgeon as well as an accurate assessment of the surgical margins by the pathologist is critical. Many studies[2] have made it clear that achieving a negative surgical margin on patients undergoing BCS is imperative. Despite this, there is no consensus on what constitutes a negative margin; varying from cancer not touching the ink in the specimen to 3cms of normal tissue around the tumor[3]. Local recurrence rates increases in patients who had persistent positive surgical margins compared to patients with negative margins[4-7].

The best cosmetic results are obtained during the primary BCS procedure. Re-excision of the breast results in a greater volume of resected tissue than with a single primary excision, which ultimately may affect cosmesis. In addition to the added costs of revision surgery, re-excision BCS leads to increased patient anxiety and a higher likelihood of infection[8]. Consequently, it is important to identify intraoperative margin assessment methods that would decrease re-excision rates.

Two intraoperative margin assessment methods are frequently reported in the literature: frozen section analysis (FSA) and touch preparation or imprint cytology (IC). FSA is the intraoperative technique that has been most widely used to analyze breast tumor excisions. It consists of freezing and sectioning the sample followed by thawing, fixation and staining. Reports indicate

that FSA may cause artifacts in the fatty tissue due to the process of freezing and thawing, resulting in loss of tissue. This technique takes approximately 30 minutes[9]. Due to the disadvantages of FSA, IC has been proposed as an alternative intraoperative method. IC is a simple and rapid method where the excised mass is oriented and pressed onto glass slides making an imprint of all 6 margins. Slides are then fixed and stained. The principle is based on the cellular surface characteristics where only malignant cells will adhere to the slides and adipose cells will not. This method has been reported to take only an average of 15 minutes to provide a diagnosis[2]. Variability in the sensitivity of the method has been reported and is related to the size of the tumor and the cytological skills of the pathologist[9]. Errors of interpretation are linked to specimen surface irregularity, dryness and presence of atypical cells[10].

Currently, there exists no systematic review that addresses the impact of these two intraoperative surgical margin assessment techniques in patients undergoing BCS. The primary objective of this work was to systematically review data published in primary studies of IC and FSA and to compare surgical re-excision rates, sensitivity and specificity of these techniques after primary BCS against, permanent histopathologic sectioning (PS).

Methods

Literature Search

The databases PubMed, Web of Knowledge, Cochrane Library and CINAHL Plus were searched from 1997 to July 1st 2011 using the strategy shown in *Figure 1*. The study period from 1997 was chosen as important cohort studies with intraoperative IC and FSA occurred after 1997 that

made an impact in the practice. References of included articles were also searched. The overall search strategy included terms for breast cancer (e.g., *breast neoplasm, breast cancer*), imprint cytology (e.g., *Touch prep* cytology, TPC, imprint cytology, intraoperative imprint cytology*), frozen section analysis (e.g., *frozen section or FS*), surgical margin (e.g., *surgical margin*, margin assess*, margin evaluat**), conserving surgery (e.g., *conserving surger*, lumpectomy, partial mastectomy*, segmental mastectomy*, quadrantectomy*) and re-excision rate (*e.g., re-excision rate, reexcision rate*) and was limited to peer-reviewed human studies. No language restrictions were applied.

Selection Criteria

Articles for inclusion and exclusion were screened by a single reviewer blinding for journal, authors, institution, and country of origin. The inclusion criteria for the overall search targeted articles with the following: 1) Individuals with cancer undergoing BCS; 2) Surgical margin assessment technique (IC, and/or FSA and/or permanent section) and re-excision rates and/ or specificity and sensitivity values for both intraoperative techniques; 3) Study sampling and methods stated and; 4) Publication was peer-reviewed. Articles that included sufficient data for cross-tabulation of the results of PS, IC and FSA were included. Articles were excluded if they analyzed or mixed data of lymph nodes, they used other intraoperative margin assessment techniques, or if studies were case reports or meeting abstracts.

Assessment of Methodologic Quality

Articles were extracted and assessed for quality following the same blinding criteria as above. Abstracted data included patient demographics, type of breast cancer, surgical margin

assessment techniques, re-excision rates during and after primary BCS, sensitivity and specificity of IC and FSA, false positive and negative cases for IC and FSA, study quality and type. Quality was assessed using components of the Strengthening the Reporting of Observational Studies in Epidemiology **(**STROBE)[15] checklist for cohort studies and the Strength of Recommendation Taxonomy (SORT)[16] numerical scale for diagnostic studies. To judge quality, information was abstracted on population source, whether the study had institutional approval, statistical methods and publication bias.

Data Synthesis and Statistical Analysis

Data were used on a per tumor basis rather than per patient basis to calculate re-excision rates, sensitivity and specificity. Some patients had more than one tumor extracted and analyzed and each tumor was considered as an independent specimen. If sensitivity and specificity data were missing, they were calculated from the available data. To avoid overlapping patient populations, data on recruitment years, data source, and geographic location was compared. If there were multiple publications of the same author with the same patient population, only the most comprehensive and relevant study was included. This resulted in the exclusion of one article[17].

Nine authors were contacted for unreported secondary information to complete the data tables and one author replied. The quality of this systematic review strictly depends on the quality of reporting of the selected studies. From the studies selected, only nine had a SORT/ STROBE score of 2 and the rest had a score of 1, suggesting the findings of this study are reliable.

The final re-excision rates among three groups: FSA, IC, and PS were compared using a general linear model with t -test. The t-test was also used to compare the intraoperative re-excision rate and final re-excision rate within IC and FSA pairs during and after primary surgery. Finally, the sensitivity and specificity between FSA and IC groups was compared using t-test. All analyses were conducted using SAS software (SAS Institute, Cary, NC).

Results

Our literature search yielded 182 potentially relevant articles, of which 67 were evaluated in full text. Most excluded studies did not report re-excision rates or mixed intraoperative methods. Of the 67 articles, 37 primary studies were eligible for inclusion in the systematic review. The number of publications doesn't match the number of patient cohort data sets included in the analysis. Four[11, 12, 13, 14] of 37 publications contained two different sets of data within the same publication, resulting in 41 patient cohort data sets for analysis. Nineteen publications were included for the analysis of permanent section, seven for IC and 15 for FSA.

Description of Studies

The 37 articles included in this systematic review were homogeneous in their primary goal. The articles measured re-excision rates or recurrence rates on patients undergoing BCS either with intraoperative IC, FSA and/or PS. All studies were conducted in medical institution-based surgical and pathology units. Nine studies used prospective cohorts of breast cancer patients and the remaining utilized retrospective cohorts. Most studies analyzed the use of intraoperative FSA and IC in all types of breast cancer. Five studies analyzed data from patients exclusively

with invasive carcinoma; three studies analyzed data from patients with *in situ* carcinoma and one included phyllodes tumors.

The studies were conducted in the United States (n=21), Canada (n=1), Brazil (n=2), Europe (n=9), Turkey (n=1), Asia (n=4), and Australia (n=1). Sample sizes ranged from 44 to 2,770 for PS studies (*Table 1*), from 12 to 1,193 for IC studies (*Table 2*) and from 54 to 1,016 for FSA studies (*Table 3*) with a median of 351, 328 and 259, respectively.

Permanent sections and frozen sections were stained with haematoxylin and eosin (H&E) in all but one study[18] in which methylene blue was used. The majority of imprint cytology studies used the Diff- Quik^{R} (Baxter Diagnostics, McGaw Park,, USA), rapid Papanicolaou as well as H&E techniques. The surgical margin results of IC and FSA were categorized as positive, suspicious/close or negative in all studies. Six articles reported the time taken to perform intraoperative imprint cytology (mean, 13 minutes; *Table 2*) and four articles reported the duration of frozen section analysis (mean, 27 minutes; *Table 3*). One FSA study[19] reported a mean of 53 minutes as they performed a total circumference sampling of the specimen which required more slides to analyze. From the IC and FSA analysis publications, cross-tabulation of results against permanent section was either reported or could be inferred from the data provided.

Re-excision rates

The total number of tumors analyzed across the studies of permanent section was 10,489 with a mean of 542. For imprint cytology, a total of 2,296 tumors were analyzed with an average of

300 specimens. Finally, 3,621 tumors were analyzed in frozen section analysis publications and 259 tumors were studied on average.

Using the general linear model, we found significant differences in final re-excision rates among the three groups with an overall p-value <0.0001. Subsequently, we performed a detailed examination with the t-test. The final re-excision rates of FSA (10% ± 6%) was significantly lower than PS (35 ± 3%) (p <0.0001). The final re-excision rate of IC (11% ± 4%) was also significantly lower than PS (35% ± 3%) (p=0.001) (*Figure 2A*). The final re-excision rates for FSA *versus* IC were not significantly different (p=0.92).

To further analyze the effects of intraoperative IC and FSA during primary BCS, we compared intraoperative re-excision rates and post-operative re-excision rates within FSA and IC. For FSA, the intraoperative re-excision rate of 27% ± 9% was significantly reduced to a final rate of 6% ± 6% (p<0.0001). Similarly, the intraoperative re-excision rate for IC (26% ± 21%) was reduced to a final re-excision rate of 4% ± 7%. However, it was not significantly different (p=0.18) due to the variation in the IC group (*Figure 2B*). We found that the intraoperative re-excision rate for FSA was not statistically different from the IC group (p=0.86).

Sensitivity and specificity

Five IC studies and nine FSA studies were used to analyze pooled intraoperative sensitivity and specificity. The sensitivity of FSA (83%±13%) *versus* IC (72%±38%) was not significantly

different (p=0.53). Similarly, the specificity of FSA (95%±8%) versus IC (97%±3%) was not significantly different (p=0.58). In IC studies, the occurrence of false positive cases was primarily due to the observation of atypical cells with characteristics of invasive components. False negative cases were due to patients presenting with DCIS, then ILC and LCIS (*Table 2*). With the intraoperative use of FSA, false positive cases were linked to atypical cells and sclerosing adenosis. Conversely, false negative cases were due to all types of malignancy (*Table 3*). Overall, there were more false negative cases when using intraoperative FSA in comparison with IC. In one study of FSA[20], false negatives correlated with younger patients and with larger tumors. In other FSA publications a correlation was found between false negative cases and patients with small volume of lesions[18, 21], micro-calcifications and neoadjuvant therapy[21].

DISCUSSION

The intraoperative use of margin assessment techniques in breast cancer patients undergoing BCS has proven to be sufficiently rapid to be used in a clinically relevant time period during the original surgical procedure. In addition, this approach can efficiently reduce but not eliminate the need for additional surgeries to attain negative margins in these patient populations. Both FSA and IC had a final pooled re-excision rate of approximately 10% which is meaningful for surgical outcomes. During surgery, IC took less than 15 minutes and FSA took less than 30 minutes to perform. The sensitivity and specificity for IC was found to be comparable to FSA. However, there was a greater degree of variation present in the sensitivity of IC among studies. Overall, for IC and FSA, most false negative cases were observed in tumors diagnosed with in-situ disease. In addition, false negative cases for FSA occurred on specimens from IDC. These

false negative cases could be related to sampling errors, size of tumor, size of lesions and the non-palpable state of the tumor.

Results of this systematic review are limited by the quality of the reporting of the studies analyzed. Both prospective and retrospective patient cohorts were reviewed, however, no randomized clinical trial data was available on this topic. Based on SORT and STROBE criteria, the quality of the studies selected was high; even though it is difficult to make comparisons when there are variations in the IC method, histopathological staining techniques and FSA specimen sectioning. The experience of the pathologists with each technique, especially cytopathological proficiency, was seldom mentioned and bias was unlikely to occur since the majority of patients had the intraoperative and histological tests performed as part of a protocol.

Implementations of IC and FSA will lead to declines in recurrence rates, surgical costs, patient anxiety and surgical complications and increases in cosmetic results, patient safety and patient satisfaction. Indeed, Uecker et al., have demonstrated the cost savings associated with intraoperative assessment of surgical margins from reduced surgical reoperations from two hospitals in Austin, TX [22]. In the hospital that performed routine PS, 60% of cases required reoperation with average costs of $22,013 per patient. Whereas in the hospital that performed intraoperative assessment, 24% of cases needed reoperation with average costs of $15,341. Likewise, a recent cost-effectiveness analysis of FSA by Osborn et al. compared patients that underwent lumpectomy with and without intraoperative margin assessment[50]. Patients that had lumpectomy without FSA, underwent a reoperation 15% to 50% of the time while patients that

had FSA executed during BCS, had a reoperation 3% of the time and the costs to provider and payer were less expensive.

The results of this review suggest that it is worthwhile for clinicians to consider adopting the use of IC and FSA if their current re-excision rates exceed 10%. The use of IC and FSA as intraoperative margin assessment tools requires a multi-disciplinary team effort among the surgeons, pathologists and radiologists. The presence of an on-site pathologist would be a requirement for performance of these procedures and may not be available at institutions where the pathology is performed at a separate location or there is limited experience with cytopathologic outcomes. In considering FSA and IC, the multi-disciplinary breast team should evaluate their current re-excision rates and if in the 10% rate range because of current practices of specimen imaging or use of intraoperative ultrasound, a change in practice may not be indicated. As new technology such as those using radiofrequency ablation and intraoperative optical imaging are refined, their performance against FSA and IC should be considered based on this systematic review.

References

1. National Institutes of Health Consensus Conference. Treatment of early-stage breast cancer. *JAMA* 1991;265:391-395.

2. Singletary SE. Surgical margins in patients with early-stage breast cancer treated with breast conservation therapy. *Am J Surg.* Nov 2002;184(5):383-393.

3. Sabel MS. Surgical considerations in early-stage breast cancer: lessons learned and future directions. *Semin Radiat Oncol.* Jan 2011;21(1):10-19.

4. Vapiwala N, Harris E, Huang W-T, et al: Long-term outcome for mammographically detected ductal carcinoma in situ managed with breast conservation treatment: Prognostic significance of reexcision. *Cancer J* 2006;12:25-32

5. Aziz D, Rawlinson E, Narod SA, et al: The role of reexcision for positive margins in optimizing local disease control after breast conserving surgery for cancer. *Breast J* 2006;12:331-7.

6. Swanson G, Rynearson K, Symmonds R: Significance of margins of excision on breast cancer recurrence. *Am J Clin Oncol* 2002;25:438-441.

7. Tartter PI, Kaplan J, Bleiweiss I, et al: Lumpectomy margins, reexcision and local recurrence of breast cancer. *Am J Surg* 2000;179:81-85.

8. Wazer DE, DePetrillo T, Schmidt-Ullrich R, et al. Factors infuencing cosmetic outcome and complication risk after conservative surgery and radiotherapy for early-stage breast carcinoma. *J Clin Oncol* 1992;10:356-363.

9. Laucirica R. Intraoperative assessment of the breast - Guidelines and potential pitfalls. *Arch. Pathol. Lab. Med.* Dec 2005;129(12):1565-1574.

10. Weinberg E, Cox C, Dupont E, et al. Local recurrence in lumpectomy patients after imprint cytology margin evaluation. *Am J Surg.* Oct 2004;188(4):349-354.

11. Cabioglu N, Hunt KK, Sahin AA, et al. Role for intraoperative margin assessment in patients undergoing breast-conserving surgery. *Ann Surg Oncol.* Apr 2007;14(4):1458-1471.

12. Camp ER, McAuliffe PF, Gilroy JS, et al. Minimizing local recurrence after breast conserving therapy using intraoperative shaved margins to determine pathologic tumor clearance. *J Am Coll Surg.* Dec 2005;201(6):855-861.

13. Loibl S, von Minckwitz G, Raab G, et al. Surgical procedures after neoadjuvant chemotherapy in operable breast cancer: results of the GEPARDUO trial. *Ann Surg Oncol.* Nov 2006;13(11):1434-1442.

14. Valdes EK, Boolbol SK, Ali I, Feldman SM, Cohen JM. Intraoperative touch preparation cytology for margin assessment in breast-conservation surgery: Does it work for lobular carcinoma? *Ann. Surg. Oncol.* Oct 2007;14(10):2940-2945.

15. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol*. 2008 Apr;61(4):344-9. PMID: 18313558.

16. Ebell MH, Siwek J, Weiss BD, et al. Strength of recommendation taxonomy (SORT): a patient-centered approach to grading evidence in the medical literature. *Am Fam Physician.* Feb 1 2004;69(3):548-556.

17. D'Halluin F, Tas P, Coue O, et al. Role of intraoperative imprint cytology for evaluation of surgical margins in breast cancer: a prospective controlled study about 400 lumpectomy. *Virchows Arch.* May 2008;452:S24-S25.

18. Rusby JE, Paramanathan N, Laws SA, Rainsbury RM. Immediate latissimus dorsi miniflap volume replacement for partial mastectomy: use of intra-operative frozen sections to confirm negative margins. *Am J Surg.* Oct 2008;196(4):512-518.

19. Fukamachi K, Ishida T, Usami S, et al. Total-circumference intraoperative frozen section analysis reduces margin-positive rate in breast-conservation surgery. *Jpn J Clin Oncol.* Jun 2010;40(6):513-520.

20. Munhoz AM, Montag E, Arruda E, et al. Immediate reconstruction following breast-conserving surgery: management of the positive surgical margins and influence on secondary reconstruction. *Breast.* Feb 2009;18(1):47-54.

21. Riedl O, Fitzal F, Mader N, et al. Intraoperative frozen section analysis for breast-conserving therapy in 1016 patients with breast cancer. *Eur J Surg Oncol.* Mar 2009;35(3):264-270.

22. Uecker JM, Bui EH, Foulkrod KH, Sabra JP. Intraoperative Assessment of Breast Cancer Specimens Decreases Cost and Number of Reoperations. *Am. Surg.* Mar 2011;77(3):342-344.

23. Arora S, Menes TS, Moung C, Nagi C, Bleiweiss I, Jaffer S. Atypical ductal hyperplasia at margin of breast biopsy--is re-excision indicated? *Ann Surg Oncol.* Mar 2008;15(3):843-847.

24. Huston TL, Pigalarga R, Osborne MP, Tousimis E. The influence of additional surgical margins on the total specimen volume excised and the reoperative rate after breast-conserving surgery. *Am. J. Surg.* Oct 2006;192(4):509-512.

25. Landercasper J, Ellis RL, Mathiason MA, et al. A Community Breast Center Report Card Determined by Participation in the National Quality Measures for Breast Centers Program. *Breast Journal.* 2010;16(5):472-480.

26. McCahill LE, Single R, Ratliff J, Sheehey-Jones J, Gray A, James T. Local recurrence after partial mastectomy: relation to initial surgical margins. *Am J Surg.* Mar 2011;201(3):374-378;discussion 378.

27. Menes TS, Tartter PI, Bleiweiss I, Godbold JH, Estabrook A, Smith SR. The consequence of multiple re-excisions to obtain clear lumpectomy margins in breast cancer patients. *Ann. Surg. Oncol.* Nov 2005;12(11):881-885.

28. Miller AR, Brandao G, Prihoda TJ, Hill C, Cruz AB, Yeh IT. Positive margins following surgical resection of breast carcinoma: Analysis of pathologic correlates. *J. Surg. Oncol.* Jun 2004;86(3):134-140.

29. Moorthy K, Asopa V, Wiggins E, Callam M. Is the reexcision rate higher if breast conservation surgery is performed by surgical trainees? *Am. J. Surg.* Jul 2004;188(1):45-48.

30. Mullenix PS, Cuadrado DG, Steele SR, et al. Secondary operations are frequently required to complete the surgical phase of therapy in the era of breast conservation and sentinel lymph node biopsy. *Am. J. Surg.* May 2004;187(5):643-646.

31. Ooi CWL, Serpell JW, Rodger A. Tumour involvement of the re-excision specimen following clear local excision of breast cancer with positive margins. *ANZ J. Surg.* Dec 2003;73(12):979-982.

32. O'Sullivan MJ, Li T, Freedman G, Morrow M. The effect of multiple reexcisions on the risk of local recurrence after breast conserving surgery. *Ann. Surg. Oncol.* Nov 2007;14(11):3133-3140.

33. Perez CA. Conservation therapy in T1-T2 breast cancer: past, current issues, and future challenges and opportunities. *Cancer J.* Nov-Dec 2003;9(6):442-453.

34. Ramanah R, Pivot X, Sautiere JL, Maillet R, Riethmuller D. Predictors of re-excision for positive or close margins in breast-conservation therapy for pT1 tumors. *Am J Surg.* Jun 2008;195(6):770-774.

35. Sanchez C, Brem RF, McSwain AR, Rapelyea JA, Torrente J, Teal CB. Factors Associated with Re-Excision in Patients with Early-Stage Breast Cancer Treated with Breast Conservation Therapy. *Am. Surg.* Mar 2010;76(3):331-334.

36. van den Broek N, van der Sangen MJ, van de Poll-Franse LV, van Beek MW, Nieuwenhuijzen GA, Voogd AC. Margin status and the risk of local recurrence after breast-conserving treatment of lobular breast cancer. *Breast Cancer Res Treat.* Sep 2007;105(1):63-68.

37. Vicini FA, Kestin LL, Goldstein NS, Baglan KL, Pettinga JE, Martinez AA. Relationship between excision volume, margin status, and tumor size with the development of local recurrence in patients with ductal carcinoma-in-situ treated with breast-conserving therapy. *J Surg Oncol.* Apr 2001;76(4):245-254.
38. Barros AC, Pinotti M, Teixeira LC, Ricci MD, Pinotti JA. Outcome analysis of patients with early infiltrating breast carcinoma treated by surgery with intraoperative evaluation of surgical margins. *Tumori.* Nov-Dec 2004;90(6):592-595.

39. Cox CE, Hyacinthe M, Gonzalez RJ, et al. Cytologic evaluation of lumpectomy margins in patients with ductal carcinoma in situ: clinical outcome. *Ann Surg Oncol.* Dec 1997;4(8):644-649.

40. D'Halluin F, Tas P, Rouquette S, et al. Intra-operative touch preparation cytology following lumpectomy for breast cancer: a series of 400 procedures. *Breast.* Aug 2009;18(4):248-253.

41. Klimberg VS, Westbrook KC, Korourian S. Use of touch preps for diagnosis and evaluation of surgical margins in breast cancer. *Ann Surg Oncol.* Apr-May 1998;5(3):220-226.

42. Cendan JC, Coco D, Copeland EM, 3rd. Accuracy of intraoperative frozen-section analysis of breast cancer lumpectomy-bed margins. *J Am Coll Surg.* Aug 2005;201(2):194-198.

43. Chen WH, Cheng SP, Tzen CY, et al. Surgical treatment of phyllodes tumors of the breast: retrospective review of 172 cases. *J Surg Oncol.* Sep 1 2005;91(3):185-194.

44. Dener C, Inan A, Sen M, Demirci S. Interoperative frozen section for margin assessment in breast conserving energy. *Scand J Surg.* 2009;98(1):34-40.

45. Ikeda T, Enomoto K, Wada K, et al. Frozen-section-guided breast-conserving surgery: implications of diagnosis by frozen section as a guide to determining the extent of resection. *Surg Today.* 1997;27(3):207-212.

46. Olson TP, Harter J, Muñoz A, Mahvi DM, Breslin T. Frozen section analysis for intraoperative margin assessment during breast-conserving surgery results in low rates of re-excision and local recurrence. *Ann Surg Oncol*. 2007 Oct;14(10):2953-60.

47. Park S, Park HS, Kim SI, Koo JS, Park BW, Lee KS. The impact of a focally positive resection margin on the local control in patients treated with breast-conserving therapy. *Jpn J Clin Oncol.* May 2011;41(5):600-608.

48. Weber S, Storm FK, Stitt J, Mahvi DM. The role of frozen section analysis of margins during breast conservation surgery. *Cancer J Sci Am.* Sep-Oct 1997;3(5):273-277.

49. Weber WP, Engelberger S, Viehl CT, et al. Accuracy of frozen section analysis versus specimen radiography during breast-conserving surgery for nonpalpable lesions. *World J Surg.* Dec 2008;32(12):2599-2606.

50. Osborn JB, Keeney GL, Jakub JW, Degnim AC, Boughey JC. Cost-effectiveness analysis of routine frozen-section analysis of breast margins compared with reoperation for positive margins. Ann Surg Oncol. 2011 Oct;18(11):3204-9. Epub 2011 Aug 23.

Appendix: Figure 1

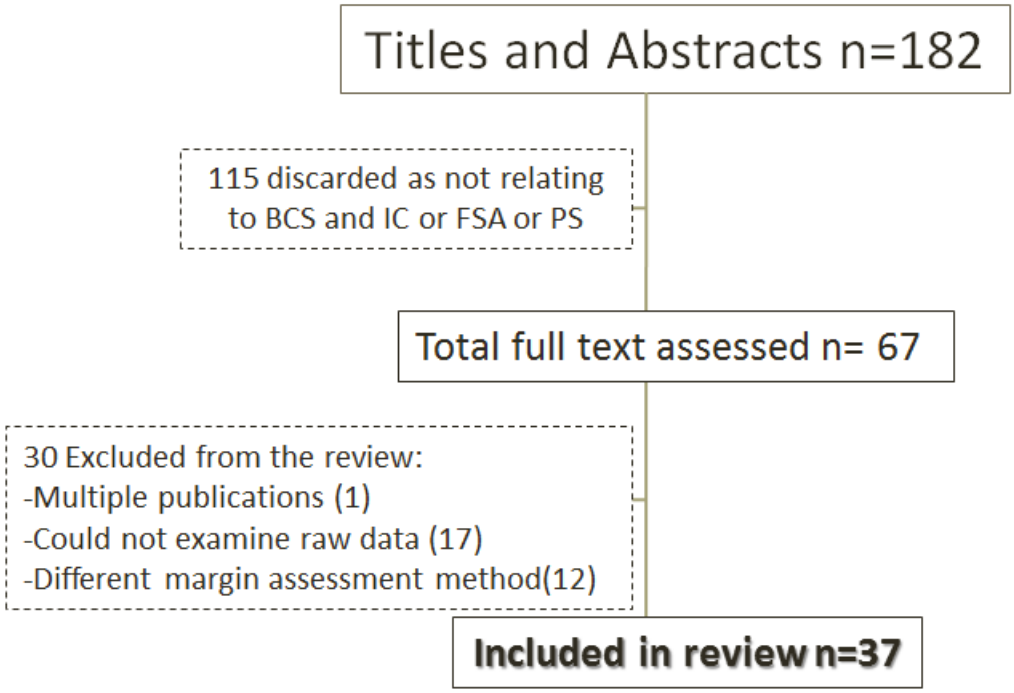

Figure 1. Flow diagram of the literature search and study selection. * The number of publications does not match the number of patient cohort data sets included in the analysis, which add to 41.

Table 1. Characteristics of permanent section studies

Author	Year	Country	Carcinoma Type	BCS	Age range	N	Re-excision rate[b]	LOE	Study type
Arora et al.[231]	2007	USA	ADH, DCIS,	Lumpectomy	42 - 87	44	55.0% (24)	1	Retrospective cohort
Aziz et al.[5]	2006	Canada	IC, DCIS	Lumpectomy	55¥	1.172	13.0% (152)	1	Retrospective cohort
Cabioglu et al.[11]	2007	USA	IC, DCIS	Wide local excision, segmental mastectomy	27 - 95	261	44.0% (116)	1	Retrospective cohort
Camp et al.*[12]	2005	USA	IC, DCIS	Lumpectomy	26 - 78	78	33.3% (26)	1	Retrospective cohort
Huston et al.[24]	2006	USA	DCIS, LCIS, IDC, ILC	Lumpectomy	30 - 91	171	29.2% (50)	2	Retrospective cohort
Landercasper et al.[25]	2010	USA	breast cancer	Lumpectomy	---	568	19.0% (108)	1	Retrospective cohort hospital report
Loibl et al.[13]	2006	Germany	ILC, IDC	Lumpectomy	26 - 78	248	27.0% (67)	1	Retrospective cohort RCT - GEPARDUO
McCahill et al.[26]	2010	USA	DCIS, IDC, ILC	Partial mastectomy	---	712	13.0% (95)	2	Retrospective cohort
Menes et al.[27]	2005	USA	DCIS, ILC, IDC	Lumpectomy	27 - 87	459	49.9% (229)	1	Retrospective cohort Prospective database
Miller et al.[28]	2004	USA	DCIS, ILC, IDC	BCS	38 - 64	143	18.2% (26)	1	Retrospective cohort
Moorthy et al.[29]	2004	UK	IDC, ILC	Lumpectomy, wide local excision, quadrantectomy	47 - 73	505	27.1% (137)	1	Prospective cohort
Mullenix et al.[30]	2004	USA	IDC, ILC	BCS	48 - 73	150	51.0% (90)	1	Retrospective cohort, prospective database
Ooi et al.[31]	2003	Australia	DCIS, ILC, IDC, IC, medullary	BCS	28 - 80	742	3.9% (29)	2	Retrospective cohort
O'Sullivan et al.[32]	2007	USA	IDC, DCIS, ILC	Lumpectomy	20 - 91	2.770	59.6% (1.651)	1	Retrospective cohort, prospective database
Perez[33]	2003	USA	ILC, IDC	Quadrantectomy, wide local excision, local excision	---	1.347	52.3% (704)	1	Retrospective cohort
Ramanah et al.[34]	2008	France	ILC, IDC	Lumpectomy, wide local excision	44 - 68	206	41.0% (84)	1	Retrospective cohort
Sanchez et al.[35]	2010	USA	IDC, ILC, DCIS, atypia	BCS	22 - 88	351	34.0% (118)	2	Retrospective cohort
van den Broek.[36]	2007	Netherlands	ILC	BCS	---	416	14.4% (60)	2	Retrospective cohort
Vicini et al.[37]	2001	USA	DCIS	Excisional biopsy	---	146	64.0% (95)	2	Retrospective cohort
Results:						**351¥**	**35% (±3%)‡**		

BCS, Breast conserving surgery; b, after first BCS; LOE, Level of evidence (SORT); N, number of samples; ADH, Atypical ductal hyperplasia; IC, Invasive carcinoma; DCIS, Ductal carcinoma in situ; LCIS, Lobular carcinoma in situ; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; ¥ sample median; ‡ pooled estimates

Table 2. Characteristics of imprint cytology studies

Author	Year	Country	Carcinoma Type	BCS	Age range	N	Intraoperative re-excision rate[a]	Re-excision rate final[b]	Sensitivity	Specificity	Time (min)	False positive cases	False negative cases	LOE	Study Type
Barros et al.[38]	2004	Brazil	IC	Quadrantectomy	28-79	102	37% (38)	0% (0)	---	---	15 - 20	13: Atypical cells present	10: Focal for DCIS (6), IC (3) and presence of carcinoma at margin in a single lymphatic space (1)	2	Prospective cohort
Cox et al.[39]	1997	USA	DCIS	Lumpectomy	30 -82	104	---	0% (1)	99%	98%		2	1: Focal for DCIS	1	Prospective database
D'Haulluin et al.**[40]	2009	France	IDC, DCIS, ILC, LCIS	Lumpectomy	29 - 88	396	38.63% (153)	12.6%(50)	88.6%	92.2%	7 - 17	25: ILC (10.2%), IDC histological grade 3 (5.6%), fibricystic mastopathy lesions	9: contact with 1 margin (4), 2mm away from margin (5). DCIS, size ≥ 30mm, histological grade of 1 or 2.	1	Retrospective cohort
Klimberg et al.[41]	1998	USA	DCIS, ILC	Lumpectomy	22 - 86	428	1.4% (6)	0% (0)	96.4%	100%	2 - 3	None	3: Focal for DCIS (2), LCIS (1). Thought to be missed by sampling error.	1	Prospective cohort
Valdes et al.*[14]	2007	USA	ILC	Lumpectomy	---	12	---	33% (4)	8.3%	98.3%	15	1: Atypical lobular hyperplasia	8: Focal for ILC (7) and ILC plus LCIS (1)	2	Prospective cohort
Valdes et al.*[14]	2007	USA	IDC	Lumpectomy	---	61	---	23% (14)	65.7%	95.5%	15	4.10%	3.3%, DCIS	2	Prospective cohort
Weinberg et al.[10]	2004	USA	DCIS, ILC, IDC	Lumpectomy	---	1,193	---	6.2% (74)	---	---	15	---	---	1	Retrospective cohort
Results:						**104¥**	**26% (±21%)‡**	**11% (±4%)‡**	**72% (±38%)‡**	**97% (±3%)‡**	**13**				

BCS, Breast conserving surgery; N, number of samples; a, during first BCS; b, after first BCS; LOE, Level of evidence (SORT); IC, Invasive carcinoma; DCIS, Ductal carcinoma in situ; LCIS, Lobular carcinoma in situ; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; * Publication used twice in analysis because of different study cohorts in same publication** Cross publication; ¥ sample median; ‡ pooled estimates.

Table 3. Characteristics of frozen section analysis studies

Author	Year	Country	Carcinoma Type	BCS	Age range	N	Intraoperative Re-excision rate[a]	Re-excision rate final[b]	Sensitivity	Specificity	Time (min)	False positive cases	False negative cases	LOE	Study Type
Cabioglu et al.[11]	2007	USA	IC, DCIS	Wide local excision, segmental mastectomy	27 - 95	110	20.0% (22)	8.8% (10)	91.7%	77.8%		---	---	1	Retrospective cohort
Camp et al*[12].	2005	USA	IC, DCIS	Lumpectomy	26 - 78	189	27.0% (51)	5.8% (11)	---	---	13	---	---	1	Retrospective cohort
Cendan et al[42].	2005	USA	DCIS, LCIS, ILC, IDC, MC	BCS	48 -71	97	44.0% (43)	19.6% (19*)	58.1%	100.0%	13	None	22: DCIS (20), LCIS (2)	1	Retrospective cohort
Chen et al.[43]	2005	Taiwan	PT	Local and wide excision	11 - 73	113		41.6% (47)	---	---		---	---	2	Retrospective cohort
Dener et al.[44]	2009	Turkey	IC	Lumpectomy	18 - 94	186	16.0% (30)	0% (0)	100.0%	100.0%		None	None	1	Retrospective cohort
Fukamachi et al.[19]	2010	Japan	IDC, DCIS, ILC, MC, AC	Wide excision, quadrantectomy	32 - 87	122	27.0% (33)	9.8% (12)	78.6%	100.0%	53*	None	---	1	Retrospective cohort
Ikeda et al[45].	1997	Japan	DCIS, IC	quadrantectomy	33 - 66	54	37% (20)	0% (0)	94%	90%		4: Atypical	1	1	Retrospective cohort
Loibl et al.[13]	2006	Germany	ILC, IDC	Lumpectomy	25 - 78	240	---	13.3% (32)	---	---		---	---	1	Retrospective cohort RCT - GEPARDUO
Munhoz et al.[20]	2009	Brazil	DCIS, ILC, IDC	Lumpectomy	23 - 71	218	28.8% (63)	5.5% (12)	---	---		---	12: IDC (7), IDC plus DCIS (2), DCIS (2). Younger age and larger tumors.	1	Retrospective cohort
Olson et al.[46]	2007	USA	IC, DCIS	Lumpectomy	27 - 89	290	24.0% (70)	5.5% (16)	73.1%	99.6%		27	17	1	Retrospective cohort
Park et al.[47]	2011	Korea	ILC, IDC, MC, TC, PC, medullary	BCS	20 - 78	705	---	13.5% (95)	---	---		---	28: Focal intraductal carcinoma	1	Retrospective cohort
Riedl et al.[21]	2009	Austria	DCIS, ILC, IDC	Lumpectomy	24 - 92*	1,016	---	9% (91)	---	---	30	---	91: IDC (88), ILC (3); Small lesions, microcalcifications, neoadjuvant therapy	1	Retrospective cohort
Rusby et al.[18]	2008	UK	DCIS, ILC, IDC, MC, TC, PC	Partial mastectomy	40 - 59	115	33.0% (38)	7% (8)	83.0%	97.0%		8: Atypia, sclerosing adenosis (3); in situ or IC (4)	9: sampling error, small volume of tissue	1	Retrospective cohort, prospective database
Weber et al.[48]	1997	USA	DCIS	Lumpectomy	---	140	15% (21)	0% (1)	91%	100%		none	3: DCIS (2), LCIS (1)	1	retrospective cohort
Weber et al[49].	2008	Switzerland	IC, DCIS, ADH	Lumpectomy	34 - 86	80	---	12.5% (10)	80.0%	87.5%		---	---	1	retrospective cohort
Results:						**163¥**	**27% (±9%)‡**	**10% (±6%)‡**	**83% (±13%)‡**	**95% (±8%)‡**	**27.25¥**				

BCS, Breast conserving surgery; N, number of samples; a, during first BCS; b, after first BCS; LOE, Level of evidence (SORT); IC, Invasive carcinoma; MC, Mucinous carcinoma; AC, Apocrine carcinoma; TC, Tubular carcinoma; PC, Papillary carcinoma; DCIS, Ductal carcinoma in situ; LCIS, Lobular carcinoma in situ; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; PT, Phyllodes tumors; ADH, Atypical ductal hyperplasia; *, personal communication, ¥ sample median; ‡ pooled estimates.

Appendix: Figure 2

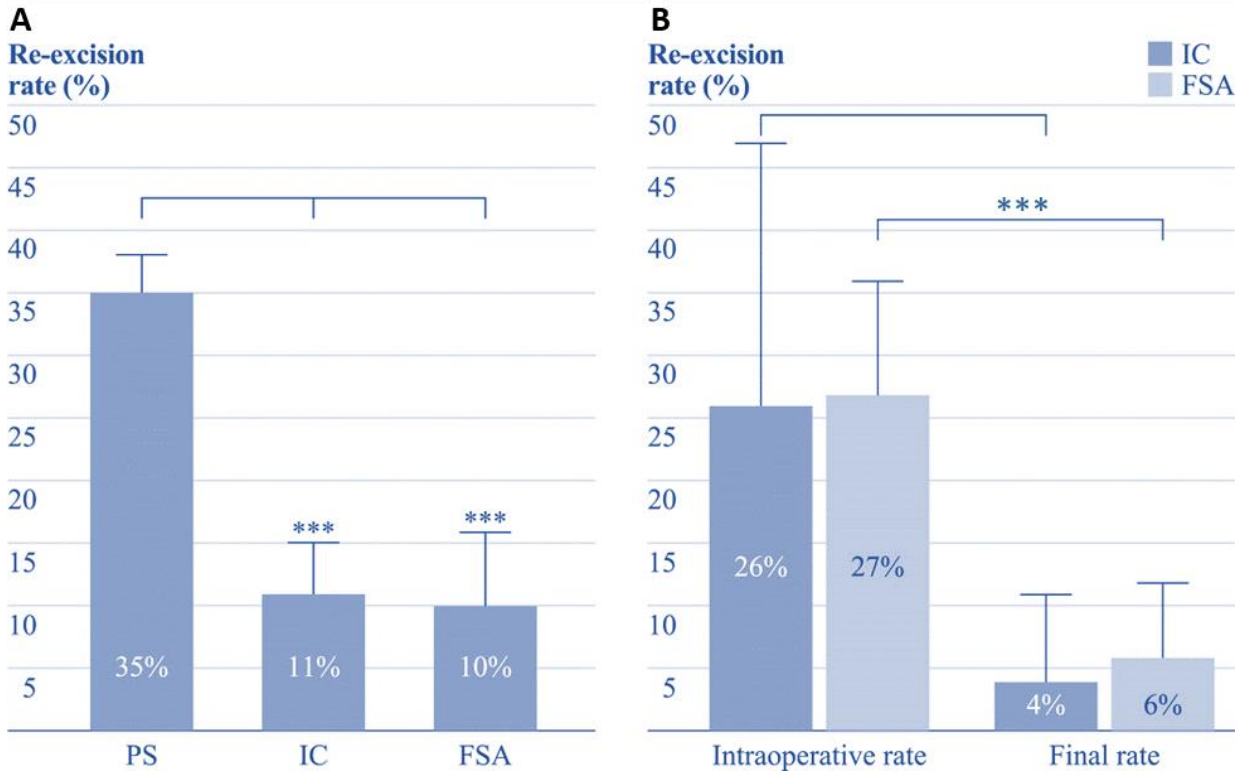

Figure 2. Graphs of re-excision rates of permanent section, imprint cytology and frozen section analysis. A. Pooled estimates of final re-excision rates after primary BCS. PS (n=19), IC (n=7) and FSA (n=15). IC *versus* PS (p=0.001) and FSA *versus* PS (p<0.0001). **B.** Pooled estimates of intraoperative re-excision rates during primary BCS versus final re-excision rates after primary BCS when using intraoperative IC and FSA as surgical margin assessment techniques. IC (n=3) and FSA (n=10). IC re-excision rates decreased from 26% to 4% (p=0.18) and FSA re-excision rates decreased from 27% to 6% (p<0.0001).

REFERENCES

1. Desantis C, Siegel R, Bandi P, Jemal A: **Breast Cancer Statistics , 2011**. *Cancer* 2011, **61**:409–418.

2. Crivellari D, Aapro MS, Leonard R, von Minckwitz G, Brain E, Goldhirsch A, Veronesi A, Muss H: **Breast cancer in the elderly**. *J Clin Oncol* 2007, **25**:1882–90.

3. Cleary MP, Grossmann ME: **Obesity and Breast Cancer: The Estrogen Connection**. *Endocrinology* 2009, **150**:2537–2542.

4. Cetin I, Cozzi V, Antonazzo P: **Infertility as a Cancer Risk Factor – A Review**. *Placenta* 2008, **29**:169–177.

5. Ha M, Mabuchi K, Sigurdson a. J, Freedman DM, Linet MS, Doody MM, Hauptmann M: **Smoking Cigarettes before First Childbirth and Risk of Breast Cancer**. *Am J Epidemiol* 2007, **166**:55–61.

6. Boyd NF, Lockwood G a., Byng JW, Tritchler DL, Yaffe MJ: **Mammographic densities and breast cancer risk**. *Cancer Epidemiol Biomarkers Prev* 1998, **7**:1133–1144.

7. McCormack V a, dos Santos Silva I: **Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis.** *Cancer Epidemiol Biomarkers Prev* 2006, **15**:1159–1169.

8. Boyd NF, Rommens JM, Vogt K, Lee V, Hopper JL, Yaffe MJ, Paterson AD: **Mammographic breast density as an intermediate phenotype for breast cancer.** *Lancet Oncol* 2005, **6**:798–808.

9. Caglayan EK, Caglayan K, Alkis I, Arslan E, Okur A, Banli O, Engin-Ustün Y: **Factors Associated with Mammographic Density in Postmenopausal Women**. *J Menopausal Med* 2015, **21**:82.

10. Fuhrman BJ, Brinton LA, Pfeiffer RM, Xu X, Veenstra TD, Teter BE, Byrne C, Dallal CM, Barba M, Muti PC, Gierach GL: **Estrogen Metabolism and Mammographic Density in Postmenopausal Women: A Cross-Sectional Study**. *Cancer Epidemiol Biomarkers Prev* 2012, **21**:1582–1591.

11. Rutter CM, Mandelson MT, Laya MB, Seger DJ, Taplin S, Page P: **Initiation , Discontinuation , and Continuing Use of Hormone Replacement Therapy**. *JAMA* 2001, **285**:171–176.

12. Ursin G, Hovanessian-Larsen L, Parisky YR, Pike MC, Wu AH: **Greatly increased occurrence of breast cancers in areas of mammographically dense tissue.** *Breast Cancer Res*

2005, **7**:R605–R608.

13. Gill JK, Maskarinec G, Pagano I, Kolonel LN: **The association of mammographic density with ductal carcinoma in situ of the breast: the Multiethnic Cohort.** *Breast Cancer Res* 2006, **8**:R30.

14. Habel LA, Dignam JJ, Land SR, Salane M, Capra AM, Julian TB: **Mammographic Density and Breast Cancer After Ductal Carcinoma In Situ**. *JNCI J Natl Cancer Inst* 2004, **96**:1467–1472.

15. Aiello EJ, Buist DSM, White E, Porter PL: **Association between mammographic breast density and breast cancer tumor characteristics.** *Cancer Epidemiol Biomarkers Prev* 2005, **14**:662–8.

16. Cil T, Fishell E, Hanna W, Sun P, Rawlinson E, Narod S a., McCready DR: **Mammographic density and the risk of breast cancer recurrence after breast-conserving surgery**. *Cancer* 2009, **115**:5780–5787.

17. Guo YP, Martin LJ, Hanna W, Banerjee D, Miller N, Fishell E, Khokha R, Boyd NF: **Growth factors and stromal matrix proteins associated with mammographic densities.** *Cancer Epidemiol Biomarkers Prev* 2001, **10**:243–8.

18. Alowami S, Troup S, Al-Haddad S, Kirkpatrick I, Watson PH: **Mammographic density is related to stroma and stromal proteoglycan expression.** *Breast Cancer Res* 2003, **5**:R129–35.

19. Li T, Sun L, Miller N, Nicklee T, Woo J, Hulse-smith L, Tsao M, Khokha R, Martin L, Boyd N: **The Association of Measured Breast Tissue Characteristics with Mammographic Density and Other Risk Factors for Breast Cancer The Association of Measured Breast Tissue Characteristics with Mammographic Density and Other Risk Factors for Breast Cancer**. 2005, **14**(February):343–349.

20. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ: **Collagen reorganization at the tumor-stromal interface facilitates local invasion.** *BMC Med* 2006, **4**:38.

21. Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, White JG, Keely PJ: **Collagen density promotes mammary tumor initiation and progression.** *BMC Med* 2008, **6**:11.

22. Liu X, Wu H, Byrne M, Jeffrey J, Krane S, Jaenisch R: **A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling**. *J Cell Biol* 1995, **130**:227–237.

23. Conklin MW, Eickhoff JC, Riching KM, Pehlke C a., Eliceiri KW, Provenzano PP, Friedl A,

Keely PJ: **Aligned collagen is a prognostic signature for survival in human breast carcinoma**. *Am J Pathol* 2011, **178**:1221–1232.

24. Daling JR, Malone KE, Doody DR, Anderson BO, Porter PL: **The relation of reproductive factors to mortality from breast cancer.** *Cancer Epidemiol biomarkers Prev* 2002, **11**:235–41.

25. Whiteman MK, Hillis SD, Curtis KM, McDonald J a., Wingo P a., Marchbanks P a.: **Reproductive History and Mortality After Breast Cancer Diagnosis**. *Obstet Gynecol* 2004, **104**:146–154.

26. Stensheim H, Moller B, van Dijk T, Fossa SD: **Cause-Specific Survival for Women Diagnosed With Cancer During Pregnancy or Lactation: A Registry-Based Cohort Study**. *J Clin Oncol* 2008, **27**:45–51.

27. Stein T, Salomonis N, Gusterson B a.: **Mammary Gland Involution as a Multi-step Process**. *J Mammary Gland Biol Neoplasia* 2007, **12**:25–35.

28. Radisky DC, Hartmann LC: **Mammary involution and breast cancer risk: transgenic models and clinical studies.** *J Mammary Gland Biol Neoplasia* 2009, **14**:181–91.

29. Schedin P, O'Brien J, Rudolph M, Stein T, Borges V: **Microenvironment of the Involuting Mammary Gland Mediates Mammary Cancer Progression**. *J Mammary Gland Biol Neoplasia* 2007, **12**:71–82.

30. Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Marusyk A, Tan A-C, Schedin P: **Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2.** *Nat Med* 2011, **17**(July 2010):1109–1115.

31. Coussens LM, Werb Z: **Inflammation and cancer**. *Nature* 2002, **420**:860–867.

32. Coussens LM, Zitvogel L, Palucka a K: **Neutralizing tumor-promoting chronic inflammation: a magic bullet?** *Science* 2013, **339**:286–91.

33. Balkwill F, Mantovani A: **Inflammation and cancer: Back to Virchow?** *Lancet* 2001:539–545.

34. O'Byrne KJ, Dalgleish a G: **Chronic immune activation and inflammation as the cause of malignancy.** *Br J Cancer* 2001, **85**:473–83.

35. Grivennikov SI, Greten FR, Karin M: **Immunity, Inflammation, and Cancer**. *Cell* 2011, **140**:883–899.

36. Kalluri R, Zeisberg M: **Fibroblasts in cancer.** *Nat Rev Cancer* 2006, **6**:392–401.

37. Orimo A, Weinberg R a.: **Stromal Fibroblasts in Cancer: A Novel Tumor-Promoting Cell Type**. *Cell Cycle* 2006, **5**:1597–1601.

38. Mueller MM, Fusenig NE: **Friends or foes - bipolar effects of the tumour stroma in cancer.** *Nat Rev Cancer* 2004, **4**:839–849.

39. Maeda H, Akaike T: **Nitric oxide and oxygen radicals in infection, inflammation, and cancer.** *Biochem Biokhimiia* 1998, **63**:854–65.

40. DeNardo DG, Coussens LM: **Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression.** *Breast Cancer Res* 2007, **9**:212.

41. de Visser KE, Eichten A, Coussens LM: **Paradoxical roles of the immune system during cancer development**. *Nat Rev Cancer* 2006, **6**:24–37.

42. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J: **A Paracrine Loop between Tumor Cells and Macrophages Is Required for Tumor Cell Migration in Mammary Tumors A Paracrine Loop between Tumor Cells and Macrophages Is Required for Tumor Cell Migration in Mammary Tumors**. 2004:7022–7029.

43. Pollard JW: **Tumour-educated macrophages promote tumour progression and metastasis.** *Nat Rev Cancer* 2004, **4**:71–78.

44. Lin EY, Nguyen A V, Russell RG, Pollard JW: **Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy.** *J Exp Med* 2001, **193**:727–40.

45. Benarafa C: **Tumor-induced inflammation alters neutrophil phenotype and disease progression**. *Breast Cancer Res* 2015, **17**:135.

46. Galdiero MR, Garlanda C, Jaillon S, Marone G, Mantovani A: **Tumor associated macrophages and neutrophils in tumor progression**. *J Cell Physiol* 2013, **228**:1404–1412.

47. Mantovani A, Cassatella M a, Costantini C, Jaillon S: **Neutrophils in the activation and regulation of innate and adaptive immunity.** *Nat Rev Immunol* 2011, **11**:519–531.

48. Mócsai A: **Diverse novel functions of neutrophils in immunity, inflammation, and beyond.** *J Exp Med* 2013, **210**:1283–99.

49. Templeton a. J, McNamara MG, Eruga B, Vera-Badillo FE, Aneja P, Ocana a., Leibowitz-Amit R, Sonpavde G, Knox JJ, Tran B, Tannock IF, Amir E: **Prognostic Role of Neutrophil-to-Lymphocyte Ratio in Solid Tumors: A Systematic Review and Meta-Analysis**. *JNCI J Natl Cancer Inst* 2014, **106**:dju124–dju124.

50. Khamis ZI, Sahab ZJ, Sang Q-XA: **Active Roles of Tumor Stroma in Breast Cancer Metastasis**. *Int J Breast Cancer* 2012, **2012**:1–10.

51. Hanahan D, Coussens LM: **Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment**. *Cancer Cell* 2012, **21**:309–322.

52. Bauer M, Su G, Casper C, He R, Rehrauer W, Friedl A: **Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast.** *Oncogene* 2010, **29**:1732–40.

53. Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld R a.: **Cancer Associated Fibroblasts Promote Tumor Growth and Metastasis by Modulating the Tumor Immune Microenvironment in a 4T1 Murine Breast Cancer Model**. *PLoS One* 2009, **4**:e7965.

54. Baron JA, Sandler RS: **Nonsteroidal anti-inflammatory drugs and cancer prevention.** *Annu Rev Med* 2000, **51**:511–23.

55. García-Rodríguez LA, Huerta-Alvarez C: **Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs.** *Epidemiology* 2001, **12**:88–93.

56. Friedman GD, Ury HK: **Initial screening for carcinogenicity of commonly used drugs.** *J Natl Cancer Inst* 1980, **65**:723–33.

57. Harris RE, Namboodiri KK, Farrar WB: **Nonsteroidal antiinflammatory drugs and breast cancer.** *Epidemiology* 1996, **7**:203–5.

58. Thun M, Namboodiri M, Heath C: **Aspirin use and reduced risk of fatal colon cancer.** *N Engl J Med* 1991, **325**:1593–1596.

59. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker S V, Robinson CR, Offerhaus GJ: **Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis.** *N Engl J Med* 1993, **328**:1313–6.

60. Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, Speizer FE: **Aspirin and the risk of colorectal cancer in women.** *N Engl J Med* 1995, **333**:609–14.

61. Schreinemachers DM, Everson RB: **Aspirin use and lung, colon, and breast cancer incidence in a prospective study.** *Epidemiology* 1994, **5**:138–46.

62. Sharpe CR, Collet JP, McNutt M, Belzile E, Boivin JF, Hanley J a: **Nested case-control study of the effects of non-steroidal anti-inflammatory drugs on breast cancer risk and stage.** *Br J Cancer* 2000, **83**:112–120.

63. Baron J a., Sandler RS, Bresalier RS, Quan H, Riddell R, Lanas A, Bolognese J a., Oxenius B, Horgan K, Loftus S, Morton DG: **A Randomized Trial of Rofecoxib for the Chemoprevention of Colorectal Adenomas**. *Gastroenterology* 2006, **131**:1674–1682.

64. Chow LWC, Yip AYS, Loo WTY, Lam CK, Toi M: **Celecoxib anti-aromatase neoadjuvant (CAAN) trial for locally advanced breast cancer**. *J Steroid Biochem Mol Biol* 2008, **111**:13–17.

65. Chow LWC, Cheng CWL, Wong JLN, Toi M: **Serum lipid profiles in patients receiving endocrine treatment for breast cancer--the results from the Celecoxib Anti-Aromatase Neoadjuvant (CAAN) Trial.** *Biomed Pharmacother* 2005, **59 Suppl 2**:S302–5.

66. Smyth EM, FitzGerald GA: **The Eicosanoids: Prostaglandins, Thromboxanes, Leukotrienes, and Related Compounds**. In *Basic and clinical Pharmacology*. 12th edition. Edited by Katzung BG, Masters SB, Trevor AJ. McGraw Hill Medical; 2012:313–329.

67. Scheiman JM, Cryer B, Kimmey MB, Rothstein RI, Riff DS, Wolfe MM: **A randomized, controlled comparison of ibuprofen at the maximal over-the-counter dose compared with prescription-dose celecoxib on upper gastrointestinal mucosal injury**. *Clin Gastroenterol Hepatol* 2004, **2**:290–295.

68. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET: **Celecoxib for the prevention of sporadic colorectal adenomas.** *N Engl J Med* 2006, **355**:873–84.

69. Elder DJ, Halton DE, Hague a, Paraskeva C: **Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression.** *Clin Cancer Res* 1997, **3**:1679–1683.

70. Brigati C, Noonan DM, Albini A, Benelli R: **Tumors and inflammatory infiltrates: Friends or foes?** *Clin Exp Metastasis* 2002, **19**:247–258.

71. Mann JR, Backlund MG, DuBois RN: **Mechanisms of Disease: inflammatory mediators and cancer prevention**. *Nat Clin Pr Oncol* 2005, **2**:202–210.

72. Wang D, Dubois RN: **Eicosanoids and cancer**. *Nat Rev Cancer* 2010, **10**:181–193.

73. Dubois RN, Abramson SB, Crofford L, Gupta R a, Simon LS, Van De Putte LB, Lipsky PE: **Cyclooxygenase in biology and disease.** *FASEB J* 1998, **12**:1063–1073.

74. Brock T, McNish R, Peters-Golden M: **Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2**. *J Biol Chem* 1999, **274**:11660–11666.

75. Diaz a, Chepenik KP, Korn JH, Reginato a M, Jimenez S a: **Differential regulation of**

cyclooxygenases 1 and 2 by interleukin-1 beta, tumor necrosis factor-alpha, and transforming growth factor-beta 1 in human lung fibroblasts. *Exp Cell Res* 1998, **241**:222–229.

76. Araki Y, Okamura S, Hussain SP, Nagashima M, He P, Shiseki M, Miura K, Harris CC: **Regulation of cyclooxygenase-2 expression by the WNT and ras pathways**. *Cancer Res* 2003, **63**:728–734.

77. Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar R: **Regulation of cyclooxygenase-2 pathway by HER2 receptor.** *Oncogene* 1999, **18**:305–14.

78. Ansari KM, Sung YM, He G, Fischer SM: **Prostaglandin receptor EP2 is responsible for cyclooxygenase-2 induction by prostaglandin E2 in mouse skin**. *Carcinogenesis* 2007, **28**:2063–2068.

79. Cui Y, Shu X, Gao Y, Cai Q, Ji B, Li H, Rothman N: **Urinary Prostaglandin E 2 Metabolite and Breast Cancer Risk**. 2014, **23**(December):2866–2874.

80. Morris PG, Zhou XK, Milne GL, Goldstein D, Hawks LC, Dang CT, Modi S, Fornier MN, Hudis CA, Dannenberg AJ: **Increased levels of urinary PGE-M, a biomarker of inflammation, occur in association with obesity, aging, and lung metastases in patients with breast cancer.** *Cancer Prev Res (Phila)* 2013, **6**:428–36.

81. Kim S, Taylor JA, Milne GL, Sandler DP: **Association between urinary prostaglandin E2 metabolite and breast cancer risk: a prospective, case-cohort study of postmenopausal women.** *Cancer Prev Res (Phila)* 2013, **6**:511–8.

82. Harris RE, Alshafie G a, Abou-issa H, Seibert K: **Chemoprevention of Breast Cancer in Rats by Celecoxib , a Cyclooxygenase 2 Inhibitor Advances in Brief Chemoprevention of Breast Cancer in Rats by Celecoxib ,**. *Cancer Res* 2000:2101–2103.

83. Alshafie GA, Abou-Issa HM, Seibert K, Harris RE: **Chemotherapeutic evaluation of Celecoxib, a cyclooxygenase-2 inhibitor, in a rat mammary tumor model.** *Oncol Rep* 2000, **7**:1377–81.

84. Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF, Hla T: **Overexpression of Cyclooxygenase-2 is Sufficient to Induce Tumorigenesis in Transgenic Mice**. *J Biol Chem* 2001, **276**:18563–18569.

85. Harris RE, Robertson FM, Abou-Issa HM, Farrar WB, Brueggemeier R: **Genetic induction and upregulation of cyclooxygenase (COX) and aromatase (CYP19): an extension of the dietary fat hypothesis of breast cancer.** *Med Hypotheses* 1999, **52**:291–2.

86. Gasparini G, Longo R, Sarmiento R, Morabito a: **Inhibitors of cyclo-oxygenase 2: a new**

class of anticancer agents? *Lancet Oncol* 2003, **4**:605–615.

87. Penseti E, Masferrer J, di Salle E: **Effect of exemestane and celecoxib alone or in combination on DBMA-induced mammary carcionma in rats.** *Breast Cancer Res* , **69**:288.

88. Richards J a., Petrel T a., Brueggemeier RW: **Signaling pathways regulating aromatase and cyclooxygenases in normal and malignant breast cells**. *J Steroid Biochem Mol Biol* 2002, **80**:203–212.

89. Chew GL, Huo CW, Huang D, Hill P, Cawson J, Frazer H, Hopper JL, Haviv I, Henderson MA, Britt K, Thompson EW: **Increased COX-2 expression in epithelial and stromal cells of high mammographic density tissues and in a xenograft model of mammographic density.** *Breast Cancer Res Treat* 2015, **153**:89–99.

90. Fornetti J, Jindal S, Middleton KA, Borges VF, Schedin P: **Physiological COX-2 Expression in Breast Epithelium Associates with COX-2 Levels in Ductal Carcinoma in Situ and Invasive Breast Cancer in Young Women**. *Am J Pathol* 2014, **184**:1–12.

91. Dranoff G: **Cytokines in cancer pathogenesis and cancer therapy.** *Nat Rev Cancer* 2004, **4**:11–22.

92. Provenzano PP, Inman DR, Eliceiri KW, Keely PJ: **Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage.** *Oncogene* 2009, **28**(August):4326–4343.

93. Ristimäki a, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J: **Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer**. *Cancer Res* 2002, **62**:632–635.

94. Kwan ML, Habel L a, Slattery ML, Caan B: **NSAIDs and breast cancer recurrence in a prospective cohort study.** *Cancer Causes Control* 2007, **18**:613–620.

95. Holmes MD, Chen WY, Schnitt SJ, Collins L, Colditz G a., Hankinson SE, Tamimi RM: **COX-2 expression predicts worse breast cancer prognosis and does not modify the association with aspirin**. *Breast Cancer Res Treat* 2011, **130**:657–662.

96. Holmes MD, Chen WY, Li L, Hertzmark E, Spiegelman D, Hankinson SE: **Aspirin intake and survival after breast cancer**. *J Clin Oncol* 2010, **28**:1467–1472.

97. Howe LR: **Inflammation and breast cancer. Cyclooxygenase/prostaglandin signaling and breast cancer.** *Breast Cancer Res* 2007, **9**:210.

98. Canney P a., Machin M a., Curto J: **A feasibility study of the efficacy and tolerability of the combination of Exemestane with the COX-2 inhibitor Celecoxib in post-menopausal patients with advanced breast cancer**. *Eur J Cancer* 2006, **42**:2751–2756.

99. Dirix LY, Ignacio J, Nag S, Bapsy P, Gomez H, Raghunadharao D, Paridaens R, Jones S, Falcon S, Carpentieri M, Abbattista a., Lobelle J-P: **Treatment of Advanced Hormone-Sensitive Breast Cancer in Postmenopausal Women With Exemestane Alone or in Combination With Celecoxib**. *J Clin Oncol* 2008, **26**:1253–1259.

100. Therasse P, Arbuck SG, Eisenhauer E a, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom a T, Christian MC, Gwyther SG: **New guidelines to evaluate the response to treatment in solid Tumors**. *JNatlCancer Inst* 2000, **92**:205–216.

101. Falandry C, Debled M, Bachelot T, Delozier T, Crétin J, Romestaing P, Mille D, You B, Mauriac L, Pujade-Lauraine E, Freyer G: **Celecoxib and exemestane versus placebo and exemestane in postmenopausal metastatic breast cancer patients: a double-blind phase III GINECO study**. *Breast Cancer Res Treat* 2009, **116**:501–508.

102. Brandão RD, Veeck J, Van de Vijver KK, Lindsey P, de Vries B, van Elssen CH, Blok MJ, Keymeulen K, Ayoubi T, Smeets HJ, Tjan-Heijnen VC, Hupperets PS: **A randomised controlled phase II trial of pre-operative celecoxib treatment reveals anti-tumour transcriptional response in primary breast cancer.** *Breast Cancer Res* 2013, **15**:R29.

103. Martin L-A, Davies GLS, Weigel MT, Betambeau N, Hills MJ, Salter J, Walsh G, A'Hern R, Dowsett M: **Pre-surgical study of the biological effects of the selective cyclo-oxygenase-2 inhibitor celecoxib in patients with primary breast cancer**. *Breast Cancer Res Treat* 2010, **123**:829–836.

104. Bundred NJ, Cramer A, Morris J, Renshaw L, Cheung KL, Flint P, Johnson R, Young O, Landberg G, Grassby S, Turner L, Baildam A, Barr L, Dixon JM: **Cyclooxygenase-2 inhibition does not improve the reduction in ductal carcinoma in situ proliferation with aromatase inhibitor therapy: Results of the ERISAC randomized placebo-controlled trial**. *Clin Cancer Res* 2010, **16**:1605–1612.

105. Ross SR: **MMTV Infectious Cycle and the Contribution of Virus-encoded Proteins to Transformation of Mammary Tissue**. *J Mammary Gland Biol Neoplasia* 2008, **13**:299–307.

106. Marcotte R, Muller WJ: **Signal Transduction in Transgenic Mouse Models of Human Breast Cancer—Implications for Human Breast Cancer**. *J Mammary Gland Biol Neoplasia* 2008, **13**:323–335.

107. Guy CT, Cardiff RD, Muller WJ: **Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.** *Mol Cell Biol* 1992, **12**:954–961.

108. Martin LJ, Melnichouk O, Guo H, Chiarelli AM, Hislop TG, Yaffe MJ, Minkin S, Hopper JL, Boyd NF: **Family history, mammographic density, and risk of breast cancer.** *Cancer*

Epidemiol Biomarkers Prev 2010, **19**:456–463.

109. Boyd NF: **Mammographic density and risk of breast cancer.** *Am Soc Clin Oncol Educ Book* 2013:57–62.

110. Pettersson A, Graff RE, Ursin G, dos Santos Silva I, McCormack V, Baglietto L, Vachon C, Bakker MF, Giles GG, Chia KS, Czene K, Eriksson L, Hall P, Hartman M, Warren RML, Hislop G, Chiarelli AM, Hopper JL, Krishnan K, Li J, Li Q, Pagano I, Rosner BA, Wong CS, Scott C, Stone J, Maskarinec G, Boyd NF, van Gils CH, Tamimi RM: **Mammographic Density Phenotypes and Risk of Breast Cancer: A Meta-analysis**. *J Natl Cancer Inst* 2014, **106** (5).

111. Denkert C, Winzer K-J, Müller B-M, Weichert W, Pest S, Köbel M, Kristiansen G, Reles A, Siegert A, Guski H, Hauptmann S: **Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma.** *Cancer* 2003, **97**:2978–2987.

112. Ristimäki A, Sivula A, Lundin J, Ristima A, Lundin M, Salminen T, Haglund C, Joensuu H: **Prognostic Significance of Elevated Cyclooxygenase-2 Expression in Breast Cancer Advances in Brief Prognostic Significance of Elevated Cyclooxygenase-2 Expression in Breast Cancer 1**. 2002:632–635.

113. Larkins TL, Nowell M, Singh S, Sanford GL: **Inhibition of cyclooxygenase-2 decreases breast cancer cell motility, invasion and matrix metalloproteinase expression.** *BMC Cancer* 2006, **6**:181.

114. Karavitis J, Hix LM, Shi YH, Schultz RF, Khazaie K, Zhang M: **Regulation of COX2 Expression in Mouse Mammary Tumor Cells Controls Bone Metastasis and PGE2-Induction of Regulatory T Cell Migration**. *PLoS One* 2012, **7**:1–11.

115. Hu M, Peluffo G, Chen H, Gelman R, Schnitt S, Polyak K: **Role of COX-2 in epithelial-stromal cell interactions and progression of ductal carcinoma in situ of the breast.** *Proc Natl Acad Sci U S A* 2009, **106**:3372–3377.

116. Yang WT, Lewis MT, Hess K, Wong H, Tsimelzon A, Karadag N, Cairo M, Wei C, Meric-Bernstam F, Brown P, Arun B, Hortobagyi GN, Sahin A, Chang JC: **Decreased TGFβ signaling and increased COX2 expression in high risk women with increased mammographic breast density**. *Breast Cancer Res Treat* 2010, **119**:305–314.

117. Harris RE, Beebe-Donk J, Doss H, Burr Doss D: **Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review).** *Oncol Rep* 2005, **13**:559–583.

118. Harris RE, Beebe-Donk J, Alshafie GA: **Cancer chemoprevention by cyclooxygenase 2 (COX-2) blockade: results of case control studies.** *Subcell Biochem* 2007, **42**:193–212.

119. Zhang Y, Coogan PF, Palmer JR, Strom BL, Rosenberg L: **Use of nonsteroidal antiinflammatory drugs and risk of breast cancer: The case-control surveillance study revisited**. *Am J Epidemiol* 2005, **162**:165–170.

120. Giardiello F, Hamilton S, Krush A, Piantadosi, S H, LM C, P B, SV R, CR O: **Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis**. *N Engl J Med* 1993, **328**:1313–1316.

121. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET: **Celecoxib for the Prevention of Sporadic Colorectal Adenomas**. *N Engl J Med* 2006, **355**:873–884.

122. Mantovani A. BF: **Inflammation and cancer: back to**. *Lancet* 2001, **357**:539–45.

123. Mantovani A, Mantovani A, Allavena P, Allavena P, Sica A, Sica A, Balkwill F, Balkwill F: **Cancer-related inflammation.** *Nature* 2008, **454**(July):436–44.

124. Mosser DM, Edwards JP: **Exploring the full spectrum of macrophage activation**. *Nat Rev Immunol* 2008, **8**:958–969.

125. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Worthen GS, Albelda SM: **Polarization of Tumor-Associated Neutrophil (TAN) Phenotype by TGF-β: "N1" versus "N2" TAN**. *Cancer Cell* 2009, **16**:183–194.

126. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown R a: **Myofibroblasts and mechano-regulation of connective tissue remodelling.** *Nat Rev Mol Cell Biol* 2002, **3**:349–363.

127. Parsonage G, Filer AD, Haworth O, Nash GB, Rainger GE, Salmon M, Buckley CD: **A stromal address code defined by fibroblasts**. 2005, **26**:150–156.

128. Descamps S, Lebourhis X, Delehedde M, Boilly B, Hondermarck H: **Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells.** *J Biol Chem* 1998, **273**:16659–16662.

129. Descamps S, Toillon R a, Adriaenssens E, Pawlowski V, Cool SM, Nurcombe V, Le Bourhis X, Boilly B, Peyrat JP, Hondermarck H: **Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways.** *J Biol Chem* 2001, **276**:17864–17870.

130. Numasaki M, Fukushi J, Ono M, Narula SK, Zavodny PJ, Kudo T, Robbins PD, Tahara H, Lotze MT: **Interleukin-17 promotes angiogenesis and tumor growth**. *Blood* 2003, **101**:2620.

131. Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, Hishinuma T, Goto J, Lotze MT, Kolls JK, Sasaki H: **IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis.** *J Immunol* 2005, **175**:6177–6189.

132. Cochaud S, Giustiniani J, Thomas C, Laprevotte E, Garbar C, Savoye A-M, Curé H, Mascaux C, Alberici G, Bonnefoy N, Eliaou J-F, Bensussan A, Bastid J: **IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2.** *Sci Rep* 2013, **3**:3456.

133. Heldin C-H: **Targeting the PDGF signaling pathway in tumor treatment.** *Cell Commun Signal* 2013, **11**:97.

134. O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, Man Y, Borges V, Schedin P: **Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species.** *Am J Pathol* 2010, **176**:1241–1255.

135. Ashok V, Dash C, Rohan TE, Sprafka JM, Terry PD: **Selective cyclooxygenase-2 (COX-2) inhibitors and breast cancer risk.** *Breast* 2011, **20**:66–70.

136. Fabi A, Metro G, Papaldo P, Mottolese M, Melucci E, Carlini P, Sperduti I, Russillo M, Gelibter A, Ferretti G, Tomao S, Milella M, Cognetti F: **Impact of celecoxib on capecitabine tolerability and activity in pretreated metastatic breast cancer: Results of a phase II study with biomarker evaluation**. *Cancer Chemother Pharmacol* 2008, **62**:717–725.

137. Gardiner SJ, Doogue MP, Zhang M, Begg EJ: **Quantification of infant exposure to celecoxib through breast milk**. *Br J Clin Pharmacol* 2006, **61**:101–104.

138. Turner S: **Role of the selective cyclo-oxygenase-2 (COX-2) inhibitors in children**. *Arch Dis Child - Educ Pract* 2004, **89**:ep46–ep49.

139. Huang W, Hennrick K, Drew S: **A colorful future of quantitative pathology: Validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays**. *Hum Pathol* 2013, **44**:29–38.

140. American Cancer Society: *Global Cancer Facts & Figures 3rd Edition.* 2015.

141. McPherson K, Steel CM, Dixon JM: **ABC of breast diseases. Breast cancer--epidemiology, risk factors and genetics.** *BMJ Br Med J* 1994, **309**:1003–1006.

142. Lakhani SR, I.O. E, Schnitt SJ, Tan PH, van de Vijver MJ: *WHO Classification of Tumours of the Breast, 4th Edition*. 4th Editio. Lyon: IARC Press; 2012.

143. Schnitt SJ: **Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy**. *Mod Pathol* 2010, **23**:S60–S64.

144. Misron NA, Looi L, Raihan N, Mustapha N: **Cyclooxygenase-2 Expression in Invasive Breast Carcinomas of No Special Type and Correlation with Pathological Profiles Suggest a Role in Tumorigenesis Rather than Cancer Progression**. *Asian Pac J Cancer Prev* 2015, **15**:1553–1558.

145. Wu WKK, Yiu Sung JJ, Lee CW, Yu J, Cho CH: **Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: An update on the molecular mechanisms**. *Cancer Lett* 2010, **295**:7–16.

146. Wang D, DuBois RN: **PPARδ and PGE2 signaling pathways communicate and connect inflammation to colorectal cancer.** *Inflamm cell Signal* 2014, **1**.

147. Bowers LW, Maximo IXF, Brenner AJ, Beeram M, Hursting SD, Price RS, Tekmal RR, Jolly CA, deGraffenried LA: **NSAID Use Reduces Breast Cancer Recurrence in Overweight and Obese Women: Role of Prostaglandin-Aromatase Interactions**. *Cancer Res* 2014, **74**:4446–4457.

148. Esbona K, Inman D, Saha S, Jeffery J, Schedin P, Wilke L, Keely P: **COX-2 modulates mammary tumor progression in response to collagen density**. *Manuscr Submitt Publ* 2015.

149. Ahn HJ, Jung SJ, Kim TH, Oh MK, Yoon H-K: **Differences in Clinical Outcomes between Luminal A and B Type Breast Cancers according to the St. Gallen Consensus 2013.** *J Breast Cancer* 2015, **18**:149–59.

150. Kim HS, Moon H-G, Han W, Yom CK, Kim WH, Kim JH, Noh D-Y: **COX2 overexpression is a prognostic marker for Stage III breast cancer**. *Breast Cancer Res Treat* 2012, **132**:51–59.

151. Van Nes JGH, De Kruijf EM, Faratian D, Van De Velde CJH, Putter H, Falconer C, Smit VTHBM, Kay C, Van De Vijver MJ, Kuppen PJK, Bartlett JMS: **COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients**. *Breast Cancer Res Treat* 2011, **125**:671–685.

152. Park K, Han S, Shin E, Kim H-J, Kim J-Y: **Cox-2 expression on tissue microarray of breast cancer.** *Eur J Surg Oncol* 2006, **32**:1093–1096.

153. Holness CL, Simmons DL: **Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins.** *Blood* 1993, **81**:1607–1613.

154. Lau SK, Chu PG, Weiss LM: **CD163: A Specific Marker of Macrophages in Paraffin-Embedded Tissue Samples**. *Am J Clin Pathol* 2004, **122**:794–801.

155. Buechler C, Ritter M, Orsó E, Langmann T, Klucken J, Schmitz G: **Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and**

antiinflammatory stimuli. *J Leukoc Biol* 2000, **67**:97–103.

156. Nguyen TT, Schwartz EJ, West RB, Warnke R a, Arber D a, Natkunam Y: **Expression of CD163 (hemoglobin scavenger receptor) in normal tissues, lymphomas, carcinomas, and sarcomas is largely restricted to the monocyte/macrophage lineage.** *Am J Surg Pathol* 2005, **29**:617–624.

157. Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake TRDJ, Reedquist KA, Tak PP, Baeten DLP: **Systematic validation of specific phenotypic markers for in vitro polarized human macrophages**. *J Immunol Methods* 2012, **375**:196–206.

158. Medrek C, Pontén F, Jirström K, Leandersson K: **The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients**. *BMC Cancer* 2012, **12**:306.

159. Li H, Yang B, Huang J, Lin Y, Xiang T, Wan J, Li H, Chouaib S, Ren G: **Cyclooxygenase-2 in tumor-associated macrophages promotes breast cancer cell survival by triggering a positive-feedback loop between macrophages and cancer cells**. *Oncotarget* 2015, **6**:29637–50.

160. Bianchini F, Massi D, Marconi C, Franchi A, Baroni G, Santucci M, Mannini A, Mugnai G, Calorini L: **Expression of cyclo-oxygenase-2 in macrophages associated with cutaneous melanoma at different stages of progression.** *Prostaglandins Other Lipid Mediat* 2007, **83**:320–8.

161. Tsai C-S, Chen F-H, Wang C-C, Huang H-L, Jung S-M, Wu C-J, Lee C-C, McBride WH, Chiang C-S, Hong J-H: **Macrophages from irradiated tumors express higher levels of iNOS, arginase-I and COX-2, and promote tumor growth.** *Int J Radiat Oncol Biol Phys* 2007, **68**:499–507.

162. Na Y-R, Yoon Y-N, Son D-I, Seok S-H: **Cyclooxygenase-2 Inhibition Blocks M2 Macrophage Differentiation and Suppresses Metastasis in Murine Breast Cancer Model**. *PLoS One* 2013, **8**:e63451.

163. Endogenous Hormones and Breast Cancer Collaborative Group: **Body Mass Index, Serum Sex Hormones, and Breast Cancer Risk in Postmenopausal Women**. *JNCI J Natl Cancer Inst* 2003, **95**:1218–1226.

164. Bredfeldt J, Liu Y, Conklin M, Keely P, Mackie T, Eliceiri K: **Automated quantification of aligned collagen for human breast carcinoma prognosis**. *J Pathol Inform* 2014, **5**:28.

165. Dittmann KH, Mayer C, Ohneseit PA, Raju U, Andratschke NH, Milas L, Rodemann HP: **Celecoxib Induced Tumor Cell Radiosensitization by Inhibiting Radiation Induced Nuclear EGFR Transport and DNA-Repair: A COX-2 Independent Mechanism**. *Int J Radiat Oncol*

2008, **70**:203–212.

166. de Pedro M, Baeza S, Escudero M-T, Dierssen-Sotos T, Gómez-Acebo I, Pollán M, Llorca J: **Effect of COX-2 inhibitors and other non-steroidal inflammatory drugs on breast cancer risk: a meta-analysis.** *Breast Cancer Res Treat* 2015, **149**:525–36.

167. Tao K, Fang M, Alroy J, Sahagian GG: **Imagable 4T1 model for the study of late stage breast cancer**. *BMC Cancer* 2008, **8**:228.

168. Aslakson CJ, Miller FR: **Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor**. *Cancer Res* 1992, **52**:1399–1405.

169. Elias AD: **Triple-Negative Breast Cancer**. *Am J Clin Oncol* 2010, **33**:637–645.

170. Chikman B, Vasyanovich S, Lavy R, Habler L, Tolstov G, Kapiev A, Halevy A, Sandbank J: **COX2 expression in high-grade breast cancer: evidence for prognostic significance in the subset of triple-negative breast cancer patients.** *Med Oncol* 2014, **31**:989.

171. White WB, Faich G, Whelton A, Maurath C, Ridge NJ, Verburg KM, Geis GS, Lefkowith JB: **Comparison of thromboembolic events in patients treated with celecoxib, a cyclooxygenase-2 specific inhibitor, versus ibuprofen or diclofenac**. *Am J Cardiol* 2002, **89**:425–430.

172. White WB, Faich G, Borer JS, Makuch RW: **Cardiovascular thrombotic events in arthritis trials of the cyclooxygenase-2 inhibitor celecoxib**The investigators had full access to the data and complete control over the design, conduct analysis, interpretation, and description of the study. The spons**. *Am J Cardiol* 2003, **92**:411–418.