The effects of flaxseed and tamoxifen on the inflammatory microenvironment in normal breast tissue and in breast cancer

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**The effects of flaxseed and tamoxifen
on the inflammatory microenvironment
in normal breast tissue and in breast cancer**

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Till Hugo, Astrid och Olle – ni är framtiden.

Abstract

Breast cancer is the most common cancer among women worldwide today. Nearly 9000 women are diagnosed with breast cancer in Sweden yearly and despite advantages in diagnostics and treatments approximately 1400 women still die from their disease every year. Breast cancer has a diverse etiology and hormonal factors and life-style factors contribute to an increased breast cancer risk. High mammographic density is also considered a risk factor but the underlying mechanisms are not fully understood. Inflammation is associated with poor survival in several malignancies and is considered a hallmark of cancer. There is evidence indicating that increased inflammation is associated with dense breast tissue and may contribute to an increased risk of breast cancer in these patients.

There is an urgent need to find risk reduction strategies in breast cancer prevention. Several studies have shown that antiestrogens significantly reduce breast cancer incidence in women with high risk of developing breast cancer and can be used for chemoprevention. These drugs may have potentially severe side effects and other strategies are needed. Dietary interventions may influence breast cancer risk without any major side effects. Studies indicate that dietary phytoestrogens may reduce breast cancer risk. The most common phytoestrogens in Western populations are lignans, mainly found in flaxseed, but results from several studies with lignans for breast cancer prevention have been inconsistent.

In this thesis we investigated the effects of tamoxifen and flaxseed on inflammatory mediators in normal breast tissue and in breast cancer. We used the microdialysis technique to sample proteins from the extracellular space *in vivo*. This technique gives us the opportunity to study proteins in their bioactive compartment *in situ* and to study changes in protein levels at different time points without affecting the tissue of interest. We also used experimental models and cell cultures to study tumor growth of human breast cancer xenografts, cancer cell proliferation and angiogenesis.

In paper I, we investigated whether tamoxifen, flaxseed, enterolactone or genestein reduced growth of human breast cancer xenografts and their association with pro-inflammatory cytokine interleukin 1 β (IL-1 β) and its antagonist interleukin 1 receptor antagonist (IL-1Ra). In paper II, we investigated whether tamoxifen and flaxseed exerted similar effects on inflammatory mediators in normal breast tissue *in vivo*. In paper III, we investigated whether osteopontin (OPN), a pro-inflammatory cytokine, was associated with dense breast tissue and breast cancer and if tamoxifen and flaxseed could alter OPN levels in normal breast tissue *in vivo*. We also investigated the correlation between OPN and inflammatory mediators in normal breast tissue and in breast cancer *in vivo*.

In conclusion, we showed that tamoxifen and flaxseed affected breast cancer growth in an experimental model and may exert an anti-inflammatory effect in breast cancer and normal breast tissue by increasing the IL-1Ra/IL-1 β ratio *in vivo*. We showed that dense breast tissue and breast cancer were associated with increased levels of OPN. Circulating estrogen did not correlate to OPN and tamoxifen and flaxseed did not affect OPN levels suggesting an estrogen independent regulation of OPN *in vivo*. These finding contributes to our understanding of how tamoxifen and flaxseed affects inflammation and the role of inflammation in the pathogenesis of breast cancer.

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Populärvetenskaplig sammanfattning

Bröstcancer är den vanligast förekommande cancersjukdom hos kvinnor idag. I Sverige insjuknar närmare 9000 kvinnor i bröstcancer varje år varav cirka 1400 kommer att dö till följd av sin sjukdom.

Det finns flera bakomliggande orsaker till att kvinnor drabbas av bröstcancer såsom hormonella faktorer och levnadsvanor. En ökad täthet i bröstvävnaden på mammografibilder, ”täta bröst”, har också kopplats till en ökad risk att drabbas av bröstcancer. Den bakomliggande mekanismen mellan täta bröst och bröstcancer är inte helt klarlagd, men det finns studier som talar för att en ökad inflammation i bröstvävnaden kan vara en förklaring. Inflammation är en känd riskfaktor för att utveckla vissa typer av cancer och inflammation är förknippad med en sämre prognos vid flera cancersjukdomar inklusive bröstcancer.

Det är viktigt att försöka hitta sätt att förebygga bröstcancer. Flera studier har visat att förebyggande medicinering med läkemedel som blockerar östrogen, antiöstrogen, och som idag används för att behandla bröstcancer, är effektiva för att skydda mot insjuknande av bröstcancer, men att de finns en risk för allvarliga biverkningar såsom livmoderkroppscancer och blodproppar med dessa läkemedel. Studier har visat att kosten möjligen kan påverka risken för att insjukna i bröstcancer och att växtöstrogener eventuellt kan ha en skyddande effekt. Det vanligaste förekommande växtöstrogenet i kosten i västvärlden är lignaner som finns i olika sädeslag, bär, nötter och frön, och då framför allt i linfrön.

Vi har undersökt hur tamoxifen, ett antiöstrogen, och linfrö påverkar tillväxten av bröstcancertumörer hos möss och vilka biologiska mekanismer som påverkar tillväxten med fokus på inflammation. Vi har även studerat hur olika proteinnivåer förändras i bröstcancertumörer och i normal bröstvävnad hos kvinnor efter behandling med tamoxifen eller ett dagligt tillskott av 25 g preparerade linfrön i kosten under sex veckor. Slutligen har vi också undersökt kvinnor med täta bröst och jämfört nivåerna av ett inflammationsprotein i bröstvävnaden med kvinnor med bröstcancer och kvinnor med icke-tät bröstvävnad. För att kunna undersöka protein i bröstvävnaden har vi använt en metod som kallas för mikrodialys. Den går i korthet ut på att man för in en mycket tunn kateter genom huden och in i bröstvävnaden. I katetern flödar en vätska som drar till sig ämnen i vävnaden och som på så vis kan samlas upp och analyseras.

Våra resultat visar att både tamoxifen och linfrö bromsar tillväxten av mänsklig bröstcancer i ett experiment på möss och att vi får en minskning av ett viktigt inflammationsprotein, interleukin 1 beta (IL-1 β), och en ökning av ett protein som hämmar IL-1 β s funktion, interleukin 1 receptor antagonist (IL-1Ra), och som sannolikt bidrar till den positiva effekten. Vi kunde också visa att tamoxifen och ett kosttillskott med linfrö hade en liknande sannolikt inflammationsdämpande effekt med en ökning av IL-1Ra i bröstvävnad hos friska kvinnor. Slutligen kunde vi visa att kvinnor med täta bröst och bröstcancer hade en likartad förhöjd nivå av ett annat viktigt inflammationsprotein, osteopontin (OPN) jämfört med icke-tät bröstvävnad. Däremot verkade nivåerna av OPN inte påverkas av behandling med tamoxifen eller ett kosttillskott av linfrö.

Sammanfattningsvis talar våra resultat för att tamoxifen eller ett kosttillskott av linfrö kan ha positiva effekter med en möjlig dämpning av vissa inflammationsproteiner i bröstvävnad. Vi

har också visat att täta bröst och bröstcancertumörer har ökade nivåer av ett viktigt inflammationsprotein, OPN, och som möjligen kan förklara den ökade risken för bröstcancer som finns hos kvinnor med täta bröst, men att detta protein inte påverkas av tamoxifen eller ett kosttillskott av linfrö. Dessa resultat kan ge en ökad förståelse för kopplingen mellan inflammation och bröstcancer och den eventuella bröstcancerförebyggande effekten av linfrö.

List of scientific papers

This thesis is based on the following papers:

1. **G. Lindahl**, N. Saarinen, A. Abrahamsson, C. Dabrosin, Tamoxifen, flaxseed, and the lignan enterolactone increase stroma- and cancer cell-derived IL-1Ra and decrease tumor angiogenesis in estrogen-dependent breast cancer. *Cancer Res* **71**, 51-60 (2011).
2. **G. Lindahl**, A. Abrahamsson, C. Dabrosin, Dietary flaxseed and tamoxifen affect the inflammatory microenvironment in vivo in normal human breast tissue of postmenopausal women. *Eur J Clin Nutr*, (2019).
3. **G. Lindahl**, A. Rzepecka, C. Dabrosin, Increased Extracellular Osteopontin Levels in Normal Human Breast Tissue at High Risk of Developing Cancer and Its Association With Inflammatory Biomarkers in situ. *Front Oncol* **9**, 746 (2019).

Abbreviations

AIs	aromatase inhibitors
BI-RADS	Breast Imaging Reporting and Data System
CAFs	cancer associated fibroblasts
CCL	chemokine (c-c motif) ligand
CKs	chemokines
COX-2	cyclooxygenase 2
CRP	c-reactive protein
CTLs	cytotoxic T cells
CXCL	chemokine (c-x-c motif) ligand
DC	dendritic cells
DNA	deoxyribonucleic acid
E2	estradiol
ECM	extra cellular matrix
ELISA	enzyme-linked immunosorbent assay
ENL	enterolactone
ER	estrogen receptor
GEN	genistein
HRT	hormone replacement therapy
HUVECs	human umbilical vein endothelial cells
IFN γ	interferon gamma
IHC	immunohistochemistry
IL-18BP	interleukin 18 binding protein
IL-1R	interleukin 1 receptor type
IL-1Ra	interleukin 1 receptor antagonist
IL-6RA	interleukin 6 receptor subtype alpha
ILs	interleukins
MBAA	multiplex bead array assay
MCF-7	Michigan Cancer Foundation-7
MD	mammographic density
MMPs	matrix metalloproteases
MVD	microvessel density
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
NPX	normalised protein expression
NSAIDs	nonsteroidal anti-inflammatory drugs
OPN	osteopontin
PAI	serine proteases inhibitor

PCR	polymerase chain reaction
PEA	proximity extension assay
s.c.	subcutaneous
SDG	secoisolariciresinol diglucoside
SERM	selective estrogen receptor modulator
SHBG	sex hormone binding globulin
sST2	soluble suppressor of tumorigenicity 2
Tam	tamoxifen
TAMs	tumor associated macrophages
Th cells	helper T cells
TIMPs	tissue inhibitors of matrix metalloproteinases
TME	tumor microenvironment
TNF	tumor necrosis factor
Treg	regulatory T cells
uPA	urokinase Plasminogen activator
VEGF	vascular endothelial growth factor

Introduction

Breast cancer is one of the oldest diseases documented throughout history. In an early Egyptian papyrus manuscript, possibly dated 1600 BC, several cases of breast cancer are described and concluded to be incurable (1). Endocrine treatment for breast cancer was introduced by Dr George Thomas Beatson, who performed a bilateral oophorectomy on a woman with recurrent breast cancer in 1895. However, the treatment was combined with sheep thyroid extract and the link between oophorectomy and breast cancer was purely empirical, and the mechanisms of action unknown. In 1897, an English surgeon, Dr Stanley Boyd, wrote a paper where he put forward the hypothesis that, “internal secretion of the ovaries in some cases favors the growth of cancer”. Years later he published his summary data and concluded that one third of his breast cancer patients benefited from the procedure although no patients were cured (2). In the late 19:th century, Dr William S. Halstead introduced a new surgical technique to treat breast cancer with a curative intent by removing the breast en bloc with the underlying pectoralis muscle and axillary lymph nodes (3). Since then, the surgical techniques have been refined and other treatment modalities have been introduced such as radiotherapy in the 1930s, adjuvant chemo- and hormonal therapy in the 70s, and since the turn of the millennium, new targeted drugs. Furthermore, in the 70s and 80s screening mammography was introduced throughout the Western world, including Sweden, and pooled estimates from several randomized control trials have shown a reduction in breast cancer mortality by at least 20 % due to screening (4). Taken together breast cancer mortality rate in the European Union has declined since the peak rate in 1989 of more than 20 /100,000 to an estimated 13.4 / 100,000 in 2020 (5). Still, breast cancer is the most common cancer among women worldwide and despite recent advantages in diagnostics and treatment, the leading cause of cancer-related death (6). In Sweden, close to 9000 women will be diagnosed with breast cancer annually, and despite a decrease in mortality rate during the last 30 years, approximately 1400 women will die from breast cancer every year (7).

The etiology of breast cancer is diverse, but long term exposure to sex steroids and genetic alterations are well-known risk factor. Estrogens play an important role in the development and progression of breast cancer and approximately 80% of all breast cancers are estrogen-receptor (ER) positive (8). The use of antiestrogens such as selective estrogens-receptor modulator (SERMs) and aromatase inhibitors (AIs) are gold standard in the treatment of ER-positive breast cancer today. SERMs are also approved as chemoprevention to women at high risk of breast cancer in several countries such as USA, Canada and the United Kingdom, but may have potentially severe side effects (9, 10).

However, other contributors such as life-style factors and chronic inflammation may also be possible causes of breast cancer. These conclusions come from migrant studies showing an increased breast cancer risk in women who move from countries with low incidence to countries with high incidence, and epidemiological studies indicating that women who regularly use anti-inflammatory drugs may have a lower breast cancer risk (11, 12).

Inflammation is considered to be a hallmark of cancer and the tumor microenvironment has become more and more recognized as a critical player in all steps of carcinogenesis (13). Stromal cells and immune cells have been shown to produce a variety of growth factors, cytokines and proteases creating an inflammatory milieu stimulating angiogenesis and tumor

proliferation. Furthermore, inflammation has been identified as a negative prognostic factor in several malignancies including breast cancer (14, 15).

Dietary habits may also affect the risk of breast cancer. Phytoestrogens are plant-derived dietary compounds that interacts with the estrogen receptor and therefore potentially can affect all processes regulated by estrogens (16). In a Western population, the most common phytoestrogens are lignans found mainly in flaxseed. Studies on lignan intake and breast cancer risk have however been inconclusive, where some studies have shown no association and others an inverse association in subpopulations of women, e.g., the postmenopausal (17, 18). To conclude, breast cancer incidence and mortality is the highest of all female malignancies despite advances in diagnostics and treatment. There is a need to further understand the underlying mechanisms of this disease and to explore possible preventive strategies. In this thesis we wanted to explore how phytoestrogens and tamoxifen (Tam) affect inflammation in breast tissue and breast cancer, hopefully elucidating a small part of this enigmatic disease. After all, every single step counts when you embark on a journey of a thousand miles.

Breast cancer and risk factors

The breast consists of different compartments, such as exocrine glands, ducts and supporting stromal and fat tissue. The breast undergoes continuous changes during a woman's different reproductive phases and reaches full maturity and function during pregnancy and lactation (19). The maintenance and differentiation of mammary gland cells is dependent on signaling from the local microenvironment by growth factors and cytokines. These interactions influence the changes that occur during development, pregnancy and lactation but also during tumorigenesis (20, 21).

Breast cancer is basically defined as the presence of a malignant tumor that originates from epithelial cells in the glands or ducts of the breast. The tumor cells proliferate and acquire the ability to invade surrounding tissues, lymph nodes and distant organs. Breast cancer is a heterogeneous disease that originates from a normal precursor cell following an accumulation of oncogenetic changes (22). It has been shown that breast cancer cells accumulate up to 1000s of mutations, but despite this multitude of mutations, breast cancers can phenotypically be divided into four major subgroups with similar clinical behavior (23). Apart from genetic changes, preclinical studies indicate that the microenvironment also affects the tumor cells and promotes or inhibits further neoplastic transformation (24). The microenvironment's role in cancer progression is furthermore illustrated by the fact that more than one third of women aged 40-50 have in situ breast cancer in autopsy studies, yet breast cancer is diagnosed in only 1% in this age group (25). This suggests that the tumor remains dormant, and that additional events in the stroma are needed for tumors to develop into clinical cancer.

The incidence of breast cancer has steadily increased during the last century and the lifetime risk of developing female breast cancer is today over 10% in the Western world. Age, reproductive history, hormonal exposure, obesity, life style factors such as high alcohol consumption and low physical activity, precancerous breast lesions, chest radiation, family history and, mutations in predisposition genes are all associated with an increased risk of

developing breast cancer (26). Mutations in predisposition genes, such as mutations in BRCA1 and BRCA2, are by far the strongest risk factor but account for only approximately 2-3 % of all breast cancer, whereas lifestyle factors, reproductive history and hormonal exposure, such as hormone replacement therapy (HRT), have a limited but significant impact on the risk increase (27).

Breast density

On mammography, epithelial cells and connective tissue attenuates x-rays more compared to surrounding adipose tissue and appears radiologically dense. Several factors such as genetics, parity, menopausal status, HRT, and diet can influence mammographic density (MD), and approximately 10 % of all women in the US are considered to have an extremely high MD corresponding to a MD $\geq 75\%$ of a mammogram (28). High MD is a risk factor for breast cancer in both pre- and postmenopausal women and women with extremely high MD have a four- to fivefold greater risk of breast cancer compared to women with low MD categorized as $<5\%$ or $<10\%$ in different studies (29, 30). There are several biological factors associated with differences in MD. Biopsies have shown more epithelial cells and/or stromal tissue, and especially a greater proportion of collagen. One plausible explanation is thus that MD correlates with epithelial tissue at risk of malignant transformation but also to a larger stromal component potentially containing inflammatory cells and mediators (31). Studies have investigated the association between inflammatory markers in breast tissue sections and MD with inconsistent results (32, 33). However, we have shown that postmenopausal women with extremely high MD have a more pro-inflammatory microenvironment compared to women with low MD which could contribute to a higher risk of developing breast cancer (34). Furthermore, we have also compared postmenopausal women with dense breast tissue to women with breast cancer and found that 26 of 32 inflammatory mediators showed similar profile (35).

There is limited knowledge of the association between MD and survival in women diagnosed with breast cancer. A recent meta-analysis indicated that high MD at diagnosis was associated with an increased risk of recurrence and higher mortality, and that a reduction in MD during breast cancer treatment is a positive predictive factor. However, due to mostly retrospective studies and different assessment methods the results have to be interpreted carefully (36).

Estrogens and the estrogen receptors

Estrogens are steroid hormones derived from cholesterol by the successive action of steroidogenic enzymes in the ovaries and through peripheral conversion of circulating precursors, androgens, in peripheral tissues (Figure 1). In women, the level of estrogen synthesis is high during the reproductive years to later decline during the transition and postmenopausal period. Estrogen exists in three forms of which 17β -estradiol (E2) is the most abundant and potent form (37). Estrogens are crucial in reproduction and normal physiological processes in both males and females, but are also associated with pathological conditions such as metabolic disorders, dementia, cardiovascular disease, osteoporosis and cancer (38). There is compelling evidence that estrogens play a major role in the development

of breast cancer and that high lifetime exposure increases the risk of breast cancer. Oophorectomized women have a significantly reduced breast cancer risk, as do women with a late onset of menarche and early menopause, and in postmenopausal women increased levels of circulating estrogens are associated with a higher risk of breast cancer (39). Furthermore, approximately 80% of all postmenopausal breast cancers are ER-positive and respond to antiestrogen treatment (8).

E2 is a small liposoluble molecule mainly synthesized in the ovaries and released into the general circulation in premenopausal women. In postmenopausal women E2 is produced by conversion of androgens by aromatase enzymes in breast, brain and fat tissue where it acts locally. E2 passively enters the cell through the plasma membrane and its actions are mainly mediated by its binding to the ER; alpha (ER α), beta (ER β) or any of their isoforms, localized mainly in the nucleus. When activated by their ligand the receptor dimerize and activate or repress gene transcription by binding to specific deoxyribonucleic acid (DNA) sequences in the genome (40).

Both ER subtypes are expressed in various tissues and while ER α primarily is expressed in female reproductive organs, ER β is mainly expressed in non-gonadal tissues (41). The expression of ER α is scarce in normal breast tissue but becomes highly upregulated in breast cancer, where it has been shown to promote tumorigenesis and progression of the disease, while the expression of ER β is decreased and even lost during breast cancer progression. Furthermore, the proliferation of ER α -positive breast cancer cells is enhanced by estrogens, which induce multiple growth factors, cyclins and cytokines involved in cell survival and cell cycle progression (38). The role of ER β in breast cancer has been debated, but several *in vitro* studies indicate a protective role of ER β through inhibited proliferation and increased apoptosis (42). ER β protective role is also supported by a meta-analysis that showed an association between ER β expression and improved disease free survival independent of ER α status (43).

The concentration of circulating E2 is also dependent on the concentration of sex hormone-binding globulin (SHBG), a serum protein synthesized in the liver in response to hormonal and non-hormonal factors such as physical activity and diet (44). SHBG regulates the bioavailable fraction of E2 and studies have shown an inverse association between SHBG and postmenopausal breast cancer risk (45).

Figure 1. Simplified overview of estrogen metabolism.

Cholesterol is converted to androgens which are subsequently aromatized to estrogens.

Estrone is reversibly converted to estradiol.

Blue ellipses: converting enzymes. 17 β -HSD: 17 β -hydroxysteroid dehydrogenases.

Antiestrogens

SERMs are molecules that bind to the ER with the capacity to exert different effects on various estrogen-related targets. Tam is the most common SERM and has been used in clinical practice for treatment of breast cancer since the mid-70s. It is metabolized by various cytochrome P450 enzymes to more active metabolites that competitively binds to both ER α and ER β and partly attenuates and inactivates downstream gene transcription (46). Tam produces different effects depending on the target tissue and exerts antagonist activity in breast but agonist activity in bone and the uterus. (47). Due to estrogens many physiological functions in different tissue such as reproductive organs, bone and cardiovascular system, there have been concerns on how to minimize possible adverse effects from these tissues (48). Tam is widely used in the treatment of ER-positive breast cancer both in the adjuvant and metastatic setting. The use of Tam as adjuvant treatment for 5 years has shown a reduction in absolute breast cancer mortality rates by 9 percentage points (24% vs 33%) in ER-positive breast cancer after 15 years, but with no benefit for patients with ER-negative cancer. However, the use of Tam also results in a slightly increased risk of endometrial cancer in the elderly population (49). Other SERMs, such as raloxifen, have been developed but none has yet shown any significant advantage over Tam. 20-30% of ER-positive breast cancers develop resistance to Tam. This mechanism is not well understood, but upregulation of membrane bound ERs may contribute to Tam resistance (50).

Another class of anti-estrogens are selective estrogen downregulators, such as fulvestrant. Fulvestrant binds to the ER but without any downstream activation of transcript factors and with a rapid degradation of the receptor and is considered a pure antagonist (48). Fulvestrant is today mainly used in treatment of metastatic breast cancer after progression of previous endocrine therapy (51).

Another endocrine approach for breast cancer treatment is to deprive ER of E2. This was previously done by oophorectomy and/or adrenalectomy but nowadays the same effect is accomplished in postmenopausal breast cancer patients with AIs that inhibit estrogen synthesis in peripheral tissue by blocking the aromatase enzyme. Several studies have shown that AIs are superior to SERMs in the treatment of postmenopausal women and is now considered gold standard in the adjuvant and metastatic setting (52, 53).

As chemoprevention in women with an increased risk of breast cancer, SERMs have been tested and approved and has shown to reduce the risk of invasive breast cancer almost by half, but with an increased risk of endometrial cancer and thromboembolic events (54, 55). AIs have also shown to be efficient in chemoprevention, and with fewer side effects than SERMs (56). However, they are not yet approved for chemoprevention by medical authorities as there are concerns regarding long term adverse effects on bone loss and cardiovascular risk.

Phytoestrogens

Phytoestrogens are plant-derived dietary compounds that represent a diverse group of naturally occurring chemicals characterized by structural similarity to E2. They bind to ERs and can potentially affect all the processes regulated by estrogens, but exert a weaker estrogenic activity than E2 (16, 57). Phytoestrogens can be divided into different groups of

which the most common are isoflavones, such as genistein (GEN) or daidzein, mostly found in soy products but also in grapes and fruits, and lignans, mainly enterolactone (ENL) and enterodiol, found in seeds, whole grains, vegetables and fruits (Figure 2) (58, 59).

Dietary habits may affect the risk of breast cancer and studies of migrant populations of Asian immigrants in USA show a higher, and increasing, incidence in breast cancer compared to their native counterparts. The increasing incidence is probably due to lifestyle factors including, among others, decreased consumption of soy products (11). There are also studies indicating that the timing of exposure to soy/isoflavones is important and that exposure early in life, i.e., before menarche, leads to a greater risk reduction of breast cancer (60).

Dietary phytoestrogen may be a non-toxic prevention strategy to reduce the incidence of breast cancer, but the evidence is not irrefutable and phytoestrogen consumption is not incorporated in the World Cancer Research Funds (WCRF) breast cancer prevention recommendations (61). Furthermore, phytoestrogens might induce potential adverse health effects since they may act as endocrine disruptors and negatively affect fertility in women (62).

Figure 2. Comparison of the chemical structures of estrogen 17β-estradiol with phytoestrogens: Isoflavones, genistein and daidzein, Lignans, enterolactone and enterodiol.

Enterolactone

In Western populations lignans are the most common type of phytoestrogen found in high concentrations in seeds, fruits and vegetables (63). Flaxseed is the richest source of lignans consumed by humans and contains the lignan secoisolariciresinol diglucoside (SDG) (59). SDG must be converted by intestinal bacteria to its active metabolites, mainly ENL, to exert a biological effect (16, 64). The metabolism of enterolignans includes conjugation, first-pass metabolism and enterohepatic recirculation both in the bowel mucosa as well as in the liver (65). Due to a high individual difference in gut microbial diversity and composition there is

an individual difference in the bioavailability of dietary enterolignans (63, 66). Previous use of antibiotics may also reduce gut microbiota which impair uptake of lignans. To estimate individual lignan consumption and metabolism, serum or urinary levels of enterolignans can be measured (67).

Phytoestrogens and breast cancer

Phytoestrogens seem to preferentially interact with ER β and may influence the risk of breast cancer by inhibiting cell proliferation, but also by initiating apoptotic events and inhibiting angiogenesis presumably in an ER-independent manner (16, 68). There might, however, be a biphasic effect of phytoestrogens exerting a stimulatory effect at low doses, but acting as an antiestrogen at higher doses suppressing cell growth. This has been shown in breast cancer cell culture where low doses of ENL induced a stimulatory effect while high doses had an inhibitory effect on cell growth *in vitro* (69). Moreover, low doses of isoflavones have shown to induce breast cancer cell proliferation through the stimulation of ER α in mouse models (70, 71). In an estrogen depleted mouse model with human breast cancer xenografts, a dietary addition of flaxseed showed no increased tumor growth compared to unexposed mice, and contrarily, an addition of dietary soy showed tumor progression (72). Phytoestrogens different effects on breast cancer growth are also supported by other models where the dietary addition of flaxseed or ENL, but not GEN, inhibited tumor growth, presumably by a vascular endothelial growth factor (VEGF)-mediated effect (73-75).

The mechanisms of action of phytoestrogens are thus not fully understood and may not only include interaction with the ER. Studies have shown that phytoestrogens suppress the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), alter the expression of proteins that control the cell cycle and apoptosis in breast cancer cell lines, and interfere with estrogen converting enzymes (68). Furthermore, flaxseed contains fatty acids, such as omega-3, which may have anticancer properties (76). An experimental model showed that flaxseed oil attenuated breast cancer growth and reduced cancer cell proliferation (77). ENL has also been shown to stimulate the production of SHBG which binds free E2 and may result in lower concentrations of circulating sex hormones thereby potentially decreasing breast cancer cell growth (78). There may also be an anti-inflammatory effect of ENL in breast cancer, as a retrospective study showed an inverse association between ENL, c-reactive protein (CRP) and mortality (79). However, an earlier meta-analysis of 20 prospective randomized studies of various conditions and diseases, including prostate cancer but not breast cancer, showed no effect of flaxseed on reducing CRP (80).

The effects of phytoestrogens on risk reduction in breast cancer have to some extent been inconclusive. Whereas a recent meta-analysis of several prospective studies of consumption of isoflavonoid containing foods and breast cancer risk suggested a reduced risk with high consumption of soy products, results from epidemiological studies on the association between lignans and/or ENL and breast cancer are more uncertain (17, 18, 67, 81, 82). In a review article from 2007 the author concludes that out of ten prospective and 18 case-control studies published, no or almost no significant associations between plasma ENL or lignan intake and breast cancer risk were reported (81). Since then, results from a large Swedish prospective

cohort study has shown a significant inverse association between lignan intake and postmenopausal breast cancer risk, especially among women using hormone replacement therapy (18). A protective effect of lignans was also demonstrated in a large case-control study and a meta-analysis showing a significant inverse association between serum levels of ENL and risk of postmenopausal breast cancer, especially in the ER-negative population (67). Conversely, a large European prospective study showed no association between lignan intake and reduced risk of breast cancer, but a significant decrease in breast cancer mortality in postmenopausal women (17, 83). Other studies support the association between high concentrations of serum ENL and reduced mortality in postmenopausal breast cancer patients (78, 84). However, the results of epidemiological trials must be interpreted cautiously since there may be limitations in the included studies, such as different self-reported questionnaires to assess exposure, different cut-off values for categorizing the study population and lack of information about important confounding factors e.g., exposure to hormone replacement therapy.

Phytoestrogens does not seem to be associated with breast density; a study of MD in postmenopausal women showed an association between high levels of ENL and a slightly increased percentage MD but of uncertain clinical significance and a recent meta-analysis showed no association between isoflavones and MD in postmenopausal women (85, 86)

The tumor microenvironment and inflammation

In 1863 the German pathologist Rudolf Virchow described leukocyte infiltration in neoplastic tissues and suggested that cancer may originate from chronic inflammation. Since then the concept of inflammation as a key event in carcinogenesis has become fully accepted and today it is estimated that approximately 25 % of all cancers are associated with chronic inflammation (87).

In 2000 Hanahan and Weinberg published a paper arguing that carcinogenesis is a multistep process of stochastic events leading to malignant transformation. They described tumor-acquired capabilities shared by most human tumors, the hallmarks of cancer, and categorized these into six categories; self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis (88). In 2011 they revised their model in the light of recent advances in cancer research and added two hallmarks, avoiding immune destruction and deregulating cellular energetics, but they also introduced a new concept of tumor initiating properties; genomic instability and mutation, and tumor-promoting inflammation (13).

Tumor development has been compared to a non-healing wound as they share many similarities such as angiogenesis, influx of inflammatory cells and remodeling of the stroma (89). Thus, the tumor microenvironment (TME) has become increasingly recognized for its collaborative interaction with tumor cells in all steps of carcinogenesis. Cancer associated fibroblasts (CAFs) are an important component of the cancer stroma. They are heterogeneous in origin, but seem mainly to be converted from resident fibroblasts by tumor released cytokines (90). CAFs have been described to play a major role in remodelling of the extracellular matrix (ECM) by expressing matrix metalloproteases (MMPs), stimulate

angiogenesis by upregulating VEGF and promote tumor proliferation via secretion of various cytokines such as tumor necrosis factor (TNF), interleukin (IL) 1 beta (IL-1 β), IL-6, osteopontin (OPN) and chemokine (c-x-c motif) ligand (CXCL) 12 (90, 91). Collagen is the main protein of the ECM and is important for the structure of the tissue as well as facilitating intracellular communication and studies have demonstrated the importance of CAFs and ECM-proteins within the stroma for cancer initiation, growth and metastatic spread (92-94).

Inflammation and the immune system

The immune system consists of two major effector pathways; the immediate non-specific innate immune response and the subsequent specialized adaptive immune response. The innate immune system includes several hematopoietic cells such as macrophages, dendritic cells (DCs), neutrophils, mastcells and natural killer (NK) cells. In addition, epithelial cells lining the surfaces of the body participate in the innate immune response together with circulating inflammatory proteins such as CRP. The innate immune response recognizes and eliminates external pathogens expressing ‘non-self’ molecules and unhealthy cells with upregulated abnormal ‘induced self’ molecules due to infection, stress and malignancies (95). The dysregulation of transcriptional factors, such as NF- κ B, is a key event in cancer related inflammation and immunity. NF- κ B may be constitutively activated in cancer cells or immune cells by genetic alterations, but is mainly activated by external factors such as hypoxia and/or pro-inflammatory cytokines, e.g., IL-1 and TNF. NF- κ B stimulates several genes, which play a major role in cell survival, resistance to cell death, migration and angiogenesis (Figure 3) (91, 96). Moreover, these transcription factors coordinate the production of inflammatory mediators including MMPs, ILs and chemokines (CKs), and as a consequence inflammatory leukocytes are recruited to the TME further sustaining the inflammation (97).

Figure 3. NF- κ B and the hallmarks of cancer.

Hallmarks and inflammatory mediators discussed in this thesis shown in black.

Adapted from: Hanahan et al., Ref 12.

Macrophages are derived from monocytes in peripheral blood and constitute a major component of the inflammatory infiltrate. They can be activated by two distinct pathways where classically activated macrophages (M1) following exposure to interferon gamma ($\text{IFN}\gamma$) and microbial products are tumoricidal, whereas alternative activated macrophages (M2) following exposure to cytokines, such as IL-4, IL-10, chemokine (c-c motif) ligand (CCL) 2 and CCL17 and 22, are considered immunoregulatory and cancer promoting (96). During cancer initiation, macrophages are characterized as more anti-tumoral (M1), but as the tumor secretes cytokines in the TME, such as IL-6, IL-10 and IL-33, the macrophages change into a more pro-tumoral M2 phenotype (98). This effect may be due to physiological changes in the TME, such as pronounced hypoxia and pH-changes, during cancer progression (96). However, the polarization of macrophages is dynamic and not absolute, resulting in a functional polymorphism of TAMs (14).

As the cells of the innate immune system neutralize tumor cells initially, they activate the adaptive immune response by presenting tumor-associated antigens to T cells. This stimulates the proliferation of cytotoxic T cells (CTLs) and augmenting T helper cells which can eliminate tumor cells and activate B-cells (Figure 4). However, TAMs and DCs in the TME may attenuate the adaptive response by recruiting immunosuppressive regulator T cells (Treg) that suppress the anti-tumoral activity of other immune cells. Furthermore, the tumor cells evade elimination by expressing transmembrane proteins, programmed death-ligands, that deactivate effector CTLs (99). The role of B-cells in the TME is not fully understood. They may act anti-tumoral by differentiating into plasma cells and produce anti-tumor antibodies but further studies are necessary (100).

Figure 4. Cells of the innate and adaptive immune system in the TME

Adapted from: www.astartebio.com

Inflammation and breast cancer

The abundance of TAMs are associated with worse overall survival in breast cancer patients and the absence of TAMs have shown a significantly reduced invasiveness and metastatic potential in an experimental breast cancer model (15, 101). Conversely, other effector cells of the immune system, such as CTLs and NK cells, may eliminate breast cancer cells and are associated with better prognosis in ER-negative breast cancer, whereas infiltrating lymphocytes are associated with reduced survival in ER-positive breast cancer (102, 103). Inflammatory cytokines may induce a more aggressive phenotype of breast cancer and elevated levels of circulating acute-phase proteins, CRP and serum amyloid A, have been found to be prognostic markers for reduced overall survival in disease free breast cancer patients after adjuvant treatment, suggesting an association between inflammation and poor prognosis (103, 104).

In addition, several experimental studies of anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), have shown reduced xenograft tumor growth in mouse models. NSAIDs induce their effects mainly by the inhibition of the enzyme cyclooxygenase (COX) 2, with subsequent reduction of COX-2 related pro-inflammatory metabolites. This may explain why NSAIDs may have a positive effect in breast cancer prevention, as COX-2 activities have been identified in various steps of breast carcinogenesis (105). The use of NSAIDs have however shown conflicting results in human studies. A large randomized trial with aspirin vs placebo in healthy women from 2005 showed no significant risk reduction of cancer in general or breast cancer in particular (106). However, a meta-analysis from 2008 showed that NSAID use was associated with reduced breast cancer risk and a more recent meta-analysis from 2015 showed a weak but significant dose-response relationship between acetylsalicylic acid and reduced breast cancer risk in premenopausal women (12, 107).

Inflammatory mediators - cytokines

The Interleukin 1 family of cytokines

Cytokines are highly localized messenger proteins which activate and mediate the immune response during inflammation. There is no clear distinction between different groups of cytokines such as ILs and CKs and several cytokines function as both. Traditionally, ILs have been recognized as mediators between immune cells and CKs as chemotactic factors.

One of the central regulators of innate immunity and inflammation is the IL-1 family of cytokines (IL-1s). They may act as alarmins, or danger signals, released as a result of injury to initiate a local inflammatory response, which under normal physiological conditions leads to tissue regeneration, but when dysregulated can cause pathologic inflammation (108). The IL-1s comprises eleven cytokines divided into three subgroups; the IL-1 subfamily, the interleukin 18 (IL-18) subfamily and the interleukin 36 (IL-36) subfamily (Table 1). Furthermore, there are ten members of the IL-1 family of receptor, including anti-inflammatory receptors and decoy receptors such as IL-1 receptor type 2 (IL-1R2) and soluble suppressor of tumorigenicity 2 (sST2) (109). The main function of IL-1s is to initiate pro-inflammatory reactions in response to tissue injury by activation of NK- κ B and subsequent expression of several other cytokines such as IL-6 and IL-8 (110). The two major IL-1

agonistic molecules are IL-1 α and IL-1 β . IL-1 α is constitutively expressed intracellularly, mainly in luminal epithelial cells, and though rarely secreted; it acts as an alarmin in response to tissue injury and initiates the innate immune system (109). IL-1 β is primarily produced by monocytes, macrophages and DCs in response to complement components, cytokines or by autocrine signaling. IL-1 β is synthesized as an inactive precursor in the cytoplasm and is activated through cleavage by caspase-1 before secretion into the extracellular space. IL-1 β is a potent mediator of inflammation and thus highly upregulated in inflammatory and malignant tissue (111).

IL-1s are also known mediators of angiogenesis. In wound healing, this effect seems to be mediated through induction of hypoxia-inducible factor-1 α and upregulation of VEGF, whereas in malignancies this seems mainly to be an effect mediated by activated NF- κ B in TAMs and the expression of IL-6, IL-8 and adhesion molecules stimulating pro-inflammatory endothelial cells (112). Moreover, in experimental models, inhibition of IL-1 β has shown greatly reduced angiogenesis (113, 114).

IL-18 is constitutively expressed by several cells including immune cells. Its main function is to modulate the immune response by activating NK cells and to enhance the adaptive immune response. Anti-tumoral effects of IL-18 have been demonstrated in animal models of early stages of inflammatory malignancies such as gastric and colon cancer. However, tumor promoting effects have been shown in advanced gastric cancer, melanoma and pancreas cancer (115). IL-18 binding protein (IL-18BP) is a natural inhibitor of IL-18 and is produced by monocytes and macrophages. In preclinical melanoma and lung cancer models, administration of IL-18BP inhibited metastasis, and thus inhibition of IL-18 may have positive effects in tumors expressing IL-18 receptors (115).

The IL-1 subfamily also includes IL-33 which is expressed mainly by various non-immune cells and acts as an alarmin triggered by allergens or parasites. IL-33 acts pro-inflammatory by inducing T cells differentiation and activating mast cells, but may also induce Tregs and thus attenuate the immune response (116). Studies have shown an overexpression of IL-33 in airway inflammatory diseases, inflammatory bowel disease and in several cancers such as colorectal, breast and lung cancer (116, 117). IL-33 is regulated by sST2, an extracellular decoy receptor that binds to IL-33 thereby preventing IL-33 signaling. Circulating sST2 is

Table 1. Interleukin 1 family of ligands and receptors

IL-1 family	Receptor	Co-receptor	Function
IL-1 α , IL-1 β	IL-1R1	IL-1R3	Pro-inflammatory
IL-1 β	IL-1R2	IL-1R3	Anti-inflammatory
IL-1Ra	IL-1R1	-	Anti-inflammatory
IL-18	IL-1R5	IL-1R7	Pro-inflammatory
IL-33	IL-1R4	IL-1R3	Pro-inflammatory
IL-36 α , β , γ	IL-1R6	IL-1R3	Pro-inflammatory
IL-36Ra	IL-1R6	IL-1R3	Anti-inflammatory
IL-37	IL-1R5	IL-1R8	Anti-inflammatory
IL-38	IL-1R6	IL-1R9	Anti-inflammatory

IL-1R4 is also known as ST2, suppressor of tumorigenicity 2

increased in intestinal and metabolic diseases and in breast cancer and, has shown to be useful as a biomarker in heart failure (118).

The IL-1 family includes a naturally occurring receptor antagonist (IL-1Ra) which antagonizes the activities of IL-1 α and IL-1 β by binding to IL-1R1 with higher affinity but without eliciting a cellular response (111). In preclinical studies, treatment with IL-1Ra has led to reduced angiogenesis and inhibited tumor development. A recombinant human IL-1Ra, anakinra, used for treatment of rheumatoid arthritis, is tested in early clinical trials on cancer patients and the addition of anakinra to chemotherapy in heavily treated patients with metastatic colorectal cancer has shown promising activity (119, 120).

The Interleukin 1 family of cytokines and breast cancer

IL-1 β is elevated in several malignancies, including breast cancer, where it has been associated with higher tumor grade, aggressiveness, angiogenesis and poor prognosis (108, 121). Its importance in the carcinogenic process of breast cancer has been shown in several experimental studies where caspase-1 knockout mice with decreased IL-1 β secretion, or mice treated with anakinra, have exhibited impaired tumor development and fewer metastases in lungs and bones. (122-124). There is data suggesting that mainly TAMs and DCs are associated with upregulated IL-1 β expression and that high level of IL-1s, including IL-1 β and IL-1Ra, in breast cancer tissue are associated with more advanced stage and worse outcome (124, 125). IL-1 β enhances the activity of estrogen producing enzymes such as aromatase and steroid sulfatase in breast cancer cell culture, presumably stimulating the production of bioactive estrogens, which may suggest an estrogen mediated effect of IL-1 β on breast cancer proliferation (126). This possible effect is furthermore supported by a positive correlation between E2 and IL-1 β , and a negative correlation between E2 and IL-1Ra, in human breast tissue (127). In a small study of 11 metastatic breast cancer patients, treatment with anakinra in combination with chemotherapy eliminated a “signature of IL-1 associated inflammation” in blood cells suggesting an enhanced anti-tumor activity of anakinra (124).

IL-18s role in breast cancer is not fully understood, but there is growing evidence that high serum levels of IL-18 are associated with worse outcome in breast cancer patients (128, 129). Serum levels of both IL-33 and sST2 have shown to be increased in patients with breast cancer and correlate to VEGF and MMPs (117). Furthermore, in a breast cancer model, IL-33 was associated with an immunosuppressive TME, accelerated tumor growth, increased angiogenesis and metastasis (130).

Interleukin 6

During acute inflammation IL-6 is mainly produced by monocytes and macrophages as a response to tissue damage and contributes to the recruitment of inflammatory cells (131). However, IL-6 is a pleiotropic cytokine with both pro- and anti-inflammatory properties. These properties are in part regulated by IL-1 β which suppresses the anti-inflammatory effects of IL-6 (132). In cancer, IL-6 is secreted by both tumor and stromal cells and is known to induce a pro-tumoral M2 phenotype of macrophages and to affect several hallmarks of

cancer, e.g., proliferation, angiogenesis, invasiveness and metastasis (98, 131). Elevated levels in serum and tissue have been observed in several malignancies and are generally associated with aggressive tumors and poor prognosis (133). IL-6 may also protect cancer cells from therapy induced damage (134). Treatment with IL-6 and IL-6 receptor inhibitors are currently being tested in several clinical cancer trials (135).

Interleukin 6 and breast cancer

The role of IL-6 in breast cancer is ambiguous. In epidemiological studies no significant correlation between circulating IL-6 and risk of developing breast cancer has been demonstrated (136). However, in breast cancer patients, high levels of serum IL-6 are associated with increased levels of VEGF and, higher tumor stage, higher metastatic burden and worse prognosis (137, 138). Conversely, expression of IL-6 within breast cancer tumors has shown an association with early stage and better overall survival (139). In cell culture, IL-6 has shown either inhibitory or promoting effects on tumor cell proliferation, in part dependent on ER-status, whereas mouse models have shown an IL-6 dependent tumor growth and metastasis (140).

Interleukin 8

Endothelial cells secrete IL-8 in both acute and chronic inflammation. IL-8 acts as a chemotactic factor for neutrophils and granulocytes and may promote angiogenesis by stimulating endothelial cell production of VEGF (141). IL-8 expression has been shown to be significantly higher in several types of malignancies and *in vivo* cancer models have shown that tumor derived IL-8 contributes to an immunosuppressive microenvironment and angiogenesis which promote tumor progression (142, 143).

Interleukin 8 and breast cancer

Studies support an estrogen-mediated effect of the regulation of IL-8 and we have previously demonstrated that E2 increased and Tam decreased IL-8 levels and that serum estrogen levels correspond to IL-8 levels in breast cancer tissue (144). Furthermore, serum levels of IL-8 correlates with more advanced disease and worse prognosis in patients with breast cancer (145, 146). Currently, the use of IL-8 neutralizing antibodies and receptor antagonists are being tested in preclinical and early clinical trials for malignant diseases but the results have so far been disappointing (142).

Osteopontin

OPN was first described by Oldberg and co-workers in Lund in 1986 as a bone specific sialoprotein isolated from a rat osteosarcoma (147). They identified an extracellular protein that facilitated the interaction between cells and minerals in the matrix suggesting a role in bone development and remodeling. Later studies showed that OPN also is present in human epithelial lining cells communicating with the external environment implicating that OPN

may have a protective role in the interaction between these surfaces and the environment (148).

Since then has OPN been identified in various tissues such as brain, liver, lung, breast, gastrointestinal tract, cardiac and kidney and can be detected in a variety of body secretions including urine, saliva and milk (149). However, OPN levels are low in healthy tissue but is upregulated in inflammation and wound healing where OPN is a key cytokine expressed by numerous cells including bone, muscle and endothelial cells contributing to the migration and activation of inflammatory cells (150). OPN is furthermore expressed by DCs, macrophages and activated T cells which enhance the inflammatory response (151).

There are both an intracellular and a secreted isoform of OPN of which the intracellular protein has been contributed to cellular processes in DCs related to migration, fusion and motility, whereas the extracellular isoform is thought to regulate the interaction with different target cells (152). Secreted OPN is subject to splicing as well as posttranslational modifications, such as proteolytic cleavage by MMPs, which results in a functional diversity (153, 154). Pro-inflammatory cytokines, such as IL-1 β , IL-10 and TNF, are reported to upregulate OPN secretion from DCs, while conversely IFN γ limits OPN expression (155).

OPN is a central mediator in inflammation and regulates the immune system at different levels. It acts as a chemotactic factor for inflammatory cells such as macrophages, neutrophils, DCs and NK cells (154). This has been shown in mouse models where the use of OPN-deficient mice and blocking antibodies greatly reduced the infiltration of macrophages and neutrophils (151, 156). OPN also plays an important role in the induction of the adaptive immune response and mediates T-cells differentiation important in the defense against bacterial infections (157). OPN is upregulated and associated with several autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus and chronic inflammatory disease such as inflammatory bowel disease and atherosclerosis (158).

OPN is produced by several different cell types in the TME, but there is limited knowledge on whether tumor-derived and stroma-derived OPN differs functionally. In the TME OPN has been shown to activate TAMs essential for promotion of tumor growth, remodeling of tissues, and suppression of antitumor immunity (98). OPN also influence angiogenesis by inducing VEGF expression in endothelial cells (159, 160). Emerging data also support OPN as a key mediator in epithelial-mesenchymal transition necessary for cancer progression and metastasis through the stimulation of CAFs and the secretion of transforming growth factor and IL-6 (161, 162).

Several meta-analyses in different malignancies such as colorectal, breast, lung, prostate cancer and studies of melanoma and glioblastoma have shown reduced overall survival for patients with high levels of OPN in plasma or serum, or increased expression in tumor tissue (163-167). Phytoestrogens may influence OPN secretion and in preclinical studies of prostate cancer, GEN has shown a biphasic dose-dependent effect on OPN expression and OPN-mediated tumor growth and survival (168, 169).

Osteopontin and breast cancer

In pre-malignant breast lesions, one study have shown that strong IHC staining of OPN-c, an isoform of OPN, is associated with a higher risk of malignant transformation and worse outcome (170). In breast cancer patients the results have been unclear. In a cohort of ER-positive early adjuvant breast cancer patients, neither OPN expression nor plasma levels were associated with reduced overall survival (171). However, a meta-analysis exploring OPN expression by immunohistochemistry (IHC) staining in breast cancer has shown that high expression of OPN is correlated with poor overall survival (166). Another study showed a negative association between OPN gene expression and overall survival in patients with breast cancer, and elevated and increasing plasma levels of OPN have also been associated with poor survival in metastatic breast cancer patients (172, 173). Furthermore, an *in vivo* model has shown that an overexpression of OPN increases lymphovascular invasion, lymph node metastases and lung micrometastases supporting OPNs role in breast cancer invasion and metastasis (174).

Chemokines

Chemotactic cytokines constitute a large family of structurally related small proteins characterized by their ability to stimulate cell migration. Today about 50 CKs, divided into four subgroups, and more than 20 receptors have been identified including decoy receptors, expressed mainly on erythrocytes, that do not elicit a response (175, 176). Membrane bound CKs form a chemotactic gradient required for directional cell migration and CK-activated receptors interact with integrins in the basement membrane resulting in a trans-endothelial migration of leukocytes. Furthermore CKs can act in synergy with other CKs or cytokines to elicit a more pronounced response. Some CKs are constitutively expressed and contribute to the maintenance of the immune system whereas inflammatory CKs are inducible and regulate leukocyte migration in response to infiltrating pathogens. Apart from their role in the inflammatory response, CKs are also critical for guiding migrating cells during embryogenesis. Nearly all tissues express CKs, but in normal breast tissue the expression is generally low (177, 178).

In tumors, both cancer and stromal cells produce CKs that contribute to both pro-tumoral activities, such as recruitment of TAMs and neutrophils, as well as anti-tumoral activities, such as recruitment of T lymphocytes and NK cells. CKs may also regulate angiogenesis and mediate metastasis (175).

There are several pro-tumoral CKs, like CXCL1-3, 5-7 and CCL2 and 5, that attract neutrophils and TAMs, promote angiogenesis and may play a role in chemotaxis and metastasis of tumor cells. CXCL12 play a distinct role in the metastatic process by attracting CK-receptor CXCR4-expressing tumor cells to distant sites. CXCL12 is released in large amounts by lung, liver, lymph nodes and bone marrow (179).

Other CKs seem to have pleiotropic effects; CXCL9-11 have anti-tumoral effects on the tumor microenvironment, like skewing TAMs to an anti-tumoral M1 phenotype, but may also have pro-tumoral effects on the tumor cells. The effects seem to depend on the CK-receptor

phenotype expression and synergistic effects of other CKs (176). CXCL4 and CXCL14 have angiostatic and anti-tumoral effects and is downregulated in cancer tissue compared to adjacent normal tissue (180). Antibodies blocking different CK-receptors, e.g., CXCR4, have shown impaired tumor growth in animal models and are being tested in different clinical trials (181).

Chemokines and breast cancer

In an analysis of gene-expression of CKs in breast cancer, 27 CKs were upregulated in breast cancer tissue compared to adjacent normal breast tissue and nine of these CKs also showed elevated levels in plasma compared to healthy controls (182). In breast cancer, CCL2 and CCL5 is highly expressed in both tumor and stromal cells and are associated with high tumor grade and poor prognosis (183). In an animal model, the levels of CCL2 and CCL5 correlated with the levels of E2, indicating an estrogen dependent mechanism. Furthermore, treatment with Tam decreased *in vivo* levels of CCL2 and CCL5 in breast tissue (184). CXCR4 expression is upregulated in patients with breast cancer and high levels have been associated with regional and distant metastasis and reduced survival (179).

Inflammatory mediators - proteases

Matrix metalloproteases

MMPs were first identified as enzymes capable of hydrolysis of collagen and were originally associated with cancer invasion due to their ability to degrade the extracellular matrix facilitating cell migration (185). Later studies have shown that MMPs also cleave non-matrix substrates contributing to the release and modification of signaling molecules (186). As such, MMPs lead to the activation of several pro-inflammatory cytokines such as TNF and IL-1 β as well as regulating chemokine gradients (187). However, the function of MMPs is often context-dependent and MMPs may also induce an anti-inflammatory response by proteolytic processing of inflammatory regulators (188).

To date 23 MMPs have been identified and most MMPs are secreted in an inactive form, subsequently activated in the extracellular space by several proteinases including plasmin and other MMPs (189). MMPs play a role in normal physiological events, such as tissue homeostasis and wound healing, but generally the expression of MMPs is low in healthy tissue. Overexpression of MMPs can induce severe tissue damage and MMP hyperactivity has been observed in many pathological conditions, such as osteoarthritis, multiple sclerosis, and cancer (187).

In cancer MMPs have been described to promote proliferation, angiogenesis and ECM degradation facilitating tumor growth, invasion and metastasis (190). However, some MMPs, such as MMP-8, seem to have a protective effect on tumor invasion and metastasis and others, such as MMP-12 and MMP-9, may exert different effects dependent on if they are secreted by tumor cells or cells in the tumor stroma such as TAMs (191). The function of MMPs is highly regulated by four tissue inhibitors of matrix metalloproteinases (TIMPs) that strongly block the activity of MMPs. Dysregulation of TIMPs have been identified in most cancers indicating their importance in MMP regulation and tumor progression. Furthermore,

decreased TIMP 3 expression has been correlated with advanced disease and poor prognosis in several cancers (192). Despite MMPs significant role in cancer, clinical trials with MMP-inhibitors in cancer treatment have all failed to reach their end points and have induced significant side effects. The poor efficacy was likely due to broad-spectrum MMP inhibition including cancer-protecting MMPs. Since these early trials, our understanding of MMPs diverse biological effects has deepened and, consequently, future development of more selective inhibitors might give better results with fewer adverse events (193).

Matrix metalloproteases and breast cancer

MMP gene expression in breast cancer has shown an association between MMP-9,-11 and -15 and poor survival (194). Furthermore, IHC-staining of MMP-2 and MMP-9 have shown significantly higher expression in breast cancer than in the surrounding breast tissue and positive staining correlates with higher tumor stage and poorer prognosis (195). MMP overexpression, especially MMP-9, have been associated with worse prognosis in several studies, but the results diverge concerning MMP-2 and its association to survival (196). Not all MMPs have purely tumor promoting effects. In mouse models MMP-8 has shown to suppress metastasis and, intriguingly, MMP-9 has been associated with tumor regression and anti-angiogenic activity in some, but not all, experimental models (197-199). Furthermore, MMP-9 was not elevated in a study by our group of human breast cancer compared to adjacent normal breast tissue *in vivo*, although MMP-1,-2,-3 were significantly elevated (35).

Urokinas plasminogen activator

The plasminogen activator system contributes to several physiological processes such as wound healing, tissue regeneration, angiogenesis and mammary gland development, but is also activated in pathological processes such as cancer progression and metastasis (200). Urokinas plasminogen activator (uPA) is one of two plasminogen activators and plays a crucial role in tumor invasion and metastasis. The uPA system consists of the serine protease uPA, a membrane anchored receptor and two inhibitors, serine proteases inhibitor-1 and -2 (PAI-1, PAI-2). uPA is activated by several proteases including plasmin and bound to its receptor uPA in turn activates plasmin in a reciprocal feedback loop. Plasmin plays a role in the degradation and remodeling of the basement membrane and the ECM as well as in the activation of growth factors and MMPs in malignancies. PAI-1 has an equivocal role in cancer with both an inhibitory effect on invasion and metastasis as well as facilitating tumor growth and dissemination (200, 201).

Urokinas plasminogen activator and breast cancer

uPA and PAI-1 have been confirmed and validated as prognostic biomarkers in breast cancer, especially in the node-negative subtype. High levels of uPA and, particularly PAI-1, have been associated with poor prognosis and have also shown to be predictive biomarkers for adjuvant chemotherapy (194, 202, 203).

Angiogenesis

Angiogenesis, the formation of new blood vessels from an existing vascular network, is considered to be one of the hallmarks of cancer and is essential for tumor growth and metastasis (13). Angiogenesis begins via the activation of endothelial cells by proangiogenic factors such as VEGF, one of the key mediators of angiogenesis, and ILs. The VEGF family comprises five structurally related factors, of which VEGFA is the most important mediator of angiogenesis, and three receptors (204). It stimulates endothelial cell growth and facilitates the invasion of the underlying matrix by upregulation of specific proteases including pro-uPA and pro-MMPs, thereby leading to the formation of new blood vessels. VEGF plays an important role in embryonic development and wound healing in healthy individuals (205). Several mediators, including IL-1s, plasmin and MMPs, but also hypoxia induce VEGF gene expression in the ECM. Under physiological conditions these factors are produced by endothelial, stromal and blood cells, but during carcinogenesis the production is upregulated by tumor cells and the ECM. Hence, the levels of VEGF are elevated in several malignancies including colorectal, lung and breast cancer and correlates with metastatic spread (206). Preclinical studies have shown that a tumor cannot grow more than 1-2 mm³ without angiogenesis and that micro-metastases remain dormant without angiogenetic activity (207, 208). VEGF has been considered to be an ideal therapeutic target due to its crucial role in tumor growth and progression. Consequently, numerous clinical trials have been performed with anti-angiogenic pharmacological agents, including monoclonal antibodies and tyrosine kinase inhibitors. As of today eleven drugs have been approved for treatment of different malignancies. The clinical effects have been modest, probably due to acquired resistance to anti-angiogenetic therapy both by the tumor cells and the stromal cells (209).

Angiogenesis and breast cancer

Estrogen stimulates the secretion of VEGF in normal human breast tissue and it has been shown in several experimental breast cancer models that E2 stimulates VEGF and angiogenesis *in vivo* (210-212). Studies have shown an association between microvessel growth in the primary tumor and metastatic lesions in breast cancer supporting the role of angiogenesis in tumor progression and metastasis (213). The impact of angiogenesis on breast cancer development was also demonstrated in a meta-analysis showing an inverse association between tumor microvessel density (MVD) and survival of breast cancer patients (214). Furthermore, studies have shown that a high level of VEGF is a negative prognostic marker in breast cancer (215, 216).

Several anti-angiogenic drugs such as the monoclonal antibody bevacizumab, targeting VEGF, and several tyrosine kinase inhibitors, targeting growth factor receptors and downstream signaling pathways, have been tested in numerous clinical trials for breast cancer, but the results have so far been disappointing with no or very limited effect on survival and as of today no anti-angiogenic drugs are considered to be standard treatment in breast cancer (217).

Aims of this thesis

Overall aim

To determine whether flaxseed and/or Tam affected the inflammatory microenvironment in normal breast tissue and in breast cancer *in vivo*.

Specific aim

- To determine whether various phytoestrogens affected experimental breast cancer growth.
- To determine the role of IL-1s in experimental breast cancer growth.
- To determine whether flaxseed exhibit similar effects as Tam in a panel of inflammatory mediators in normal breast tissue *in vivo*.
- To determine whether extracellular OPN was altered in normal breast tissue with different densities and in breast cancer *in vivo*.
- To determine whether flaxseed or Tam could modify OPN levels in normal breast tissue *in vivo*.
- To determine whether OPN correlated with a panel of inflammatory mediators in normal breast tissue and breast cancer *in vivo*.

Comments on material and methods

This section gives an outline of the techniques and procedures used in the papers that comprise this thesis. For detailed information on experimental protocols used in the different studies, please see the materials and methods section of each paper.

Cell culture

The use of breast cancer cell lines has greatly contributed to our knowledge on breast cancer and is widely used in cancer research. Studies have confirmed that many cancer cell lines capture the genomic diversity of their original cancers and that it is plausible to use them as *in vitro* model systems (218). Most cell lines are from metastatic tumors, and establishment of new cell lines is complex with low success rate. Furthermore, attempts to culture cells from primary tumors have so far often been unsuccessful. There are several positive aspects of using cell lines; they are usually easily cultured, provide an indefinite source of biological material and are free from other contaminating cells. Another advantage of using commercial established human cancer cell lines is the consistency and reproducibility of results when applying the same protocol. There are also potential problems such as cross-contamination between cell lines and microbial contamination. However, advances in molecular biology have made it possible to use DNA profiling for cell line authentication to ascertain that the cell lines are not misidentified or cross-contaminated. Microbial contamination of cell cultures continues to be problematic and antibiotics are often used to control the growth of bacterial and fungal contaminants, but may interfere with metabolism of sensitive cells and mask infections by mycoplasma why all active cell cultures must be tested regularly for mycoplasma contamination (219).

In this thesis (paper I) we used cell cultures with Michigan Cancer Foundation-7 (MCF-7) cells, human umbilical vein endothelial cells (HUVECs), and macrophages as specified below.

Michigan Cancer Foundation-7

MCF-7 is a well characterized, commonly used E2-sensitive breast cancer cell line normally considered to be poorly aggressive and with low metastatic potential. MCF-7 cells comprise a large number of individual phenotypes which are maintained during culturing. They express high levels of ER α , but low levels of ER β , and are dependent on E2 in order to proliferate and, conversely, E2 depletion leads to a reduced proliferation rate and an increased ER-expression (220). Therefore, MCF-7 cells are considered suitable for studying the association between E2 and cancer cell progression.

In our first study (paper I) we used MCF-7 cells from the American Type Culture Collection (ATCC). The cells were immediately expanded and stored as stocks in liquid nitrogen after delivery. Every cell culture started with a frozen stock from early passage to minimize the risk of cross-contamination and genomic instability.

The choice of cell culture media is important, and may affect the success of cell culture experiments. Different cell types have highly specific growth requirements. Unless stated

otherwise, we used Dulbecco's Modified Eagle's Medium (DMEM), a commonly used medium that contains no proteins or growth promoting agent and that requires supplementation with 5-10% Fetal Bovine Serum to be a complete medium (221).

Human umbilical vein endothelial cells

HUVECs are primary endothelial cell types commonly used for studying angiogenesis *in vitro*. This model recapitulates all of the early stages of angiogenesis including proliferation and migration of endothelial cells (222). Studies have shown sex-gender differences in cell proliferation and protein expression which must be taken in consideration when designing a study (223).

We prepared HUVECs from anonymously donated female fresh umbilical cords. The cords were filled with collagenase, digested at 37°C, and detached cell mixture was centrifuged. The cell pellets were re-suspended and cultured in M199 medium. HUVECs are known to have a limited lifespan and all experiments were conducted on cells from passages 2-3.

Macrophages

Monocytes were isolated from human venous blood, seeded in 24-well plates and incubated for two hours to allow macrophage adhesion. Nonattached cells were removed by washing. The cells were exposed to E2, Tam, ENL or GEN during 24 hours. The macrophages were analyzed for ER α expression and the medium was analyzed for IL-1Ra.

Mouse models

Mouse models have been essential in our understanding of pathological processes in several diseases, including malignancies, and in drug development. The athymic nude mouse has a gene mutation resulting in lack of the thymus and a defective differentiation and proliferation of T-lymphocytes. Due to this natural immunosuppression, nude mice tolerate xenografts without any transplant rejection, which make them suitable candidates for simple and fast testing of the efficacy of anticancer agents (224). When studying the TME, the use of xenografts make it possible to determine if proteins of interest originate from stromal or cancer cells using species-specific antibodies. Furthermore despite the mouse's immunosuppression, the innate immune system is intact which makes it possible to study the effects of macrophages, neutrophils and NK cells in the TME. However, the model has some limitations as the tumor lacks its original microenvironment and fails to reflect the real *in vivo* situation with a competent immune system. This may result in a limited predictability when experimental results are applied in a clinical setting (224, 225).

In our first study (paper I) athymic female mice (Balb/cA nu/nu, 6–7 weeks old) were housed in a pathogen-free isolation facility and administered sterilized diet and water *ad libitum*. The animals were oophorectomized under anesthesia and implanted with E2 pellets providing E2 concentrations that represent physiological levels equivalent to circulating levels of E2 in postmenopausal women. Four days after surgery, MCF-7 cells (5×10^6 cells) were injected

subcutaneously (s.c.) in mammary pads in both flanks of the mice. After two weeks, the growth of the s.c. xenograft tumor area was measured using a caliper twice a week.

In the first experiment, mice were divided into four dietary treatment groups (including one control group) and fed basal diet with the addition of GEN, ENL or ground flaxseeds.

In the second experiment, mice with xenograft tumors of similar sizes were divided in two groups, one group treated with s.c. injections of 1 mg Tam every second day and one control group injected with vehicle only.

In the third experiment, mice with xenograft tumors of similar size were treated with anakinra, human recombinant IL-1Ra, 5 mg injections daily or with solvent injections as controls.

Microdialysis was performed at the end of each experiment.

Human subjects

Before start of the studies, we obtained local ethics committee approval and all participating women gave informed consent.

In our second study (paper II) we included 28 postmenopausal women (Figure 5). All 28 women had a normal breast examination and mammography prior to the interventions. Each procedure included one microdialysis catheter inserted in the upper lateral quadrant of the breast and one catheter inserted in abdominal sub cutaneous fat as a reference. Venous blood was drawn from all subjects at the time for microdialysis.

15 women had undergone surgical resection of the breast for early breast cancer.

Microdialysis was performed in the contralateral unaffected breast immediately before start of

Figure 5. Female subjects in paper II and paper III, grouped according to examination/intervention.

Blue squares = microdialysis in normal breast tissue.

Red square = microdialysis in breast cancer and in adjacent normal breast tissue.

*Patients included in paper II.

**Microdialysis was performed in two consecutive luteal phases and hence counted twice (n=8) as normal breast tissue in paper III

adjuvant Tam treatment (20 mg/day) and again after six weeks of treatment. The remaining 13 women were healthy volunteers who added 25 g of ground flaxseed daily to their regular diet for six weeks. Microdialysis was performed before and after six weeks of flaxseed consumption. No one was using flaxseed in their regular diet or had been on antibiotics the last three months. In our third study (paper III) 89 postmenopausal and 14 premenopausal women were included (Figure 5). In 13 women with newly diagnosed breast cancer, one microdialysis catheter was inserted in the tumor and one in the adjacent normal breast tissue. In 42 healthy postmenopausal women, mammograms were scored according to the Breast Imaging Reporting and Data System (BI-RADS) density scale and women with non-dense tissue (n=20), BI-RADS A, or extremely dense tissue (n=20), BI-RADS D, were selected (226). Two patients were misclassified and not included in the analysis of OPN in dense vs non-dense breast tissue. Microdialysis was performed in the breast and abdominal subcutaneous fat. In a total of 21 postmenopausal women who underwent breast cancer surgery, microdialysis was performed before and during Tam treatment as previously described. Two patients were omitted from the second analysis due to non-compliance. 13 postmenopausal women added dietary flaxseed for six weeks and 10 premenopausal women added flaxseed for one menstrual cycle. Microdialysis was performed prior and after the interventions. Furthermore, four unexposed women were investigated in two consecutive luteal phases. None of the healthy women had any history of breast cancer or had used hormone replacement therapy, sex steroid contraceptives, anti-estrogen therapy or antibiotics within the past three months.

The microdialysis technique

The microdialysis method was developed during the mid-1960s to study neurotransmitters. The technique mimics the function of a capillary blood vessel where extracellular molecules are continuously sampled by passive diffusion (227). The system includes a double lumen catheter connected to a pump with constant flow of an isotonic perfusion fluid with added colloids to reduce ultrafiltration by an increased osmotic pressure. At the tip of the catheter is a semi-permeable membrane which can be placed within the tissue of interest. The collected microdialysate reflects the composition of the extracellular fluid and the analyte concentration in the dialysate is a relative measure of the concentration in the extracellular fluid (Figure 6) (228). The sampling of a certain molecule is dependent on the flow rate, pore size and the length of the semi-permeable membrane of the catheter to reach equilibrium (229, 230). The technique has been extensively used in numerous studies and has proven to be an important method for a greater understanding of pharmacokinetics and metabolism in different tissue (230). Moreover, microdialysis reflects the concentration of molecules/proteins at the site of interest better than measurements in blood (231, 232). There are however two potential drawbacks that must be considered; partial recovery and depletion of the molecule of interest near the sampling site (229). In our studies we present raw data and compare differences in analyte concentration over time and therefore do not need to know the recovery percentage or the absolute protein levels in the tissue. The risk of protein depletion compromising our data is minimized as we use an ultraslow flow of the perfusion fluid and as we compare dialysate

from samples collected at the same time point after calibration, and thus possible depletion of proteins would be expected to be the same.

Microdialysis has shown to be safe and reliable and histological examinations of the affected tissue after the procedure have not shown any signs of complications such as edema, bleeding or inflammatory reactions adjacent to the catheter (229). It has been shown that implantation of the catheter at different position in breast tissue in the same individual gives reproducible data, indicating that the method is reliable for intra-individual comparison over time (233).

Prof C Dabrosin has improved the technique for studying the microenvironment in breast tissue and the method has been used in several studies by her research group. Throughout the studies in this thesis we have used catheters with a membrane pore size of 100 kDa and a length of 20 mm in human breast tissue and abdominal fat tissue, or 10 mm in breast cancer tumors, and 4 mm in mice xenograft tumors. The perfusion rate was 0.5 $\mu\text{l}/\text{min}$ in human studies and 0.6 $\mu\text{l}/\text{min}$ in mouse models. To establish steady state and to avoid any method related contamination of the samples, an equilibration period of 30 to 60 minutes was applied before collecting dialysate for analysis. To analyze if the changes in protein levels were breast tissue specific we also collected, and compared the results with, microdialysate from abdominal subcutaneous fat tissue.

Figure 6. The principle of microdialysis.

The collected microdialysate reflects the composition of the extracellular fluid; the molecules of interest pass through the semi-permeable membrane of the microdialysis catheter by passive diffusion and into a continuous flow of perfusion fluid.

Originally from: CMA/Microdialysis AB, Stockholm, Sweden.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) uses the concept of antigen-antibody interaction to detect small quantities of antigens such as proteins, peptides and hormones in a fluid. A primary antibody binds to a specific antigen, the protein of interest, and the antigen – antibody complex is subsequently detected by an enzyme-coupled antibody which reacts with a chromogenic substrate creating a visible color change that can be assessed by a colorimetric reading. To calculate the amount of bound protein to the primary antibody, the color intensity of the sample is compared with a standard curve with known concentrations of the antigen.

The technique is easy to use, inexpensive, and has a high sensitivity and specificity. ELISA

has some limitations; it's a singleplex method that only detects and measures one protein at the time, and there is a risk of interference, i.e., cross-reactivity between antibodies and antigens, which can cause a false result. This can, however, be avoided by adding a blocking reagent before executing the assay (234).

We used commercial human and mouse immunoassay kits for the detection and measuring of IL-1 α , IL-1 β and IL-1Ra in paper I. In this study we used a sandwich ELISA with capture antibodies precoated in the wells of an ELISA plate. The sample is added to the wells followed by a wash, an addition of a conjugated enzyme-labeled antibody and finally addition of a chromogenic substrate. This method has a high sensitivity and specificity and is suited for analysis of complex samples (figure 7).

In paper III we used a competitive ELISA to measure E2. Sample target proteins and a known amount of enzyme labeled target proteins were added to an ELISA plate which was pre-coated with capture antibodies. Unbound proteins were removed by washing and a chromogenic substrate was added. High antigen concentration in the sample results in low concentration of remaining antibodies and a low output signal (Figure 7). This method is preferred when measuring small proteins where two antibodies cannot bind to the protein in a sandwich ELISA. This method was also used in paper II and III to measure ENL, but with a fluoroimmunoassay that detects emission of light instead of measuring color intensity.

Figure 7. The principles of sandwich and competitive ELISA

Adapted from: www.ruo.mbl.co.jp/bio/e/support/method/elisa.html

Luminex®

Luminex® is a multiplex bead array assay (MBAA) that provides quantitative measurements of large numbers of analytes. Depending on the system used, MBAA allows simultaneous quantification of up to 100s of analytes. The technique resembles ELISA but captures antigens onto spherical beads, not flat surfaces, with specific antibodies and uses fluorescence

as reporter system instead of colorimetric substrates. In brief, first you add different color coded beads with specific capture antibodies to the sample. Then you add an analyte specific detection antibody, and finally a reporter molecule. The beads are gated through a dual laser flow cytometer where one laser classifies the bead and one determines the magnitude of the reporter molecule derived signal (Figure 8) (235).

Several studies have shown good correlation between MBAA and ELISA measurements, but often with poor agreement of quantitative values. Furthermore, multiplexing increases the risk of interference and each analyte should ideally be tested for non-reactivity against all antibodies in the multiplex array (236).

We used Luminex® to measure IL-1 β and IL-1Ra in paper II.

Figure 8. The principle of Luminex®

Different color coded beads with specific capture antibodies bind to a target protein. Analyte specific detection antibodies and reporter molecules are added. The beads are gated through a dual laser flow cytometer where one laser classifies the bead and one determines the magnitude of the reporter molecule derived signal.

Adapted from: www.thermofisher.com

Immunohistochemistry

IHC is a commonly used, fast and reliable method in search for cell or tissue proteins while preserving tissue histology. The basic principle behind the technique is relatively simple with detection of tissue antigens by specific antibodies followed by a colored histochemical reaction visible by light microscopy or fluorescence. The statistically significant correlation between the results from the quantification of IHC and protein levels have been demonstrated through measurements by methods such as Western blotting (237).

Primary antibodies are either polyclonal or monoclonal. Polyclonal antibodies are produced by immunized animals, such as mouse, rabbit, goat, and may be more heterogeneous with several different antibodies to a target protein. Polyclonal antibodies have a higher affinity but lower specificity than monoclonal antibodies. Monoclonal antibodies are produced by hybrids of B-cells from mice immunized with antigen and myeloma cells. Monoclonal antibodies have a high specificity with low risk of cross-reactivity (238).

A secondary antibody directed against the primary antibody is conjugated to a label enzyme such as horseradish peroxidase which in the presence of a chromogen 3,3'-diminobenzidine produce a brown color and allows visualization. The tissue might be subject to counterstaining to produce contrast making the interpretation easier, but without interfering with the staining itself (238). To ensure that you will have a correct reading it's important to have a positive primary antibody control, that is a specimen containing the target molecule in its known location, and a negative secondary antibody control using your tissue of interest, but replacing the primary antibody with normal serum and observe that no labeling occurs. These controls should run in parallel with the experiment, however, primary antibody control is only necessary for each new antibody used and does not have to be repeated in each experiment (239).

The method has several pitfalls and the required steps must follow an established protocol in order to avoid non-desirable interactions. There are pre-analytic variables such as tissue fixation, preparation of slides and antigen retrieval. Fixation, mainly in formaldehyde and subsequent inclusion in paraffin, is necessary to preserve tissue over time, but may alter protein biochemistry by cross-links so that the epitope of interest is masked. Antigen retrieval is a way to reduce this chemical modification. One commonly used method is heat-induced epitope retrieval using heat, e.g., a pressure cooker, to reverse cross-links and restore the structure of the epitope. Another pitfall is related to the interpretation of the slides, including the selection of antibodies and reading the slides (237, 240). Interpreting the results of IHC depends on qualitative observation and requires a manual scoring system and therefore two issues have to be considered, false positive signals and false negative signals. The first might be the result of nonspecific background signaling that represents the binding of antibodies by mechanisms other than specific binding to their epitope on the target antigen, which can be avoided by adding a blocking reagent, or it might be due to inappropriately high antibody concentrations. The most common causes of false-negative immunostaining are poor tissue fixation, not optimized epitope retrieval method or too diluted antibodies (240).

IHC was used in our first study (paper I) to stain IL-1 α and IL-1 β (monoclonal mouse anti-human), ER α (monoclonal rabbit anti-mouse), the macrophage marker F4/80 (monoclonal rat anti-mouse) and anti-von Willebrand's factor (polyclonal rabbit anti-human with cross reactivity with mice) in human breast cancer xenografts in mice, and ER α (monoclonal rabbit anti-human) in human monocytes.

Proliferation – MTS assay

In our first study (paper I) we evaluated the effects of IL-1Ra and E2 on MCF-7 or HUVEC proliferation. We used the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS assay), a non-toxic, colorimetric method for indirect quantification of viable cells. MTS, a water soluble salt, is absorbed by metabolically active cells and converted by mitochondrial dehydrogenase to insoluble purple formazan crystals. As the amount of the converting enzyme is highly stable in a given cell population the color intensity is proportional to the number of viable cells (241). MCF-7 or HUVECs were seeded in 96 well plates and incubated with IL-1Ra and/or E2. Proliferation was analyzed after three days by absorbance detection using an ELISA reader set at 490 nm.

Proximity extension assay

Proximity extension assay (PEA) is a multiplex immunoassay that combines the detection specificity of antibodies with the amplification power of polymerase chain reactions (PCR). Two antibodies labelled with a unique DNA oligonucleotide bind different epitopes on a target protein bringing the nucleotides together. The nucleotides hybridize and serve as a template for a DNA polymerase extension step creating a unique DNA signature for the target protein, quantitatively proportional to the initial concentration of the protein. The DNA signature is followed by amplification and quantification by a high-throughput real-time PCR detection system (Figure 9) (242). The generated fluorescent signal corresponds with the amount of protein in the sample.

Figure 9. The principle of proximity extension assay.

Two antibodies labelled with a unique DNA oligonucleotide bind different epitopes on a target protein bringing the nucleotides together. The nucleotides hybridize and serve as a template for DNA polymerase creating a unique DNA signature for the target protein followed by amplification and quantification.

Adapted from: www.bioxpedia.com/technologies

There are several advantages with this method. Only a small volume of 1 μ l per sample is needed to potentially detect and measure small quantities of 92 different proteins simultaneously with high specificity and sensitivity. Furthermore, the risk of false positive signals generated by unspecific cross-reactivity is eliminated since only correctly matched DNA signatures generate a signal (243).

In this thesis, we used PEA for protein analyses in paper II and paper III. The analyses were performed by an external company using commercial available standard panels (Olink Proteomics, Uppsala, Sweden). To get a group of coherent inflammatory proteins, we used the inflammation, cardiometabolic, cardiovascular II and cardiovascular III panel to analyze our samples. The data was provided in the arbitrary unit normalized protein expression (NPX). The data should be used for relative quantification, i.e., comparisons of the levels of the same protein between different samples, but not for comparisons of absolute levels between different proteins.

Statistics

In paper I, we performed Student's *t*-test to compare means in groups of two and one-way ANOVA with Tukey's *post hoc* test in groups of three or more under the assumption of normal distribution. Fishers's exact test was used to compare nominal variables such as immunohistochemical scoring.

In paper II and III, the data was considered non-normally distributed and Wilcoxon signed rank test was used for pairwise comparisons and Mann Whitney *U*-test for comparison of independent unpaired samples. Correlations were calculated using Spearman's rank correlation test, or the identical method, Pearson's correlation between ranks.

In paper I and II, data was presented as means $\pm$ SEM and in paper III mainly as scatter diagrams with regression lines. All statistical tests were two-sided and in all papers $p < 0.05$ was considered statistically significant.

Statistical analyses were performed using GraphPad Prism software 5.0 and 7.0

Results and discussion

Breast cancer is the most common female cancer worldwide today. There is an urgent need to better understand the etiology and pathogenesis of the malignant transformation in cancer in general, and in breast cancer in particular. Hence, it is important to find effective preventive strategies to reduce the risk of breast cancer without compromising quality of life. There are several studies showing that antiestrogens, SERMs and AIs, have a protective effect on breast cancer risk and can be used for chemoprevention (54-56). These drugs come with potentially severe side effects and other strategies are needed. Phytoestrogens may be a non-toxic alternative, but epidemiological studies have so far been inconclusive (17, 81). However, some studies show an association between lignan intake, the main phytoestrogen in flaxseed, or its bioactive metabolite ENL, and reduced breast cancer risk (18, 67).

Inflammation is considered an enabling characteristic of cancer as described by Hanahan et al. and impacts several of the hallmarks of cancer such as sustained proliferation, limited cell death, angiogenesis and extracellular matrix remodelling facilitating invasion and metastasis (13). Several malignancies, such as gastric and colon cancer, are preceded by chronic inflammation, which supports the concept of inflammation as a major risk factor in cancer progression (87). The role of inflammation in cancer development is also suggested by epidemiological studies showing potential effects of NSAIDs in reducing the risk of colon and breast cancer (12, 217, 244). Furthermore, inflammation in manifest tumors is, with almost no exceptions, associated with more aggressive disease and worse prognosis (14, 15, 90).

The main pro-inflammatory cytokine IL-1 β is known to stimulate cancer cells and cells in the TME with the subsequent expression of other pro-inflammatory mediators such as IL-6 and IL-8, IL-1s, CKs, MMPs, and VEGF (91, 110). IL-1 β is highly upregulated in several malignancies, including breast cancer, and is associated with higher tumor stage and poor prognosis (110, 111). OPN is another key inflammatory cytokine with functional diversity regulating inflammation, chemotaxis and angiogenesis. OPN is upregulated in several malignancies and is associated with poor outcome and, overexpression of OPN in breast cancer may be associated with poor overall survival (163-167).

In paper I, we aimed to investigate the effects of Tam and phytoestrogens on ER-positive breast cancer growth and whether they affected IL-1 β and IL-1Ra. We set up human breast cancer xenograft mouse models where we exposed groups of mice to dietary flaxseed (n=14), ENL (n=20) and GEN (n=15), and in a second experiment to Tam (n=8), and compared tumor growth with an unexposed control group (n=23, n=9). Flaxseed, ENL and Tam significantly impaired or reduced estrogen dependent tumor growth whereas GEN had no effect on tumor development. These results are in line with previous experiments done by our research group and others that have shown that flaxseed and ENL impair breast cancer tumor growth, presumably by an anti-angiogenetic effect, but that GEN and soy have no effect on, or stimulate, tumor progression (69-71). ENL and flaxseed showed similar reduction of tumor growth which suggests that the effects of flaxseed may be mediated by ENL. However, mice fed flaxseed showed a slightly greater growth reduction than mice fed ENL which may be related to other components in flaxseed such as fatty acids. These may also exert anticancer

effects as shown in an experimental model where flaxseed oil attenuated breast cancer growth and reduced cancer cell proliferation in a mouse model (77).

We used microdialysis to investigate the TME and observed that impaired or decreased tumor growth was associated with a significantly decreased secretion of stromal-derived IL-1 β and a significant increase of stromal-derived and cancer-derived IL-1Ra (Figure 10). We observed a significant association between impaired tumor growth *in vivo* and decreased MVD in IHC-staining of tumor sections which suggests that the effect on tumor growth in our model was mediated by decreased angiogenesis. In addition, we observed a significant decreased proliferation rate in cell culture of HUVECs exposed to E2 and IL-1Ra, whereas the proliferation rate of MCF-7 was unaffected. This supports the observation of an IL-1Ra/IL-1 β mediated anti-angiogenetic effect, and not a cytotoxic effect on tumor cells, reducing tumor growth. Moreover, to determine the effect of IL-1Ra on tumor growth we set up another model and exposed mice with manifest tumors to anakinra (n=6), a recombinant human IL-1Ra, which resulted in reduced tumor growth compared to an untreated control group (n=7). MVD was also significantly decreased supporting our conclusion that the effect was mediated by inhibited angiogenesis. These findings are in concordance with other studies that have shown an IL-1 dependent angiogenesis in experimental models and that inhibition of IL-1 β correlates with reduced tumor growth (113, 114).

Figure 10. Microdialysis for *in vivo* sampling of extracellular stroma- and cancer cell-derived IL-1s in tumor tissue.

A – C, Mice allocated to four different diet groups: BD – basal diet, flax – flaxseed, ENL – enterolactone, GEN – genestein: A, extracellular murine IL-1 β ; B, extracellular murine IL-1Ra; C, extracellular human IL-1Ra; n=5 to 7 in each group.

D – F, Untreated mice vs mice treated with tamoxifen (Tam):

D, extracellular murine IL-1 β ; E, extracellular murine IL-1Ra; F, extracellular human IL-1Ra; n=5 to 10 in each group.

P-values: *, p <0.05; **, p <0.01; ***, p <0.0001.

We have previously shown decreased VEGF levels in the TME with subsequent reduced MVD in xenograft mouse models exposed to ENL (73). This suggests that the correlation between MVD and IL-1 β and IL-1Ra observed in our study may be mediated by VEGF. IL-1 β is known to promote angiogenesis via NK- κ B activation in TAMs resulting in an increased secretion of VEGF, IL-6 and IL-8 (112). Thus, it would be expected that decreased levels of IL-1 β and increased levels of IL-1Ra would reduce the levels of angiogenic factors. We could identify an abundance of ER-positive TAMs in the TME by IHC-staining of tumor sections. Exposure to flaxseed, ENL and Tam decreased stroma-derived IL-1 β and increased stroma-derived IL-1Ra supporting the notion that cells in the TME may be targeted by our interventions and hence contribute to decreased tumor growth.

There are several limitations in using an immunocompromised mouse model with a MCF-7 cell line-derived xenograft. The model does not reflect the true biological conditions in human breast cancer; MCF-7 xenografts have limited cellular diversity and do not metastasise and, even though the mice have an upregulated innate immune response, they lack the adaptive immune response and hence T cell immunity.

However, mouse models are important research tools to investigate proof of mechanism; as in our study, the effects of phytoestrogens on breast cancer growth and their association with IL-1 β and IL-1Ra. Furthermore, using a xenograft model with human cancer cells in mice make it possible to apply species-specific antibodies to determine whether different proteins are secreted from cancer cells or cells in the stroma. This gives us a better understanding of the interaction between our interventions, cancer cells and tumor associated cells in the stroma.

In paper II we aimed to explore whether our results from paper I, with an assumed association between flaxseed, ENL or Tam and IL-1s in an experimental breast cancer model, could be verified in normal human breast tissue. This was in part already suggested in a previous study by our group that showed that dietary flaxseed in premenopausal women and adjuvant Tam in breast cancer patients increased IL-1Ra, but not IL-1 β in breast tissue *in vivo* (127). However, we wanted to explore if there was an association between adjuvant Tam treatment in postmenopausal breast cancer patients and IL-1s and/or other pro-inflammatory mediators. Moreover, we wanted to investigate whether flaxseed exerted the same effects on the tissue microenvironment in healthy postmenopausal women possibly attenuating potentially harmful inflammation. While large studies have shown a chemopreventive effect of Tam, results from epidemiological studies have been somewhat conflicting on whether lignans and/or ENL reduce breast cancer risk or not (18, 55, 67, 81, 83). This might, in part, be due to methodological difficulties in how to estimate lignan intake and/or differences in effect in different subgroups according to menopausal status, breast cancer ER status etc.

We performed microdialysis in normal breast tissue and in abdominal subcutaneous fat in 28 postmenopausal women before adjuvant treatment with Tam (n=15) or a dietary addition of 25 g ground flaxseed daily (n=13). The procedure was repeated after six weeks. Lignans must be converted by gut microbiota to its bioactive metabolite ENL to exert any effect (16). Not all humans have this capability, and 2 out of 13 women in our study were considered non-converters, as ENL levels in serum did not increase after exposure to dietary flaxseed.

However, a lack of increased ENL levels in plasma may also be an effect of low compliance to supplementary flaxseed and they were therefore excluded from the analysis. The main results are summarized in Table 2.

Concerning IL-1Ra and IL-1 β levels in breast tissue, our data is to some extent in agreement with the results from paper I with significantly increased IL-1Ra levels after the interventions. However, no significant change in IL-1 β levels was detected, but the IL-1Ra/IL-1 β ratio was significantly increased by both flaxseed and Tam suggesting a similar overall anti-inflammatory effect. These data corroborates the results from a previous study by our group in premenopausal women showing increased levels of IL-1Ra after flaxseed intake (127). We also found a significant correlation between the inflammatory mediators including IL-1Ra/IL-1 β ratio and ENL in serum, indicating that this was an ENL mediated effect. To further explore the effects of flaxseed and Tam and to get a coherent picture of important inflammatory mediators we complemented our analyses with PEA of the microdialysate which allow highly specific and sensitive multiplex analyses of low quantities of proteins in small sample volumes (243).

These results were inconsistent, with a significant reduction of IL-18 and IL-8 with Tam and a significant increase of IL-1R2, IL-18, sST2 and MMP-9 with flaxseed. All changes were breast tissue specific except for the increase of MMP-9 which could be detected in abdominal s.c. fat tissue as well (Table 2).

Table 2. Summary of the effects on inflammatory mediators after interventions with Flaxseed or Tam in normal breast tissue.

Mediators Interventions	IL- β	IL-1Ra	IL-1Ra IL-1 β	IL-1R1	IL-1R2	IL-18	IL-18BP	IL-33	sST2	IL-6	IL-6RA	IL-8	MMP-1	MMP-2	MMP-3	MMP-9
Tamoxifen	—	↑*	↑**	—	—	↓	—	—	—	—	—	↓	—	—	—	—
Flaxseed	—	↑**	↑**	—	↑	↑	—	—	↑	—	—	—	—	—	—	↑*

↑: significant increase, ↓: significant decrease, —: no significant change. P-values equals $p < 0.05$, unless *, $p < 0.01$, or **, $p < 0.001$.

IL-8 stimulates angiogenesis and acts as chemotactic, immunosuppressive factor and, is associated with more advanced disease and worse prognosis in breast cancer (142, 144, 146). The role of IL-18 in cancer is however more ambiguous as it seems to be highly context dependent and may exert both anti-tumoral and pro-tumoral effects (115). However, several studies in breast cancer patients have shown an association with high serum levels of IL-18 and worse outcome (128, 129). IL-1R2 is decoy receptor acting on IL-1 β and sST2 is a decoy receptor that inhibits IL-33, a pro-inflammatory cytokine associated with tumor progression in breast cancer (118, 130). An increase of IL-1R2 and sST2 may suggest an anti-inflammatory effect. However, sST2 is increased in several pathological conditions, including breast cancer, and the clinical interpretation of increased sST2 levels remains to be clarified (117, 118, 245). MMPs are mainly thought to contribute to carcinogenesis, angiogenesis and metastasis. However, some MMPs have anti-tumoral effects and others may exert either pro- or anti-tumoral effects dependent their context (191). MMP-9 has been associated with poor outcome in breast cancer patients but some experimental models have shown anti-tumoral effects on xenografts in mice (195, 196, 198, 199). Furthermore, in a previous study by our group, no

elevated levels of MMP-9 were measured in human breast cancers compared to adjacent normal breast tissue *in vivo*, whereas other MMPs were significantly elevated in breast cancer compared to adjacent normal breast tissue (35). This underlines the importance of studying the inflammatory mediators in their bioactive compartment. For example, cytokines have short half-lives and are consumed locally and, the levels circulating in blood are therefore often below the limits of detection of various assays (246).

Our data showed that both flaxseed and Tam increase the IL-1Ra/IL-1 β ratio suggesting in part a similar anti-inflammatory effect. As IL-1 β is a key inflammatory initiator one could expect that an increased IL-1Ra/IL-1 β ratio, suggesting a more anti-inflammatory microenvironment, would correspond to a reduction of other pro-inflammatory mediators. The effects of Tam with decreased IL-8 and IL-18 may suggest an anti-inflammatory mechanism involved in chemoprevention. However, the effects of flaxseed on other pro-inflammatory mediators diverge and are difficult to interpret as some effects may be anti-inflammatory and others may be pro-inflammatory and thus no firm conclusions can be drawn on possible net effects on the inflammatory microenvironment.

IL-1 β is highly upregulated in inflammatory and malignant tissue but not constitutively expressed during healthy conditions (111). In our study we investigated apparently normal breast tissue, and thus the levels of IL-1 β and other inflammatory mediators may not be as upregulated as in a more inflammatory environment. These differences in expression of inflammatory mediators are to some extent illustrated by one of our previous studies that showed that 23 of 32 inflammatory proteins were significantly increased in breast cancer compared to adjacent normal breast tissue (35). Hence, the possibility to detect any significant changes in inflammatory mediators in normal breast tissue may to some extent be limited.

Microdialysis gives us a unique possibility to examine proteins in their bioactive compartment and to study changes in concentration at different time points *in vivo*. By combining microdialysis with the high sensitivity of PEA we can detect low levels of proteins in the microenvironment. This gives us the opportunity to understand how different intervention, such as Tam treatment or dietary interventions, influence paracrine signalling in the tissue of interest *in situ* compared to other inexact methods such as IHC, which only gives a rough estimate of the expression of proteins in tissue sections *ex vivo*, or the measurement of proteins in serum which reflect protein metabolism in the whole body and not only in the tissue of interest. Furthermore, methods to detect proteins in tissue such as IHC staining and mRNA quantification techniques are only proxy methods to measure protein abundance in tissue and do not take into account any post-translational or extracellular modification of the protein. Inflammatory mediators exert their effects extracellularly and several inflammatory mediators such as MMPs and uPA are secreted in inactive forms that require extracellular activation. Thus, to only measure the abundance of proteins in tissue does not fully reflect the levels of active proteins in their bioactive compartment.

The likelihood of a successful protein sampling with microdialysis is related to properties of the catheter membrane, the flow of the perfusate, and to properties of the proteins themselves, such as weight, structure and charge. There is also a risk that the procedure cause bleeding or inflammation and thus may affect the tissue of interest. There may be a concern that the

dialysate does not correctly reflect the protein composition of the extracellular space. However, by using ultraslow flow of the perfusate and a long equilibrium period before collecting the dialysate we minimize the risk of an unreliable and contaminated recovery. Microdialysis has been extensively used by our research group and others and has proven to be an important method in understanding the physiological changes in the microenvironment *in vivo*, giving us new insights into the pathogenesis of cancer.

In paper III we focused on breast tissue at high risk of developing cancer, i.e., postmenopausal women with extremely high MD, and expanded our analysis to include more inflammatory mediators. We compared the data with analyses from our previous cohorts of women receiving flaxseed or Tam and a cohort of women with breast cancer. Extremely high MD has in several meta-analyses shown a four- to fivefold increased risk of breast cancer (29, 30). Although the pathogenesis is not fully understood, it has been suggested that increased inflammation may be one contributing factor, but the evidence is inconsistent; while one study showed no association between MD and the expression of inflammatory markers, another study showed an association between high MD and the expression of IL-6 in all women and, an association with the expression of TNF, CRP and IL-8 in premenopausal women (32, 33). This corroborates an *in vivo* study of inflammatory mediators in the microenvironment in healthy postmenopausal women where we found significant associations between reduced IL-1Ra/IL-1 β ratio, increased levels of IL-6, IL-8, VEGF and CCL2 in breast tissue with extremely high MD compared to breast tissue with low MD (34). We have also shown that out of 32 pro-inflammatory mediators assessed, 26 proteins exhibited similar profiles in breast cancer and dense breast tissue suggesting a pro-inflammatory microenvironment as a possible initiator of malignant transformation in dense breast tissue (35).

In paper III we specifically aimed to study OPN. In concordance with other studies showing increased circulating OPN levels or overexpression in tumor slides of breast cancer, we found that OPN levels *in situ* were significantly increased in breast cancer compared to normal adjacent breast tissue (n=13) (172, 173). Furthermore OPN levels were significantly increased in breast tissue with extremely high MD (n=20) compared to breast tissue with low MD (n=20). However, no effect on OPN levels could be detected after Tam or flaxseed in normal breast tissue and there was no correlation between circulating E2 levels and OPN levels. In normal breast tissue (n=94), OPN levels were significantly correlated with several inflammatory mediators including cytokines IL-6,-8, CXCL1,10,11 and proteases MMP-1,-2,-3,-7,-12 and uPA, and angiogenetic factor VEGF (Table 3).

OPN correlated with fewer inflammatory mediators in breast cancer tissue than in normal breast tissue, and correlated only to CXCL9 and MMP-1,-2,-3,-12 (table 3).

Several CKs are upregulated in breast cancer and specifically CCL2 and CCL5 are associated with higher tumor grade and poor prognosis (182-184). uPA is associated with poor prognosis and is validated as a prognostic biomarker in breast cancer (194, 202). However, CCL2, CCL5 and uPA did not correlate with OPN in our material, but this may be due to a small and relatively heterogeneous group of breast cancer tumors. OPN was significantly upregulated in

breast cancer compared to normal adjacent breast tissue and likewise in breast tissue with extremely high MD compared to low MD. This suggests that OPN may contribute not only to cancer progression but also to the malignant transformation of breast tissue at risk. Furthermore, OPN correlated with several inflammatory mediators in general and several proteases in particular as shown in table 3. This indicates a close association between OPN and proteases which may enhance remodelling of the ECM and promote tumor progression, angiogenesis and metastasis as shown in previous studies (247, 248). Neither Tam nor flaxseed altered the levels of OPN in our study, and there was no correlation between E2 and OPN. These findings suggest an E2-independent regulation of OPN and that flaxseed and Tam exert their effects by other mechanisms than by affecting OPN in breast cancer and in normal breast tissue. However, OPN is upregulated in breast cancer and in breast tissue at risk in our study and has in some studies been associated with reduced survival among breast cancer patients. Hence, OPN may be an interesting novel target in treatment and risk reduction strategies of breast cancer.

Table 3. Significant correlations between OPN and inflammatory mediators in normal breast tissue and in breast cancer.

	Cytokines	Proteases	Angiogenic factors
Normal breast tissue Positive correlation	IL-6,-8 CXCL1, 9, 10, 11	MMP-1,-2,-3,-7,-12 uPA	VEGF
Normal breast tissue No correlation	CCL2, 5	MMP-9	
Breast cancer Positive correlation	CXCL 9	MMP-1,-2,-3,-12	
Breast cancer No correlation	IL-6,-8 CXCL1, 10, 11 CCL2, 5	MMP-7,-9 uPA	VEGF

P-values in normal breast tissue: $p < 0.0001$ for all mediators except for IL-8, $p < 0.01$.

P-values in breast cancer: CXCL9, $p < 0.001$; MMP-1, $p < 0.01$; MMP-2 and MMP-3, $p < 0.05$; MMP-12, $p < 0.0001$.

Conclusions

Flaxseed and tamoxifen may reduce inflammation and affect breast cancer progression by increasing IL-1Ra/IL-1 β ratio in the breast tissue microenvironment.

In paper I, flaxseed, ENL and Tam, but not GEN significantly reduced human breast cancer growth in an experimental mouse model by decreased angiogenesis mediated by decreased levels of stroma cell derived IL-1 β and increased levels of stroma- and cancer cell derived IL-1Ra.

In paper II, flaxseed and Tam exerted in part similar possible anti-inflammatory effects in normal breast tissue by increasing IL-1Ra/IL-1 β ratio *in vivo*.

Increased breast cancer risk in dense breast tissue with extremely high MD may in part be mediated by increased levels of OPN.

In paper III, both breast cancer and dense breast tissue expressed significantly increased levels of OPN compared to adjacent normal breast tissue and non-dense breast tissue with low MD *in vivo*. OPN correlated with MMPs and CXCL9 in both breast cancer and normal breast tissue. In addition, a wider range of inflammatory mediators were correlated with OPN in normal breast tissue than in breast cancer.

Regulation of OPN is E2-independent in normal breast tissue *in vivo*.

In paper III, there was no correlation between E2 levels in serum and OPN levels in breast tissue or any effect of flaxseed or Tam on OPN levels suggesting an E2-independent regulation of OPN. Other strategies to decrease OPN levels than interacting with E2/ER signaling may be investigated.

Future perspectives

In this thesis we have shown that flaxseed and Tam reduce experimental breast cancer growth and may exert anti-inflammatory effects in normal breast tissue by affecting the key pro-inflammatory cytokines IL-1s. We have also shown that the pro-inflammatory cytokine OPN was elevated in breast tissue with extreme high MD compared to normal breast tissue indicating an inflammatory milieu in dense breast tissue that may contribute to an increased risk of breast cancer in these women.

As breast cancer is the female malignancy with the highest mortality worldwide, there is an urgent need to find better biomarkers in screening and new strategies for chemoprevention. While large randomized trials have shown that Tam is effective as chemoprevention for breast cancer, but with potentially severe side effects, epidemiological studies on the effect of lignans, i.e., ENL, on breast cancer risk have been more inconclusive.

Inflammation is a known risk factor in several malignancies. Extremely high MD on mammograms may be associated with a more pro-inflammatory tissue microenvironment and have shown to increase the risk of breast cancer four to fivefold compare to low MD.

In future breast cancer prevention studies, it would be of interest to identify a subpopulation of women with breast tissue showing signs of increased inflammation, e.g., high MD, and within randomized trials offer them anti-inflammatory interventions. Diet modifications, such as the addition of lignans, anti-inflammatory drugs, such as NSAIDs, or selective cytokine inhibitors may be possible alternatives.

As part of translational research in such a study it may be interesting to apply the microdialysis technique in a smaller cohort of women to study the effects of the interventions in the breast tissue microenvironment. By doing so, we could better identify alterations of the inflammatory mediators *in situ*. This would give us a better understanding of the mechanisms behind the effects of our interventions compared to protein measurements in regular blood test or protein detection in tissue samples. The dialysate reflects the actual composition of proteins in the extracellular space that may promote cancer initiation, progression and angiogenesis. To identify key proteins of inflammation increases our knowledge on possible new therapeutic targets and may help us to refine our treatments.

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References

1. F. Ades, K. Tryfonidis, D. Zardavas, The past and future of breast cancer treatment-from the papyrus to individualised treatment approaches. *Ecancermedicalscience* **11**, 746 (2017).
2. R. R. Love, J. Philips, Oophorectomy for breast cancer: history revisited. *J Natl Cancer Inst* **94**, 1433-1434 (2002).
3. S. Zurrida, U. Veronesi, Milestones in breast cancer treatment. *Breast J* **21**, 3-12 (2015).
4. B. L. Niell, P. E. Freer, R. J. Weinfurtnr, E. K. Arleo, J. S. Drukteinis, Screening for Breast Cancer. *Radiol Clin North Am* **55**, 1145-1162 (2017).
5. G. Carioli *et al.*, Trends and predictions to 2020 in breast cancer mortality in Europe. *Breast* **36**, 89-95 (2017).
6. Globocan. (International Agency for Research on Cancer, <http://gco.iarc.fr>), vol. 2019.
7. E. Johansson, Cancer i siffror 2018. Socialstyrelsen 2018.
8. F. Lumachi, D. A. Santeufemia, S. M. Basso, Current medical treatment of estrogen receptor-positive breast cancer. *World journal of biological chemistry* **6**, 231-239 (2015).
9. F. Li, J. Dou, L. Wei, S. Li, J. Liu, The selective estrogen receptor modulators in breast cancer prevention. *Cancer Chemother Pharmacol* **77**, 895-903 (2016).
10. A. Goldhirsch *et al.*, Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. *Annals of oncology : official journal of the European Society for Medical Oncology* **20**, 1319-1329 (2009).
11. J. Lee, K. Demissie, S. E. Lu, G. G. Rhoads, Cancer incidence among Korean-American immigrants in the United States and native Koreans in South Korea. *Cancer Control* **14**, 78-85 (2007).
12. B. Takkouche, C. Regueira-Mendez, M. Etminan, Breast cancer and use of nonsteroidal anti-inflammatory drugs: a meta-analysis. *J Natl Cancer Inst* **100**, 1439-1447 (2008).
13. D. Hanahan, R. A. Weinberg, Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
14. W. Hu *et al.*, Tumor-associated macrophages in cancers. *Clin Transl Oncol* **18**, 251-258 (2016).
15. X. Zhao *et al.*, Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. *Oncotarget* **8**, 30576-30586 (2017).
16. A. V. Sirotkin, A. H. Harrath, Phytoestrogens and their effects. *Eur J Pharmacol* **741**, 230-236 (2014).
17. R. Zamora-Ros *et al.*, Dietary flavonoid and lignan intake and breast cancer risk according to menopause and hormone receptor status in the European Prospective Investigation into Cancer and Nutrition (EPIC) Study. *Breast Cancer Res Treat* **139**, 163-176 (2013).
18. R. Suzuki *et al.*, Dietary lignans and postmenopausal breast cancer risk by oestrogen receptor status: a prospective cohort study of Swedish women. *Br J Cancer* **98**, 636-640 (2008).
19. J. Russo, I. H. Russo, Development of the human breast. *Maturitas* **49**, 2-15 (2004).
20. J. L. Inman, C. Robertson, J. D. Mott, M. J. Bissell, Mammary gland development: cell fate specification, stem cells and the microenvironment. *Development* **142**, 1028-1042 (2015).
21. L. Kass, J. T. Erler, M. Dembo, V. M. Weaver, Mammary epithelial cell: influence of extracellular matrix composition and organization during development and tumorigenesis. *Int J Biochem Cell Biol* **39**, 1987-1994 (2007).
22. A. Skibinski, C. Kuperwasser, The origin of breast tumor heterogeneity. *Oncogene* **34**, 5309-5316 (2015).
23. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70 (2012).
24. T. Sakakura, Y. Suzuki, R. Shiurba, Mammary stroma in development and carcinogenesis. *J Mammary Gland Biol Neoplasia* **18**, 189-197 (2013).
25. M. Nielsen, J. L. Thomsen, S. Primdahl, U. Dyreborg, J. A. Andersen, Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies. *Br J Cancer* **56**, 814-819 (1987).

26. K. Rojas, A. Stuckey, Breast Cancer Epidemiology and Risk Factors. *Clin Obstet Gynecol* **59**, 651-672 (2016).
27. S. E. Singletary, Rating the risk factors for breast cancer. *Ann Surg* **237**, 474-482 (2003).
28. S. S. Nazari, P. Mukherjee, An overview of mammographic density and its association with breast cancer. *Breast Cancer* **25**, 259-267 (2018).
29. N. F. Boyd *et al.*, Mammographic density and the risk and detection of breast cancer. *N Engl J Med* **356**, 227-236 (2007).
30. V. A. McCormack, I. dos Santos Silva, Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* **15**, 1159-1169 (2006).
31. N. F. Boyd *et al.*, Breast tissue composition and susceptibility to breast cancer. *J Natl Cancer Inst* **102**, 1224-1237 (2010).
32. M. Hanna *et al.*, Association between expression of inflammatory markers in normal breast tissue and mammographic density among premenopausal and postmenopausal women. *Menopause* **24**, 524-535 (2017).
33. G. Maskarinec *et al.*, Mammographic density and breast tissue expression of inflammatory markers, growth factors, and vimentin. *BMC Cancer* **18**, 1191 (2018).
34. A. Abrahamsson *et al.*, Dense breast tissue in postmenopausal women is associated with a pro-inflammatory microenvironment in vivo. *Oncoimmunology* **5**, e1229723 (2016).
35. A. Abrahamsson, A. Rzepecka, C. Dabrosin, Equal Pro-inflammatory Profiles of CCLs, CXCLs, and Matrix Metalloproteinases in the Extracellular Microenvironment In Vivo in Human Dense Breast Tissue and Breast Cancer. *Front Immunol* **8**, 1994 (2017).
36. I. H. Kanbayti, W. I. D. Rae, M. F. McEntee, E. U. Ekpo, Are mammographic density phenotypes associated with breast cancer treatment response and clinical outcomes? A systematic review and meta-analysis. *Breast* **47**, 62-76 (2019).
37. J. Cui, Y. Shen, R. Li, Estrogen synthesis and signaling pathways during aging: from periphery to brain. *Trends Mol Med* **19**, 197-209 (2013).
38. M. Jia, K. Dahlman-Wright, J. A. Gustafsson, Estrogen receptor alpha and beta in health and disease. *Best Pract Res Clin Endocrinol Metab* **29**, 557-568 (2015).
39. H. Samavat, M. S. Kurzer, Estrogen metabolism and breast cancer. *Cancer Lett* **356**, 231-243 (2015).
40. C. Thomas, J. A. Gustafsson, The different roles of ER subtypes in cancer biology and therapy. *Nat Rev Cancer* **11**, 597-608 (2011).
41. K. F. Koehler, L. A. Helguero, L. A. Haldosen, M. Warner, J. A. Gustafsson, Reflections on the discovery and significance of estrogen receptor beta. *Endocr Rev* **26**, 465-478 (2005).
42. A. Strom *et al.*, Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A* **101**, 1566-1571 (2004).
43. J. Liu *et al.*, Impact of estrogen receptor-beta expression on breast cancer prognosis: a meta-analysis. *Breast Cancer Res Treat* **156**, 149-162 (2016).
44. M. A. Thaler, V. Seifert-Klauss, P. B. Lippa, The biomarker sex hormone-binding globulin - from established applications to emerging trends in clinical medicine. *Best Pract Res Clin Endocrinol Metab* **29**, 749-760 (2015).
45. N. L. Dimou *et al.*, Sex hormone binding globulin and risk of breast cancer: a Mendelian randomization study. *Int J Epidemiol* **48**, 807-816 (2019).
46. D. P. Cronin-Fenton, P. Damkier, T. L. Lash, Metabolism and transport of tamoxifen in relation to its effectiveness: new perspectives on an ongoing controversy. *Future Oncol* **10**, 107-122 (2014).
47. M. Dutertre, C. L. Smith, Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *J Pharmacol Exp Ther* **295**, 431-437 (2000).

48. S. J. Howell, S. R. Johnston, A. Howell, The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. *Best Pract Res Clin Endocrinol Metab* **18**, 47-66 (2004).
49. C. Davies *et al.*, Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **378**, 771-784 (2011).
50. N. Nass, T. Kalinski, Tamoxifen resistance: from cell culture experiments towards novel biomarkers. *Pathol Res Pract* **211**, 189-197 (2015).
51. F. Poggio *et al.*, Role of fulvestrant in the treatment of postmenopausal metastatic breast cancer patients. *Expert Rev Clin Pharmacol* **9**, 1153-1161 (2016).
52. Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. *Lancet* **386**, 1341-1352 (2015).
53. L. Gibson, D. Lawrence, C. Dawson, J. Bliss, Aromatase inhibitors for treatment of advanced breast cancer in postmenopausal women. *Cochrane Database Syst Rev*, Cd003370 (2009).
54. B. Fisher *et al.*, Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* **90**, 1371-1388 (1998).
55. J. Cuzick *et al.*, Selective oestrogen receptor modulators in prevention of breast cancer: an updated meta-analysis of individual participant data. *Lancet* **381**, 1827-1834 (2013).
56. J. Cuzick *et al.*, Anastrozole for prevention of breast cancer in high-risk postmenopausal women (IBIS-II): an international, double-blind, randomised placebo-controlled trial. *Lancet* **383**, 1041-1048 (2014).
57. I. Rietjens, J. Louisse, K. Beekmann, The potential health effects of dietary phytoestrogens. *Br J Pharmacol* **174**, 1263-1280 (2017).
58. I. Paterni, C. Granchi, F. Minutolo, Risks and benefits related to alimentary exposure to xenoestrogens. *Crit Rev Food Sci Nutr* **57**, 3384-3404 (2017).
59. L. U. Thompson, B. A. Boucher, Z. Liu, M. Cotterchio, N. Kreiger, Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestanol. *Nutr Cancer* **54**, 184-201 (2006).
60. A. Warri, N. M. Saarinen, S. Makela, L. Hilakivi-Clarke, The role of early life genistein exposures in modifying breast cancer risk. *Br J Cancer* **98**, 1485-1493 (2008).
61. W. C. R. F. A. I. f. C. Research, Ed. (World Cancer Research Fund, <https://www.wcrf.org/dietandcancer/breast-cancer>, 2018.), vol. 2019.
62. C. Bennetau-Pelissero, Risks and benefits of phytoestrogens: where are we now? *Curr Opin Clin Nutr Metab Care* **19**, 477-483 (2016).
63. J. M. Landete *et al.*, Bioactivation of Phytoestrogens: Intestinal Bacteria and Health. *Critical reviews in food science and nutrition* **56**, 1826-1843 (2016).
64. M. Axelsson, J. Sjoval, B. E. Gustafsson, K. D. Setchell, Origin of lignans in mammals and identification of a precursor from plants. *Nature* **298**, 659-660 (1982).
65. A. F. Brito, Y. Zang, A Review of Lignan Metabolism, Milk Enterolactone Concentration, and Antioxidant Status of Dairy Cows Fed Flaxseed. *Molecules* **24**, (2018).
66. S. Possemiers, S. Bolca, E. Eeckhaut, H. Depypere, W. Verstraete, Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS microbiology ecology* **61**, 372-383 (2007).
67. A. K. Zaineddin *et al.*, Serum enterolactone and postmenopausal breast cancer risk by estrogen, progesterone and herceptin 2 receptor status. *Int J Cancer* **130**, 1401-1410 (2012).
68. S. M. Mense, T. K. Hei, R. K. Ganju, H. K. Bhat, Phytoestrogens and breast cancer prevention: possible mechanisms of action. *Environ Health Perspect* **116**, 426-433 (2008).
69. L. Q. Wang, Mammalian phytoestrogens: enterodiol and enterolactone. *J Chromatogr B Analyt Technol Biomed Life Sci* **777**, 289-309 (2002).

70. A. Uifalean, S. Schneider, C. Ionescu, M. Lalk, C. A. Iuga, Soy Isoflavones and Breast Cancer Cell Lines: Molecular Mechanisms and Future Perspectives. *Molecules* **21**, E13 (2015).
71. S. Qiu, C. Jiang, Soy and isoflavones consumption and breast cancer survival and recurrence: a systematic review and meta-analysis. *Eur J Nutr*, (2018).
72. N. M. Saarinen, K. Power, J. Chen, L. U. Thompson, Flaxseed attenuates the tumor growth stimulating effect of soy protein in ovariectomized athymic mice with MCF-7 human breast cancer xenografts. *Int J Cancer* **119**, 925-931 (2006).
73. M. Bergman Jungstrom, L. U. Thompson, C. Dabrosin, Flaxseed and its lignans inhibit estradiol-induced growth, angiogenesis, and secretion of vascular endothelial growth factor in human breast cancer xenografts in vivo. *Clin Cancer Res* **13**, 1061-1067 (2007).
74. N. M. Saarinen, A. Abrahamsson, C. Dabrosin, Estrogen-induced angiogenic factors derived from stromal and cancer cells are differently regulated by enterolactone and genistein in human breast cancer in vivo. *Int J Cancer* **127**, 737-745 (2010).
75. C. Dabrosin, J. Chen, L. Wang, L. U. Thompson, Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. *Cancer Lett* **185**, 31-37 (2002).
76. A. Calado, P. M. Neves, T. Santos, P. Ravasco, The Effect of Flaxseed in Breast Cancer: A Literature Review. *Front Nutr* **5**, 4 (2018).
77. J. S. Truan, J. M. Chen, L. U. Thompson, Flaxseed oil reduces the growth of human breast tumors (MCF-7) at high levels of circulating estrogen. *Mol Nutr Food Res* **54**, 1414-1421 (2010).
78. P. Seibold *et al.*, Enterolactone concentrations and prognosis after postmenopausal breast cancer: assessment of effect modification and meta-analysis. *International journal of cancer* **135**, 923-933 (2014).
79. S. Jaskulski *et al.*, Circulating enterolactone concentrations and prognosis of postmenopausal breast cancer: assessment of mediation by inflammatory markers. *Int J Cancer* **143**, 2698-2708 (2018).
80. G. Y. Ren *et al.*, Effect of Flaxseed Intervention on Inflammatory Marker C-Reactive Protein: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Nutrients* **8**, 136 (2016).
81. H. Adlercreutz, Lignans and human health. *Crit Rev Clin Lab Sci* **44**, 483-525 (2007).
82. T. T. Zhao *et al.*, Dietary isoflavones or isoflavone-rich food intake and breast cancer risk: A meta-analysis of prospective cohort studies. *Clin Nutr* **38**, 136-145 (2019).
83. C. Kyro *et al.*, Pre-diagnostic polyphenol intake and breast cancer survival: the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. *Breast Cancer Res Treat* **154**, 389-401 (2015).
84. K. Buck *et al.*, Serum enterolactone and prognosis of postmenopausal breast cancer. *J Clin Oncol* **29**, 3730-3738 (2011).
85. A. Stuedal *et al.*, Plasma levels of enterolactone and percentage mammographic density among postmenopausal women. *Cancer Epidemiol Biomarkers Prev* **14**, 2154-2159 (2005).
86. E. Mareti, C. Abatzi, D. Vavilis, I. Lambrinoudaki, D. G. Goulis, Effect of oral phytoestrogens on endometrial thickness and breast density of perimenopausal and postmenopausal women: A systematic review and meta-analysis. *Maturitas* **124**, 81-88 (2019).
87. F. R. Balkwill, A. Mantovani, Cancer-related inflammation: common themes and therapeutic opportunities. *Seminars in cancer biology* **22**, 33-40 (2012).
88. D. Hanahan, R. A. Weinberg, The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
89. H. F. Dvorak, Tumors: wounds that do not heal-redux. *Cancer Immunol Res* **3**, 1-11 (2015).
90. K. Shiga *et al.*, Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers (Basel)* **7**, 2443-2458 (2015).
91. K. Taniguchi, M. Karin, NF-kappaB, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol* **18**, 309-324 (2018).

92. K. Pietras, A. Ostman, Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* **316**, 1324-1331 (2010).
93. M. W. Pickup, J. K. Mouw, V. M. Weaver, The extracellular matrix modulates the hallmarks of cancer. *EMBO Rep* **15**, 1243-1253 (2014).
94. S. D. Soysal, A. Tzankov, S. E. Muenst, Role of the Tumor Microenvironment in Breast Cancer. *Pathobiology* **82**, 142-152 (2015).
95. S. E. Turvey, D. H. Broide, Innate immunity. *J Allergy Clin Immunol* **125**, S24-32 (2010).
96. A. Mantovani, A. Sica, Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol* **22**, 231-237 (2010).
97. A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation. *Nature* **454**, 436-444 (2008).
98. J. Kim, J. S. Bae, Tumor-Associated Macrophages and Neutrophils in Tumor Microenvironment. *Mediators Inflamm* **2016**, 6058147 (2016).
99. F. S. Varn, D. W. Mullins, H. Arias-Pulido, S. Fiering, C. Cheng, Adaptive immunity programmes in breast cancer. *Immunology* **150**, 25-34 (2017).
100. M. C. A. Wouters, B. H. Nelson, Prognostic Significance of Tumor-Infiltrating B Cells and Plasma Cells in Human Cancer. *Clin Cancer Res* **24**, 6125-6135 (2018).
101. L. M. Coussens, Z. Werb, Inflammation and cancer. *Nature* **420**, 860-867 (2002).
102. C. Denkert *et al.*, Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol* **19**, 40-50 (2018).
103. X. Jiang, D. J. Shapiro, The immune system and inflammation in breast cancer. *Mol Cell Endocrinol* **382**, 673-682 (2014).
104. B. L. Pierce *et al.*, Elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients. *J Clin Oncol* **27**, 3437-3444 (2009).
105. E. Yiannakopoulou, Aspirin and NSAIDs for breast cancer chemoprevention. *Eur J Cancer Prev* **24**, 416-421 (2015).
106. N. R. Cook *et al.*, Low-dose aspirin in the primary prevention of cancer: the Women's Health Study: a randomized controlled trial. *Jama* **294**, 47-55 (2005).
107. S. Kim *et al.*, Lifetime use of nonsteroidal anti-inflammatory drugs and breast cancer risk: results from a prospective study of women with a sister with breast cancer. *BMC Cancer* **15**, 960 (2015).
108. A. M. Lewis, S. Varghese, H. Xu, H. R. Alexander, Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *J Transl Med* **4**, 48 (2006).
109. C. A. Dinarello, Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev* **281**, 8-27 (2018).
110. A. Weber, P. Wasiliew, M. Kracht, Interleukin-1 (IL-1) pathway. *Sci Signal* **3**, cm1 (2010).
111. C. Garlanda, C. A. Dinarello, A. Mantovani, The interleukin-1 family: back to the future. *Immunity* **39**, 1003-1018 (2013).
112. E. Fahey, S. L. Doyle, IL-1 Family Cytokine Regulation of Vascular Permeability and Angiogenesis. *Front Immunol* **10**, 1426 (2019).
113. E. Voronov *et al.*, IL-1 is required for tumor invasiveness and angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 2645-2650 (2003).
114. D. Bar, R. N. Apte, E. Voronov, C. A. Dinarello, S. Cohen, A continuous delivery system of IL-1 receptor antagonist reduces angiogenesis and inhibits tumor development. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18**, 161-163 (2004).
115. M. Fabbi, G. Carbotti, S. Ferrini, Context-dependent role of IL-18 in cancer biology and counter-regulation by IL-18BP. *Journal of leukocyte biology* **97**, 665-675 (2015).

116. F. Y. Liew, J. P. Girard, H. R. Turnquist, Interleukin-33 in health and disease. *Nature reviews. Immunology* **16**, 676-689 (2016).
117. Z. P. Yang *et al.*, The Association of Serum IL-33 and sST2 with Breast Cancer. *Dis Markers* **2015**, 516895 (2015).
118. B. Griesenauer, S. Paczesny, The ST2/IL-33 Axis in Immune Cells during Inflammatory Diseases. *Front Immunol* **8**, 475 (2017).
119. C. A. Dinarello, Why not treat human cancer with interleukin-1 blockade? *Cancer Metastasis Rev* **29**, 317-329 (2010).
120. N. Isambert *et al.*, Fluorouracil and bevacizumab plus anakinra for patients with metastatic colorectal cancer refractory to standard therapies (IRAFU): a single-arm phase 2 study. *Oncoimmunology* **7**, e1474319 (2018).
121. C. Chavey *et al.*, Oestrogen receptor negative breast cancers exhibit high cytokine content. *Breast cancer research : BCR* **9**, R15 (2007).
122. B. Guo, S. Fu, J. Zhang, B. Liu, Z. Li, Targeting inflammasome/IL-1 pathways for cancer immunotherapy. *Scientific reports* **6**, 36107 (2016).
123. I. Holen *et al.*, IL-1 drives breast cancer growth and bone metastasis in vivo. *Oncotarget* **7**, 75571-75584 (2016).
124. T. C. Wu *et al.*, IL1 Receptor Antagonist Controls Transcriptional Signature of Inflammation in Patients with Metastatic Breast Cancer. *Cancer Res* **78**, 5243-5258 (2018).
125. L. Jin *et al.*, Expression of interleukin-1beta in human breast carcinoma. *Cancer* **80**, 421-434 (1997).
126. S. Honma *et al.*, The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells. *Endocr J* **49**, 371-377 (2002).
127. A. Abrahamsson, V. Morad, N. M. Saarinen, C. Dabrosin, Estradiol, tamoxifen, and flaxseed alter IL-1beta and IL-1Ra levels in normal human breast tissue in vivo. *The Journal of clinical endocrinology and metabolism* **97**, E2044-2054 (2012).
128. I. H. Park *et al.*, Tumor-derived IL-18 induces PD-1 expression on immunosuppressive NK cells in triple-negative breast cancer. *Oncotarget* **8**, 32722-32730 (2017).
129. N. Inoue *et al.*, High Serum Levels of Interleukin-18 Are Associated With Worse Outcomes in Patients With Breast Cancer. *Anticancer Res* **39**, 5009-5018 (2019).
130. I. P. Jovanovic *et al.*, Interleukin-33/ST2 axis promotes breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells. *International journal of cancer* **134**, 1669-1682 (2014).
131. X. Yao *et al.*, Targeting interleukin-6 in inflammatory autoimmune diseases and cancers. *Pharmacology & therapeutics* **141**, 125-139 (2014).
132. S. Radtke *et al.*, Cross-regulation of cytokine signalling: pro-inflammatory cytokines restrict IL-6 signalling through receptor internalisation and degradation. *J Cell Sci* **123**, 947-959 (2010).
133. N. Kumari, B. S. Dwarakanath, A. Das, A. N. Bhatt, Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol* **37**, 11553-11572 (2016).
134. D. Conze *et al.*, Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. *Cancer Res* **61**, 8851-8858 (2001).
135. D. E. Johnson, R. A. O'Keefe, J. R. Grandis, Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nature reviews. Clinical oncology*, (2018).
136. K. Heikkilä *et al.*, Associations of circulating C-reactive protein and interleukin-6 with cancer risk: findings from two prospective cohorts and a meta-analysis. *Cancer Causes Control* **20**, 15-26 (2009).
137. K. Raghunathachar Sahana, P. Akila, V. Prashant, B. Sharath Chandra, M. Nataraj Suma, Quantitation of Vascular Endothelial Growth Factor and Interleukin-6 in Different Stages of Breast Cancer. *Reports of biochemistry & molecular biology* **6**, 33-39 (2017).

138. R. Salgado *et al.*, Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer* **103**, 642-646 (2003).
139. C. Dethlefsen, G. Højfeldt, P. Højman, The role of intratumoral and systemic IL-6 in breast cancer. *Breast Cancer Res Treat* **138**, 657-664 (2013).
140. Q. Chang *et al.*, The IL-6/JAK/Stat3 feed-forward loop drives tumorigenesis and metastasis. *Neoplasia* **15**, 848-862 (2013).
141. D. Martin, R. Galisteo, J. S. Gutkind, CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. *The Journal of biological chemistry* **284**, 6038-6042 (2009).
142. C. Alfaro *et al.*, Interleukin-8 in cancer pathogenesis, treatment and follow-up. *Cancer treatment reviews* **60**, 24-31 (2017).
143. Q. Liu *et al.*, The CXCL8-CXCR1/2 pathways in cancer. *Cytokine & growth factor reviews* **31**, 61-71 (2016).
144. C. Bendrik, C. Dabrosin, Estradiol increases IL-8 secretion of normal human breast tissue and breast cancer in vivo. *Journal of immunology (Baltimore, Md. : 1950)* **182**, 371-378 (2009).
145. I. H. Benoy *et al.*, Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**, 7157-7162 (2004).
146. J. Milovanovic, N. Todorovic-Rakovic, T. Vujasinovic, Z. Abu Rabi, Interleukin 8 in progression of hormone-dependent early breast cancer. *J Biosci* **42**, 265-274 (2017).
147. A. Oldberg, A. Franzen, D. Heinegard, Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci U S A* **83**, 8819-8823 (1986).
148. L. F. Brown *et al.*, Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. *Mol Biol Cell* **3**, 1169-1180 (1992).
149. H. J. Cho, H. S. Kim, Osteopontin: a multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Curr Atheroscler Rep* **11**, 206-213 (2009).
150. C. M. Giachelli, S. Steitz, Osteopontin: a versatile regulator of inflammation and biomineralization. *Matrix Biol* **19**, 615-622 (2000).
151. C. M. Giachelli, D. Lombardi, R. J. Johnson, C. E. Murry, M. Almeida, Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. *Am J Pathol* **152**, 353-358 (1998).
152. M. L. Shinohara, H. J. Kim, J. H. Kim, V. A. Garcia, H. Cantor, Alternative translation of osteopontin generates intracellular and secreted isoforms that mediate distinct biological activities in dendritic cells. *Proc Natl Acad Sci U S A* **105**, 7235-7239 (2008).
153. L. A. Shevde, R. S. Samant, Role of osteopontin in the pathophysiology of cancer. *Matrix Biol* **37**, 131-141 (2014).
154. S. A. Lund, C. M. Giachelli, M. Scatena, The role of osteopontin in inflammatory processes. *J Cell Commun Signal* **3**, 311-322 (2009).
155. A. Del Prete, S. Scutera, S. Sozzani, T. Musso, Role of osteopontin in dendritic cell shaping of immune responses. *Cytokine Growth Factor Rev*, (2019).
156. A. Koh *et al.*, Role of osteopontin in neutrophil function. *Immunology* **122**, 466-475 (2007).
157. A. C. Renkl *et al.*, Osteopontin functionally activates dendritic cells and induces their differentiation toward a Th1-polarizing phenotype. *Blood* **106**, 946-955 (2005).
158. S. R. Rittling, R. Singh, Osteopontin in Immune-mediated Diseases. *Journal of dental research* **94**, 1638-1645 (2015).

159. S. Kale *et al.*, Osteopontin signaling upregulates cyclooxygenase-2 expression in tumor-associated macrophages leading to enhanced angiogenesis and melanoma growth via alpha9beta1 integrin. *Oncogene* **33**, 2295-2306 (2014).
160. J. Dai *et al.*, A humanized anti-osteopontin antibody inhibits breast cancer growth and metastasis in vivo. *Cancer immunology, immunotherapy : CII* **59**, 355-366 (2010).
161. C. E. Weber *et al.*, Osteopontin mediates an MZF1-TGF-beta1-dependent transformation of mesenchymal stem cells into cancer-associated fibroblasts in breast cancer. *Oncogene* **34**, 4821-4833 (2015).
162. A. N. Kothari *et al.*, Osteopontin-A Master Regulator of Epithelial-Mesenchymal Transition. *J Clin Med* **5**, (2016).
163. G. F. Weber, G. S. Lett, N. C. Haubein, Osteopontin is a marker for cancer aggressiveness and patient survival. *Br J Cancer* **103**, 861-869 (2010).
164. M. Zhao, F. Liang, B. Zhang, W. Yan, J. Zhang, The impact of osteopontin on prognosis and clinicopathology of colorectal cancer patients: a systematic meta-analysis. *Sci Rep* **5**, 12713 (2015).
165. T. Kiss *et al.*, The role of osteopontin expression in melanoma progression. *Tumour Biol* **36**, 7841-7847 (2015).
166. Y. Y. Xu, Y. Y. Zhang, W. F. Lu, Y. J. Mi, Y. Q. Chen, Prognostic value of osteopontin expression in breast cancer: A meta-analysis. *Molecular and clinical oncology* **3**, 357-362 (2015).
167. J. Wei *et al.*, Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. *J Clin Invest* **129**, 137-149 (2019).
168. R. Mentor-Marcel, C. A. Lamartiniere, I. A. Eltoum, N. M. Greenberg, A. Elgavish, Dietary genistein improves survival and reduces expression of osteopontin in the prostate of transgenic mice with prostatic adenocarcinoma (TRAMP). *J Nutr* **135**, 989-995 (2005).
169. L. H. El Touny, P. P. Banerjee, Identification of a biphasic role for genistein in the regulation of prostate cancer growth and metastasis. *Cancer Res* **69**, 3695-3703 (2009).
170. K. Walaszek, E. E. Lower, P. Ziolkowski, G. F. Weber, Breast cancer risk in premalignant lesions: osteopontin splice variants indicate prognosis. *Br J Cancer* **119**, 1259-1266 (2018).
171. V. H. Bramwell *et al.*, Assessment of osteopontin in early breast cancer: correlative study in a randomised clinical trial. *Breast Cancer Res* **16**, R8 (2014).
172. A. Psyrri *et al.*, Association of osteopontin with specific prognostic factors and survival in adjuvant breast cancer trials of the Hellenic Cooperative Oncology Group. *J Transl Med* **15**, 30 (2017).
173. V. H. Bramwell *et al.*, Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. *Clin Cancer Res* **12**, 3337-3343 (2006).
174. A. L. Allan *et al.*, Role of the integrin-binding protein osteopontin in lymphatic metastasis of breast cancer. *Am J Pathol* **169**, 233-246 (2006).
175. J. Vandercappellen, J. Van Damme, S. Struyf, The role of CXC chemokines and their receptors in cancer. *Cancer Lett* **267**, 226-244 (2008).
176. G. D'Agostino, V. Cecchinato, M. Uguccioni, Chemokine Heterocomplexes and Cancer: A Novel Chapter to Be Written in Tumor Immunity. *Front Immunol* **9**, 2185 (2018).
177. J. Wang, H. Knaut, Chemokine signaling in development and disease. *Development* **141**, 4199-4205 (2014).
178. M. I. Palacios-Arreola *et al.*, The role of chemokines in breast cancer pathology and its possible use as therapeutic targets. *J Immunol Res* **2014**, 849720 (2014).
179. C. Xu, H. Zhao, H. Chen, Q. Yao, CXCR4 in breast cancer: oncogenic role and therapeutic targeting. *Drug Des Devel Ther* **9**, 4953-4964 (2015).
180. E. Marcuzzi, R. Angioni, B. Molon, B. Cali, Chemokines and Chemokine Receptors: Orchestrating Tumor Metastasis. *International journal of molecular sciences* **20**, (2018).

181. M. Vela, M. Aris, M. Llorente, J. A. Garcia-Sanz, L. Kremer, Chemokine receptor-specific antibodies in cancer immunotherapy: achievements and challenges. *Front Immunol* **6**, 12 (2015).
182. D. Narita *et al.*, Altered levels of plasma chemokines in breast cancer and their association with clinical and pathological characteristics. *Neoplasma* **63**, 141-149 (2016).
183. G. Soria, A. Ben-Baruch, The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Cancer Lett* **267**, 271-285 (2008).
184. S. Svensson *et al.*, CCL2 and CCL5 Are Novel Therapeutic Targets for Estrogen-Dependent Breast Cancer. *Clin Cancer Res* **21**, 3794-3805 (2015).
185. L. A. Liotta *et al.*, Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* **284**, 67-68 (1980).
186. C. C. Lynch, L. M. Matrisian, Matrix metalloproteinases in tumor-host cell communication. *Differentiation; research in biological diversity* **70**, 561-573 (2002).
187. L. Nissinen, V. M. Kahari, Matrix metalloproteinases in inflammation. *Biochim Biophys Acta* **1840**, 2571-2580 (2014).
188. B. Fingleton, Matrix metalloproteinases as regulators of inflammatory processes. *Biochimica et biophysica acta* **1864**, 2036-2042 (2017).
189. F. L. Fonseca, B. da Costa Aguiar Alves, L. A. Azzalis, T. M. Belardo, Matrix Metalloproteases as Biomarkers of Disease. *Methods Mol Biol* **1579**, 299-311 (2017).
190. E. S. Radisky, D. C. Radisky, Matrix metalloproteinases as breast cancer drivers and therapeutic targets. *Front Biosci (Landmark Ed)* **20**, 1144-1163 (2015).
191. M. D. Martin, L. M. Matrisian, The other side of MMPs: protective roles in tumor progression. *Cancer metastasis reviews* **26**, 717-724 (2007).
192. H. W. Jackson, V. Defamie, P. Waterhouse, R. Khokha, TIMPs: versatile extracellular regulators in cancer. *Nat Rev Cancer* **17**, 38-53 (2017).
193. R. E. Vandenbroucke, C. Libert, Is there new hope for therapeutic matrix metalloproteinase inhibition? *Nat Rev Drug Discov* **13**, 904-927 (2014).
194. D. M. Roy, L. A. Walsh, Candidate prognostic markers in breast cancer: focus on extracellular proteases and their inhibitors. *Breast Cancer (Dove Med Press)* **6**, 81-91 (2014).
195. H. Li, Z. Qiu, F. Li, C. Wang, The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. *Oncology letters* **14**, 5865-5870 (2017).
196. F. Ren *et al.*, Overexpression of MMP Family Members Functions as Prognostic Biomarker for Breast Cancer Patients: A Systematic Review and Meta-Analysis. *PLoS One* **10**, e0135544 (2015).
197. E. S. Radisky, M. Raeeszadeh-Sarmazdeh, D. C. Radisky, Therapeutic Potential of Matrix Metalloproteinase Inhibition in Breast Cancer. *J Cell Biochem* **118**, 3531-3548 (2017).
198. C. Bendrik, J. Robertson, J. Gaudie, C. Dabrosin, Gene transfer of matrix metalloproteinase-9 induces tumor regression of breast cancer in vivo. *Cancer research* **68**, 3405-3412 (2008).
199. K. S. Leifler *et al.*, Inflammation induced by MMP-9 enhances tumor regression of experimental breast cancer. *Journal of immunology (Baltimore, Md. : 1950)* **190**, 4420-4430 (2013).
200. A. H. Mekawy, M. H. Pourgholami, D. L. Morris, Involvement of urokinase-type plasminogen activator system in cancer: an overview. *Med Res Rev* **34**, 918-956 (2014).
201. L. Tang, X. Han, The urokinase plasminogen activator system in breast cancer invasion and metastasis. *Biomed Pharmacother* **67**, 179-182 (2013).
202. J. A. Foekens *et al.*, The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. *Cancer Res* **60**, 636-643 (2000).
203. N. Harbeck *et al.*, Ten-year analysis of the prospective multicentre Chemo-N0 trial validates American Society of Clinical Oncology (ASCO)-recommended biomarkers uPA and PAI-1 for therapy decision making in node-negative breast cancer patients. *Eur J Cancer* **49**, 1825-1835 (2013).

204. S. Koch, L. Claesson-Welsh, Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med* **2**, a006502 (2012).
205. P. Carmeliet, VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4-10 (2005).
206. N. Ferrara, VEGF as a therapeutic target in cancer. *Oncology* **69 Suppl 3**, 11-16 (2005).
207. L. Holmgren, M. S. O'Reilly, J. Folkman, Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature medicine* **1**, 149-153 (1995).
208. J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature medicine* **1**, 27-31 (1995).
209. K. Zirlik, J. Duyster, Anti-Angiogenics: Current Situation and Future Perspectives. *Oncology research and treatment* **41**, 166-171 (2018).
210. C. Dabrosin, Positive correlation between estradiol and vascular endothelial growth factor but not fibroblast growth factor-2 in normal human breast tissue in vivo. *Clin Cancer Res* **11**, 8036-8041 (2005).
211. C. Dabrosin, P. J. Margetts, J. Gaudie, Estradiol increases extracellular levels of vascular endothelial growth factor in vivo in murine mammary cancer. *Int J Cancer* **107**, 535-540 (2003).
212. C. Dabrosin, K. Palmer, W. J. Muller, J. Gaudie, Estradiol promotes growth and angiogenesis in polyoma middle T transgenic mouse mammary tumor explants. *Breast Cancer Res Treat* **78**, 1-6 (2003).
213. C. Sun *et al.*, Tumor angiogenesis and bone metastasis - Correlation in invasive breast carcinoma. *J Immunol Methods* **452**, 46-52 (2018).
214. B. Uzzan, P. Nicolas, M. Cucherat, G. Y. Perret, Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Res* **64**, 2941-2955 (2004).
215. G. Gasparini *et al.*, Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst* **89**, 139-147 (1997).
216. B. K. Linderholm *et al.*, Significantly higher levels of vascular endothelial growth factor (VEGF) and shorter survival times for patients with primary operable triple-negative breast cancer. *Ann Oncol* **20**, 1639-1646 (2009).
217. K. C. Aalders, K. Tryfonidis, E. Senkus, F. Cardoso, Anti-angiogenic treatment in breast cancer: Facts, successes, failures and future perspectives. *Cancer Treat Rev* **53**, 98-110 (2017).
218. P. Mirabelli, L. Coppola, M. Salvatore, Cancer Cell Lines Are Useful Model Systems for Medical Research. *Cancers (Basel)* **11**, (2019).
219. Y. Reid, D. Storts, T. Riss, L. Minor, in *Assay Guidance Manual*, G. S. Sittampalam *et al.*, Eds. (Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD), 2004).
220. S. Comsa, A. M. Cimpean, M. Raica, The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. *Anticancer Res* **35**, 3147-3154 (2015).
221. M. Arora, in *MATER METHODS 2013*;3:175. (2013), pp. <https://www.labome.com/method/Cell-Culture-Media-A-Review.html>.
222. S. P. Crampton, J. Davis, C. C. Hughes, Isolation of human umbilical vein endothelial cells (HUVEC). *J Vis Exp*, 183 (2007).
223. R. Addis *et al.*, Human umbilical endothelial cells (HUVECs) have a sex: characterisation of the phenotype of male and female cells. *Biol Sex Differ* **5**, 18 (2014).
224. I. Szadvari, O. Krizanov, P. Babula, Athymic nude mice as an experimental model for cancer treatment. *Physiol Res* **65**, S441-S453 (2016).
225. E. A. Sausville, A. M. Burger, Contributions of human tumor xenografts to anticancer drug development. *Cancer Res* **66**, 3351-3354, discussion 3354 (2006).
226. D. A. Spak, J. S. Plaxco, L. Santiago, M. J. Dryden, B. E. Dogan, BI-RADS((R)) fifth edition: A summary of changes. *Diagn Interv Imaging* **98**, 179-190 (2017).

227. U. Ungerstedt, Microdialysis--principles and applications for studies in animals and man. *J Intern Med* **230**, 365-373 (1991).
228. X. Ao, J. A. Stenken, Microdialysis sampling of cytokines. *Methods* **38**, 331-341 (2006).
229. A. de la Pena, P. Liu, H. Derendorf, Microdialysis in peripheral tissues. *Advanced drug delivery reviews* **45**, 189-216 (2000).
230. M. Hammarlund-Udenaes, Microdialysis as an Important Technique in Systems Pharmacology-a Historical and Methodological Review. *The AAPS journal* **19**, 1294-1303 (2017).
231. C. Dabrosin, Microdialysis - an in vivo technique for studies of growth factors in breast cancer. *Front Biosci* **10**, 1329-1335 (2005).
232. S. Garvin, C. Dabrosin, In vivo measurement of tumor estradiol and vascular endothelial growth factor in breast cancer patients. *BMC Cancer* **8**, 73 (2008).
233. C. Dabrosin, Technical aspects of microdialysis of human breast. *Scand J Clin Lab Invest* **61**, 269-272 (2001).
234. S. D. Gan, K. R. Patel, Enzyme immunoassay and enzyme-linked immunosorbent assay. *J Invest Dermatol* **133**, e12 (2013).
235. G. Harris, W. Chen, Profiling of Cytokine and Chemokine Responses Using Multiplex Bead Array Technology. *Methods Mol Biol* **2024**, 79-94 (2019).
236. M. F. Elshal, J. P. McCoy, Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods* **38**, 317-323 (2006).
237. L. L. Matos, D. C. Trufelli, M. G. de Matos, M. A. da Silva Pinhal, Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights* **5**, 9-20 (2010).
238. J. A. Ramos-Vara, Technical aspects of immunohistochemistry. *Vet Pathol* **42**, 405-426 (2005).
239. R. W. Burry, Controls for immunocytochemistry: an update. *J Histochem Cytochem* **59**, 6-12 (2011).
240. A. M. Gown, Diagnostic Immunohistochemistry: What Can Go Wrong and How to Prevent It. *Arch Pathol Lab Med* **140**, 893-898 (2016).
241. P. Nowak-Sliwinska *et al.*, Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis* **21**, 425-532 (2018).
242. C. Greenwood *et al.*, Proximity assays for sensitive quantification of proteins. *Biomol Detect Quantif* **4**, 10-16 (2015).
243. E. Assarsson *et al.*, Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One* **9**, e95192 (2014).
244. S. Umezawa *et al.*, Chemoprevention of colorectal cancer: Past, present, and future. *Cancer Sci* **110**, 3018-3026 (2019).
245. T. Schluter, C. Schelmbauer, K. Karram, I. A. Mufazalov, Regulation of IL-1 signaling by the decoy receptor IL-1R2. *J Mol Med (Berl)* **96**, 983-992 (2018).
246. N. Aziz, Measurement of Circulating Cytokines and Immune-Activation Markers by Multiplex Technology in the Clinical Setting: What Are We Really Measuring? *For Immunopathol Dis Therap* **6**, 19-22 (2015).
247. R. Agnihotri *et al.*, Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). *J Biol Chem* **276**, 28261-28267 (2001).
248. R. Das, S. Philip, G. H. Mahabeleshwar, A. Bulbule, G. C. Kundu, Osteopontin: it's role in regulation of cell motility and nuclear factor kappa B-mediated urokinase type plasminogen activator expression. *IUBMB Life* **57**, 441-447 (2005).

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"Barn's burnt down
Now
I can see the moon"

MIZUTA MASAHIDE [1657-1723]