Estradiol, tamoxifen, and flaxseed regulate the inflammatory microenvironment in normal human breast tissue *in vivo* via IL-1β and IL-1Ra

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Abstract

Sex steroid exposure increases the risk of breast cancer by unclear mechanisms. Diet modifications may be one breast cancer prevention strategy. The pro-inflammatory cytokine family of IL-1s is implicated in cancer progression via effects on angiogenesis- and growth factors. If estrogen regulates IL-1s in normal human breast tissue or if these cytokines may be attenuated by diet is unknown. Here, microdialysis was performed in healthy women under various hormone exposures, tamoxifen therapy, and diet modifications. Microdialysis was also executed in breast cancers of women before surgery. Breast tissue biopsies from reduction mammoplasties were cultured. We show a significant positive correlation between estradiol and *in vivo* levels of IL-1β both in breast tissue and abdominal subcutaneous fat whereas IL-1Ra exhibited a significant negative correlation with estradiol in breast tissue. Tamoxifen or a dietary addition of 25 grams of flaxseed/day resulted in significantly increased levels of IL-1Ra in the breast. These results were confirmed in *ex vivo* culture of breast biopsies. Immunohistochemistry of the biopsies did not reveal any changes in cellular content of the IL-1s suggesting that mainly the secreted levels were affected. In breast cancer patients, intra-tumoral levels of IL-1β were significantly higher compared with normal adjacent breast tissue. We conclude that IL-1s may be under the control of estrogen *in vivo* and that these cytokines may be attenuated by anti-estrogen therapy and diet modifications. The increased IL-1β levels in breast cancers of women strongly suggest IL-1 as a potential therapeutic target in breast cancer.

Introduction

Inflammation is one of the hallmarks of cancer initiation and progression (1). The inflammatory response may be both pro- and antitumorigenic depending on type of activation of different immune cells in the tissue and depending on the stage of cancer progression. Several mediators are involved in the regulation of the immune response including the interleukin-1 (IL-1) family. IL-1α and IL-1β are the two major proinflammatory cytokines of this family (2, 3). The naturally occurring soluble receptor antagonist, IL-1Ra, which binds to the IL-1 receptor without any agonist effect, may antagonize the effect of these proteins (2). In cancer, IL-1s may contribute to angiogenesis, tumor proliferation, and local invasion (4, 5) and IL-1 α or IL-1 β knockout mice have been shown to exhibit dramatically impaired ability of tumor development and angiogenesis (6). We, and others have shown that treatment with IL-1Ra leads to tumor regression, decreased the inflammatory response and inhibited tumor development in mice (7-9)

The majority of breast cancers express the estrogen receptor (ER), and reducing the effect of estrogens is a corner stone in the medical treatment of breast cancer today (10, 11). Long-term exposure to sex steroids such as estrogens also plays an important role in the development breast cancer (12, 13) but the mechanisms behind this correlation are still not fully explored. Lifestyle factors such as diet has been identified as a possible contributor to the variation of breast cancer incidence in Eastern and Western societies (14, 15). In Eastern societies, where the incidence of breast cancer is low, the diet contains large amounts of phytoestrogens, and migrant studies support that genetics is not responsible for this difference in incidence. Phytoestrogens are plant compounds structurally similar to estrogen, which may have both agonistic and antagonistic actions in breast cancer dependent on the type and dose ingested (16, 17). Lignans, one class of phytoestrogens, are found in high amounts in flaxseed

and several recent studies have shown that, dietary plant lignans or their enterolignan metabolites have been associated with reduced breast cancer risk and increased overall survival of breast cancer patients (18-23). Hence, there is a need to explore the mechanisms involved in the correlations found in these epidemiological studies and diet modification may be one way to explore as a preventive strategy.

We have previously shown that several angiogenesis regulators are estrogen dependent in normal human breast tissue *in vivo*. To our knowledge there are no studies of possible estrogen regulation of IL-1s in normal human breast tissue *in vivo*. The aims of our study were to explore whether sex steroids affected the levels of IL-1s in normal human breast tissue *in vivo*, and to explore whether these cytokines could be affect by tamoxifen treatment or diet modifications. Moreover, we wanted to investigate if these cytokines were present in breast cancer tumors of women *in vivo*.

Materials and Methods

Subjects

The regional ethical review board of Linköping approved the study and all women gave their informed consent. A total of 41 women were included for microdialysis investigations. Twenty women were pre-menopausal (age 20-32 years) healthy volunteers. Of these 20 women, six were investigated in the follicular and luteal phases of one menstrual cycle, five were investigated in two consecutive luteal phases, and nine were investigated in one unexposed luteal phase and one flaxseed exposed luteal phase. All of the premenopausal women had a history of regular menstrual cycles (cycle length 27-34 days) and had not used any antibiotics or sex steroid containing contraceptive within past three months. Eleven women were postmenopausal (age 58-78 years) and were investigated in their normal unaffected breast after surgery for early breast cancer. All women had been off sex steroid containing medication such hormone replacement therapy for more than three months. Another ten women, age 51-86 years, diagnosed with breast cancer were investigated before surgery with microdialysis in tumor tissue and normal adjacent breast tissue. Tumor histology, size, immunohistochemistry of estrogen (ER)- and progesterone (PR) receptors, and Nottingham histological grade (NHG) according to Elston Ellis scoring system were determined at the Department of Pathology and Cytology, University Hospital of Linköping and summarized in Table 1.

Biopsies from human breast tissue were obtained from pre-menopausal women, without ongoing hormonal treatment, undergoing routine reduction mammoplasty.

Experimental design

All women were omnivore without any flaxseed in their daily diet. Microdialysis was performed in breast tissue and abdominal subcutaneous fat and blood samples were collected at the start of each microdialysis investigation.

Of the controls, six premenopausal women were investigated in the follicular and the luteal phase in one menstrual cycle and five were investigated during two consecutive luteal phases of two menstrual cycles. The luteinizing hormone (LH) peak was monitored in urine samples and after the peak the first microdialysis was performed within 5-10 days. Nine women were included in the flaxseed group and were investigated during two consecutive luteal phases. After the first microdialysis session the women added 25 g of freshly ground flaxseed/day to their diet. The next microdialysis investigation was performed at the same day after the LH-peak as in the first un-exposed cycle i.e. if the first microdialysis was performed at day 5 after the LH-peak the next microdialysis investigation was also performed at day 5.

In the tamoxifen exposed group all women had been treated for early breast cancer and tamoxifen 20 mg/day had been prescribed as an adjuvant therapy. Tamoxifen treatment to early breast cancer patients is a standard regimen in low risk patients in Sweden. All had had a normal breast examination and mammography on the contra-lateral side. Before the start of tamoxifen, microdialysis was performed. After six weeks of tamoxifen treatment the women returned for the second microdialysis.

Another ten postmenopausal breast cancer patients were investigated prior surgery. One microdialysis catheter was inserted intratumorally in the breast cancer and one in normal adjacent breast tissue.

Prior to insertion of the microdialysis catheters 0.5 ml lidocain (10 mg/ml) was administrated intracutaneously. Of the women investigated in normal breast tissue one microdialysis catheter was placed in abdominal subcutaneous fat and one in the upper lateral quadrant of the breast and directed towards the nipple as previously described (24-27). At the next microdialysis investigation the catheters were inserted at the same locations as in the previous session. The premenopausal women were investigated in the left breast whereas the women treated for breast cancer were investigated in their healthy unaffected breast. In the patients

investigated prior surgery one microdialysis catheter was inserted intratumorally and one in adjacent, macroscopically normal breast tissue. For women investigated in their unaffected normal breast a microdialysis catheter (CMA 71/Microdialysis AB, Solna, Sweden), which consists of a tubular dialysis membrane (100,000 atomic mass cut-off, 20 mm membrane lenght) glued to the end of a double-lumen tube was used. The catheters were inserted guided by a splitable introducer (CMA Microdialysis AB). In breast cancer patients investigated in their affected breast prior surgery, microdialysis catheters as described above but with a membrane length of 10 mm guided by a catheter for intravenous use (Venflon, 1.4 mm; BOC Ohmeda AB, Helsingborg, Sweden) were used. The catheters were connected to a microinfusion pump (CMA 107, CMA/Microdialysis AB) and perfused with NaCl 154 mmol/L and hydroxyethyl starch 60g/l (Voluven®, Fresenius Kabi, Uppsala, Sweden) or in the patients investigated prior surgery NaCl (154 mM) and dextran-70 (40 g/l), at a perfusion rate of 0.5 µl/min. The solution entered the catheter through the outer tube and left it through the inner tube, from which it was collected. After a 30-min equilibration period, the outgoing perfusate containing extracellular proteins was collected and stored at –70°C for subsequent analysis.

Microdialysis is a sampling technique, which allows continuous sampling of the extracellular fluid by passive diffusion of molecules over a semi-permeable membrane. The recovery i.e. the amount of substances from the tissue that diffuse into the perfusion fluid depends on the membrane properties, the flow rate, tissue temperature, and the size of the compound of interest. Diffusion of low molecular substances over the dialysis membrane has been shown to be almost complete at low flow rates using a 30 mm long dialysis membrane (28). However, for larger molecules the recovery over the membrane decreases and the measured levels in the microdialysis sample cannot be considered as the absolute concentrations in the tissue. Therefore, all microdialysis values are given as original raw data.

Breast tissue culture

Tissue biopsies containing epithelium, stroma, and adipose tissue, were produced by using an 8 mm biopsy punch (Kai Europe GmbH, Solingen, Germany) and placed in a 12-well plate (Costar, Cambridge, MA, USA). Serum-free medium was used consisting of a 1:1 mixture of nutrient mixture F-12 (GIBCO, Paisley, UK) and Dulbecco's modified Eagle's medium without phenol red (GIBCO, Paisley, UK) supplemented with transferrin (10 μg/ml; Sigma), insulin (1 μg/ml; Sigma), and bovine serum albumin (0.2 mg/ml; Sigma) with or without physiological levels of 10^{-9} M estradiol (17β-estradiol; E2; Sigma), a combination of 10^{-9} M estradiol and 10^{-8} M progesterone (P4; Sigma) (E2 + P4), or tamoxifen 10^{-6} M. The control group was incubated in media supplemented with the vehicle, ethanol, equivalent to the hormone treated groups (0.001%). The biopsies were treated for 7 days at 37°C in a humidified atmosphere containing 5% CO_2 and the medium was changed every day. After the seventh day of incubation, the medium from each biopsy was collected and stored at –70°C for subsequent analysis. The biopsies were formalin fixed and embedded in paraffin for immunohistochemistry.

Immunohistochemistry

Formalin-fixed, paraffin-embedded normal breast tissue biopsies were cut into 4 μm sections. Sections were de-paraffinized and high temperature antigen retrieval was performed prior staining with mouse anti human IL-1α (5μg/ml), mouse anti human IL-1β (25μg/ml), or goat anti human IL-1Ra (15μg/ml), purchased from R&D Systems Minneapolis MN. The secondary antibodies bovine anti-goat (diluted 1:200, Santa Cruz Biotechnology) and goat anti-mouse (Envision HRP, DAKO, Carpinteria, CA) were used for detection. All the tissue sections were counterstained using Mayer's hematoxylin (Histolab, Göteborg, Sweden) and mounted with PERTEX (Histolab). Negative controls showed no staining. Slides were analysed using an Olympus BX43 microscope and digital images were collected using

Olympus DP72 camera and CellSens Dimension software (Olympus Life Science Europe GmbH, Hamburg, Germany).

Determinations of estradiol, progesterone, and IL-1s

Plasma was collected using a plastic tube containing spray dried K2 EDTA as an anticoagulant, and were spun down and frozen at –70°C within 20 minutes of collection.

Microdialysates, plasma samples, and media from the breast tissue biopsies, were analyzed using commercial quantitative immunoassay kits; human IL-1α, IL-1β, and IL-1Ra (Quantikine®, R&D Systems, Minneapolis, MN), Estradiol and progesterone were analyzed using ELISA kits from Calbiotech, Spring Valley, CA. The sensitivity of the estradiol assay was 1.47 pmol/l and the intra-assay variation 4.6-10%, and the sensitivity of the progesterone assay was 0.7 nmol/l and the intra-assay variation 2-5.3%.

Lignan analyses

Serum samples were prepared for the analyses with a previously described method (29) with slight modifications. Briefly, samples were hydrolyzed at 37°C in 0.2 M sodium acetate buffer pH 5 containing 0.2 U/mL of β-glucuronidase and 2 U/mL of sulphatase and extracted twice with diethyl ether. The extracts evaporated to dryness were re-dissolved in methanol and purified with QAE-Sephadex A-25 in acetate form as earlier described (30). Chromatographic conditions for serum lignan analyses were as described before (31). The lignans in the batch of flaxseed used were determined as previously described (32).

Statistics

Data are expressed as mean±SEM. Student's t-test, ANOVA, and Pearson's correlation coefficient were used as appropriate. A $p<0.05$ was considered as statistically significant.

Results

Characteristics of the subjects

There were no subsequent complications after the microdialysis experiments.

Of the six premenopausal investigated in early follicular phase and luteal phase the hormone levels suggested that all women had had an ovulatory menstrual cycle; estradiol levels increased from 94±20 pmol/L to 322±53 pmol/L and the progesterone levels increased from 1.9±0.3 nmol/L to 26±8 nmol/L, n=6.

There were no significant difference between estradiol and progesterone levels in the five premenopausal women investigated in two luteal phases; estradiol levels were 334±45 pmol/L in the first luteal phase and 314±61 pmol/l in the second luteal phase, p=0.8, n=5, and the progesterone levels were 13±3.9 nmol/l and 11±3 nmol/l, p=0.5, n=5.

In the flaxseed exposed nine women investigated in unexposed luteal phase and then in the luteal phase after flaxseed ingestion the levels of sex steroids exhibited no significant differences; estradiol levels in the unexposed luteal phase were 392±36 pmol/l and in the exposed cycle 425±47 pmol/l, p=0.6, and the progesterone levels 20±3.4 nmol/l and 24±3.8 nmol/L, p=0.4, n=9. In the eleven postmenopausal women treated for breast cancer that were investigated in their healthy unaffected breast the estradiol levels before and after tamoxifen treatment were 79±5 pmol/l and 84±4 pmol/l respectively, p=0.5, and the progesterone levels 1.0±0.2 nmol/l and 0.7±0.2 nmol/l respectively, p=0.4, n=11.

Plasma levels of estradiol in the ten women with breast cancer investigated in cancer tissue and adjacent normal breast tissue were 80±20 pmol/l and progesterone levels 1.0±0.01 nmol/l.

The 25 g of flaxseed contained 44.32 mg of secoisolariciresinol, 0.92 mg of pinoresinol, 0.46 mg of isolariciresinol, 0.40 mg of matairesinol, and 0.24 mg of lariciresinol.

IL-1β increased and IL-1Ra decreased in breast tissue during estradiol exposure

To correlate extracellular levels of the IL-1s, data from unexposed healthy breast tissues were compared i.e. before start of tamoxifen treatment in the postmenopausal women and in unexposed menstrual cycle in the premenopausal flaxseed group, in the two unexposed luteal phases as well as in the follicular and luteal phases of the premenopausal controls. All premenopausal women were investigated in both breast tissue and subcutaneous fat whereas of the eleven postmenopausal patients treated for breast cancer and investigated in their normal healthy breast, nine were investigated in both in the breast and the fat and two women were investigated in breast tissue only. Hence, in women investigated in their normal unaffected breast, 42 microdialysis investigations in unexposed breast tissue and 40 in unexposed fat tissue were performed.

There were no detectable levels of IL-1α and IL-1β in plasma whereas IL-1Ra was measurable in all women. In microdialysates all IL-1s were detectable. IL-1α did not correlate with estradiol either in breast tissue, $r=0.05$, $p=0.7$ or fat, $r=0.02$, $p=0.9$. Fig 1x. There were significant positive correlations between estradiol levels and extracellular IL-1β both in breast tissue, $r=0.36$, $p<0.05$, $n=42$, and subcutaneous fat, $r=0.35$, $p<0.05$, $n=40$, Fig Xx. IL-1β in breast tissue did also correlate with progesterone levels, $r=0.43$, $p<0.01$. In contrast to IL-1β, there was a strong significant negative correlation between IL-1Ra and estradiol in breast tissue, $r=-0.67$, $p<0.0001$, $n=42$, Fig Xx. In fat tissue IL-1Ra and estradiol did not correlate, $r=0.14$, $p=0.4$, $n=40$, Fig 1x. IL-1Ra did not correlate with progesterone in either tissue.

In the six women investigated during follicular and luteal phases of the menstrual cycle, it was possible to compare intra-individual levels of the IL-1s during physiological changes of hormone levels. IL-1α did not change either in breast tissue or fat tissue during the menstrual cycle, Fig 2A. In the case of IL-1β, the levels increase significantly in breast tissue in the luteal phase when the levels of estradiol and progesterone were increased, $p<0.05$, $n=6$, Fig XB, whereas there were no changes in fat tissue during the menstrual cycle. IL-1Ra decreased

significantly in the luteal phase in breast tissue, $p<0.05$, $n=6$, with no changes in fat tissue, Fig XC.

To further explore if the correlations observed in microdialysates were a direct cause of estradiol and or progesterone exposure whole breast tissue biopsies from reduction mammoplasties of premenopausal women were cultured in presence or absence of estradiol and estradiol+progesterone in physiological levels seen during the menstrual cycle and the levels of IL-1s released into culture media were analyzed. There were no changes in IL-1α levels after hormone exposure, Fig 2D. However, IL-1β increased significantly after estradiol treatment, Fig 2E, and the addition of progesterone did not alter the levels. IL-1Ra decreased in presence of estradiol, Fig 2F and the addition of progesterone did not alter the levels compared to estradiol alone. Immunohistochemistry revealed no differences in cellular staining of IL-1α, IL-1β, or IL-1Ra with either treatment.

IL-1Ra increased significantly in breast tissue after the addition of flaxseed to the diet

Next, we sought to investigate if flaxseed added to the diet altered the levels of IL-1s in the breast. A daily addition of 25 g of ground flaxseed lead to increased levels of the mammalian lignans secoisolarisiresinol, enterodiol, and enterolactone in serum; secoisolariciresinol increased from non-detectable to 14±8, enterodiol increased from 4.6±1.3 nmol/l to 85±20 nmol/l, and enterolactone increased from 27±7 nmol/l to 189±33 nmol/l confirming compliance with the flaxseed addition. Nine premenopausal women were investigated in two consecutive luteal phases, one unexposed and the next flaxseed exposed. As controls, five women were investigated in two consecutive unexposed luteal phases. In the control women no significant changes were detected in either tissue of any IL-1. The addition of flaxseed did not change the levels of IL-1α and IL-1β in either breast or fat tissue. IL-1Ra did, however, increase significantly in breast tissue after the addition of flaxseed, $p<0.01$, $n=9$, Fig 3x.

Tamoxifen increased IL-1Ra significantly in breast tissue

Thereafter, IL-1s were investigated before and after tamoxifen treatment. Again, no changes were seen with IL-1α and IL-1β in either tissue but IL-1Ra did increase significantly in breast tissue after six weeks of tamoxifen therapy, $p<0.001$, $n=11$, Fig 4x. To verify these *in vivo* results breast tissue biopsies were cultured and exposed to tamoxifen *ex vivo*. This experiment confirmed the *in vivo* result with significantly increased levels of IL-1Ra after tamoxifen and no changes of IL-1α and IL-1β after treatment, Fig 4x. We also performed immunohistochemistry to explore whether the intracellular levels altered by tamoxifen treatment. The staining revealed no significant changes with either treatment of cellular IL-1s.

IL-1β significantly increased in breast cancer compared with normal adjacent breast tissue

To further investigate whether IL-1s levels were found in breast cancers of patients and if the levels were altered in breast cancer tissue compared with normal adjacent breast, microdialysis was performed before surgery in ten women. These patients were investigated with a catheter with a ten mm membrane in both the tumor and adjacent tissue to ensure that the whole membrane was within the tumor tissue. Therefore, the presented levels cannot be directly compared to the results given from the volunteers investigated with a 20 mm membrane. There was a significantly increase of IL-1β within the breast cancer compared to adjacent normal breast tissue, $p<0.01$, $n=10$, Fig 5x. IL-1α and IL-1Ra levels were unaltered between the tissues.

Discussion

In the present study we show in normal human breast tissue that estradiol affect the extracellular levels of two members of the IL-1 family of cytokines, namely IL-1β and IL-1Ra. Estradiol increased the levels of IL-1β and decreased the levels of IL-1Ra suggesting that hormone exposure induced a pro-inflammatory environment in the breast. Our *in vivo* correlations were verified *ex vivo* by culture of breast tissue biopsies where estradiol increased IL-1β levels and decreased IL-1Ra. The breast biopsy culture also revealed that progesterone had no additional effect compared to estradiol exposure alone suggesting minor importance of progesterone on the regulation of this protein in the breast. In premenopausal women the pro-inflammatory profile of IL-1s could be counteracted by a diet addition of 25 g of ground flaxseed for one menstrual cycle. IL-1Ra increased significantly in breast tissue whereas IL-1β remained at similar levels as before the addition of flaxseed. The IL-1Ra levels in breast tissue increased by over 50% after flaxseed ingestion, which was in the same range and even slightly higher than in women after tamoxifen therapy where IL-1Ra increased by approximately 40%. Immunohistochemistry did not reveal any changes of stained area or staining intensity with either treatment suggesting that the hormones and treatments mainly affected the secreted levels of IL-1s. In breast cancer tumors the IL-1s exhibited a pro-inflammatory pattern with more than three times higher IL-1β levels compared to adjacent normal breast tissue with IL-1Ra levels not significantly higher in tumor tissue than the adjacent normal breast.

The IL-1s have been shown to be important for cancer progression but also in earlier stages of carcinogenesis as IL-1 α or IL-1 β knockout mice have been shown to exhibit dramatically impaired ability of tumor development (6). Several studies have shown that tumor-promoting effects of IL-1β can be inhibited by IL-1Ra in experimental models (6, 7, 33).

This has been supported by clinical studies which have revealed that high levels of IL-1Ra and low levels of IL-1 at the tumor site in breast cancer patients correlates with improved prognosis (34). High IL-1β concentration within the tumor microenvironment has also been associated aggressive phenotype with a higher recurrence rates (5, 35). In our present study we show, to our knowledge, for the first time measurements of these cytokines in the extracellular space, the bioactive site for these proteins. Our results show that in breast cancer of women the balance between IL-1β and IL-1Ra shifts towards a pro-inflammatory, pro-angiogenic environment with increased levels of IL-1β compared to the normal adjacent breast. This supports that the IL-1s may be involved in tumor progression and viable targets for interventions. Moreover, we have recently shown that the anti-estrogen tamoxifen or a diet with flaxseed to tumor bearing mice decreased the levels of IL-1β from the stroma of experimental breast cancer and increased the levels of IL-1Ra leading to tumor regression and decreased angiogenesis (9). This further supports the importance of the IL-1 family of cytokines being important players in breast cancer and that these targets may be under the influence of estrogen regulation as well as modifiable with diet interventions. This lead us to investigate if these proteins were present in normal breast tissue and whether sex steroids and/or flaxseed could influence their levels. As either IL-1α or IL-1β were detectable in blood samples estrogen dependency of these compounds have not been possible to investigate *per se*, hence previous studies have used and approach with LPS stimulated isolated monocytes from donors (36). In several previous studies, we have shown that microdialysis enables measurement of proteins in the target tissue despite un-detectable or very low levels in blood samples (25-27, 37-39). Here we show that the levels of extracellular IL-1β were much higher than the levels of IL-1α, an expected finding, as IL-1α is mainly an intracellular protein. IL-1β levels showed a significant positive correlation with estradiol both in breast tissue and subcutaneous fat, which supports previous studies that have shown an enhanced IL-1β

promotor activity by estradiol (40). To our knowledge there are no previous studies of tissue levels of IL-1Ra in the breast. Our data revealed a down-regulation of IL-1Ra by estradiol in breast tissue both *in vivo* and *ex vivo*. This is in line with studies of macrophages where estradiol has been shown to lower the transcript levels of IL-1Ra (41)

In breast tissue, autopsy investigations have shown that approximately fifteen percent of women present *in situ* tumors whereas only 1% of the same age group is diagnosed with breast cancer (42). Events in the stroma, such as an angiogenic switch and/or inflammatory reactions are key in the initiation, growth, and progression of cancer and may convert *in situ* tumors into a clinical cancer disease (1). Exposure to sex steroids increases the risk of breast cancer while early oophorectomy may reduce the risk of this disease by up to 60% to the cost of severe side effects such as osteoporosis and cardiovascular disease as well as vasomotor symptoms, urogenital atrophy, and decrease quality of life (43, 44). The anti-estrogen tamoxifen reduces the incidence of breast cancer by more than 40% but may induce severe side effects such as endometrial cancer and thromboembolism (45, 46) and is not registered as a preventive drug in all countries. Less toxic and safe breast cancer preventive strategies such as diet modifications need to be developed. Specific phytoestrogens may be one successful route as very recent epidemiological data has shown reduced breast cancer risk and increased overall survival of breast cancer patients with high serum levels of dietary plant lignans (18-23). We have recently shown in experimental breast cancer models as well as in normal breast tissue that a diet of flaxseed affects several angiogenesis regulators towards angiogensis inhibition (9, 47-49). Here we add another mechanism of actions by flaxseed by the attenuation of IL-1 levels and we also show alterations of the inflammatory response by tamoxifen and phytoestrogens (50, 51). The increase of IL-1Ra in breast tissue by flaxseed and tamoxifen was equally potent. Flaxseed has previously shown exhibit powerful biological activity in breast cancer patients where a daily addition of 25 g of flaxseed for 30 days

attenuated several biological tumor markers (52). Moreover, The importance of IL-1s as viable targets in breast cancer is shown by our data from breast cancers of patients where we demonstrate, for the first time, that the balance of extracellular IL-1β and IL-1Ra in is altered into a pro-inflammatory pattern.

In conclusion, our data revealed an estrogen dependent increase of IL-1β and a decrease of IL-1Ra in normal human breast tissue *in vivo*. The levels of IL-1Ra in normal human breast tissue *in vivo* increased significantly both by a diet of flaxseed and by tamoxifen therapy. Diet modifications may exert potent biological activities in breast tissue, which merit further mechanistic studies in order to elucidate safety and effects as breast cancer prevention strategies. In breast cancers of women before surgery a pro-inflammatory environment was detected by increased levels of IL-1β and unaltered levels of IL-1Ra suggesting blockade of IL-1 as a therapeutic option useful to explore in the clinic.

Table 1 Tumor characteristics

Patient#	Age	Tumor size (mm) and type	Grade (NHG)	ER (%)	PR (%)
1	67	18 lobular	2	50	100
2	59	9 ductal	2	80	10
3	51	20 ductal	3	100	0
4	86	60 ductal	3	80	80
5	83	20 ductal	2	100	100
6	66	20 ductal	2	80	0
7	56	17 ductal	2	100	0
8	68	15 lobular	2	75	0
9	65	50 lobular	2	75	50
10	57	21 ductal	3	75	0

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Figure legends

Figure 1

Estradiol increases extracellular IL-1β and decreases IL-1Ra in normal human breast tissue.

Women were investigated with microdialysis for sampling of extracellular proteins *in vivo* in normal breast tissue and subcutaneous fat. Blood samples were drawn at the time of investigation. 31 microdialysis investigations were performed in premenopausal healthy volunteer women during different phases of the menstrual cycle. Postmenopausal women were investigated in their un-affected breast before start of tamoxifen as an adjuvant for early breast cancer, and of these, nine of these were investigated in both breast tissue and subcutaneous fat and two patients were investigated in breast tissue only.

IL-1a and IL-1b were not detectable in the blood samples whereas IL-1Ra was detectable at low concentrations in all women.

A. IL-1α did not correlate with estradiol either in breast tissue, $r=0.05$, $p=0.7$, $n=42$ or fat tissue, $r=0.02$, $p=0.9$, $n=40$.

B. There were significant positive correlations between estradiol levels and extracellular IL-1β both in breast tissue, $r=0.36$, $p<0.05$, $n=42$, and subcutaneous fat, $r=0.35$, $p<0.05$, $n=40$,

C. IL-1Ra exhibited a strong significant negative correlation between and estradiol in breast tissue, $r=-0.67$, $p<0.0001$, $n=42$, whereas no correlation was found in fat tissue $r=0.14$, $p=0.4$, $n=40$. Plasma IL-1Ra did not correlate with estradiol.

Figure 2

IL-1β increased and IL-1Ra decreased in normal human breast tissue in the luteal phase of the menstrual cycle

A. Six women were investigated with microdialysis in breast tissue and subcutaneous fat during the follicular and luteal phases of one menstrual cycle in order to compare intra-individual levels of the IL-1s during physiological changes of hormone levels.

*p<0.05 compared to the follicular phase, n=6.

B. To verify the *in vivo* findings, normal human breast tissue biopsies from women undergoing reduction mammoplasty were cultured in the presence of E_2 (10^{-9} mol/L), a combination of E_2 and P4 (E_2 + P4; 10^{-9} and 10^{-8} mol/L, respectively), or serum-free medium alone supplemented with hormone solvent (control) for 7 days and the media was analyzed for its content of IL-1s. *p<0.05, **p<0.01 compared to control biopsies, n=6 in each group.

C. Immunohistochemistry of the breast biopsies cultured as described above. No changes in staining area or intensity were detected with either treatment. Representative tissue sections from the different treatment groups are shown. Bars 20μm

Figure 3

Flaxseed increased the levels of IL-1Ra in breast tissue

Five healthy premenopausal volunteers were investigated with microdialysis in normal breast tissue and abdominal subcutaneous fat during two consecutive luteal phases and nine premenopausal women before and after the addition of 25 g of ground flaxseed/day (Flax) to their diet.

A. There were no significant changes in luteal phase one and two in any IL-1.

B. The levels of IL-1Ra increased significantly after the addition of flaxseed to the diet.

**p<0.01, n=9.

Figure 4

Tamoxifen increased the levels of IL-1Ra in normal human breast tissue

A. Nine women were investigated with microdialysis in their un-affected normal breast tissue and abdominal subcutaneous fat and two women were investigated with microdialysis in breast tissue only before and after the addition of tamoxifen 20 mg daily as an adjuvant for early breast cancer.

***p<0.001 compared with un-exposed tissue, n=11.

B. To verify the *in vivo* findings, normal human breast tissue biopsies from women undergoing reduction mammoplasty were cultured in the presence of tamoxifen (10^{-6} mol/L), or serum-free medium alone supplemented with hormone solvent (control) for 7 days and the media was analyzed for its content of IL-1s. **p<0.01 compared to control biopsies, n=6 in each group.

C. Immunohistochemistry of the breast biopsies cultured as described above. No changes in staining area or intensity were detected with either treatment. Representative tissue sections from the different treatment groups are shown. Bars 20μm

Figure 5

IL-1β in vivo *was increased in breast cancers compared with adjacent normal breast in patients investigated prior surgery*

Ten postmenopausal breast cancer patients were investigated with microdialysis before surgery. One catheter was inserted into tumor tissue and another in adjacent normal breast tissue in the affected breast. IL-1s were analyzed in the microdialysates. **p<0.01, n=10.

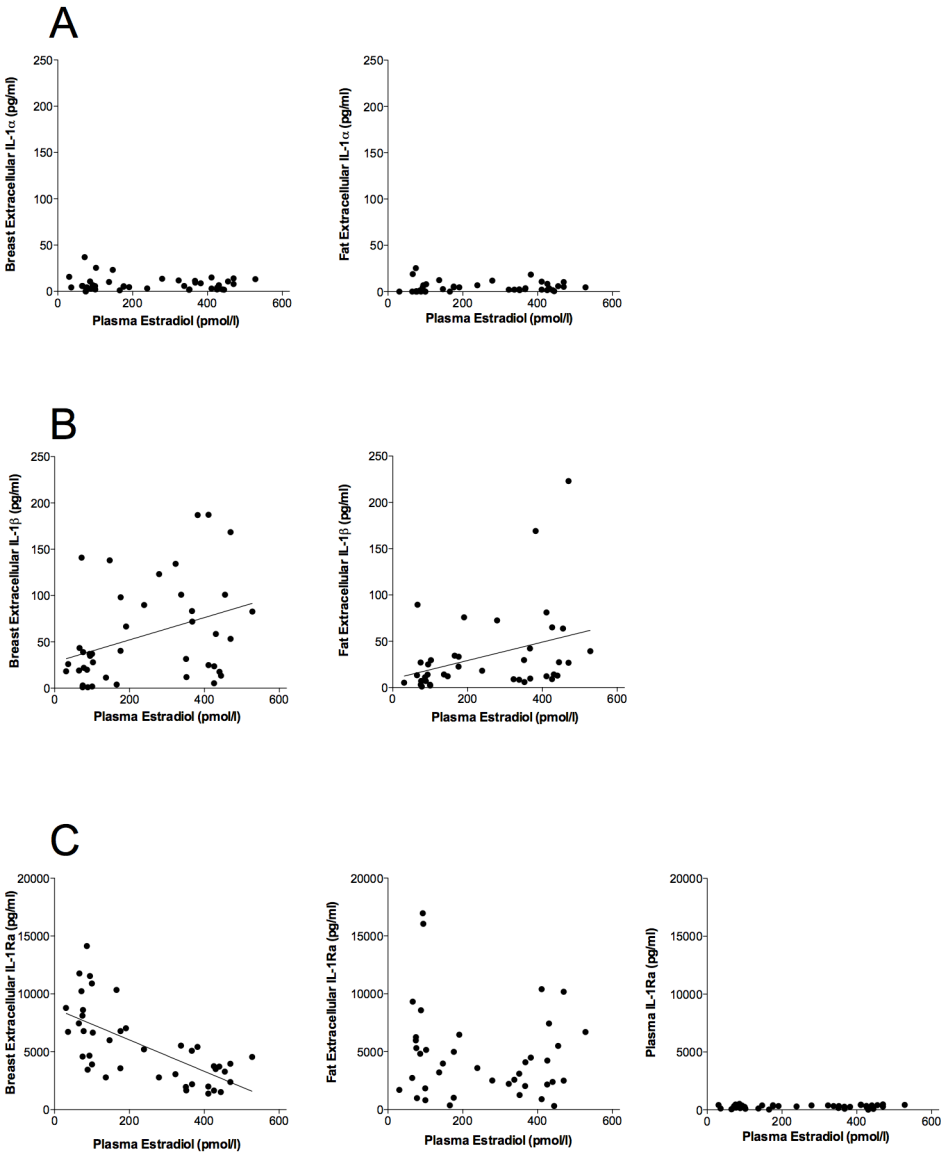

A
Breast Extracellular IL-1α (pg/ml)
Plasma Estradiol (pmol/l)
Fat Extracellular IL-1α (pg/ml)
Plasma Estradiol (pmol/l)
B
Breast Extracellular IL-1β (pg/ml)
Plasma Estradiol (pmol/l)
Fat Extracellular IL-1β (pg/ml)
Plasma Estradiol (pmol/l)
C
Breast Extracellular IL-1Ra (pg/ml)
Plasma Estradiol (pmol/l)
Fat Extracellular IL-1Ra (pg/ml)
Plasma Estradiol (pmol/l)
Plasma IL-1Ra (pg/ml)
Plasma Estradiol (pmol/l)

Figure 1

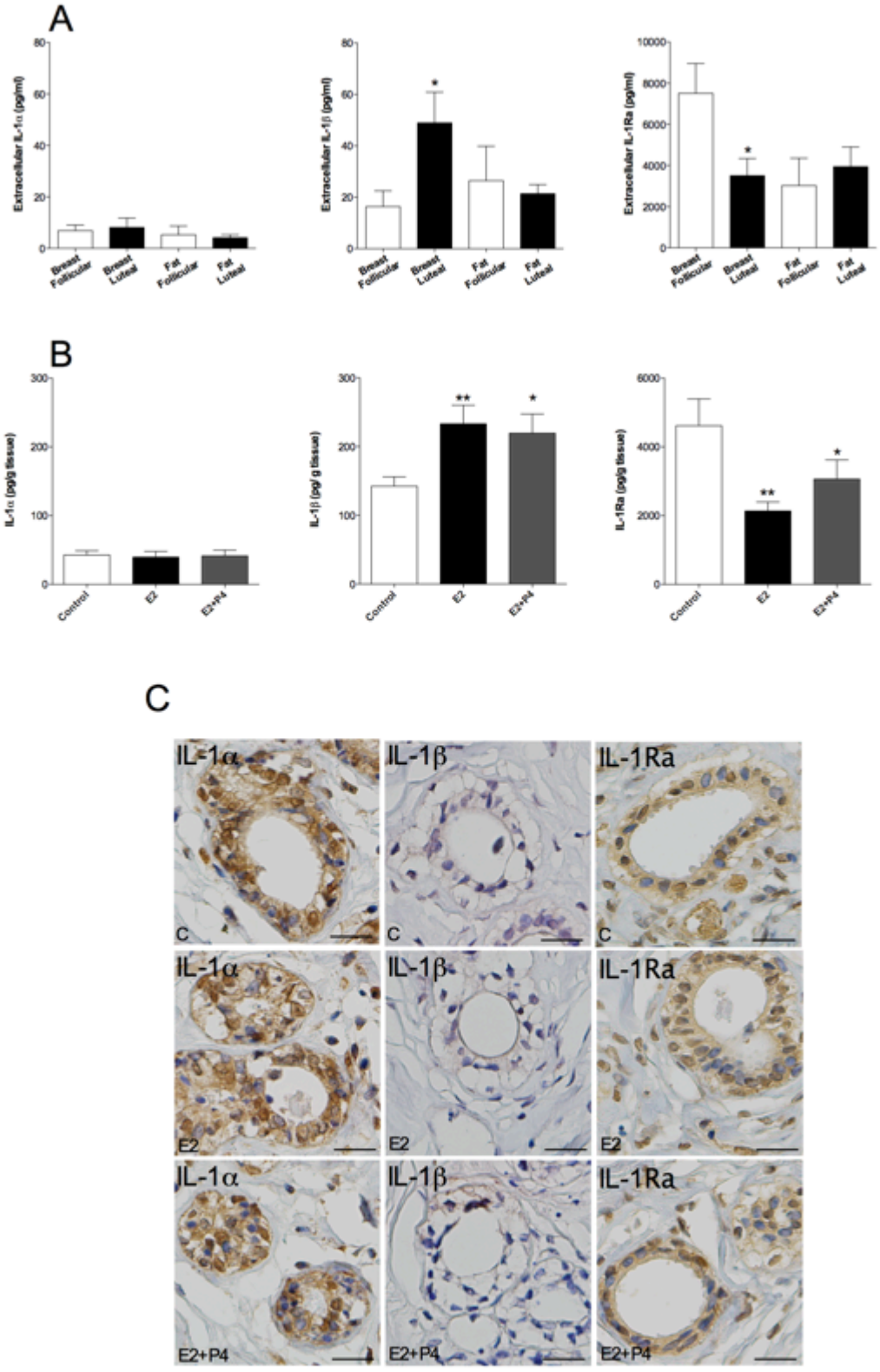
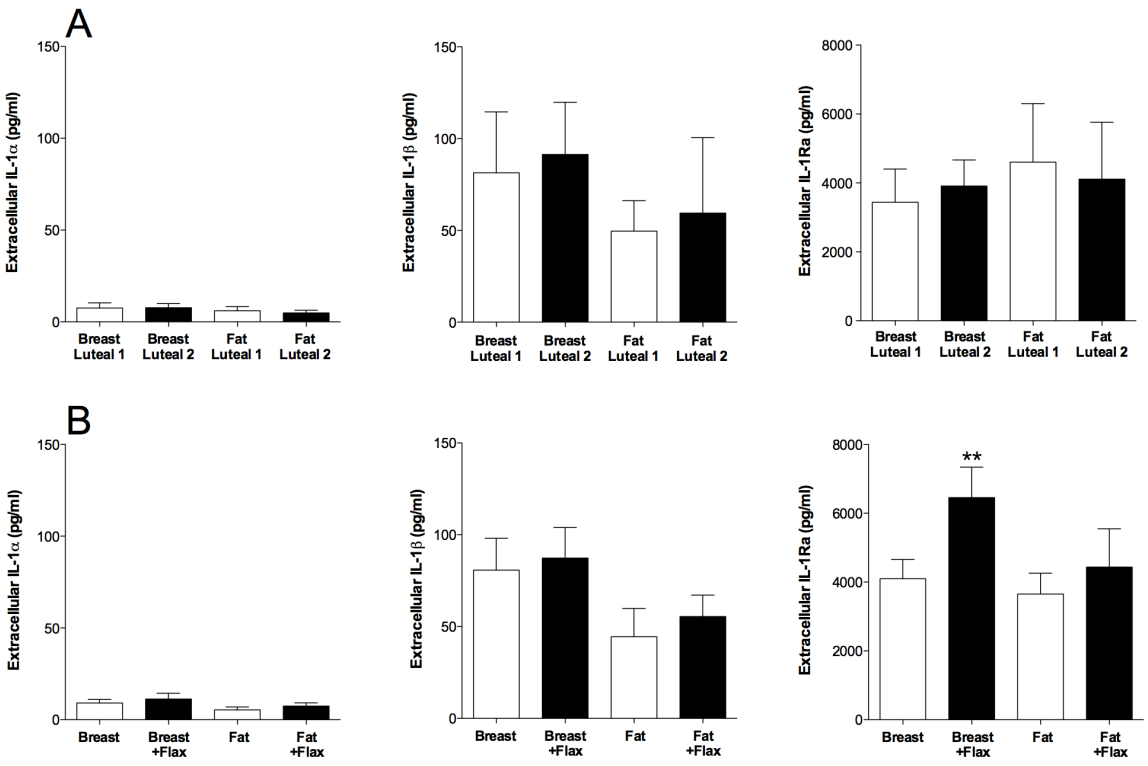

A
Extracellular IL-1α (pg/ml)
Extracellular IL-1β (pg/ml)
Extracellular IL-1Ra (pg/ml)
Breast Follicular
Breast Luteal
Fat Follicular
Fat Luteal
B
IL-1α (pg/g tissue)
IL-1β (pg/g tissue)
IL-1Ra (pg/g tissue)
Control
E2
E2+P4
C
IL-1α
IL-1β
IL-1Ra
C
E2
E2+P4

Figure 2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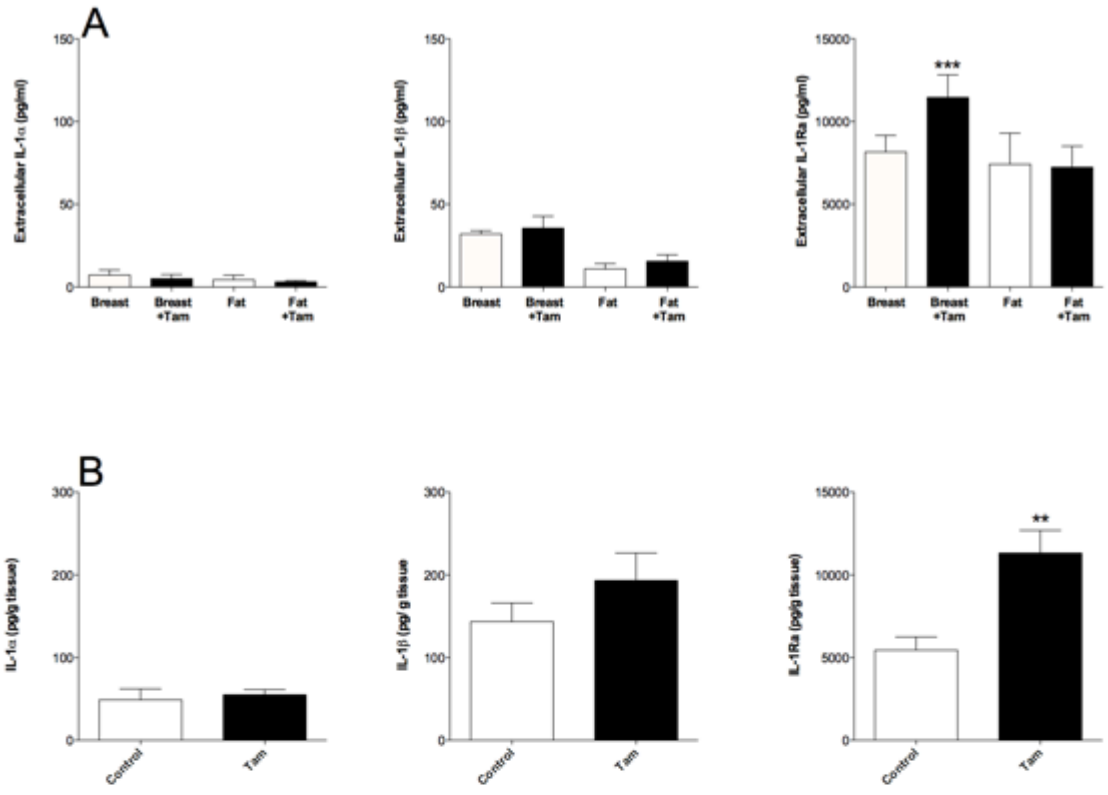
Extracellular IL-1α (pg/ml)
150
100
50
0
Breast Luteal 1
Breast Luteal 2
Fat Luteal 1
Fat Luteal 2
Extracellular IL-1β (pg/ml)
Extracellular IL-1Ra (pg/ml)
8000
6000
4000
2000
B
Breast
Breast +Flax
Fat
Fat +Flax
**

Figure 3


A
Extracellular IL-1α (pg/ml)
Extracellular IL-1β (pg/ml)
Extracellular IL-1Ra (pg/ml)
***
Breast
Breast +Tam
Fat
Fat +Tam
B
IL-1α (pg/g tissue)
IL-1β (pg/ g tissue
IL-1Ra (pg/g tissue)
**
Control
Tam

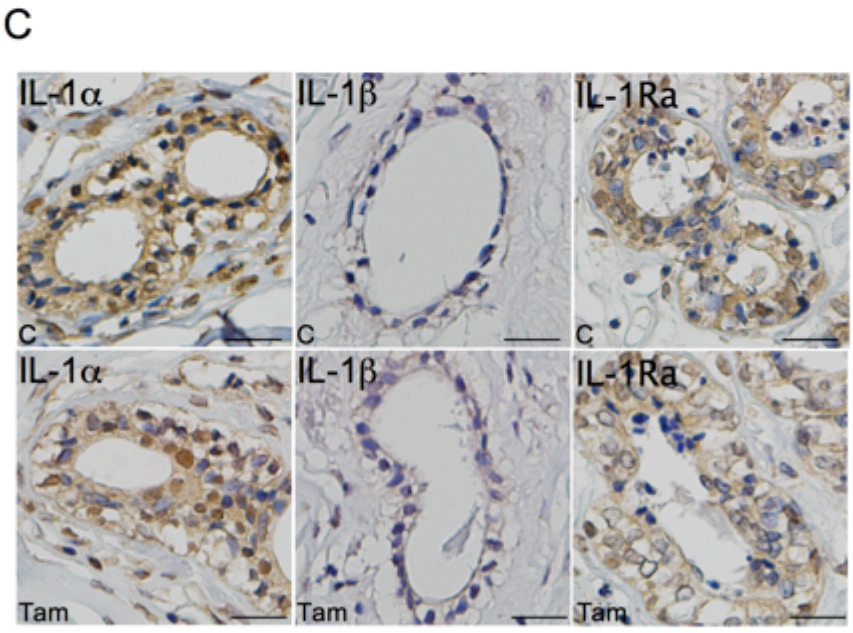

C
IL-1α
IL-1β
IL-1Ra
C
Tam

Figure 4

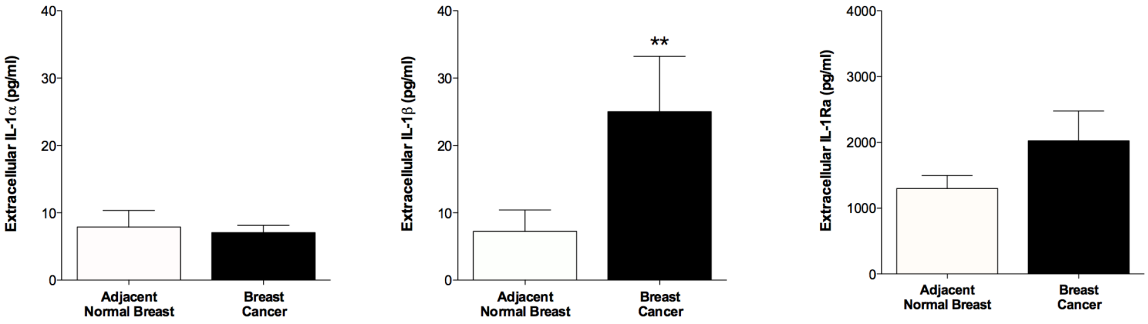

Extracellular IL-1α (pg/ml)
40
30
20
10
0
Adjacent Normal Breast
Breast Cancer
Extracellular IL-1β (pg/ml)
**
Extracellular IL-1Ra (pg/ml)
4000
3000
2000
1000

Figure 5