Postmenopausal Dense Breasts Maintain Premenopausal Levels of GH and Insulin-like Growth Factor Binding Proteins in Vivo

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Postmenopausal dense breasts maintain premenopausal levels of GH and insulin-like growth factor binding proteins *in vivo*

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Abstract

CONTEXT: Dense breast tissue is associated with 4-6 times higher risk of breast cancer by poorly understood mechanisms. No preventive therapy for this high-risk group is available. After menopause breast density decreases due to involution of the mammary gland. In dense breast tissue this process is halted by undetermined biological actions. Growth hormone (GH) and insulin-like binding proteins (IGFBPs) play major roles in normal mammary gland development but their roles in maintaining breast density are unknown.

OBJECTIVE: To reveal *in vivo* levels of GH, IGFBPs, and other pro-tumorigenic proteins in the extracellular microenvironment in breast cancer, in normal breast tissue with various breast density in postmenopausal women, and premenopausal breasts. We also sought to determine possible correlations between these determinants.

SETTING AND DESIGN: Microdialysis was used to collect extracellular *in vivo* proteins intratumorally from breast cancers before surgery and from normal human breast tissue from premenopausal women and postmenopausal women with mammographic dense or nondense breasts.

RESULTS: Estrogen receptor positive breast cancers exhibited increased extracellular GH ($P<0.01$). Dense breasts of postmenopausal women exhibited similar levels of GH as premenopausal breasts and significantly higher levels than in nondense breasts ($P<0.001$). Similar results were found for IGFBP-1, -2, -3, and -7 ($P<0.01$) and for IGFBP-6 ($P<0.05$). Strong positive correlations were revealed between GH and IGFBPs and pro-tumorigenic matrix metalloproteinases, urokinase-type plasminogen activator, IL-6, IL-8, and VEGF in normal breast tissue.

CONCLUSIONS: GH pathways may be targetable for cancer prevention therapeutics in postmenopausal women with dense breast tissue.

Précis

Postmenopausal dense breasts exhibited similar GH and IGFBPs levels as premenopausal breasts suggesting their role in failed involution and increased breast cancer risk.

Introduction

Besides being a systemically secreted pituitary hormone growth hormone (GH) may be produced locally in tissues where it exerts autocrine/paracrine effects. In breast cancer, local synthesis of GH has been detected in all cell types including normal epithelial cells, breast cancer cells, fibroblasts, myoepithelium, inflammatory cells and endothelial cells; in particular, metastatic breast cancer cells have been found to highly express GH (1,2). Autocrine production of GH may confer an invasive phenotype among breast epithelial cells by inducing an epithelial-mesenchymal-transition (EMT) and increasing the secretion of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), and inflammatory cytokines (3-6). GH has been implicated in the progression of cancer including breast cancer, but there is a poor correlation between circulating GH and breast cancer risk (7,8).

Circulating GH is a major stimulator of insulin-like growth factors (IGFs) in the liver. The physiological effects of IGF depend on its binding to IGF binding proteins (IGFBPs), which are also regulated by that GH (9). Additionally, the IGFBPs can perform other IGF independent actions in tissues on proliferation and apoptosis (9). A good correlation between IGF/IGFBP and an increased risk of estrogen receptor positive (ER+) breast cancer has been established suggesting that the cancer risk associated with GH may be mediated via the IGF/IGFBP axis (10).

Liver secretion of GH may be affected by estradiol (11) and cumulative exposure to sex steroids including estradiol is an established risk factor for breast cancer although the exact biological mechanisms have not been identified (12). How estradiol and GH intercorrelate locally in breast tissue is remains unknown.

Another major risk factor for breast cancer is increased mammographic density (13). As reviewed by Boyd *et al*, several cohort studies, comprising women 35-70 years of age, have revealed a 3-6 fold increased risk of breast cancer in women with more than 50% dense area compared to women with less than 5% dense area after adjustment for other risk factors such as age, parity, and family history (13). Additionally, the absolute amount of nondense area seems to be independently and inversely associated with breast cancer risk (14). It has been estimated that approximately 30% of all breast cancer can be attributed to the group of women with greater than 50% mammographic dense area (13,15). To identify the group of women with the highest 5-year risk of breast cancer, breast density measures can be combined with age, race, family history, and previous breast biopsy for the selection of women needing additional screening imaging modalities (16,17).

Nondense breast tissue contains higher amounts of fat, but the amounts of epithelial cells, which account for 1-6% of the tissue, seems to be unrelated to breast density (13,18-20). Recent studies suggest that dense breast tissue may be associated with increased inflammation, altered metabolic profiles, and a microenvironment similar to that found in breast cancer (21-24). Breast density is also related to age, with higher mammographic density in premenopausal women. After menopause the breast undergoes an involution in which the glandular tissue and stroma are gradually replaced by adipose tissue, a normal process that may be halted by local inflammation (25,26). Whether local breast tissue GH is involved in these processes or associated with local inflammation has not been examined. Revealing the biological pathways underlying the association between dense breast tissue and breast cancer risk is key for discovering appropriate preventative strategies.

Here, we investigated the microenvironment of breast tissue with high biological activity and very little involution; breast tissue of premenopausal nulliparous women compared with breast tissue of women with a high risk of developing breast cancer (i.e., dense breast tissue of

postmenopausal women). As a control, breast tissue at the end stage of involution and complete replacement of adipose tissue in postmenopausal women, a nondense breast tissue with very low risk of developing breast cancer, was used. Additionally, to determine if the analyzed molecules were affected in human breast cancers, a cohort of women with ongoing breast cancer was included.

We showed that dense breast tissue of postmenopausal women maintained similar levels of GH as premenopausal women and that GH was strongly correlated with IGFBPs, proteases, and angiogenic and inflammatory proteins. Extracellular GH and IGFBPs were increased in breast cancers. Our results suggest that GH pathways may be targetable as prevention therapeutics for postmenopausal women with dense breast tissue.

Materials and Methods

Subjects

The Regional Ethical Review Board of Linköping, Sweden approved the study, which was carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent. Seventy-one women were included in the study. Of these, 10 women, without BRCA-1 or BRCA-2 mutations, with ongoing breast cancer, were investigated with microdialysis before surgery. The women were consecutively recruited regardless of their breast density. Forty-two healthy postmenopausal women (ages 55–74 years) were consecutively recruited from the mammography screening program at Linköping University Hospital. Their regular screening mammograms were categorized according to the Breast Imaging Reporting and Data System (BI-RADS) density scale as either entirely fatty nondense (BI-RADS A) or extremely dense (BI-RADS D) (27). Additionally, 19 nulliparous premenopausal women (ages 20–32 years) with a history of regular menstrual cycles (cycle length, 27–34 days) were investigated in the luteal phase of the menstrual cycle. None of the healthy volunteer women had a history of breast

cancer. In addition, none of the women were currently using (or had used within the past 3 months) hormone replacement therapy, sex steroid-containing contraceptives, anti-estrogen therapies, including selective estrogen receptor modulators, or degraders.

Microdialysis procedure

Prior to insertion of the microdialysis catheters 0.5 mL lidocaine (10 mg/mL) was administrated intracutaneously. Microdialysis catheters (M Dialysis AB, Stockholm, Sweden), which consisted of a tubular dialysis membrane (diameter 0.52mm, 100,000 atomic mass cut-off) glued to the end of a double-lumen tube were inserted via a splitable introducer (M Dialysis AB), connected to a microinfusion pump (M Dialysis AB) and perfused with 154 mmol/L NaCl and 60g/L hydroxyethyl starch (Voluven®; Fresenius Kabi, Uppsala, Sweden), at 0.5 μ L/min. The women with ongoing breast cancer were investigated with 10 mm long membranes; one catheter was inserted within the cancer tissue and the other into normal adjacent breast tissue. The healthy volunteer women were investigated with 20-mm long microdialysis membranes, which was placed in the upper lateral quadrant of the left breast and directed towards the nipple, as previously described (28-38).

After a 60-min equilibration period, the outgoing perfusate was stored at -80°C for subsequent analysis.

Protein quantifications

The microdialysis samples were analyzed using a multiplex proximity extension assay (PEA, Olink Bioscience, Uppsala Sweden). In brief, 1 μ L sample was incubated in the presence of proximity antibody pairs tagged with DNA reporter molecules. Once the pair of antibodies was bound to their corresponding antigens, the respective DNA tails formed an amplicon by proximity extension, which was quantified by high-throughput real-time PCR (BioMark™ HD System; Fluidigm Corporation, South San Francisco, CA, USA). The generated fluorescent signal directly correlated with protein abundance. The output from the Proseek Multiplex

protocol was correlated in quantitation cycles (Cq) produced by the BioMark Real-Time PCR Software. To minimize variation within and between runs, the data were normalized using both an internal control (extension control) and an interplate control, and transformed using a predetermined correction factor. The pre-processed data were provided in the arbitrary unit normalized protein expression (NPX) on a $\log_2$ scale, which were then linearized by using the formula 2^{NPX} . A high NPX value corresponded to a high protein concentration. Values represented a relative quantification meaning that no comparison of absolute levels between different proteins could be made.

Estradiol

Estradiol levels in the microdialysis samples were analyzed using a high sensitivity immunoassay kit (DRG International, Springfield Township, NJ, USA).

Statistical analyses

Statistical analyses were performed using nonparametric Wilcoxon matched-pairs signed rank tests or Kruskal Wallis tests followed by unpaired Mann-Whitney U tests when more than two groups were compared as the data was non-normally distributed. Spearman's correlation test was used for calculations of correlations. All tests were two-sided. A $P < 0.05$ was considered statistically significant. Statistics were performed with Prism 7.0 (GraphPad, San Diego, CA, USA).

Results

Increased extracellular GH levels in vivo in ER+ breast cancers

There were no complications after the microdialysis investigations. We found significantly higher levels of extracellular GH in breast cancer tissue compared to normal adjacent breast tissue ($P<0.01$; Fig. 1A). All breast cancers were estrogen receptor positive (ER+); clinically routine determinations of tumor histology, size, immunohistochemistry for ER, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and Nottingham histological grade (NHG) according to the Elston Ellis scoring system are shown in Table 1.

Increased levels of GH in in postmenopausal dense breasts and premenopausal breasts compared to postmenopausal nondense breasts

After being initially categorized as having either dense (BI-RADS D) or completely nondense (BI-RADS A) breast tissue, two women were reclassified as having intermediate density. The final dense and nondense groups therefore comprised 20 women each. The two women with intermediate density were, however, included in the correlations analyses because these were performed regardless of breast density.

We detected significantly higher levels of extracellular GH in dense breast tissue of postmenopausal women compared to nondense breast tissue of postmenopausal women ($P<0.001$; Fig. 1B). Premenopausal breast tissue exhibited similar levels of extracellular GH as postmenopausal dense breast tissue (Fig. 1B).

As expected, the local extracellular breast tissue estradiol levels were significantly higher in premenopausal women than in postmenopausal women, whereas no differences were found in local estradiol levels between dense and nondense breast tissue from postmenopausal women (Fig. 1C). We found a moderate correlation between local estradiol and GH levels ($n=61$, $r=0.38$, $P<0.01$; Fig. 1D).

Increased extracellular levels in vivo of IGFBP-2, -3, -6, and -7 in ER+ breast

To the best of our knowledge, no previous data exist regarding extracellular IGFBPs of human breast cancers *in vivo*. Therefore, the next step was to determine extracellular IGFBP levels in ER+ breast cancers. As shown in Fig. 2 left column, no significant difference was found of IGFBP-1 in cancerous tissue compared to normal adjacent breast tissue. However, IGFBP-2, -3, -6, and -7 all exhibited significantly increased levels in the ER+ breast cancers (Fig. 2 left column).

Increased levels of IGFBPs in postmenopausal dense breast tissue compared to postmenopausal nondense breasts with similar levels as in premenopausal breasts and positive correlations between GH and IGFBPs in normal breast tissue

In the postmenopausal groups, IGFBP-1, -2, -3, -6, and -7 were significantly increased in dense breast tissue as compared to in nondense breast tissue (Fig. 2 middle column). IGFBP-1, -2, -3, and -7 levels were similar between postmenopausal dense breast tissue and premenopausal breast tissue (Fig. 2 middle column). Additionally, we found that IGFBP-6 levels were significantly increased in premenopausal breast tissue compared to dense breast tissue of postmenopausal women (Fig. 2 middle column).

Because GH is believed to be involved in the local regulation of IGFBPs, we analyzed whether any correlations between GH and these proteins were present locally in normal human breast tissue (n=61). Indeed, we found a significant correlation between GH and all measured IGFBPs (Fig 2 right column). We detected the strongest correlations between GH and IGFBP-2 ($r=0.69$, $P<0.0001$) and IGFBP-3 ($r=0.65$, $P<0.0001$; Fig 2 right column).

Increased levels of IL-6, IL-8 and VEGF in dense breast tissue compared to nondense breasts

As recently reported in a mixed cohort of ER+ and ER- breast cancers (24), increased levels of IL-8 and VEGF were found in the ER+ breast cancer cohort whereas no difference for IL-6 was detected (Fig. 3 left column)

Our data revealed significantly higher levels IL-6, IL-8, and VEGF in premenopausal breast tissue compared to both postmenopausal groups as shown in Fig. 3 middle column. However, dense breast tissue of postmenopausal women exhibited increased levels of all three proteins compared to nondense breasts (Fig. 3 middle column). Strong positive correlations were found between GH and all three proteins as shown in Fig. 3 right column.

Increased levels of proteases in postmenopausal dense breasts compared to postmenopausal nondense breasts with similar profiles as in premenopausal breasts

We have previously shown that, with the exception of matrix metalloproteinase (MMP)-9, several extracellular proteases were increased in a mixed cohort of ER- and ER+ breast cancers (24). Our present data of ER+ breast cancers revealed increased levels of MMP-1, -2, -3, and urokinase-type plasminogen activator (uPA) in cancerous tissue compared to normal adjacent breast tissue whereas MMP-9, and -12 were unaltered in breast cancer (Fig. 4 left column).

No differences between premenopausal breast tissues and dense breast tissues were detected for MMP-1, -2, -3, and -12 but all of these proteases were significantly increased in dense breast tissue as compared to nondense breast tissue in postmenopausal women (Fig 4 middle column). MMP-9 was significantly increased in premenopausal breast tissues as compared to both postmenopausal groups, but no differences were found between dense and nondense breast tissues of postmenopausal women (Fig 4 middle column). The uPA level was highly significantly increased in premenopausal breast tissues as compared to both postmenopausal groups and a significant increase was found in dense breast tissue as compared to nondense breast tissue (Fig 4 middle column). Strong positive correlations were found between GH and all proteases as shown in Fig. 4 right column.

Significant correlations between extracellular in vivo breast GH and IGFBPs and inflammatory- and angiogenic proteins and proteases

Several of the molecules detected in this study share pathways and their regulation is a result of an intricate interaction. As shown in Table 2, this is indeed the case in the normal breast tissue microenvironment where almost all proteins exhibit significant correlations with each other with a few exceptions; MMP-9 seems to be uninvolved in the regulation of extracellular IGFBP-1, IGFBP-7, and MMP-1, and MMP-2 did not exhibit any correlation with IGFBP-1. Additionally, estradiol did not correlate with IGFBP-1 but did so with all other protein levels.

Discussion

Our data described here demonstrated increased extracellular levels of GH in ER+ breast cancers. Similar to breast cancer, noncancerous breast tissue with intrinsically higher risk of developing breast cancer i.e. dense breast tissue of postmenopausal women exhibited increased levels of GH as compared to low-risk nondense breast tissue. GH levels in dense breast tissue were similar to those found in breast tissues of premenopausal women. Additionally, IGFBPs, proinflammatory and proangiogenic factors, as well as several proteases in dense breast tissue of postmenopausal women exhibited a similar expression pattern as in premenopausal breast tissue.

Breast density is related to age with higher mammographic density in premenopausal women. The normal physiological development of the mammary gland after menopause is an age-related lobular involution, which is characterized by loss of glandular tissue by atrophy and progressive replacement with adipose tissue (39-41). Lack of lobular involution has been associated with increased mammographic density, but the timing of the mammographic assessment is important; in the first stage of the involution process epithelial tissue is replaced with radiographically dense stroma, whereas the adipose replacement occurs at the final stage only (42,43). However, less than 10% of the mammary tissue comprises epithelial cells, and the data regarding the amount of epithelial cells in dense vs. nondense breast tissue is inconclusive (13,18-20). Thus, mammographic density reflects the stroma rather than glandular tissue. Other known risk factors for breast cancer, unrelated to hormone exposure, may also affect the extent of involution, but the exact biological basis for involution, as well as mammographic density, is yet to be determined (25). Our data suggest that mammographic density in the postmenopausal cohort was unrelated to hormone exposure as the local estradiol levels were similar in dense and nondense groups.

In addition to sex steroids, several hormones and growth factors are essential regulators of normal breast tissue development (44). GH is a key director in the mammary gland development during puberty (44). Its role in other stages of normal breast physiology is less understood. In experimental systems, increased GH levels in local breast have been linked to breast carcinogenesis by stimulation of an oncogenic transformation of mammary stem cells and enhancement of epithelial mesenchymal transition (EMT) (45). Our data showing significantly increased GH in ER+ tumors *in vivo* indeed suggested a role of local extracellular GH in human breast cancer. This was previously unrecognized because, as a secreted protein, expression of GH has been difficult to detect by immunohistochemistry or *in situ* hybridization. The high levels detected in dense breast tissue, with a high risk of developing breast cancer, also suggested that the expected normal decrease of GH after menopause, as seen in nondense breasts, was altered for some reason.

GH has a major impact on the regulation of the IGF/IGFBP axis, which, in turn, has been associated with an increased risk of ER+ breast cancer (10). We showed here that GH exhibited significant positive correlations with IGFBP-1,-2,-3,-6, and -7, suggesting an interaction of these proteins locally in normal human breast tissue. In addition to regulating IGF bioactivities, IGFBPs have been shown to have IGF-independent effects via different mechanisms (46,47). The role of IGFBP-1 in breast cancer is controversial, but epidemiological studies do not support a causative role in breast cancer; although, IGF sequestering may result in decreased mitogenic signaling (48). Our data of 10 tumors, pairwise compared to adjacent normal tissue, do not support a role for IGFBP-1 in breast cancers, as the levels were unaltered in cancerous tissues. However, a larger sample size in future studies may result in a different outcome.

IGFBP-1 may still have a role in the regulation of normal breast tissue and possibly in carcinogenesis, as the levels were increased in premenopausal and dense breasts compared to nondense breast tissue, and significant correlations with several pro-tumorigenic proteins were

detected. Among the IGFBPs that have been associated with pro-tumorigenic actions are IGFBP-2, -3, and -6, which was further supported by our findings of increased extracellular levels of these IGFBPs in ER+ breast cancers. IGFBP-2, -3, and -6 were also increased in dense breast tissue, with similar levels detected in premenopausal breasts. IGFBP-2 interacts with integrin receptors, increases VEGF production, and increases angiogenesis, resulting in pro-tumorigenic actions (49,50). This is consistent with our data showing a highly significant positive correlation with IGFBP-2 and VEGF locally in breast tissue. Although IGFBP-3 dual inhibitory and stimulatory effects of different signaling pathways are important in cancer, these proteins have been associated with poor prognosis in breast cancer (51). In addition to being the major binding protein of circulating IGFs, IGFBP-3 may also directly interact with other growth factors, potentiating proliferation of breast epithelial cells (52). Our current results revealed a significant positive correlation between IGFBP-3, VEGF, IL-6, IL-8, and all proteases, suggesting that IGFBP-3 contributed to a pro-tumorigenic microenvironment in human breast tissue. Regarding IGFBP-6 and IGFBP-7, the literature is sparse. IGFBP-6 has been suggested to be a tumor suppressor as it is decreased in cancerous tissue and may inhibit angiogenesis and cancer cell migration (53,54). IGFBP-7 has been reported as a player in cancer progression by affecting EMT and cell adhesion, also acting as a tumor suppressor in other cancer forms including breast cancer (55,56). This was in contradiction with our present data where both IGFBP-6 and IGFBP-7 were significantly increased in ER+ breast cancer and breast tissue with high risk of developing the disease. Moreover, both proteins exhibited strong positive correlations with other established pro-angiogenic and protumorigenic factors such as VEGF, inflammatory proteins, and proteases. One major disadvantage in studies of soluble proteins is that protein and mRNA levels have been determined in whole tissues, reflecting cellular levels and not the levels in locations where the proteins are biologically active (i.e., the

extracellular space). Our present data analyzed the bioactive site of these proteins, which may explain some of the discrepancies from previous data.

Here, our data demonstrated a strong correlation of GH and VEGF, which was consistent with previous data reporting direct and indirect regulations of GH on VEGF secretion (57,58). Similarly, both direct and indirect roles of GH and GH releasing hormone, respectively, in the inflammatory response have been suggested in several cancer forms (58-60). Our *in vivo* data showing a positive correlation of GH and IL-6 and IL-8 suggested a link between GH and inflammation.

The function of GH in the regulation of proteinases has been demonstrated in several previous studies; in experimental breast cancer and endometrial cancer, it was determined that GH upregulated MMP-2 and MMP-9 in a paracrine fashion (3,61). Additionally, GH has been shown to increase migration, invasion, and metastatic ability via increased MMP activity in melanoma cells (62). These previous data corroborated our human *in vivo* data of significant positive correlations between GH and MMP-1, -2, -3, -9, -12, and uPA.

We conclude that *in vivo* extracellular GH was increased in human ER+ breast cancers and in normal breast tissue at high risk of developing breast cancer, in dense breast tissue in postmenopausal women. GH levels in dense breast tissue were similar to those in premenopausal breasts. Additionally, IGFBPs, inflammatory biomarkers, and the proteases were increased in dense breasts with comparable levels as in premenopausal breasts and all these protein increases correlated significantly with GH. Our data revealed an intricate interplay of GH, IGFBPs, and protumorigenic proteins in normal breast tissue and suggested that the normal involution is altered in dense breast tissue via GH related pathways. These pathways may be targetable in novel prevention therapeutics for postmenopausal women with dense breast tissue.

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Figure legends

Figure 1. Extracellular levels of growth hormone (GH) and estradiol in vivo in breast cancer and in normal breast tissue in pre- and postmenopausal women.

Ten women diagnosed with breast cancer underwent microdialysis before surgery. One catheter was inserted within the tumor and, as the control, another catheter was used in normal adjacent breast tissue. Postmenopausal women with either dense (n=20) or nondense (n=20) breast tissue, categorized by screening mammograms, and two women with intermediate density breasts underwent microdialysis of their left breast. Nineteen premenopausal women underwent microdialysis of their left breast in the luteal phase of the menstrual cycle. Microdialysis samples were analyzed for protein abundance and estradiol as described in the materials and methods section.

A. Extracellular GH in estrogen receptor (ER)+ breast cancers and normal adjacent breast tissue. The inset shows individual data without the outlier data. Data analyzed with Wilcoxon's matched-pairs signed rank tests. ** $P < 0.01$.

B. Extracellular GH in normal human breast tissue. Data analyzed with the Kruskal-Wallis test ($P < 0.0001$), followed by the Mann-Whitney U test. *** $P < 0.001$, **** $P < 0.0001$. Data are displayed as Box plots with median and 10–90 percentile.

C. Extracellular local levels of estradiol in breast tissue. Data analyzed with the Kruskal-Wallis test ($P < 0.0001$), followed by the Mann-Whitney U test; **** $P < 0.0001$. Data are displayed as Box plots with median and 10–90 percentile.

D. Scatterplot illustrates correlations between ranked GH and ranked estradiol levels in normal human breast tissue; n=61, Spearman's correlation coefficient (r values).

Figure 2. Extracellular levels of insulin-like growth factor binding proteins (IGFBPs) in ER+ breast cancers and in normal breast tissue in pre- and postmenopausal women and their correlation with GH.

Ten women diagnosed with breast cancer underwent microdialysis before surgery. One catheter was inserted within the tumor and, as the control, another catheter was used in normal adjacent breast tissue. Postmenopausal women with either dense (n=20) or nondense (n=20) breast tissue, categorized by screening mammograms, and two women with intermediate density breasts underwent microdialysis of their left breast. Nineteen premenopausal women underwent microdialysis of their left breast in the luteal phase of the menstrual cycle. Microdialysis samples were analyzed for protein abundance as described in the materials and methods section.

Left column: Extracellular IGFBPs in ER+ breast cancers and normal adjacent breast tissue.

Data analyzed with Wilcoxon's matched-pairs signed rank test. ** $P<0.01$, *** $P<0.001$.

Middle column: Extracellular IGFBPs in normal human breast tissue. Data analyzed with Kruskal-Wallis tests followed by the Mann-Whitney U test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Data are displayed as Box plots with median and 10–90 percentile.

Right column: Scatterplot illustrates correlations between ranked GH and ranked IGFBPs in normal human breast tissue; n=61, Spearman's correlation coefficient (r values).

Figure 3. Extracellular levels of IL-6, IL-8, and VEGF in vivo in normal breast tissue in pre- and postmenopausal women and their correlation with GH.

Ten women diagnosed with breast cancer underwent microdialysis before surgery. One catheter was inserted within the tumor and, as the control, another catheter was used in normal adjacent breast tissue. Postmenopausal women with either dense (n=20) or nondense (n=20) breast tissue, categorized by screening mammograms, and two women with intermediate density breasts underwent microdialysis of their left breast. Nineteen premenopausal women underwent

microdialysis of their left breast in the luteal phase of the menstrual cycle. Microdialysis samples were analyzed for protein abundance as described in the materials and methods section.

Left column: Extracellular IL-6, IL-8, and VEGF in ER+ breast cancers and normal adjacent breast tissue. Data analyzed with Wilcoxon's matched-pairs signed rank test. $^{**}P<0.01$.

Middle column: Extracellular levels of IL-6, IL-8, and VEGF in normal human breast tissue. Data analyzed with the Kruskal-Wallis test followed by the Mann-Whitney U test. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Data are displayed as box plots with median and 10–90 percentile.

Right column: Scatterplot illustrates correlations between ranked GH and ranked IL-6, IL-8, and VEGF, respectively, in normal human breast tissue; $n=61$, Spearman's correlation coefficient (r values).

Figure 4. Extracellular levels of proteases in vivo in normal breast tissue in pre- and postmenopausal women and their correlation with GH.

Ten women diagnosed with breast cancer underwent microdialysis before surgery. One catheter was inserted within the tumor and, as the control, another catheter was used in normal adjacent breast tissue. Postmenopausal women with either dense ($n=20$) or nondense ($n=20$) breast tissue, categorized by screening mammograms, and two women with intermediate density breasts underwent microdialysis of their left breast. Nineteen premenopausal women underwent microdialysis of their left breast in the luteal phase of the menstrual cycle. Microdialysis samples were analyzed for protein abundance as described in the materials and methods section.

Left column: Extracellular proteases in ER+ breast cancers and normal adjacent breast tissue. Data analyzed with Wilcoxon's matched-pairs signed rank test. $^{*}P<0.05$, $^{**}P<0.01$.

Middle column: Extracellular levels of proteases in normal human breast tissue. Data analyzed with the Kruskal-Wallis test followed by the Mann-Whitney U test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Data are displayed as box plots with median and 10–90 percentile.

Right column: Scatterplot illustrates correlations between ranked GH and ranked proteases in normal human breast tissue; n=61, Spearman's correlation coefficient (r values).

Table 1. Characteristics of patients subjected to intratumoral microdialysis. All cancers were HER-2 negative.

Patient	Age	Tumor size (mm)	Grade (NHG)	ER (%)	PR (%)
1	48	30	2	100	100
2	52	25	3	>50	10-50
3	61	25	2	95	95
4	62	21	2	95	95
5	66	60	2	100	60
6	68	24	2	>50	>50
7	70	22	2	>50	>50
8	73	30	2	100	<5
9	78	19	2	95	50
10	78	28	2	>50	>50

ER=estrogen receptor, PR=progesterone receptor, HER-2=human epidermal growth factor receptor 2, NHG=Nottingham histological grade

1 **Table 2.**
2 Correlations between local *in vivo* extracellular breast insulin-like growth factor binding proteins (IGFBP), matrix metalloproteinases (MMP), urokinase-type plasminogen
3 activator (uPA), vascular endothelial growth factor (VEGF), and interleukins (IL), estradiol (E2), and growth hormone (GH) in normal human breast tissue.
4

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-6	IGFBP-7	MMP-1	MMP-2	MMP-3	MMP-9	MMP-12	uPA	VEGF	IL-8	IL-6	E2
IGFBP-2	***0.61														
IGFBP-3	***0.48	***0.72													
IGFBP-6	**0.35	***0.66	***0.87												
IGFBP-7	***0.49	***0.66	***0.81	***0.83											
MMP-1	***0.50	**0.42	***0.64	***0.50	***0.63										
MMP-2	0.25	***0.45	***0.78	***0.81	***0.69	***0.49									
MMP-3	***0.46	***0.59	***0.70	***0.54	***0.64	***0.70	***0.60								
MMP-9	0.16	**0.38	*0.39	*0.31	0.18	0.20	**0.40	*0.37							
MMP-12	***0.52	***0.64	***0.55	***0.50	***0.55	***0.45	**0.37	***0.51	***0.46						
uPA	**0.40	***0.58	***0.66	***0.63	***0.56	***0.50	***0.55	***0.55	***0.60	***0.66					
VEGF	***0.60	***0.67	***0.72	***0.59	***0.63	***0.62	***0.55	***0.70	***0.54	***0.69	***0.79				
IL-8	***0.53	***0.52	***0.43	**0.36	*0.41	***0.46	*0.26	**0.34	***0.51	***0.57	***0.67	***0.71			
IL-6	**0.40	***0.61	***0.60	***0.51	***0.44	**0.42	***0.45	***0.49	***0.74	***0.65	***0.76	***0.74	***0.68		
E2	0.13	*0.32	***0.47	***0.50	*0.27	**0.39	***0.48	*0.29	***0.45	*0.38	***0.69	***0.44	**0.34	***0.52	
GH	***0.48	***0.68	***0.65	***0.49	***0.49	***0.65	**0.40	***0.60	***0.50	***0.65	***0.56	***0.72	***0.54	***0.72	**0.38

n=61. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

Data presented as Spearman's correlation coefficient (r values). Nonsignificant values in bold.