Platelet Activation In Situ in Breasts at High Risk of Cancer: Relationship with Mammographic Density and Estradiol

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Platelet activation *in situ* in breasts at high risk of cancer: relationship with mammographic density and estradiol

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Abstract

CONTEXT: High mammographic density in postmenopausal women is an independent risk factor for breast cancer by undetermined mechanisms. No preventive therapy for this risk group is available. Activated platelets release growth factors that modulate the microenvironment into a pro-tumorigenic state. Estrogens may affect the risk of breast cancer and platelet function. Whether platelets are activated *in situ* in breast cancer or in normal breast tissue at high risk of breast cancer and the association to estradiol remains elusive.

OBJECTIVE: To investigate whether platelets are activated *in situ* in breast cancers and in dense breast tissue of postmenopausal women and explore correlations between estradiol, released platelet factors, and inflammatory proteins.

SETTING AND DESIGN: Sampling of *in vivo* proteins was performed using microdialysis in a total of 71 women; 10 with breast cancer, 42 healthy postmenopausal women with different breast densities, and 19 premenopausal women.

RESULTS: Our data demonstrate increased levels of coagulation factors in dense breast tissue similar to that found in breast cancers, indicating excessive platelet activation. Premenopausal breasts exhibited similar levels of coagulation factors as postmenopausal dense breasts. Out of 13 coagulations factors that were upregulated in dense breasts, 5 exhibited significant correlations with estradiol both locally in the breast and systemically. In breast tissue, positive correlations between coagulation factors and key inflammatory proteins and matrix metalloproteinases were detected.

CONCLUSIONS: Breast density, not estradiol, is the major determinant of local platelet activation. Inactivation of platelets may be a therapeutic strategy for cancer prevention in postmenopausal women with dense breasts.

Introduction

Platelets have dynamic biological roles in a wide range of pathophysiological events including wound healing and cancer progression (1). Inflammation, increased cell proliferation, extracellular matrix (ECM) remodelling and migration are fundamental features of the wound healing process and hallmarks of cancer (2). Activated platelets may contribute to primary cancer growth via release of growth factors in the local microenvironment. When platelets adhere to defects in the endothelial lining of vessel walls, they are activated and aggregate to form a primary hemostatic plug and the coagulation cascade is initiated. This physiological process is tightly regulated to prevent thrombogenic complications. The activated platelets secrete a plethora of proteins, including pro-inflammatory and angiogenic factors, that modify the tissue microenvironment and provide proper "soil" for cancer growth (3). Tightly intertwined crosstalk occurs between the hemostatic and inflammatory systems; pro-inflammatory proteins trigger the hemostatic system, including platelet activation, which releases additional pro-inflammatory cytokines leading to recruitment of immune cells into the tissue that exacerbate inflammation (4). During inflammation and platelet activation, several pro-angiogenic proteins are released that contribute to increased vascular leakiness and increased angiogenesis, including platelet-derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs) (5-7). Although it is well known that cancer patients display elevated platelet counts and are prone to thrombotic events and that platelets contribute to metastatic spread (8), the role of activated platelets during the early steps of carcinogenesis remains unknown. Estradiol may affect hemostasis via effects on platelet function, as well as affecting the liver production of several coagulation factors (9,10). Additionally, cumulative exposure to sex steroids including estradiol is an established risk factor for breast cancer but the exact biological mechanisms remain undetermined (11).

Another major independent risk factor for breast cancer is increased mammographic density (12). Women with > 50% dense area of the breasts have a 4–6 fold increased risk of breast cancer compared to women with < 10% dense area (12). Additionally, the absolute amount of nondense area is independently and inversely associated with breast cancer risk (13). Although it has been suggested that women with > 50% dense area comprise 30% of all breast cancer cases (12,14), there are no specific preventive measures available for these women. While the epidemiological evidence is compelling, very little is known about the biological differences between dense and nondense breast tissues, which is necessary to identify novel preventive therapeutics. Recent studies suggest that dense breast tissue is associated with an altered metabolic profile and an inflammatory microenvironment similar to that found in breast cancer (15-18). Activation of the hemostatic system, including platelet activation, is a well-known systemic trait in cancer patients, including breast cancer patients (19). Whether and how the hemostatic system is activated *in vivo* in the microenvironment human breast cancer microenvironment and in normal breast tissues at high risk of cancer i.e. dense breast tissue in postmenopausal women and the possible relationship with estradiol remains unknown.

Here, we present new evidence suggesting that dense breast tissue in postmenopausal women, which has inherently high risk of breast cancer, was associated with increased platelet activation *in situ* similar to that found in established breast cancers. Several components of the hemostatic system correlated significantly with key inflammatory proteins and MMPs in the breast. Whereas estradiol affected some of the coagulation factors, breast density seems to play a more prominent role for the local control of coagulation and platelet activation. Our results provide novel biological understanding of the microenvironment of dense breast tissue and identify platelet activation as a new potential molecular target for the prevention of breast cancer in postmenopausal women with dense breast tissue.

Material and Methods

Subjects

The Regional Ethical Review Board of Linköping, Sweden approved this study, which was carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent. Forty-two healthy postmenopausal women (ages 55–74 years) were consecutively recruited during their regular mammography screening at Linköping University Hospital. The screening mammograms were categorized according to the Breast Imaging Reporting and Data System (BI-RADS) density scale as either entirely fatty non-dense (BI-RADS A) or extremely dense (BI-RADS D) (20). After re-evaluation of the mammograms one woman in each group were miss-categorized and therefore excluded from the group-wise comparisons between dense and nondense groups but included in the correlation analyses. Additionally, 19 nulliparous premenopausal women (ages 20–32 years) with a history of regular menstrual cycles (cycle length, 27–34 days) were investigated the luteal phases of the menstrual cycle. None of the healthy volunteer women had a history of breast cancer.

Furthermore, 10 postmenopausal women (ages 52–78 years) who had ongoing breast cancer were recruited and investigated before surgery. All of the cancers were estrogen- and progesterone receptor positive and HER-2 receptor negative, the tumor size ranged from 19–60 mm, and the Nottingham histological grades were 2 or 3 according to the Elston Ellis scoring system determined at the Department of Pathology and Cytology, University Hospital of Linköping. Nine of the women were free of axillary metastases and one woman had a positive sentinel node.

Importantly, none of the women were currently using (or had used within the past 3 months) any sex steroid containing drugs or implants, such as hormone replacement therapy, sex steroid containing contraceptives, or anti-estrogen therapies, such as selective estrogen receptor modulators or degraders.

Microdialysis Procedure

Microdialysis was been performed as previously described (17,18,21-23). In brief, 0.5 mL lidocaine (10 mg/mL) was administrated intracutaneously prior the insertion of the microdialysis catheters. The microdialysis catheter (71/M Dialysis AB, Stockholm, Sweden) consisting of a tubular dialysis membrane (diameter 0.52 mm, 100,000 atomic mass cut-off) glued to the end of a double-lumen tube (80 mm long × 0.8 mm in diameter), was inserted via a splitable introducer (M Dialysis AB) connected to a microinfusion pump (M Dialysis AB) and perfused with 154 mmol/L NaCl and 60 g/L hydroxyethyl starch (Voluven®, Fresenius Kabi, Uppsala, Sweden) at a perfusion rate of 0.5 μL/min. In the healthy postmenopausal women with various breast densities and in the premenopausal women, 20 mm long microdialysis membranes were placed in the upper lateral quadrant of the left breast directed towards the nipple. In the women with breast cancer, microdialysis catheters with a 10 mm long membrane were inserted within the breast cancer tissue and another one was inserted within adjacent normal breast tissue. After a 60 min equilibration period, the outgoing perfusate was stored at −80°C for subsequent analysis.

Protein quantification

The microdialysis samples were analyzed using a multiplex proximity extension assay (PEA, Olink Bioscience, Uppsala Sweden). Briefly, 1 μL sample was incubated with 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 correlated directly with protein abundance. The output from the Proseek Multiplex protocol was correlated to quantitation cycles (Cq) by the BioMark's 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 were subsequently transformed using a pre-determined correction factor. The pre-processed data were provided as arbitrary unit normalized protein expressions (NPX) on a $\log_2$ scale and were then linearized using the 2^{NPX} formula. A high NPX value corresponded to a high protein concentration. The values were relative quantifications, thus no comparisons of absolute levels of the 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).

Platelet preparation and analyses

Blood was collected into vacutainers containing acid-citrate-dextrose (ACD-A vacuette tube, Greiner Bio-One, Kremsmünster, Austria) and centrifuged to obtain platelet rich plasma (PRP), which was diluted in HEP buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EGTA) containing 1 µM prostaglandin-E1 (Sigma-Aldrich, MO, USA) to prevent platelet activation. PRP was centrifuged and platelet pellets were carefully washed in 10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA and 1% dextrose. Platelets resuspended in Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES) containing 5 mM glucose and 3 mg/ml of bovine serum albumin were seeded in 96 U-shaped well plates at 1.8 x 10^7 cells/well and preincubated at 37°C for 10 min with 1 nM 17-β-estradiol (E2) (Sigma-Aldrich, MO, USA) ± 10 nM progesterone (Sigma-Aldrich, MO, USA) or left untreated. Thereafter, 0.1 U/ml thrombin (Sigma-Aldrich) was added for 10 min at 37°C. Plates were centrifuged at +4°C and supernatants were frozen for subsequent ELISA assays; u-plasminogen activator/urokinase (uPA), soluble uPA receptor (suPAR) and plasminogen activator inhibitor-1 (PAI) (R&D Systems Inc. Minneapolis, USA)

and serine protease inhibitor 5 (SERPINA5) (RayBiotech Peachtree Corners, GA, USA) performed according to manufactures instructions.

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. Correlations were analyzed using Spearman's correlation test. All tests were two-sided. A $P<0.05$ was considered statistically significant. Statistics were performed with Prism 8.0 (GraphPad, San Diego, CA, USA).

Results

No difference in local estradiol levels in postmenopausal women with dense or nondense breast tissue

No significant difference was detected of local estradiol levels in breast tissue, median (25-75 percentiles), between postmenopausal women with dense or nondense breasts; 38 pmol/l (28-48) vs. 37 pmol/l (34-44) respectively. The levels in breast tissue of premenopausal women were 205 pmol/l (170-234). Similarly, no significant difference was detected of local estradiol levels in abdominal subcutaneous fat, median (25-75 percentiles), between postmenopausal women with dense or nondense breasts; 38 pmol/l (31-49) vs. 36 pmol/l (35-46) respectively. The levels in abdominal subcutaneous fat of premenopausal women were 202 pmol/l (155-235).

Breast cancer, dense breast tissue in postmenopausal women, and premenopausal breasts tissue display markers of platelet activation in situ

Platelet activation is driven by different pathways stimulated by agonists, such as von Willebrand factor (VWF). In normal hemostasis, VWF is released by endothelial cells to initiate platelet activation and, subsequently, by alpha granules of activated platelets themselves. Significantly increased levels of extracellular VWF were observed in breast cancer and dense breast tissues (Figure 1A). Premenopausal breast levels of VWF were similar to those found in dense breasts. Thrombin is a potent platelet agonist that binds and cleaves the extracellular domain of protease-activated receptors (PARs) during platelet activation. Levels of secreted PAR1 were significantly higher in breast cancer tissue than in adjacent normal breast tissue and in dense breast tissue than in nondense breast tissue. Premenopausal breasts exhibited similar levels as dense breasts (Figure 1B). Several other proteins, including P-selectin, CD40 ligand (CD40L), and platelet-derived growth factor subunits A (PDGFA) and B (PDGFB) are associated with platelet activation. Expression of all of these markers was significantly higher

214 in breast cancer tissue than in normal tissue and higher in dense breast tissue than in nondense
215 breast tissue (Figure 1C-F). With the exception of PDGFB all of these proteins were similar in
216 premenopausal breast tissues and postmenopausal dense breasts.

217 ***Enhanced activation of the coagulation system in breast cancer, dense breasts in***
218 ***postmenopausal women, and premenopausal breasts tissue***

219 As a consequence of the initial platelet adhesion and activation, the coagulation
220 cascade is triggered. The coagulation cascade can be activated via the intrinsic and/or the
221 extrinsic pathway that ultimately converge on the activation of Factor X (FX) (24). The
222 extrinsic pathway is initiated by tissue injury when Factor VII (FVII) leaves circulation and
223 accumulates in the tissue, whereas the intrinsic (contact) pathway is triggered by Factor XI
224 (FXI). Our data showed significantly increased levels of FVII and FXI in breast cancer tissue
225 indicating activation of both the intrinsic and the extrinsic coagulation pathways (Figure 2A-
226 B). There was a three-fold increase in extracellular levels of FVII in dense breast tissue when
227 compared with nondense breast tissue, whereas there was no difference in the FXI level
228 suggesting that the extrinsic pathway but not the intrinsic pathway is activated in dense breasts
229 (Figure 2A-B). In premenopausal breasts the levels of FVII were similar to those detected in
230 postmenopausal dense breasts whereas no differences were found in FXI levels (Figure 2A-B).

231 ***Activation of anticoagulant mechanisms in breast cancer, dense breasts in postmenopausal***
232 ***women, and premenopausal breasts tissue***

233 During activation of the coagulation cascade, levels of many anticoagulant proteins also
234 increase to maintain the hemostatic balance. Protein C (PROC) controls blood coagulation by
235 inhibiting FV and FVII. PROC activity is regulated by plasma serine protease inhibitor 5
236 (SERPINA5), which has both pro- and anti-coagulation activities, and by the tissue factor
237 pathway inhibitor (TFPI), which also controls tissue factor (TF) activity. All of these proteins
238 were significantly upregulated in breast cancer and dense breast tissues (Figure 3A-C). In

premenopausal breasts the levels of PROC and TFPI were significantly increased as compared to both dense and nondense breasts in postmenopausal women. SERPINA5, on the other hand, was significantly lower compared to dense and nondense breasts in postmenopausal women (Figure 3A-C).

Fibrinolysis, another key step in coagulation, limits the activity of the coagulation cascade by dissolving blood clots via plasmin and thus inhibits excess thrombotic formations. Plasmin is generated from plasminogen by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) and its generation is inhibited by plasminogen activator inhibitor-1 (PAI). Although tPA levels did not differ in breast cancer vs. normal and in dense breast vs. nondense breast tissues or premenopausal breasts (Figure 3D), both uPA and PAI levels were significantly higher in breast cancer and dense breast tissues (Figure 3E-F). In premenopausal breasts, the uPA levels were significantly higher than in both dense and nondense postmenopausal breasts, whereas the levels of PAI in premenopausal breast were comparable to postmenopausal dense breasts (Figure 3E-F).

The soluble form of the urokinase plasminogen activator receptor (suPAR) has also been shown to increase lysis of fibrin clots (25). Consistently, secretion of suPAR was significantly increased in both breast cancer and dense breast tissue. Premenopausal breasts exhibited similar levels as in dense postmenopausal breasts (Figure 3G).

Correlations between coagulation factors and inflammatory and angiogenic proteins in the normal breast tissue microenvironment

Comparative studies have shown that various steps of hemostasis and immunity are co-regulated and intertwined in cancerous tissue and during injury. Whether this relationship is present in normal tissue is unknown. To test this hypothesis, we evaluated associations between the pro-inflammatory cytokines and angiogenic proteins IL-6, IL-8, TGF-β, and VEGF and hemostatic biomarkers in normal breast tissue. Our data demonstrated that

IL-6, TGF-β, and VEGF correlated positively with all hemostatic proteins except SERPINA5 and tPA. IL-8 levels also correlated positively with levels of the hemostatic factors with the few exceptions of SERPINA5, tPA, FXI, SELP, and PDGFB (Figure 4).

Correlations between coagulation factors and MMPs in the normal breast tissue microenvironment

Another group of platelet-derived molecules that control the tissue microenvironment are MMPs. Platelets contain and release several MMPs, including MMP-1, MMP-2, MMP-3, and MMP-9 (7). Additionally, platelets may enhance the secretion of MMPs from immune cells in the microenvironment (26). As shown in Figure 5, significant correlations were found between MMP-1,-2, -3, and -9 and the majority of coagulations factors.

Correlations between coagulation factors and estradiol levels locally in abdominal subcutaneous fat and in breast tissue

Finally, we investigated whether the findings in dense breast tissue were a result of local events in the breast microenvironment or an inherently systemic difference between the women. Microdialysis was therefore performed in abdominal subcutaneous fat also. As shown in Table 1, the levels of uPA, PAI, suPAR, SERPINA5, and PROC were significantly altered in abdominal subcutaneous fat of premenopausal women whereas there were no differences in fat tissue between the two groups of postmenopausal women. This led us to investigate whether estradiol was involved in the regulation of these factors. Indeed, we found that in abdominal subcutaneous fat, local levels of estradiol correlated significantly with these five factors (Figure 6A). In breast tissue these five proteins, and in addition TFPI, also correlated significantly with local breast estradiol levels (Figure 6B).

Sex steroids may affect platelet phenotypes both by genomic effects on bone marrow derived megacaryocytes and by non-genomic effect directly on platelets, which lack cell nucleus. To elucidate whether estradiol alone or in presence of progesterone affected platelets *per se*,

platelets were exposed to the hormones *in vitro.* Of the five proteins that correlated with estradiol uPA, PAI, suPAR, SERPINA5 may be release by platelets in the tissue whereas PROC is mainly produced in the liver. As shown in Figure 6C the release of suPAR increased and SERPINA5 decreased after estradiol exposure whereas progesterone had no additional effects on platelet function. These results corroborate the *in vivo* data. uPA was below the detection limit and PAI was unaltered after hormone exposure.

Sustained coagulation-inflammation loop in dense breast tissue

In figure 7 the results are summarized in a graphical abstract.

Discussion

Although a high mammographic density is an established independent risk factor for breast cancer, the molecular mechanisms underlying this risk has not yet been determined. More knowledge is required to design preventive therapeutic interventions. In this study, we found that, similar to breast cancers, dense breast tissue displayed platelet activation and upregulation of coagulation and fibrinolysis pathway components. Indeed, dense breast tissue in postmenopausal women exhibited similar levels of the factors as premenopausal breasts. Some of the factors correlated with estradiol both systemically and locally in the breast but breast density was the major determinant for the regulation of the proteins. To the best of our knowledge, this is the first report showing similar *in vivo* coagulation factor profiles in dense breasts and breast cancers.

Increased coagulation has been associated with cancer since 1865 when an association of excessive blood coagulation and cancer progression was described (27). Additionally, numerous studies have shown that plasma levels of different platelet activation biomarkers and coagulation factors, such as soluble P-selectin, soluble CD40L, and VWF, are significantly higher in cancer patients, including breast cancer patients, suggesting that coagulation systems are activated during cancer progression (28-31). Platelets may also sustain tumor dissemination by protecting tumor cells in the circulation from the normal immune response, thus increasing the metastatic capacity of cancer cells (32). Previous studies have primarily described the systemic coagulation effects, rather than the local mechanisms, during cancer progression. Experimental studies have revealed that platelets create a microenvironment supportive of cancer cell survival by secreting a plethora of cytokines, chemokines, ECM remodeling proteases, growth and angiogenic factors (26), however, how these proteins are released in human cancers *in vivo* is undetermined. Indeed, our data corroborate previous experimental results and systemic effects; the local breast cancer microenvironment exhibited protein levels suggestive of platelet activation and induction of coagulation. Additionally, anticoagulant

factors, including components of fibrinolysis, were also higher in the breast cancer microenvironment. Interestingly, the uPA level was higher, whereas the tPA level was unaltered in the breast cancers. We interpret this as a consequence of tPA primarily being involved in blood clotting in the systemic circulation, whereas uPA is important for local remodeling of the ECM (33). Here, we report striking similarities of platelet activation and coagulation between the microenvironment of breast cancers and the microenvironment of healthy dense breast tissue. Similar to the breast cancer microenvironment, dense breast tissue exhibited increased levels of VWF, PAR1, P-selectin, CD40L, PDGFA, PDGFB, FVII, SERPINA5, TFPI, PROC, uPA, suPAR, and PAI whereas FXI was unaffected in dense breasts but up-regulated in breast cancer.

Coagulopathy may be related to estrogen therapies; the risk of thromboembolism seems to be increased with oral preparations rather than other routes of administration due to the first passage effect on liver function (10). How endogenous estradiol affects the coagulation system is elusive. Our present data suggests that estradiol may indeed affect suPAR, uPA, PAI, PROC, and SERPINA5 as the levels of these proteins correlated significantly with estradiol both locally in the breast and in abdominal subcutaneous fat, which we interpret as systemic effects. Increased plasma levels of suPAR in women compared with men has recently been reported corroborating our results (34). PROC, which is produced in the liver, exhibited significant correlations with estradiol suggesting hormonal liver effects. Interestingly, our data revealed a significant negative correlation with estradiol and SERPINA5, which is mainly produced in the liver in women and in male genitalia. The majority of the coagulation factors were increased in premenopausal breasts but unrelated to estradiol. Thus, breast density *per se* seems to be more important than hormone levels for the regulation of coagulation factors in the breast microenvironment. This is supported by several studies showing that, after adjustments of body mass index, endogenous levels of estrogens, androgens, progesterone, and prolactin lack

association with mammographic breast density (12,35). Regarding exogenous hormones, the combination of estrogens (E) and progestins (P) to postmenopausal women seems to be associated with increased mammographic density compared to E only therapy (36). However, while the combined therapy of E+P is associated with increased risk of estrogen receptor positive (ER+) breast cancer, high mammographic density has mainly been associated with equal risk of both ER+ and ER- breast cancer in postmenopausal women and with ER- breast cancer in premenopausal women (37). Additionally, other steroid hormone signaling pathways may also affect the breast cancer progression; activation of the glucocorticoid receptor (GR) may increase ER- breast cancer progression whereas GR expression in ER+ breast cancer may improve progression free survival (38,39). A link between GR and inflammatory pathways via TGF-β has also been demonstrated in ER- breast cancer (39). How glucocorticoids and/or GR affect breast density is yet to be determined.

Premenopausal women have high mammographic breast density and the normal physiological process after menopause is an involution with loss of glandular tissue, which is replaced with adipose tissue resulting in decreased mammographic density (40-42). Why or how this process is halted in some women is elusive. One reason may be that a stiff extracellular microenvironment affects the activation of the estrogen receptor including ligand-independent actions, thereby sustaining the expression of proteins that were induced by previous estradiol exposure (43,44). This supports our results suggesting that breast density *per se* plays a more critical role than estradiol exposure for the local activation of platelets and coagulation.

In contrast with breast cancers, only components of the extrinsic pathway were upregulated in dense breasts indicating that platelet activation and coagulation is initiated by external stimuli, such as increased tissue pressure induced by dense collagen (45). Indeed, it has been demonstrated that mechanical properties may affect platelet function; platelets can mechanosense the stiffness of the underlying fibrin/fibrinogen substrate leading to increased

platelet activation and adhesion that enhances pro-coagulant activity (46). Additionally, components of the immune system are sensitive to changes in the ECM, for example, macrophages grown on stiff substrates produce more proinflammatory mediators than macrophages cultured on soft substrates, and many ECM proteins contain cryptic domains that affect immune and pro-inflammatory cell behavior (47-49). Previous data indicate that mammographic dense breast tissue is associated with a local proinflammatory microenvironment including increased levels of VEGF and IL-8 and MMPs (16-18,50,51). These proteins increase vascular permeability, disrupt the endothelial barrier which in turn will expose ECM components, and enhance extravasation of immune cells leading to the recruitment of activated platelets. During activation, platelets release granules containing a plethora of factors that modulate the microenvironment and attract innate immune cells to the site creating a vicious circle (52-57). These interactions have primarily been shown in cancerous tissues, and it has been much less clear whether these interactions are present in normal tissues. Our present data show that this tight interdependent relationship between inflammation, angiogenesis, and hemostasis is also present in normal breast tissue. We hypothesize that breast density itself may trigger the coagulation system to act in concert with the inflammatory cascade creating an inflammation-coagulation cycle resulting in a pro-tumorigenic microenvironment in dense breasts (summarized in Figure 7). Thus, when atypical cells arise in the breast epithelium, the extracellular microenvironment in dense breasts, exhibiting a triggered coagulation system, would be more permissive for continuous expansion and invasion of these cells leading to increased risk of developing clinically significant breast cancer in dense breasts. Our data clearly shows that the changes detected in dense breasts are tissue-specific because no differences were found between the dense breast and nondense breast groups in the extracellular environment of abdominal subcutaneous fat.

Nonsteroidal inflammatory drugs (NSAIDs), such as acetylsalicylic acid (aspirin) inhibits platelet aggregation and activation by inhibiting cyclooxygenase activity (58). Low-dose aspirin may reduce the incidence of colorectal cancer by one-third, but the effect on breast cancer prevention has been less encouraging (59). In a large population study of women without risk factors, alternate-day low-dose aspirin use did not reduce breast cancer incidence, whereas a small study of women with benign breast disease who reported aspirin use suggested a reduced risk of breast cancer (60,61). Additionally, epidemiological data of women with early stage breast cancer suggested beneficial effects of low-dose aspirin (62). To date, there are no randomized data on low-dose aspirin use as a preventative therapy for women with a high risk of breast cancer. Our data has unraveled a molecular justification for such trials.

We conclude that dense breast tissue in postmenopausal women is associated with increased platelet activation *in vivo* similar to what was detected in human breast cancers. Premenopausal breasts exhibited similar levels of coagulation factors as postmenopausal dense breasts, but the majority of these factors were unrelated to estradiol suggesting that breast density *per se* is a major determinant for activation of platelets and the coagulation system. Our data revealed significant correlations between inflammatory proteins and coagulation factors supporting intertwined local regulation in normal breast tissue. Targeting platelet activation with aspirin or other pathways in the coagulation system may be a feasible approach to developing preventative therapeutics for women with a high risk of developing breast cancer, such as postmenopausal

Author contributions

CD designed the project and performed all microdialysis investigations. CD and SM analyzed the data and prepared and finally approved the manuscript

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Data availability

The data generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1. *Markers of platelets activation are upregulated in breast cancers and in dense breast tissue*

A total of 69 women were investigated; 10 breast cancer patients underwent microdialysis before surgery with one catheter inserted into the breast cancer and another into adjacent normal breast tissue and 40 postmenopausal healthy volunteer women, attending the regular mammography-screening program categorized as either having dense (n=20, dark green bars) or nondense breasts (n=20, light green bars) and 19 premenopausal women (yellow bars) underwent microdialysis of their left breast. Data represents extracellular local protein abundance in linear values (2^{NPX} as described in the Methods section). Kruskal-Wallis followed by Mann Whitney U-test was used for unpaired samples (healthy breast tissues) and Wilcoxon matched-pairs signed-rank for paired samples (breast cancer vs. normal adjacent breast). A. Extracellular local levels of von Willebrand's factor (VWF), B. Extracellular local levels of proteinase-activated receptor 1 (PAR1), C. Extracellular local levels of P-selectin, D. Extracellular local levels of soluble CD40 ligand (CD40L), E. Extracellular local levels of platelet-derived growth factor subunit A (PDGFA), F. Extracellular local levels of platelet-derived growth factor subunit B (PDGFB). Data are displayed as box plots with median and 10–90 percentile.

ns = not significance, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Figure 2. *Increased levels of coagulation factors in breast cancer and dense breast tissue*

A total of 69 women were investigated; 10 breast cancer patients underwent microdialysis before surgery with one catheter inserted into the breast cancer and another into adjacent normal breast tissue and 40 postmenopausal healthy volunteer women, attending the regular mammography-screening program categorized as either having dense (n=20, dark green bars)

or nondense breasts (n=20, light green bars) and 19 premenopausal women (yellow bars) underwent microdialysis of their left breast. Data represents extracellular local protein abundance in linear values (2^{NPX} as described in the Methods section). Kruskal-Wallis followed by Mann Whitney U-test was used for unpaired samples (healthy breast tissues) and Wilcoxon matched-pairs signed-rank for paired samples (breast cancer vs. normal adjacent breast). A. Extracellular local levels of Factor VII (FVII), B. Extracellular local levels of Factor XI (FXI). Data are displayed as box plots with median and 10–90 percentile. $^{**}P < 0.01$.

Figure 3. *Increased levels of fibrinolysis components in breast cancers and dense breast tissue* A total of 69 women were investigated; 10 breast cancer patients underwent microdialysis before surgery with one catheter inserted into the breast cancer and another into adjacent normal breast tissue and 40 postmenopausal healthy volunteer women, attending the regular mammography-screening program categorized as either having dense (n=20, dark green bars) or nondense breasts (n=20, light green bars) and 19 premenopausal women (yellow bars) underwent microdialysis of their left breast. Data represents extracellular local protein abundance in linear values (2^{NPX} as described in the Methods section). Kruskal-Wallis followed by Mann Whitney U-test was used for unpaired samples (healthy breast tissues) and Wilcoxon matched-pairs signed-rank for paired samples (breast cancer vs. normal adjacent breast). A. Extracellular local levels of vitamin K-dependent protein C (PROC), B. Extracellular local levels of plasma serine protease inhibitor 5 (SERPINA5), C. Extracellular local levels of tissue factor pathway inhibitor (TFPI), D. Extracellular local levels of Tissue plasminogen activator (tPA), E. Extracellular local levels of Urokinase-type plasminogen activator (uPA), F. Extracellular local levels of plasminogen activator inhibitor 1 (PAI), G. Extracellular local levels of soluble urokinase plasminogen activator receptor (suPAR). Data are displayed as box plots with median and 10–90 percentile.

654 ns = not significance, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

655

656 **Figure 4.** *Correlations between inflammatory mediators and coagulation factors in normal*
657 *human breast tissue in situ*

658 A total 42 postmenopausal healthy volunteer women, attending the regular mammography-
659 screening program and 19 healthy premenopausal women underwent microdialysis of their left
660 breast for sampling of extracellular proteins *in vivo*. Proteins were quantified using a multiplex
661 proximity extension assay as described in the Methods section. Interleukin (IL), transforming
662 growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), von Willebrand's
663 factor (VWF), proteinase-activated receptor 1 (PAR1), P-selectin, soluble CD40 ligand
664 (CD40L), Platelet-derived growth factor subunit A (PDGFA), Platelet-derived growth factor
665 subunit B (PDGFB), Factor VII (FVII), Factor XI (FXI), tissue factor pathway inhibitor (TFPI),
666 vitamin K-dependent protein C (PROC), tissue plasminogen activator (tPA), urokinase-type
667 plasminogen activator (uPA), soluble urokinase plasminogen activator receptor (suPAR),
668 plasminogen activator inhibitor 1 (PAI), plasma serine protease inhibitor 5 (SERPINA5). Bars
669 represent Spearman's Rank correlation coefficient. White bars, not significant; *$P < 0.05$,
670 **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

671

672 **Figure 5.** *Correlations between matrix metalloproteinases (MMPs) and coagulation factors in*
673 *normal human breast tissue in situ*

674 A total 42 postmenopausal healthy volunteer women, attending the regular mammography-
675 screening program and 19 healthy premenopausal women underwent microdialysis of their left
676 breast for sampling of extracellular proteins *in vivo*. Proteins were quantified using a multiplex
677 proximity extension assay as described in the Methods section. Matrix metalloproteinases
678 (MMP), von Willebrand's factor (VWF), proteinase-activated receptor 1 (PAR1), P-selectin,

679 soluble CD40 ligand (CD40L), Platelet-derived growth factor subunit A (PDGFA), Platelet-
680 derived growth factor subunit B (PDGFB), Factor VII (FVII), Factor XI (FXI), tissue factor
681 pathway inhibitor (TFPI), vitamin K-dependent protein C (PROC), tissue plasminogen
682 activator (tPA), urokinase-type plasminogen activator (uPA), soluble urokinase plasminogen
683 activator receptor (suPAR), plasminogen activator inhibitor 1 (PAI), plasma serine protease
684 inhibitor 5 (SERPINA5). Bars represent Spearman's Rank correlation coefficient. White bars,
685 not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$
686
687 **Figure 6.**
688 *Correlations between local estradiol levels and coagulation factors in abdominal subcutaneous*
689 *fat and normal human breast tissue in situ*
690 A total 42 postmenopausal healthy volunteer women, attending the regular mammography-
691 screening program and 19 healthy premenopausal women underwent microdialysis of their left
692 breast and abdominal subcutaneous for sampling of extracellular proteins and estradiol *in vivo*.
693 Proteins and estradiol (E2) were quantified as described in the Methods section. von
694 Willebrand's factor (VWF), proteinase-activated receptor 1 (PAR1), P-selectin, soluble CD40
695 ligand (CD40L), Platelet-derived growth factor subunit A (PDGFA), Platelet-derived growth
696 factor subunit B (PDGFB), Factor VII (FVII), Factor XI (FXI), tissue factor pathway
697 inhibitor (TFPI), vitamin K-dependent protein C (PROC), tissue plasminogen activator (tPA),
698 urokinase-type plasminogen activator (uPA), soluble urokinase plasminogen activator receptor
699 (suPAR), plasminogen activator inhibitor 1 (PAI), plasma serine protease inhibitor 5
700 (SERPINA5).
701 A. Correlations between local levels of estradiol (E2) and proteins in abdominal subcutaneous
702 fat.
703 B. Correlations between local levels of estradiol (E2) and proteins in normal breast tissue.

Bars represent Spearman's Rank correlation coefficient. White bars, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

C. Platelets were isolated and exposed to estradiol (E2) ± progesterone (P) or without hormones (Control) and activated with thrombin and the release of proteins was quantified as described in the materials and methods. uPA was not detectable. PROC is mainly produced in the liver and was therefore not analyzed.

*$P < 0.05$ compared to control, Student's t-test.

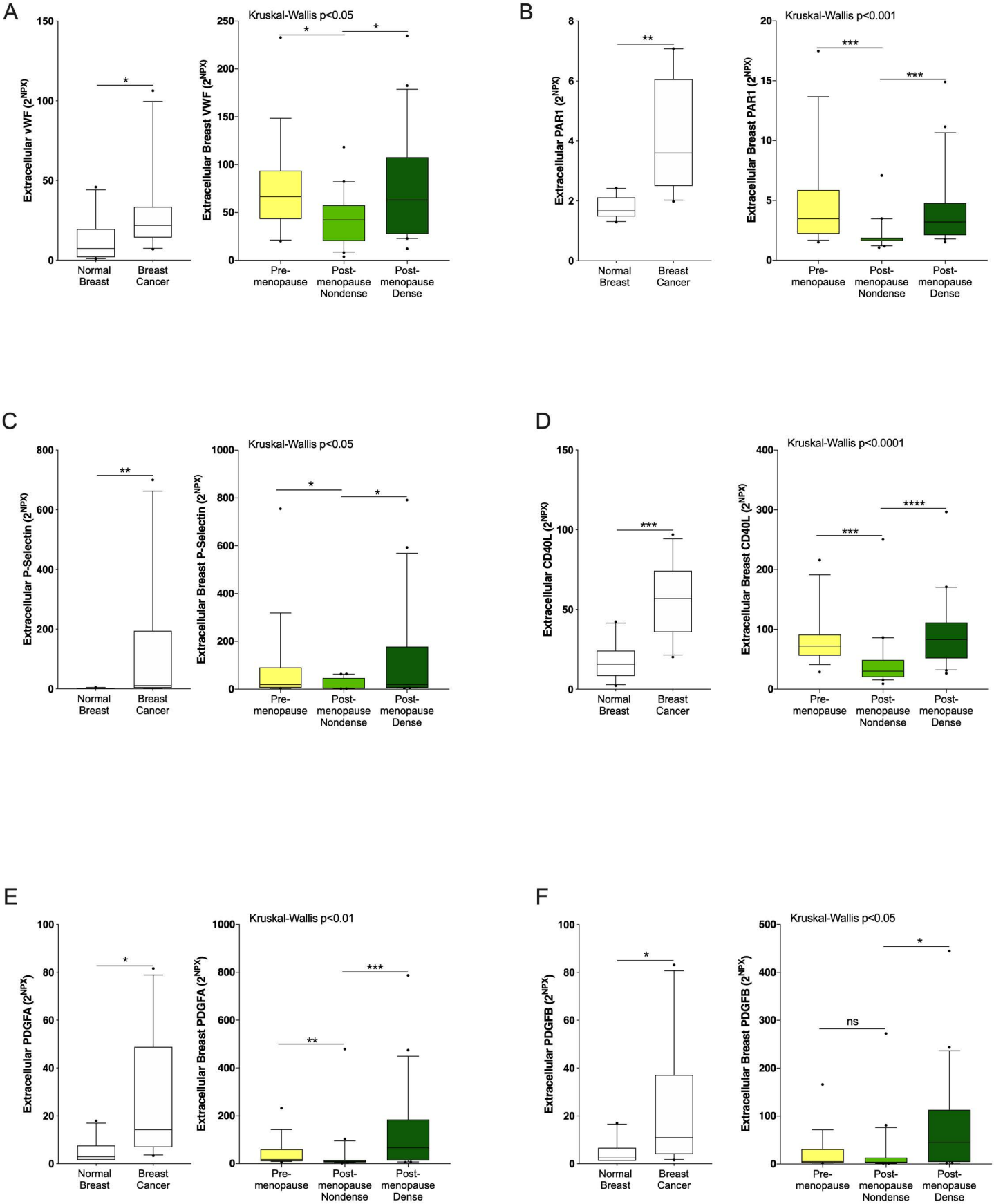
Figure 7. *Coagulation-inflammation loop in breast tissues with varying densities*

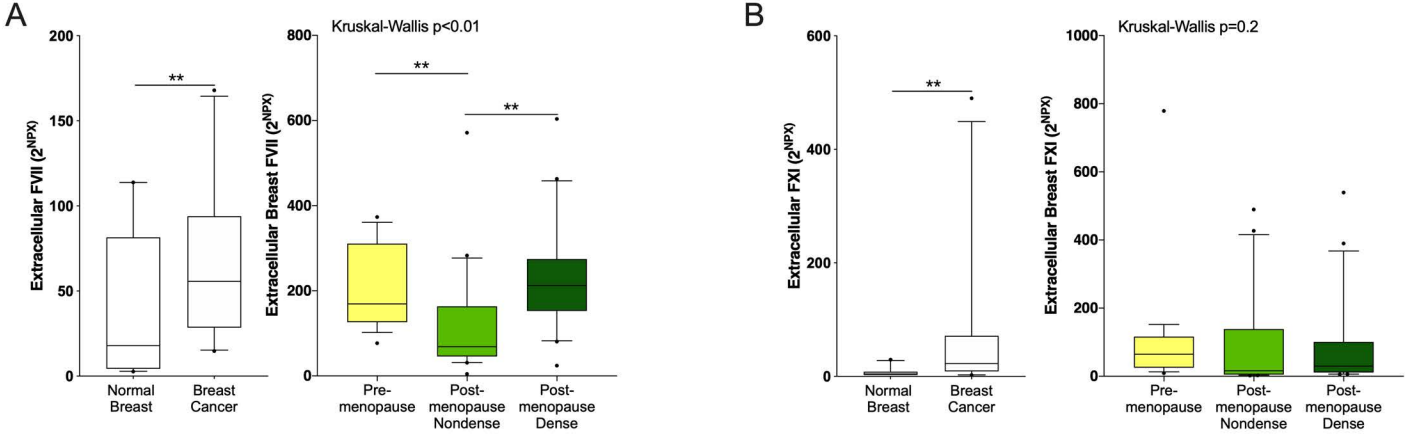
Nondense breast tissue (left) consists mainly of adipose tissue and low levels of inflammatory cytokines, MMPs and factors released from activated platelets. Dense breast tissue (right) on the other hand comprise higher amounts of collagen, stromal tissue and less adipose tissue and increased levels of inflammatory cytokines, MMPs and factors released from activated platelets. Higher breast density triggers the coagulation system; high abundance of pro-angiogenic factors increase the vascular permeability allowing platelets to mechanosense the increased stiffness, which in turn triggers the release of factors contained in the platelet granules starting a procoagulant cascade and recruitment of inflammatory cells to the site. In concert with other cell types in the tissue including immune cells, this will lead to an inflammatory cascade that creates an inflammation-coagulation cycle. Persistent inflammation and platelet activation will further promote tissue remodeling and fibrosis resulting in a pro-tumorigenic microenvironment. Less inflammation-coagulation interactions are present in nondense breast tissue because concentrations of the various factors are lower. Thus, if atypical cells arise in the breast epithelium, the microenvironment in dense breasts will be more permissive for continued growth and invasion and subsequent development into clinically significant breast cancer.

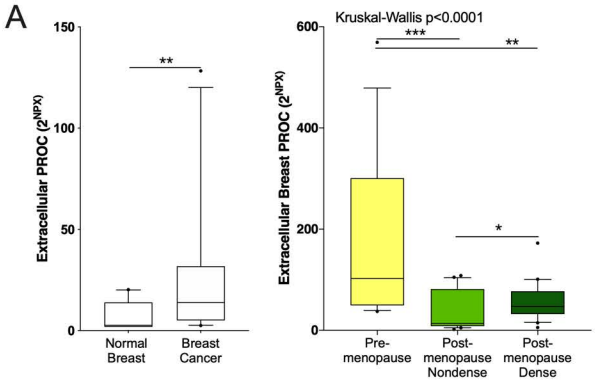
Table 1. Levels of extracellular proteins in microdialysis samples from subcutaneous abdominal fat tissue in postmenopausal women with dense or nondense breast tissue and premenopausal women. Median values (25-75 percentiles).

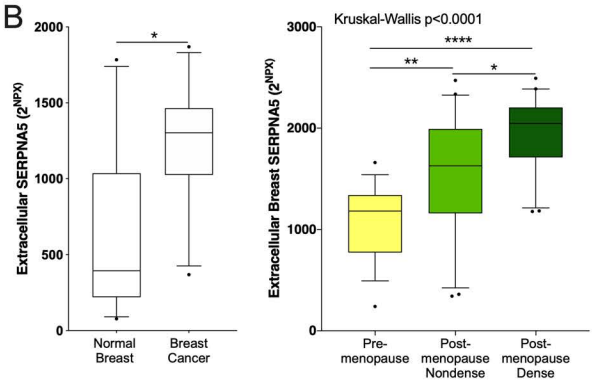
Protein (2^{NPX})	Nondense (*n*=20)	Dense (*n*=19)	Premenopausal (*n*=22)	*P*-value Kruskal-Wallis
PDGFA	7.7 (4.6-12.6)	10 (6.6-16)	18 (7-38)	0.1
PDGFB	2.9 (1.7-6.7)	3.5 (1.9-6.8)	5.1 (2-12)	0.3
PAR1	1.7 (1.5-2.1)	2 (1.7-2.6)	1.7 (1.4-2.7)	0.2
CD40L	38 (17-45)	46 (36-55)	42 (27-73)	0.3
P-Selectin	4.7 (3-11)	5.5 (4.7-21)	20 (3.9-34)	0.1
uPA	122 (54-264)	201 (113-327)	675 (306-1049)	<0.0001
PAI	74 (33-146)	128 (66-194)	168 (82-651)	0.04
suPAR	1413 (1019-2769)	2013 (1419-2966)	3852 (1943-5727)	0.01
tPA	37 (15-118)	94 (32-142)	54 (17-79)	0.2
FVII	76 (40-165)	144 (64-220)	160 (88-226)	0.1
VWF	28 (11-82)	38 (25-69)	70 (31-133)	0.1
FXI	17 (4-32)	23 (8-79)	46 (15-93)	0.1
SERPINA5	1828 (1478-1991)	1896 (1733-2031)	1053 (836-1334)	<0.0001
TFPI	267 (142-349)	343 (191-416)	305 (160-546)	0.3
PROC	18 (4-53)	36 (19-61)	67 (42-116)	0.01

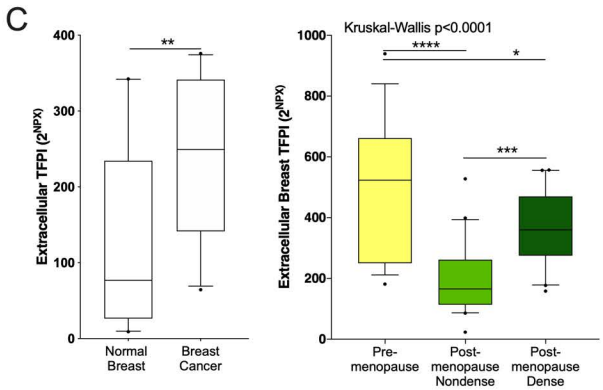
von Willebrand's factor (VWF), proteinase-activated receptor 1 (PAR1), P-selectin, soluble CD40 ligand (CD40L), Platelet-derived growth factor subunit A (PDGFA), Platelet-derived growth factor subunit B(PDGFB), Factor VII (FVII), Factor XI (FXI), tissue factor pathway inhibitor (TFPI), vitamin K-dependent protein C (PROC), tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), soluble urokinase plasminogen activator receptor (suPAR), plasminogen activator inhibitor 1 (PAI), plasma serine protease inhibitor 5 (SERPINA5).

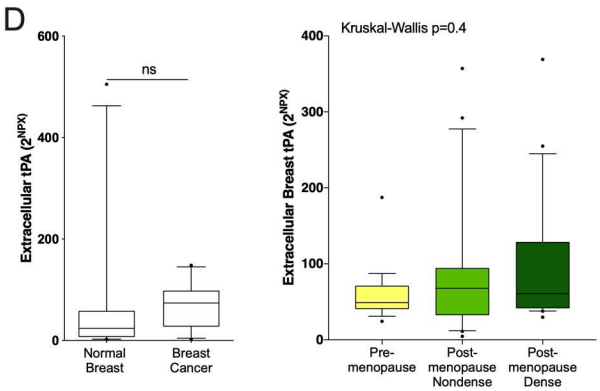

A
Extracellular vWF (2^NPX)
Normal Breast
Breast Cancer
Extracellular Breast VWF (2^NPX)
Kruskal-Wallis p<0.05
Pre-menopause
Post-menopause Nondense
Post-menopause Dense
B
Extracellular PAR1 (2^NPX)
Extracellular Breast PAR1 (2^NPX)
Kruskal-Wallis p<0.001
C
Extracellular P-Selectin (2^NPX)
Extracellular Breast P-Selectin (2^NPX)
Kruskal-Wallis p<0.05
D
Extracellular CD40L (2^NPX)
Extracellular Breast CD40L (2^NPX)
Kruskal-Wallis p<0.0001
E
Extracellular PDGFA (2^NPX)
Extracellular Breast PDGFA (2^NPX)
Kruskal-Wallis p<0.01
F
Extracellular PDGFB (2^NPX)
Extracellular Breast PDGFB (2^NPX)
Kruskal-Wallis p<0.05
ns

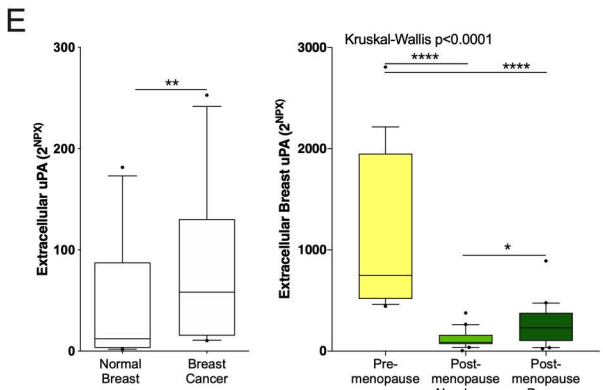

A
Extracellular FVII (2^NPX)
200
150
100
50
0
**
Normal Breast
Breast Cancer
Extracellular Breast FVII (2^NPX)
Kruskal-Wallis p<0.01
800
600
400
200
0
**
**
Pre-menopause
Post-menopause Nondense
Post-menopause Dense
B
Extracellular FXI (2^NPX)
600
400
200
0
**
Normal Breast
Breast Cancer
Extracellular Breast FXI (2^NPX)
Kruskal-Wallis p=0.2
1000
800
600
400
200
0
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

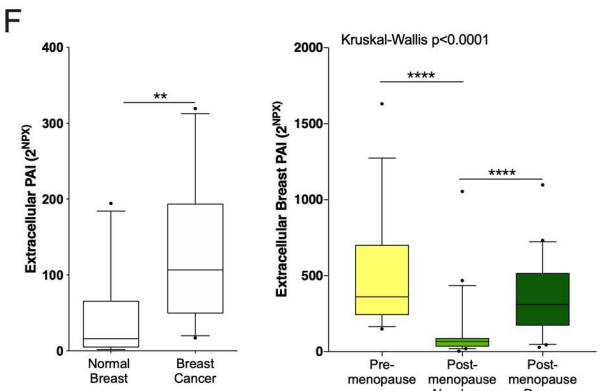

A
Extracellular PROC (2^NPX)
**
Normal Breast
Breast Cancer
Extracellular Breast PROC (2^NPX)
Kruskal-Wallis p<0.0001
***
**
*
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

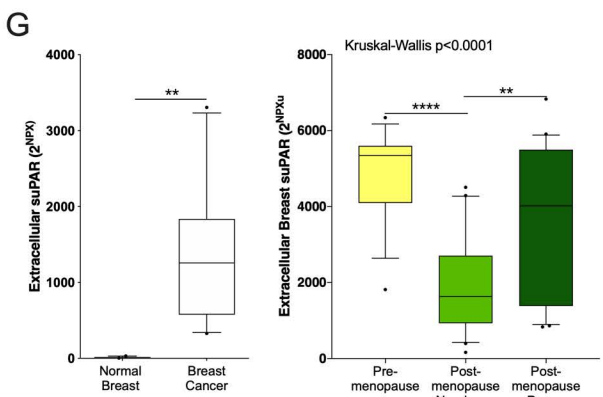

B
Extracellular SERPNA5 (2^NPX)
*
Normal Breast
Breast Cancer
Extracellular Breast SERPNA5 (2^NPX)
Kruskal-Wallis p<0.0001
****
**
*
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

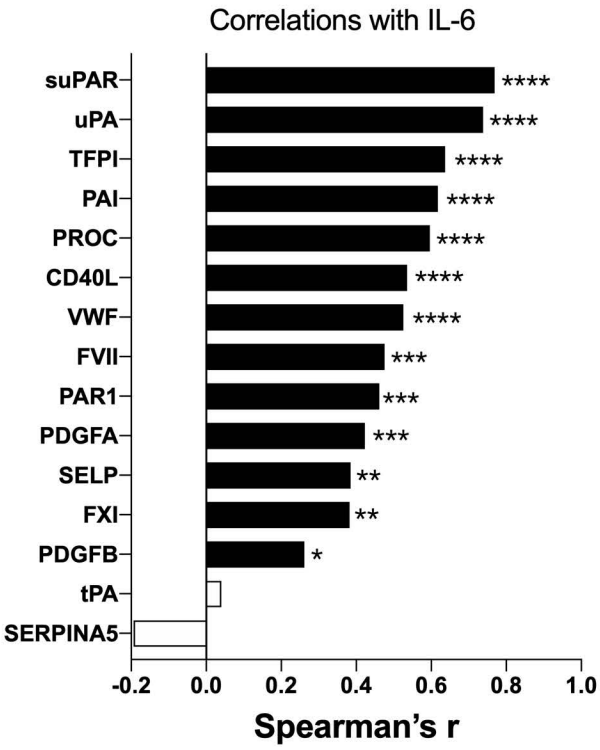

C
Extracellular TFPI (2^NPX)
**
Normal Breast
Breast Cancer
Extracellular Breast TFPI (2^NPX)
Kruskal-Wallis p<0.0001
****
*
***
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

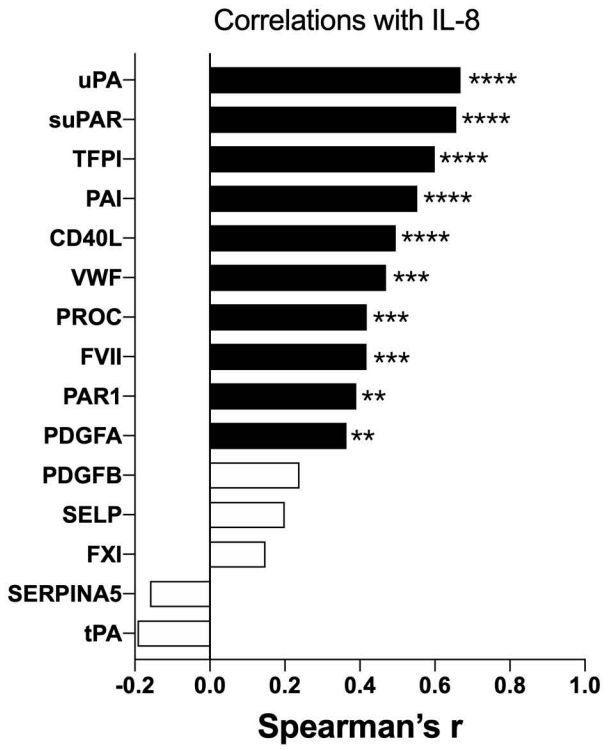

D
Extracellular tPA (2^NPX)
ns
Normal Breast
Breast Cancer
Extracellular Breast tPA (2^NPX)
Kruskal-Wallis p=0.4
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

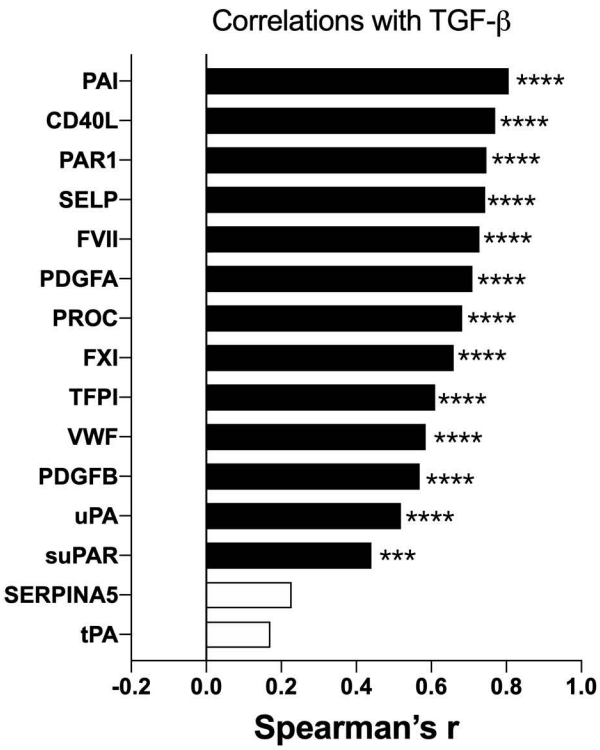

E
Extracellular uPA (2^NPX)
**
Normal Breast
Breast Cancer
Extracellular Breast uPA (2^NPX)
Kruskal-Wallis p<0.0001
****
****
*
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

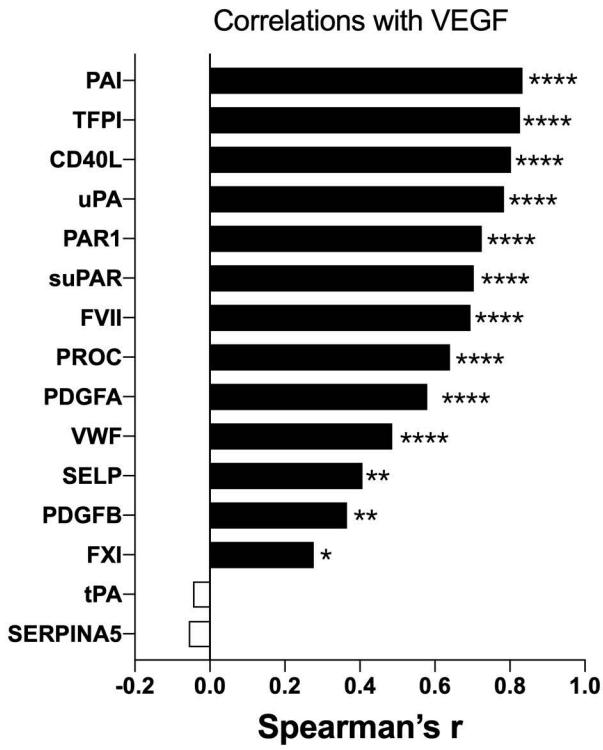

F
Extracellular PAI (2^NPX)
**
Normal Breast
Breast Cancer
Extracellular Breast PAI (2^NPX)
Kruskal-Wallis p<0.0001
****
****
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

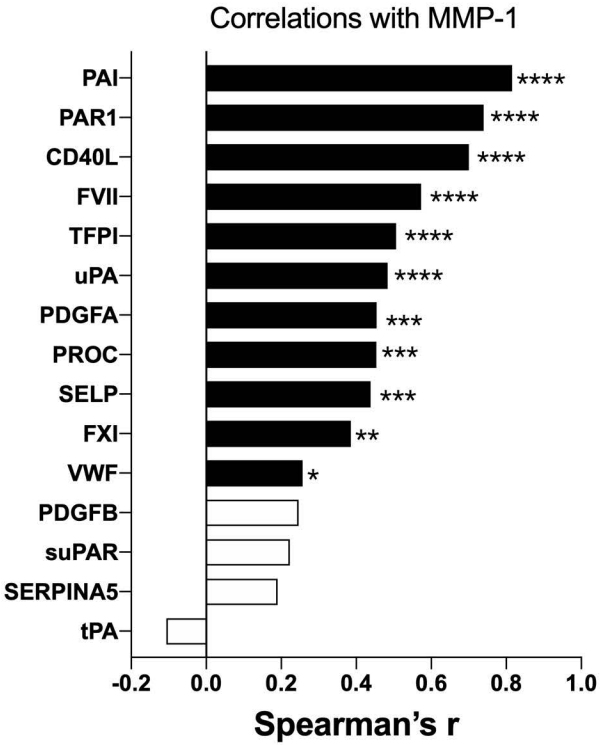

G
Extracellular suPAR (2^NPX)
**
Normal Breast
Breast Cancer
Extracellular Breast suPAR (2^NPXu
Kruskal-Wallis p<0.0001
****
**
Pre-menopause
Post-menopause Nondense
Post-menopause Dense

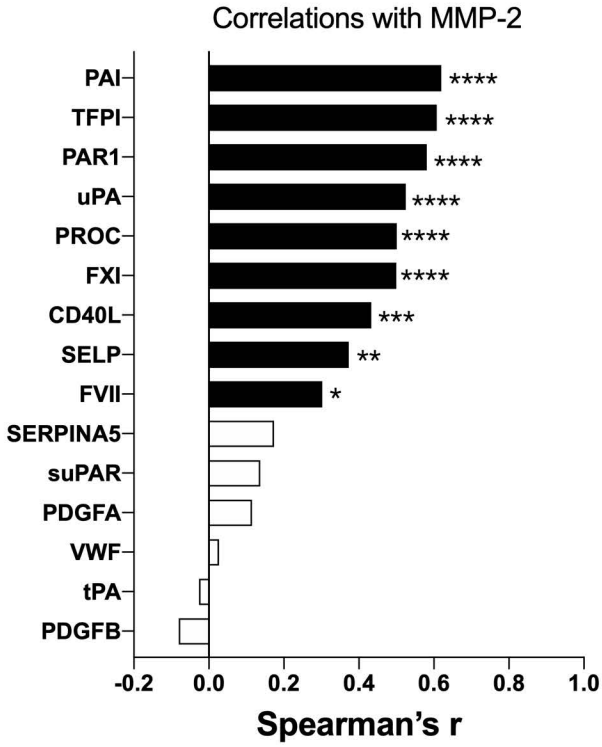

Correlations with IL-6
suPAR ****
uPA ****
TFPI ****
PAI ****
PROC ****
CD40L ****
VWF ****
FVII ***
PAR1 ***
PDGFA ***
SELP **
FXI **
PDGFB *
tPA
SERPINA5
-0.2 0.0 0.2 0.4 0.6 0.8 1.0
Spearman's r

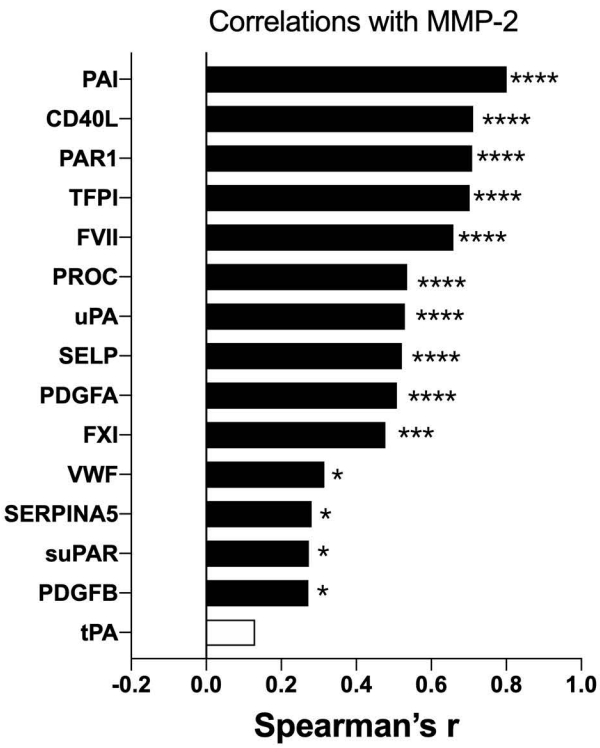

Correlations with IL-8
uPA ****
suPAR ****
TFPI ****
PAI ****
CD40L ****
VWF ***
PROC ***
FVII ***
PAR1 **
PDGFA **
PDGFB
SELP
FXI
SERPINA5
tPA
-0.2 0.0 0.2 0.4 0.6 0.8 1.0
Spearman's r

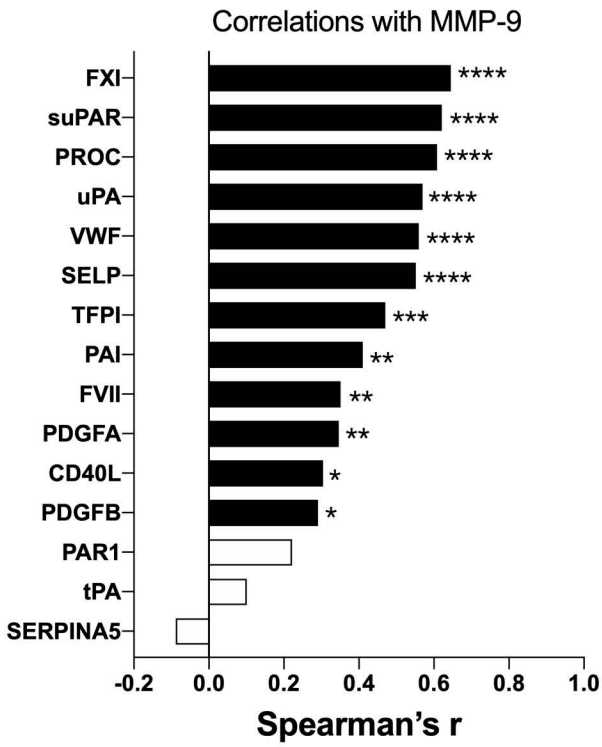

Correlations with TGF-β
PAI ****
CD40L ****
PAR1 ****
SELP ****
FVII ****
PDGFA ****
PROC ****
FXI ****
TFPI ****
VWF ****
PDGFB ****
uPA ****
suPAR ***
SERPINA5
tPA
-0.2 0.0 0.2 0.4 0.6 0.8 1.0
Spearman's r

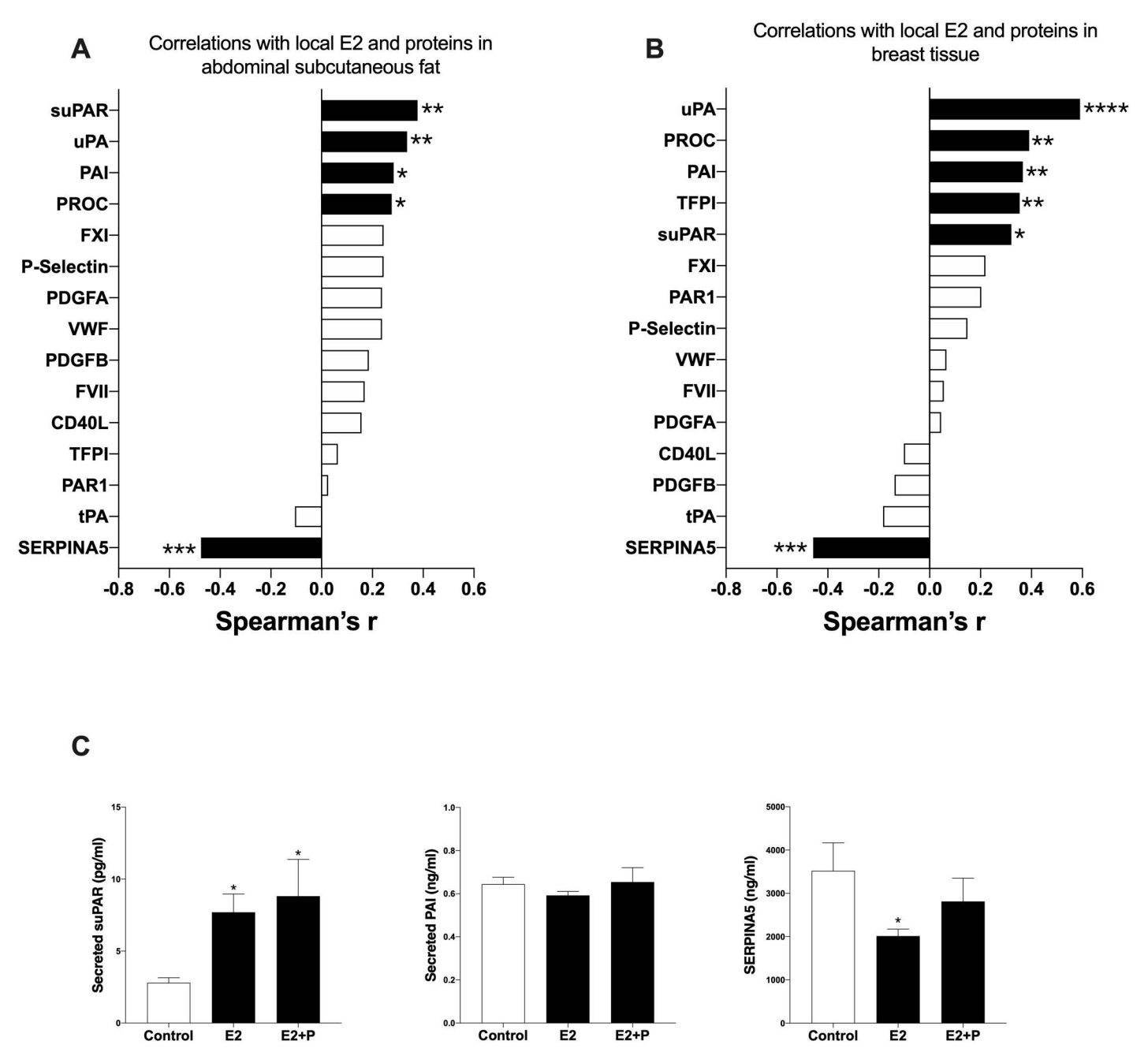

Correlations with VEGF
PAI ****
TFPI ****
CD40L ****
uPA ****
PAR1 ****
suPAR ****
FVII ****
PROC ****
PDGFA ****
VWF ****
SELP **
PDGFB **
FXI *
tPA
SERPINA5
-0.2 0.0 0.2 0.4 0.6 0.8 1.0
Spearman's r

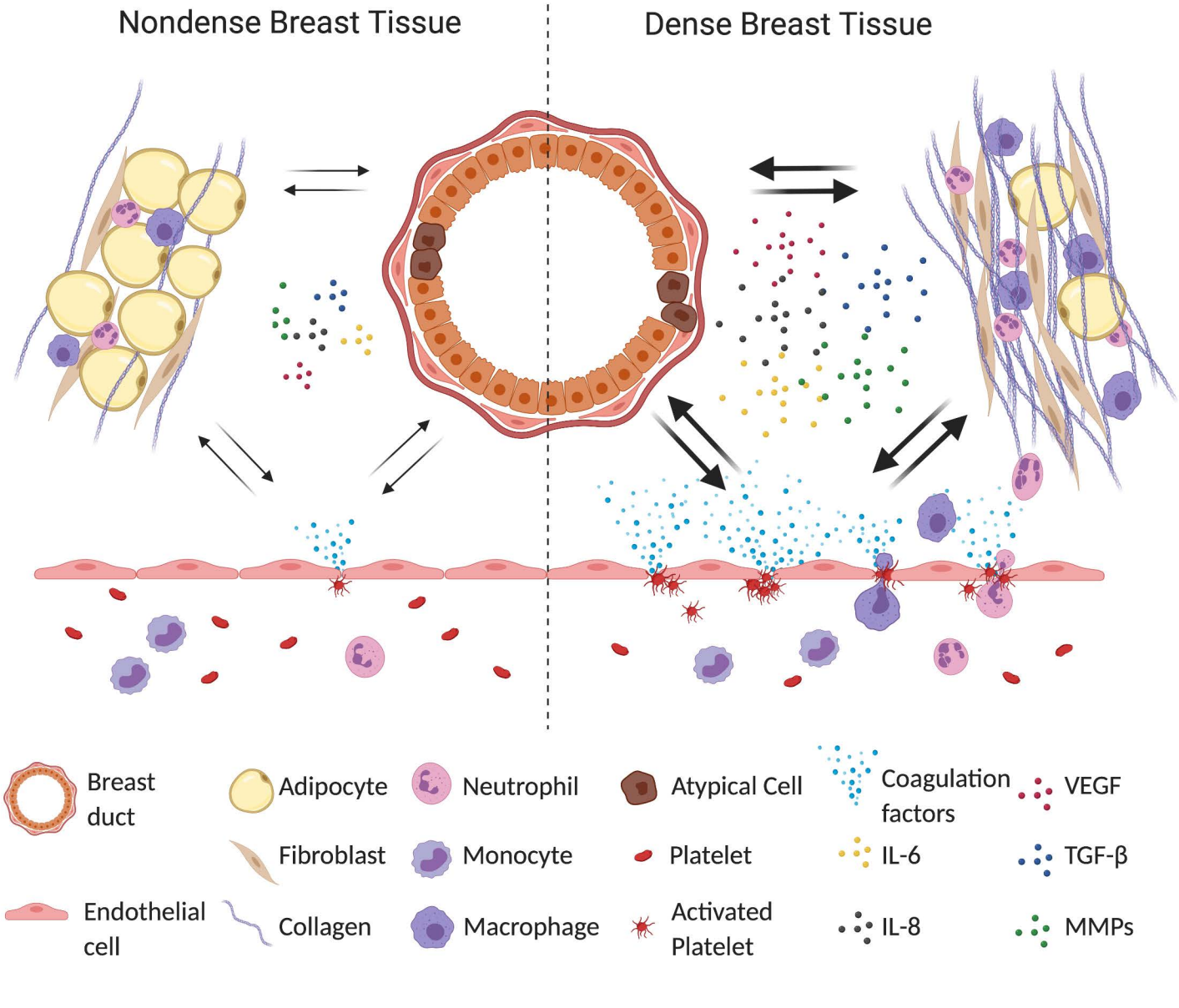

Correlations with MMP-1
PAI
PAR1
CD40L
FVII
TFPI
uPA
PDGFA
PROC
SELP
FXI
VWF
PDGFB
suPAR
SERPINA5
tPA
****
****
****
****
****
****
***
***
***
**
*
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Spearman's r


Correlations with MMP-2
PAI
TFPI
PAR1
uPA
PROC
FXI
CD40L
SELP
FVII
SERPINA5
suPAR
PDGFA
VWF
tPA
PDGFB
****
****
****
****
****
****
***
**
*
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Spearman's r


Correlations with MMP-2
PAI
CD40L
PAR1
TFPI
FVII
PROC
uPA
SELP
PDGFA
FXI
VWF
SERPINA5
suPAR
PDGFB
tPA
****
****
****
****
****
****
****
****
***
*
*
*
*
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Spearman's r


Correlations with MMP-9
FXI
suPAR
PROC
uPA
VWF
SELP
TFPI
PAI
FVII
PDGFA
CD40L
PDGFB
PAR1
tPA
SERPINA5
****
****
****
****
****
****
***
**
**
**
*
*
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Spearman's r


A
Correlations with local E2 and proteins in abdominal subcutaneous fat
suPAR
uPA
PAI
PROC
FXI
P-Selectin
PDGFA
VWF
PDGFB
FVII
CD40L
TFPI
PAR1
tPA
SERPINA5
**
**
*
*
***
-0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6
Spearman's r
B
Correlations with local E2 and proteins in breast tissue
uPA
PROC
PAI
TFPI
suPAR
FXI
PAR1
P-Selectin
VWF
FVII
PDGFA
CD40L
PDGFB
tPA
SERPINA5
****
**
**
**
*
***
-0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6
Spearman's r
C
Secreted suPAR (pg/ml)
15
10
5
0
*
*
Control
E2
E2+P
Secreted PAI (ng/ml)
1.0
0.8
0.6
0.4
0.2
0.0
Control
E2
E2+P
SERPINA5 (ng/ml)
5000
4000
3000
2000
1000
0
*
Control
E2
E2+P


Nondense Breast Tissue
Dense Breast Tissue
Breast duct
Adipocyte
Neutrophil
Atypical Cell
Coagulation factors
VEGF
Fibroblast
Monocyte
Platelet
IL-6
TGF-β
Endothelial cell
Collagen
Macrophage
Activated Platelet
IL-8
MMPs