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Title: Systemic Chemokine Levels in Breast Cancer Patients and Their Relationship with Circulating Menstrual Hormones

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Abstract

Introduction: The chemokines Stromal Cell-Derived Factor-1α (SDF-1α/CXCL12) and Monocyte Chemotactic Protein-1 (MCP-1/CCL2) have been implicated in breast cancer progression. We recently reported elevated systemic MCP-1 in breast cancer patients. This study investigated circulating levels of SDF-1α in breast cancer patients, and addressed potential hormonal regulation of these two potent chemokines. Methods: SDF-1α levels were determined by ELISA in 114 breast cancer patients and 85 controls, and correlated with clinical data. Blood samples were collected from 36 healthy premenopausal volunteers weekly for four weeks to measure Luteinising Hormone (LH), Follicular Stimulating Hormone (FSH), Oestradiol and Progesterone using a Bayer ADVIA® Centaur Immunoassay system, in parallel with SDF-1α and MCP-1. CXCL12 expression was determined using RQ-PCR in primary tumour stromal cells (n=16) harvested at surgery. Results: Plasma SDF-1α was significantly higher in breast cancer patients than age-matched controls and had a significant correlation with tumour grade and epithelial subtype. Investigation of menstrual variations of these chemokines revealed lower SDF-1α levels in the mid-luteal phase of the menstrual cycle and a significant positive correlation with circulating Oestradiol. MCP-1 levels showed no correlation with menstrual hormones. There was a trend towards increased CXCL12 expression in tumour compared to normal stromal cells. Conclusions: The elevated level of SDF-1α detected in breast cancer patients, and it's correlation with prognostic indicators, highlights the importance of this chemokine in disease progression. Elucidation of factors influencing chemokine secretion supports clarification of their role in tumourigenesis.

Key Words: Breast Cancer; Menstrual Hormones; Monocyte Chemotactic Protein-1 (MCP-1/CCL2); Prognosis; Stromal Cell-derived Factor 1-α (SDF-1α/CXCL12).

Introduction

Breast cancer development and progression is influenced by intrinsic properties of the tumour cells, as well as macro-environmental factors, with an intensive interplay existing between the tumour cells and signalling molecules such as chemokines [1]. Chemokines are chemotactic cytokines that act by altering the function of target cells in a paracrine, autocrine or endocrine manner. Chemokine gradients are central to the movement of cells in many normal and pathological processes by inducing cytoskeletal rearrangement, adhesion and directional migration. Chemokine receptor expression and activation on malignant cells have previously been shown to promote growth, survival and migration of cancer cells within and from the primary tumour site, and thereby contribute to locoregional and metastatic spread [2, 3]. Alterations in the systemic concentration of such chemokines may have potential for use as a marker of disease prognosis and monitoring, as in the case of other established tumour biomarkers, such as Prostate Specific Antigen or CA 15.3 [4, 5].

Two chemokines which have recently been highlighted as having roles in breast cancer are Stromal Cell Derived Factor-1α (SDF-1α) and Monocyte Chemotactic Protein-1 (MCP-1) [6-8]. Interaction of SDF-1α with its main receptor, CXCR4, has a pivotal role to play in increased survival of cancer cells within the tumour, establishment of a tumour promoting chemokine network, as well as the directed migration of cancer cells to sites of metastasis [6]. Normal breast tissues express very low levels of CXCR4, whereas breast neoplasms overexpress the receptor, with the highest expression reported in the most invasive tumour types [6, 7, 9]. Although CXCR4 was previously believed to be the sole receptor for SDF-1α, more recently CXCR7 (RDC1, CCX-CKR2) has also been shown to be a target for this chemokine [10, 11]. CXCR7 promotes cell survival, growth, and adhesion *in vitro* and *in vivo* and has been shown to be expressed in several tumour cell lines, including the breast tumour lines MCF-7 and MDA-MB-435 [10]. Within the primary tumour, local production of SDF-1α promotes the growth of breast cancer cells, as well as the initial stages of angiogenesis [12]. Additionally, sites to which breast cancer cells preferentially metastasise, such as bone, liver, brain and lung, produce abundant amounts of SDF-1α [6]. Previous reports have focused on SDF-1α and CXCR4 expression in breast cancer tissues primarily using immunohistochemistry and PCR based techniques [6]. Li et al [9] demonstrated by immunohistochemistry and RQ-

PCR that HER2-neu upregulates the expression of CXCR4, which is required for HER2-neu enhanced invasion, migration, adhesion and metastasis to the lung and also that CXCR4 expression is correlated with overall patient survival in breast cancer, which has important clinical implications. Signalling through CXCR4 may contribute to critical pathways that determine the survival, invasion, or growth of disseminated HER2-neu-expressing breast cancer cells. HER2-neu could enhance the expression of CXCR4, which was required for metastatic colonization by HER2-neu expressing cells [9, 13].

In a recent multi-center study by Hassan et al [14], low plasma SDF-1α was found to be predictive of distant metastasis and an independent prognostic marker in breast cancer patients. This led to the hypothesis that elevated circulating SDF-1α levels in breast cancer patients may act to retain tumour cells in the circulation and prevent them from homing to their metastatic sites, with lower levels promoting establishment of metastases [14], thereby highlighting SDF-1α as a potential prognostic marker in breast cancer.

Monocyte Chemotactic Protein-1 is a 76-amino acid protein and is well established as a chemotactic factor regulating the recruitment of monocytes/macrophages and other inflammatory cells to sites of inflammation. MCP-1 is not only secreted by many activated inflammatory or immune cells, but also by a variety of human and murine tumour cells [15]. Recent research has implicated MCP-1 as an active participant in the tumour microenvironment, influencing factors such as tumour-associated macrophages, growth, angiogenesis and metastasis [8, 16, 17]. We have previously reported a trend toward higher levels of circulating MCP-1 in breast cancer patients compared to age-matched controls [18], with no significant correlation to commonly used prognostic indicators identified. A consensus has yet to be reached as to whether circulating MCP-1 is a positive or negative prognostic indicator in breast cancer patients [19, 20].

Circulating chemokines can potentially be regulated by endogenous hormones. The relationship between SDF-1α and circulating menstrual hormones has never previously been investigated, although it is known to be hormonally regulated at a cellular level and to mediate the proliferative action of Oestrogen [21]. Given that SDF-1α is one of the key factors implicated in the local micrometastatic milieu, alterations in the circulating levels of such a potent chemokine in a particular phase of

the menstrual cycle may suggest a decreased potential for micrometastasis establishment in that phase. The question of hormonal regulation of systemic MCP-1 has previously been raised, but not fully elucidated [22]. This laboratory previously reported [18] higher levels of MCP-1 in postmenopausal versus premenopausal breast cancer patients, suggesting underlying hormonal control of this chemokine. Premenopausal levels of both chemokines may correlate with phase of menstrual cycle and thereby influence timing of surgical intervention, as has been previously reported with other cytokines, such as Vascular Endothelial Growth Factor (VEGF) [23]. Both SDF-1α and MCP-1 have been implicated in the progression of breast cancer, having potential roles in angiogenesis and initiation of metastasis. The factors controlling these potent chemokines undoubtedly warrant further investigation.

The aim of this study was to investigate whether circulating SDF-1α levels are altered in breast cancer patients compared to age-matched healthy controls. A potential relationship between SDF-1α, MCP-1 and circulating menstrual hormones was also investigated.

We report that levels of SDF-1α were significantly elevated in breast cancer patients compared to age matched controls, and exhibited a significant positive correlation with two well established indicators of prognosis, tumour grade and epithelial subtype. Examination of systemic levels of SDF-1α and MCP-1 throughout the menstrual cycle revealed that, while serum MCP-1 levels did not differ significantly across the menstrual cycle, plasma SDF-1α levels were significantly lower in the mid-luteal phase and showed a significant correlation with circulating Oestradiol.

Materials & Methods

Study cohort

Following ethical committee approval and written informed consent, preoperative blood samples were collected prospectively from 114 consecutive breast cancer patients (36 pre-menopausal, 78 post-menopausal) and 85 age-matched controls (34 pre-menopausal, and 51 post-menopausal) (Table 1). All patients had histologically confirmed breast cancer and patients with benign disease or distal metastatic disease were excluded. Forty six patients had axillary node positive disease at the time of recruitment. One patient in the breast cancer cohort had received neo-adjuvant chemotherapy. Ten premenopausal patients were taking the Oral Contraceptive Pill

and five postmenopausal patients were on Hormone Replacement Therapy at the time of recruitment. Post-menopausal status was defined as amenorrhea for more than 12 months. Control samples were collected from healthy women attending an outpatient facility, with no current or previous malignancy or inflammatory condition.

To study the relationship between SDF-1α, MCP-1 and menstrual hormones, plasma and serum samples were taken from 36 premenopausal healthy females, on a weekly basis for four consecutive weeks, i.e. 144 samples in total. Measurement of SDF-1α, MCP-1, FSH, LH, Oestradiol and Progesterone were performed on each individual sample as described below. Demographic data such as age, date of last menstrual period and current medications was also collected from this group.

Blood collection

Whole blood was collected in a Vacuette EDTA K3E blood bottle (Grenier Bio-one) for plasma (SDF-1α detection) and Vacutainer Serum Separator Tubes II (Becton Dickinson) for serum (MCP-1 detection). Upon receipt, samples for serum collection were allowed to clot for 30 minutes and then all samples were centrifuged at 2000rpm @ 4°C for 10mins in a Sorvall RT6000D centrifuge. Plasma/serum was removed, aliquoted and stored at -20°C until required.

Chemokine Detection

Levels of SDF-1α and MCP-1 in patient plasma and serum samples were measured using Quantikine® Enzyme Linked Immunosorbent Assay (ELISA) kits (R & D Systems) according to the manufacturer's protocol. Briefly, this involves use of microplates that have been precoated with an antibody specific for the target chemokine. Samples or standards are applied to the wells for a fixed period of time, aspirated, and the wells washed repeatedly. A secondary target-specific conjugate is then added for a fixed time period, followed by substrate solution and a "stop" solution. The optical density of each well is then read on a microplate reader at 450nm wavelength, and the chemokine concentration in each sample determined using the standard curve. This permitted direct quantification of the levels of SDF-1α and MCP-1. All samples were analysed in duplicate.

Analysis of menstrual status

Serum Luteinising Hormone (LH), Follicular Stimulating Hormone (FSH), Oestradiol and Progesterone were measured by direct chemiluminescence, using Siemens ADVIA® Centaur™ Immunoassay System. LH peak was taken to denote mid-cycle, while the mid-luteal phase was marked by a peak in systemic Progesterone. Other phases of the cycle were extrapolated from these two time points.

Culture of Primary Stromal Cells

Following ethical committee approval and written informed consent, fresh specimens of human breast cancer were harvested from patients undergoing surgery. Breast tissue obtained from reduction mammaplasty served as normal controls. Primary stromal cell cultures, both tumour and normal, were prepared from the fresh tissue samples, as previously described [24]. Briefly, tissues were washed in Phosphate Buffered Saline (PBS) supplemented with 200 IU/ml Penicillin/200mg/ml Streptomycin, minced finely using crossed scalpels, then digested in 0.1% Collagenase Type III (Biochem Corp) at 37°C for 12-18 hrs in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Stromal cells were isolated from mixed cell populations by differential centrifugation and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Heat Inactivated FBS (HiFBS). Medium was changed twice weekly and cells passaged every 7-10 days.

Analysis of SDF-1α/CXCL12 Gene Expression

Total RNA was isolated from primary breast cancer stromal cells (n=16), and normal stromal cells (n=4) using the RNeasy® Mini Kit (QIAGEN Ltd.) according to manufacturer's instructions, and included an on-column DNase treatment step. RNA concentration and integrity were determined using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA), and 2100 BioAnalyser (Agilent Technologies, Waldbronn, Germany), respectively. First strand cDNA was generated from 1μg of total RNA by reverse transcription using SuperScript III reverse transcriptase enzyme (Invitrogen, Carlsbad, CA, USA). cDNA samples were amplified and analysed by real-time quantitative PCR (RQ-PCR) using the ABI Prism 7000 (Applied Biosystems, Warrington, UK). Taqman® Universal Master Mix was used as well as Taqman® Gene Expression Assays (Applied

Biosystems) designed for the target gene (SDF-1α/CXCL12), and endogenous RNA reference genes Hypoxanthine Guanine Phosphoribosyltransferase-1 (HPRT1) and Glucoronidase beta (GUSB). The comparative CT method for relative quantification was used, allowing determination of the quantity of SDF-1α/CXCL12 in stromal cells normalised to HPRT1 and GUSB, and levels in tumours cells expressed relative to normal stromal cells. Results were expressed in a linear form using the formula $2^{-\Delta\Delta CT}$.

Statistical Analysis

Data are presented as Mean ± SEM. Data were analysed using the software package SPSS 14.0 for Windows. Results with a $P<0.05$ were considered statistically significant. All tests were two-tailed. Levene's test confirmed equal variance of observations in each group and permitted parametric data to be compared using a student's unpaired t-test. Normality was confirmed using the Kolmogorov-Smirnov test. Linear regression analysis was preformed to investigate the relationship between plasma SDF-1α levels and commonly used clinical prognostic indicators. Pearson correlation and multiple regression analysis were used to identify any relationship between systemic chemokine levels, menstrual hormones and phase of menstrual cycle.

Results

Systemic SDF-1α levels

SDF-1α levels were measured in plasma samples harvested from 114 breast cancer patients (preoperative) (Table 1), and 85 age-matched controls (34 premenopausal, 51 postmenopausal). Plasma SDF-1α was significantly higher in the breast cancer cohort (2448 ± 49pg/mL) than in healthy controls (2141 ± 58pg/mL, $p<0.00005$) (Figure 1a). When divided on the basis of menopausal status, within the breast cancer group, postmenopausal patients had significantly higher SDF-1α than premenopausal patients, and this difference was not reflected in the control cohort ($p<0.05$, Figure 1a). There was no significant difference in grade or size of tumours between pre- and post-menopausal groups. Only one patient had received neoadjuvant chemotherapy prior to recruitment (SDF-1α 2213.9pg/mL). Ten premenopausal patients were taking

the Oral Contraceptive Pill (Mean SDF-1α 2450.11pg/mL), and five postmenopausal patients were on Hormonal Replacement Therapy (Mean SDF-1α 2485.12pg/mL).

Clinical Correlations

The histological tumour profile of patients included in this study reflected that of a typical breast cancer cohort (Table 1), with the majority of ductal histology. Analysis of clinical data revealed a significant relationship between the prognostic indicator tumor grade and plasma SDF-1α levels (Pearson correlation coefficient=0.194, $p<0.05$) (Figure 1b), with higher plasma SDF-1α levels associated with tumours of higher grade. Tumours were also classified according to epithelial subtype. As expected, the majority of tumours were Luminal A (Table 1). Increasing plasma SDF-1α levels were found to have a significant relationship with the subtypes associated with poorer prognosis, i.e. Basal and Her2 (Pearson correlation coefficient=0.260, $p<0.05$) (Figure 1c). A potential relationship between SDF-1α and available Nottingham Prognostic Index (NPI) scores was also investigated and a non-significant positive correlation was found (Pearson correlation coefficient = 0.146, $p=0.227$, results not shown).

Hormonal Control of Chemokines

The mean age of the premenopausal control cohort was 25 years (Median = 23 years). All cycles were found to be ovulatory, that is, each showed an LH surge followed by an appropriate mid-luteal peak of Progesterone. Nine patients were taking an oral contraceptive pill at the time of the study and this had no significant impact on circulating SDF-1α or MCP-1 levels. Plasma SDF-1α was significantly lower in the mid-luteal phase of the menstrual cycle (2157 ± 60 pg/ml, $p<0.05$) than other time points, late luteal/early follicular (2387 ± 69 pg/ml), mid-follicular (2267 ± 65 pg/ml), and mid-cycle (2349 ± 71 pg/ml) (Figure 2a). SDF-1α displayed a significant correlation with Oestradiol ($r = 0.213$, $p<0.005$) throughout the cycle (Figure 2b), with SDF-1α at it's lowest when Oestradiol was at its highest. MCP-1 levels did not differ significantly across the menstrual cycle and did not show any significant correlation to menstrual hormones (Figure 2c).

Analysis of SDF-1α/CXCL12 Gene Expression

Following separation into individual stromal cell populations, total RNA was extracted and the SDF-1α/CXCL12 expression level was quantified in tumour stromal cells (n=16, labelled A-P) compared to normal stromal cells (n=4) harvested at reduction mammaplasty (Fig. 3). Results are expressed as Relative Quantity (Log_{10}) with the levels of expression in each sample corrected to the level of endogenous control (GUS & HPRT), and expressed relative to that detected in normal stromal cells, represented by the baseline of the graph. Although variable, there was an overall trend towards increased SDF-1α gene expression in tumour compared to normal stromal cells.

Discussion

Prognostic markers are markers that have been found to have a robust association with some clinical outcome, such as overall survival or recurrence-free survival [4]. It remains crucially important to define more sensitive and specific indicators of prognosis that reflect the complicated and heterogeneous nature of breast cancer, and could identify patients at greatest risk of disease progression. Lymph node status, tumour size and grade, epithelial subtype and lymphovascular invasion are among the prognostic indicators currently employed to predict an individual's risk of progression [25].

The data presented here reveals that breast cancer patients have a significantly higher level of plasma SDF-1α than healthy age-matched controls. Studies have shown that CXCR4 is present on platelets, and so to measure circulating levels of free SDF-1α, platelet free plasma was used [26]. Plasma SDF-1α levels in our control group (2141 ± 58 pg/mL) were similar to those previously reported in healthy subjects (2192 ± 76.4 pg/mL) [27]. Circulating levels of SDF-1α have recently been reported in patients with multiple myeloma [28] and colorectal cancer [29]. In multiple myeloma patients, levels of SDF-1α were found to positively correlate with the degree of bone marrow angiogenesis, while in colorectal cancer, low circulating levels correlated with higher tumour grade and CXCR4 expression [28, 29]. In an extensive study Hassan et al [14] recently reported low plasma SDF-1α as a predictive marker of distant breast cancer metastasis and poorer survival, which contrasts with the results presented here. Although the cohort studied here is smaller, there were also significant differences in study design. Their patient cohort was divided based on plasma SDF-

1α into “Lo” (<2295 pg/mL), “Mid” (2296 – 2557 pg/Ml) and “Hi” (>2557 pg/mL) with the relative risk of dying from breast cancer 5.17-fold higher in the “Lo” versus “High” group. The values found in the “Lo” group are similar to those found in normal healthy controls reported in our study and by others [27], and also this “Lo” group was not compared to a healthy age-matched control group. Low plasma SDF-1α was also associated with younger age at time of surgery, however menstrual or menopausal status was not specified. The increased levels detected in breast cancer patients versus age-matched controls in the current study had a significant predictive relationship to tumour grade (I-III), an indicator of the degree of tumour cell differentiation and a well established predictor of prognosis.

Breast cancer epithelial subtypes identified by gene microarray studies capture the heterogeneity of breast cancer and have a statistically robust relationship with disease-free and overall survival [25]. Plasma SDF-1α levels also had a significant association with epithelial subtype, with higher SDF-1α levels seen in those classically associated with poorer prognosis, such as basal subtype. The relationship reported here between plasma SDF-1α and these subtypes further highlights the potential significance of this chemokine in disease progression. It is noteworthy that plasma levels also displayed a relationship with Nottingham Prognostic Index (NPI) values, which takes into account tumour size, grade and lymph node status [30], however this relationship did not achieve statistical significance ($p = 0.227$). The elevated pre-operative levels in breast cancer patients highlights the potential for SDF-1α as a novel diagnostic or prognostic biomarker for this disease. Further research is required to determine whether any relationship exists between SDF-1α and end-points such as disease free survival and overall survival.

It is well established that SDF-1α is secreted by many different cell types, so undoubtedly there are other systemic factors at play in this group, such as the already firmly established constitutive expression of SDF-1α at the primary sites of breast cancer metastasis including bone, liver, brain and lung tissues [6]. In agreement with previous reports [12, 31], in the current study we detected increased levels of SDF-1α gene expression in stromal cells derived from primary breast tumours, relative to the levels detected in normal breast tissue harvested at reduction mammaplasty. Hassan et al [14] found no significant difference in SDF-1α levels before and after tumour

resection in a cohort of 22 patients. Further research is required to determine the true contribution of tumour-derived SDF-1α to systemic levels of the chemokine.

Postmenopausal patients had significantly higher plasma SDF-1α than pre-menopausal breast cancer patients, a difference that was not seen in the corresponding control groups. SDF-1α has been shown to be an Oestrogen receptor-regulated gene and mediator of the mitogenic effects of Oestradiol in ovarian and breast cancer cell lines [21]. The relationship between systemic SDF-1α and circulating hormones has never previously been investigated. In this study, based on 36 healthy premenopausal subjects and 144 samples in total, a cyclical variation in circulating SDF-1α in relation to the phases of the menstrual cycle was demonstrated, suggesting a potential relationship between this chemokine and menstrual hormones, while no such relationship was observed with MCP-1. The level of SDF-1α was lowest at mid-luteal phase while Oestradiol levels were highest. The elevated systemic levels in postmenopausal compared to premenopausal breast cancer patients may be explained by a deregulation of Oestrogen control in this group, potentially mediated by Oestrogen receptor coactivators/corepressors whose expression is altered in breast cancer [32]. The importance of this novel finding is increased in the light of a study by Holdaway et al [33] which has shown that the hormone profile of the menstrual cycle is maintained in patients with breast cancer. Thus it is reasonable to extrapolate these findings in normal premenopausal women to those with breast cancer.

The controversy of timing of surgical intervention in premenopausal breast cancer patients was initiated by Hrushesky et al [34] and has since been supported by 4 major studies, all favouring the second half of the menstrual cycle as optimal for surgery [35-38]**.** Our findings indicate that Oestradiol may cause down-regulation of SDF-1α in this phase. Given that SDF-1α is one of the key factors implicated in the local micrometastatic milieu, alterations in the circulating levels of such a potent chemokine in a particular phase of the menstrual cycle may suggest a decreased potential for micrometastasis establishment in that phase.

A previous study investigated MCP-1 levels through the menstrual cycle in a small group (n=18, one sample per subject) of healthy premenopausal subjects who were not on oral contraceptives and found a significant decrease in MCP-1 levels from the follicular to the luteal phase of the cycle [22]. Clearly the number of women in each phase of the cycle was small. In the current study, over the 144 samples taken, there

was no correlation found between circulating MCP-1 and menstrual hormones, with no cyclical variation observed. This suggests differing control mechanisms operating between these two important chemokines.

The results presented here showing elevated SDF-1α in breast cancer patients, and it's association with commonly used prognostic indicators, highlights the potential importance of this chemokine in disease progression. Further study of SDF-1α and its receptors, both systemically and within the tumour microenvironment, may ultimately support prediction of metastatic potential, monitor response to treatment, or advance therapeutic options for this disease. Increased elucidation of the intrinsic factors that regulate this potent chemokine may have implications in terms of timing surgical intervention in premenopausal breast cancer patients and lead to increased understanding of the biology of chemokine regulation.

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Table 1. Patient Characteristics

	Breast Cancer Patients
Number of subjects	
Premenopausal	36
Postmenopausal	78
Mean Age (years)	
Premenopausal	42
Postmenopausal	58
Histology	
Ductal	81
Lobular	16
Other	14
Unknown	3
Epithelial Subtype	
Luminal A	73
Luminal B	10
Her2	4
Basal	6
Unknown	21
Grade	
I	13
II	54
III	39
Unknown	8

Table 1 Demographics of breast cancer cohort.

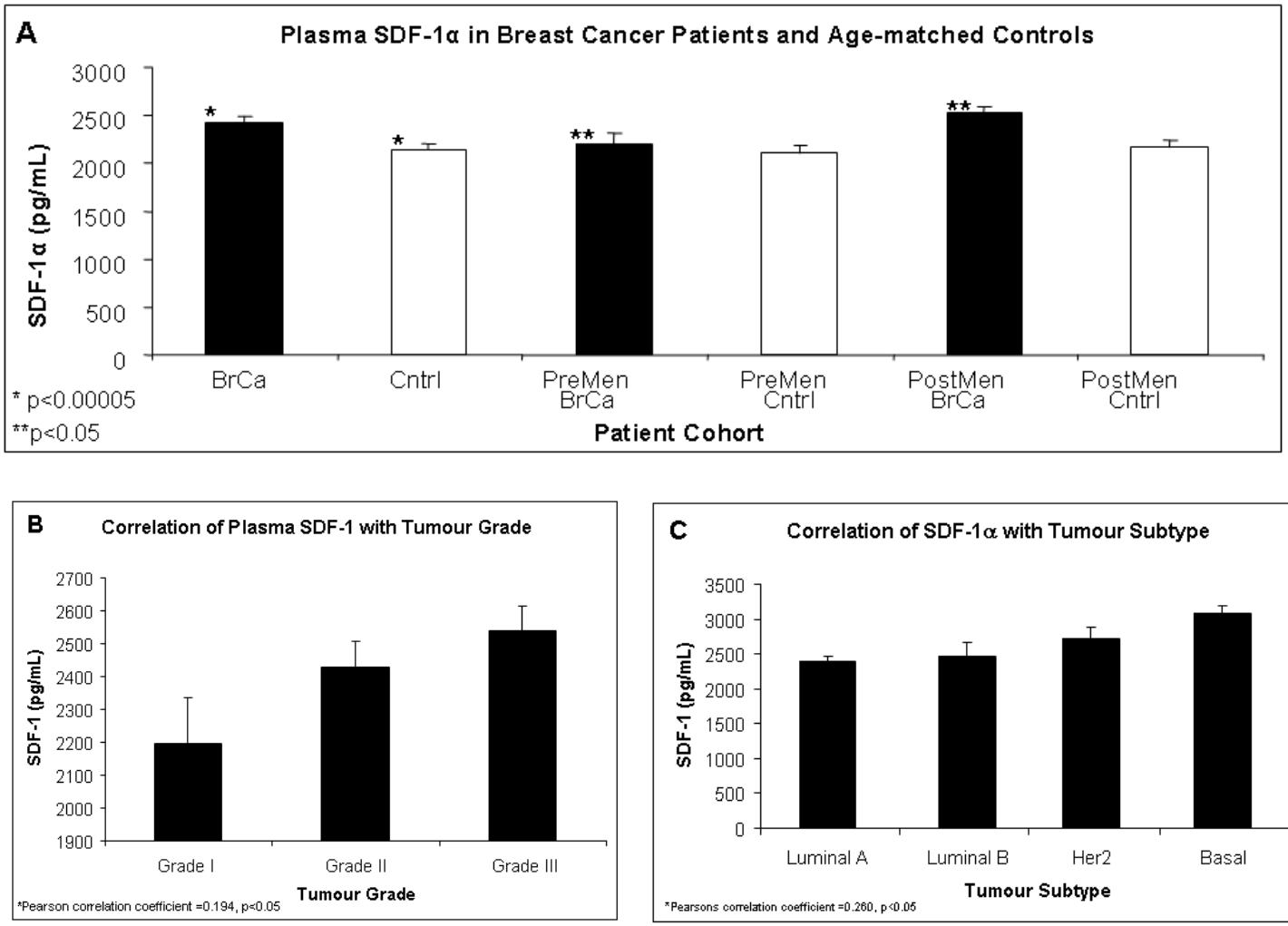

Fig 1 (a) Graph showing plasma SDF-1α levels in 114 breast cancer patients (BrCa) (preoperative) and 85 age-matched controls (Cntrl). Plasma SDF-1α was significantly higher in breast cancer patients (2448 ± 49pg/mL) than in healthy controls (2141 ± 58pg/mL, $p<0.00005$). Within the breast cancer cohort, SDF-1α levels were significantly higher in postmenopausal (PostMen, n=78) compared to pre-menopausal patients (PreMen, n=36) ($p<0.05$) which was not reflected in the control group. **(b), (c)** Analysis of patient clinical data. A significant positive correlation was found between the prognostic indicator tumor grade (ranked I-III) and plasma SDF-1α levels ($p<0.05$) **(b)**. Tumours were also classified according to epithelial subtype, and increasing SDF-1α was found to correlate with subtypes associated with poorer prognosis ($p<0.05$) **(c)**.

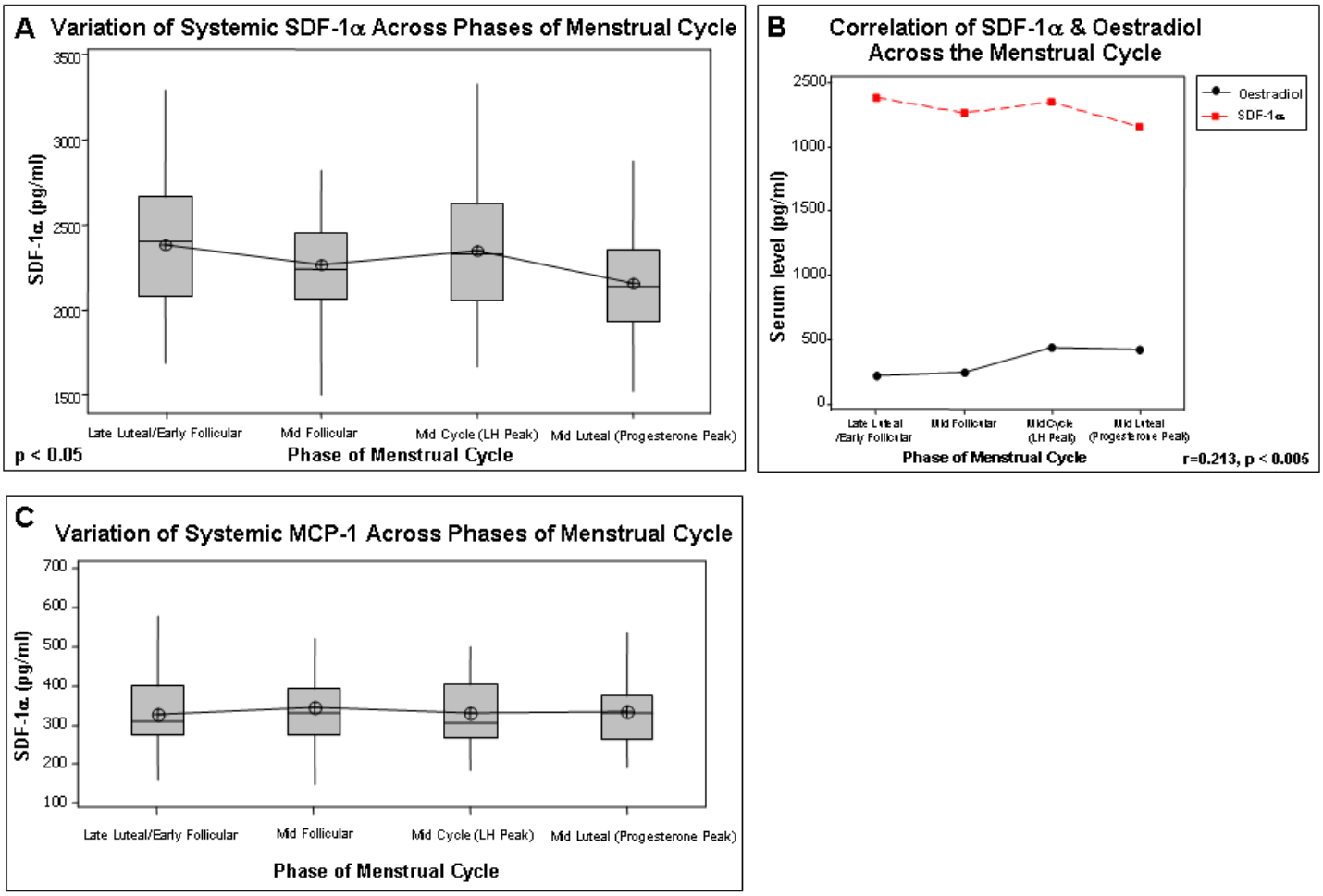

Fig 2 Relationship between chemokines and circulating menstrual hormones. **(a)** Variation of systemic SDF-1α across phases of menstrual cycle. The levels of SDF-1α were significantly lower in the mid-luteal phase ($p<0.05$). **(b)** Correlation of systemic SDF-1α and Oestradiol across the menstrual cycle. SDF-1α was found to have a significant correlation with Oestradiol (Pearson correlation coefficient =0.213, $p<0.005$). **(c)** Variation of systemic MCP-1 in phases of menstrual cycle. MCP-1 did not show any cyclical variation across the menstrual cycle.

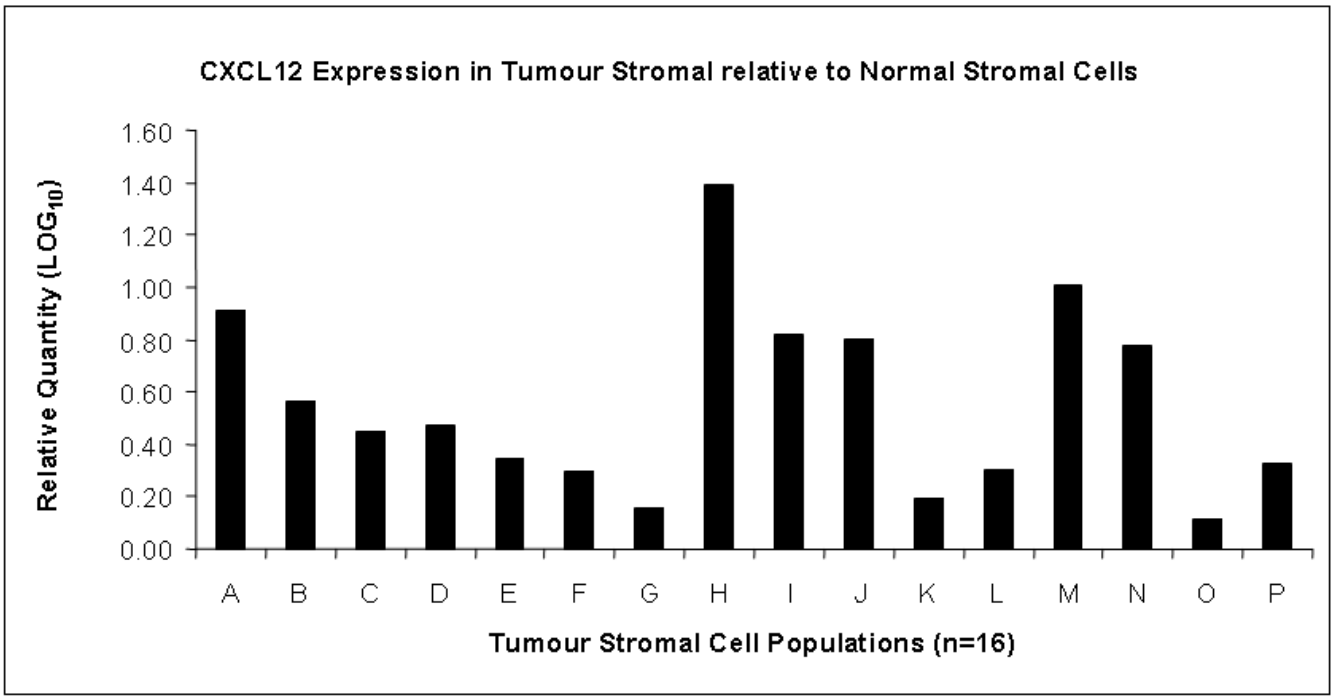

Fig 3 Analysis of SDF-1α/CXCL12 Gene Expression in Tumour Stromal compared to Normal Stromal Cells. The level of expression of SDF-1α was quantified in tumour stromal cells (A-P, n=16) compared to normal stromal cells harvested at reduction mammaplasty (n=4), represented by the baseline.