Serum Vascular Endothelial Growth Factor in Breast Cancer

G.J. BYRNE[1], G. MCDOWELL[2], R. AGARAWAL[1], G. SINHA[1], S. KUMAR[3] and N.J. BUNDRED[1]

[1]*Department of Surgery, South Manchester University Hospitals Trust, Wythenshawe Hospital, Southmoor Road, Manchester;*
[2]*Department of Clinical Biochemistry, Manchester Royal Infirmary, Oxford Road, Manchester;*
[3]*Division of Regenerative Medicine, The University of Manchester, Stopford Building, Oxford Road, Manchester, U.K.*

Abstract. *Many studies have reported elevated serum concentrations of vascular endothelial growth factor (VEGF) in patients with cancer and claimed that the measurement of circulating VEGF is a surrogate marker of angiogenesis and/or metastasis. To determine the value of VEGF measurement in the diagnosis and prognosis of breast cancer, we measured levels in women with and without breast cancer. Platelet-depleted plasma VEGF levels were measured in premenopausal women at four-day intervals across the menstrual cycle, postmenopausal women and postmenopausal women who had undergone hysterectomy. Platelet-depleted plasma VEGF was also measured in pre- and postmenopausal women with early breast cancer (EBC) and levels compared with intratumoral levels, clinicopathological prognostic parameters and microvessel density. Levels of VEGF were determined using ELISA and immuno-histochemistry. Microvessel density was determined by immunohistochemical CD34 staining. Plasma VEGF in premenopausal women remained stable across the menstrual cycle except for a peak between days 8 and 12. VEGF levels in postmenopausal women were higher than in premenopausal women unless postmenopausal women had undergone hysterectomy. Amongst premenopausal women, levels of VEGF were high in 22 EBC patients when compared to normal premenopausal controls. No correlation was found between plasma and intratumoral VEGF, clinicopathological prognostic parameters or tumour microvessel density. The origin of circulating VEGF differs between pre- and postmenopausal women. Its measurement is unlikely to provide clinically useful diagnostic and prognostic information in women with early and advanced breast cancer.*

Correspondence to: Dr. G. McDowell, Department of Clinical Biochemistry, Manchester Royal Infirmary, Oxford Road, Manchester, M13 9WL, U.K. Tel: +44 161 276 6728, Fax: +44 161 276 4585, e-mail: garry.mcdowell@manchester.ac.uk

Key Words: VEGF, breast cancer, diagnosis, prognosis.

Angiogenesis, the formation of new blood vessels from an existing circulation, is a prerequisite for tumour growth and metastasis (1-3). Vascular endothelial growth factor (VEGF) is a potent angiogenic cytokine in normal tissues and tumours (4-7) stimulating endothelial cell proliferation *in vitro* and inducing angiogenesis *in vivo* (8-10). VEGF is overexpressed in breast cancer tissue when compared to normal breast tissue (11-13) and levels of VEGF in these tissues correlate with disease-free and overall survival (14, 15). Moreover, levels of VEGF in breast cancer tissue correlate with microvessel density, the current standard measurement of tumoral angiogenesis (11, 16, 17).

A soluble form of VEGF ($VEGF_{121}$) is detectable in the circulation and studies have demonstrated elevated serum levels of VEGF in patients with solid tumours including uterine, ovarian, colorectal, lung and brain cancers (7, 18-21) leading some to hypothesise that serum VEGF measurement may have predictive value for the progression of disease in patients with solid tumours. Circulating levels of VEGF may represent a surrogate marker for angiogenesis, aiding the decision process involved in the targeting of chemotherapy.

VEGF is overexpressed in follicular fluid prior to ovulation (22) and two studies have reported that serum VEGF demonstrates a cyclical variation across the menstrual cycle (23, 24) implying that levels of circulating VEGF may, in normal women, be influenced by levels of ovarian hormones.

Experimental analysis of circulating VEGF levels by ELISA has revealed a wide discrepancy between levels in serum and in plasma. This observed difference has recently been explained by the discovery of large quantities of VEGF in megakaryocytes and platelets (25). Moreover, serum levels of VEGF correlate with platelet numbers (26) and VEGF is released into the serum by activated platelets following routine phlebotomy (27). Thus, it would seem reasonable to analyse circulating VEGF levels in plasma samples rather than serum. For this reason, this study

focuses on VEGF levels in plasma which has been immediately depleted of platelets following venesection.

The source of circulating VEGF in normal women and women with pre and postmenopausal breast cancer remains unclear. We sought to determine the prognostic significance and source of elevated VEGF levels in women with breast cancer and to determine whether its measurement could be used as a surrogate marker of angiogenesis.

Materials and Methods

VEGF in platelet-depleted and platelet-rich plasma; effect of platelet release on plasma VEGF concentration. Venous blood samples were obtained from twelve healthy male (n=6) and female (n=6) volunteers aged between 19 and 47 years. Each sample of venous blood was separated into two citrated and one EDTA bottle. One of the citrated tubes was immediately plunged into ice and taken to the laboratory where the sample was centrifuged at 4˚C for twenty minutes at 3500 xg. The supernatant was removed and re-centrifuged for 20 mins at 3500 xg at 4˚C following which the platelet-depleted plasma (PDP) was aliquoted and the last 0.5 ml discarded. PDP was immediately frozen and stored at –70˚C until assay. The other citrated sample and the EDTA sample were centrifuged at 1500 xg for 10 minutes, and the platelet-rich plasma aliquoted and stored at –70˚C until assay. A small aliquot of the platelet-rich and platelet-depleted plasma was sent to the hospital haematology laboratory for estimation of platelet concentration by standard clinical laboratory methods.

Normal pre- and postmenopausal women. VEGF was measured in PDP prepared as described above in nine premenopausal female volunteers. Serum follicle stimulating hormone (FSH) and luteinising hormone (LH) were also analysed using routine clinical laboratory methods. Samples were collected on the first day of menstruation and then at intervals of four days. In agreeing to participate in the study, women were asked to disclose their normal cycle length and regularity and women with cycle lengths longer than 32 days or shorter than 26 days or those with irregular menstruation were excluded from the study. The median age for pre-menopausal women was 29 (range 22-43) years. Women were deemed to have entered the luteal phase of the menstrual cycle when FSH levels fell below 10 mIU/ml or following a LH surge.

PDP-VEGF was measured in 32 postmenopausal women. Postmenopausal was defined as a period of not less than two years since last menstruation. Twelve of these women had undergone hysterectomy without salpingo-oophorectomy, whilst eight had undergone hysterectomy with bilateral salpingo-oophorectomy.

Women with breast cancer. PDP VEGF was measured in ninety-one women with stage T1 to T3 early breast cancer (EBC) immediately prior to surgery. Twenty-two were premenopausal (median age 41 years, range 28-49) whilst 69 were postmenopausal (median age 68 years, range 48-83). Levels of VEGF were compared to normal pre- and postmenopausal levels, known clinicopathological prognostic features of the disease, intratumoral VEGF expression and microvessel density. Microvessel density was assessed by an independent pathologist following immunohisto-chemical staining of the CD34 antigen in paraffin-embedded blocks of primary tumour.

A further 22 women with advanced breast cancer (assessed by UICC criteria) had a single estimation of serum VEGF. Ethical approval was obtained for this study from the South Manchester Local Research Ethics Committee and all participants gave written informed consent.

Analysis of VEGF.

Analysis of circulating levels by ELISA. VEGF was assayed by commercial ELISA according to manufacturers instructions (R and D systems, Oxon, UK). In duplicate, sample (100 μl) diluted with 100 μl of assay diluent was incubated with the capture antibody for 2 hours at room temperature. Following washing steps (x3), 200 μl of detection antibody conjugate was added and the assay incubated for 2 hours at room temperature. The plate was again washed (x3) and the detection substrate added and incubated for 25 minutes at room temperature. Stop solution (25 μl) was added and the optical density read at 450 nm.

Semiquantification of intratumoral VEGF by immunocytochemistry. Paraffin-embedded sections of 7 μm were stained for VEGF protein. Slides were dewaxed in xylene and exogenous peroxidase blocked with hydrogen peroxide mixed in methylated spirit for 30 minutes. Slides were pretreated with chymotrypsin (0.01% for 20 min) and rinsed in TBS. Non-specific binding was blocked by immersing slides in rabbit serum for two hours. Mouse monoclonal VEGF antibody (VEGF monoclonal. Calbiochem. Poole, Dorset, UK) diluted to 1:50 with 1% bovine serum albumin was added to each slide and the slides incubated at 4˚C overnight.

Sections were washed with TBS and incubated with biotinylated goat anti-mouse immunoglobulin antibodies and streptavidin-biotin complex (DAKO, Copenhagen, Denmark). The slides were washed in TBS followed by treatment with filtered 3,3 diaminobenzidine tetrahydrochloride (DAB) chromogen solution for 15 min and the nuclei counterstained with haematoxylin. Staining of tumours was assessed by an independent pathologist by a technique previously described (28). Tumours were assessed semi-quantitatively for the number of tumour cells staining (0=0%; 1=<20%; 2=20-80%; 3=>80% and the density of stain (0=none; 1=weak; 2=moderate; 3=strong). The two assessment scores were multiplied to give a total score between 0 and 9.

Microvessel count: assessment of angiogenesis. Formalin-fixed and paraffin-embedded tissues were cut into 5 μm-thick sections and mounted on polylysine-coated slides. Sections were deparaffinized by xylene and dehydrated by a graded series of ethanol. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 for 30 minutes, slides were microwaved for 10 minutes, then cooled to room temperature for 20 minutes and incubated with 5% bovine serum albumin for 30 minutes to block non-specific staining. Duplicate sections were incubated overnight with the primary specific biotinylated antibodies against CD34 (diluted to 1:100; Serotec Ltd, Oxford, UK), followed by incubation with HRP-conjugated streptavidin (1:100 dilution) for 20 minutes. Antibody binding was visualized with 3,3-diaminobenzidine tetrahydro-chloride, and sections were lightly counterstained with Mayer's hematoxylin (29). Microvessel density was quantified by light microscopy without knowledge of patient details. The most vascular areas in a tumour (hot-spots) were located at low magnification and the vessels were counted using a Chalkley point eyepiece graticule at x400 magnification (30). Any brown-staining EC or

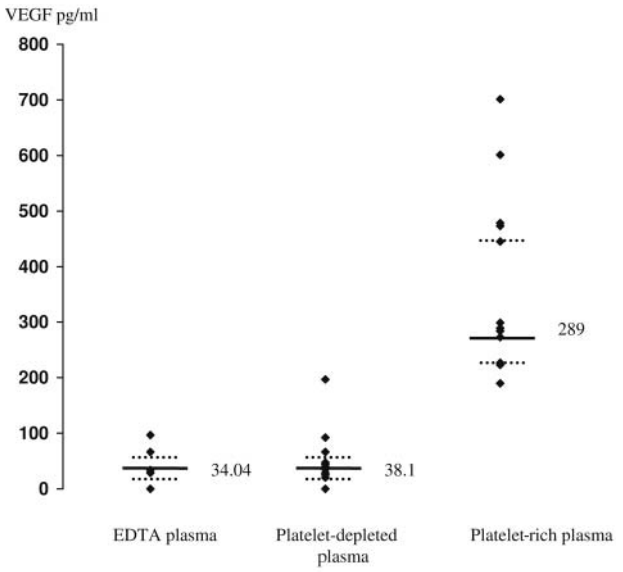

Figure 1. *Measurement of VEGF in EDTA plasma, platelet-depleted plasma and platelet-rich plasma. Results expressed as median and interquartile range. Only five of the twelve (41.6%) samples of EDTA plasma demonstrated detectable levels of VEGF, rising to eleven of twelve (91.6%) of the PDP samples. Levels of VEGF in PDP were five-fold lower than in platelet-rich plasma suggesting that platelet activation results in a significant contamination of platelet-rich plasma with platelet-bound VEGF. No correlation was observed between levels of VEGF in normal platelet-rich plasma and levels in PDP.*

group of cells in contact with a spot in a graticule was counted as an individual vessel. The mean of four Chalkley counts for each tumour was calculated and used in statistical analysis. Microvessel density was assessed without knowledge of plasma VEGF levels.

Statistical analysis. The median (M) and interquartile range (IQR) were calculated for all sets of variables and all *p*-values were derived using the Mann Whitney *U*-test for non-parametric data. Correlations between variable data were calculated using the Spearman correlation analysis. A *p*-value of less than or equal to 0.05 was taken as the level of statistical significance.

Results

VEGF in EDTA plasma, platelet-depleted plasma and platelet-rich plasma (Figure 1). Median levels of VEGF in the twelve healthy volunteers were 289 pg/ml (IQR=227-473) in platelet-rich plasma compared to 38.1 pg/ml (IQR=25.2-47.3) in platelet-depleted plasma (PDS) ($p<0.001$). VEGF was detectable in eleven of twelve (91%) platelet-depleted plasma samples compared to five of twelve (41.6%) EDTA plasma samples ($\chi^2=15.4$, $p=0.00043$). The median level of VEGF in EDTA plasma samples was 34.04 pg/ml (IQR 28.7-58.4). No correlation was observed between levels in platelet-depleted and platelet-rich plasma. Platelet counts of $<4x10^9$/l were found in all platelet-depleted plasma samples.

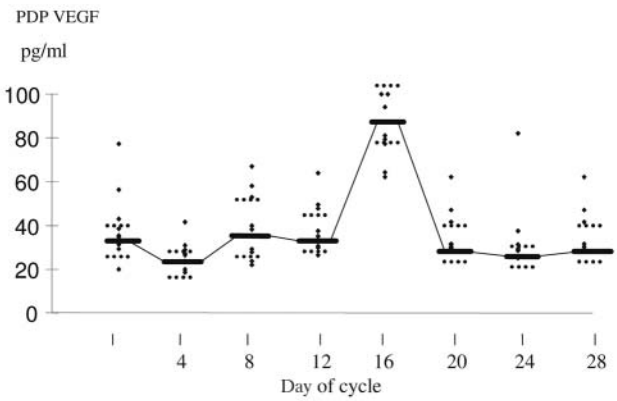

Figure 2. *Platelet-depleted plasma VEGF across the menstrual cycle. Results expressed as median and interquartile range. No difference in levels was detected between follicular and luteal phases of the cycle but the was a significant peak ($p<0.05$, Mann-Whitney) in levels at mid-cycle.*

VEGF in normal premenopausal and postmenopausal women. Platelet-depleted plasma VEGF remained stable throughout the menstrual cycle (M=30.4 pg/ml, IQR= 17.4-40.0) except for a single peak (M=104.5 pg/ml, IQR=70.1-130.7) occurring in mid-cycle (M=15 days, IQR=10-18 days) ($p<0.001$) (Figure 2). There was no difference in levels between the follicular (M= 31.4 pg/ml, IQR= 2l.5-78.9) and luteal (M= 35.26 pg/ml, IQR=22.75-55.67) phases of the menstrual cycle ($p=0.94$). Higher levels of platelet-depleted plasma VEGF were seen in normal post-menopausal (M=80.4 pg/ml, IQR=47.7-197.1) women than in premenopausal women ($p=0.028$) (Figure 3) but levels were lower in women who had undergone hysterectomy (M=28.4 pg/ml, IQR=20.5-38.8) ($p<0.01$ *vs.* normal post-menopausal controls). No difference in VEGF levels was detected between women whose ovaries were preserved after hysterectomy (median=29.7 pg/ml, IQR=19.7-40.1) and those who had undergone oopherectomy (median 27.44 pg/ml, IQR=22.3-39.0)

Platelet-depleted plasma VEGF in women with early breast cancer (Figure 4). Median levels of VEGF in women with postmenopausal breast cancer (median=142.4 pg/ml, IQR=100.1-210.2) were higher than normal postmenopausal controls but the difference was not significant. For menopausal women with early breast cancer who had undergone hysterectomy (n= 27, median=98.4 pg/ml, IQR=76.2-108.4) had lower levels of PDP VEGF than women who had not (n=42, median=189.7 pg/ml, IQR=143.78-224.9), ($p<0.01$).

Approximately five times as much circulating VEGF was seen in premenopausal women with early breast cancer (M=157.1 pg/ml, IQR=128.8-197.58) compared with their healthy premenopausal controls (median=38.1 pg/ml IQR=25.2-47.3) ($p<0.01$)). No correlation was observed

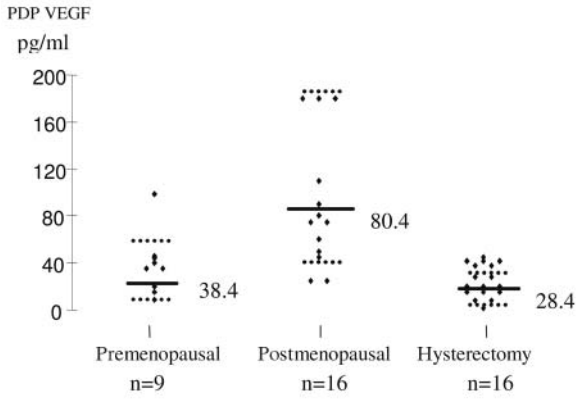

Figure 3. *Platelet-depleted plasma VEGF in normal pre- and postmenopausal women compared to postmenopausal women following hysterectomy. Results expressed as median and interquartile range. Levels of PDP VEGF in postmenopausal women who had undergone hysterectomy were lower than non-hysterectomised, postmenopausal women ($p<0.05$ Mann Whitney).*

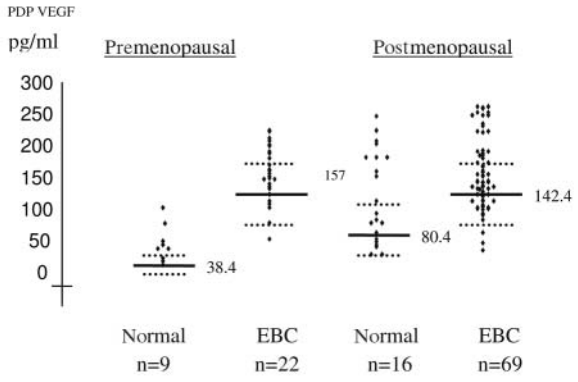

Figure 4. *Plateletdepleted plasma VEGF in pre- and postmenopausal early breast cancer compared to normal pre- and postmenopausal controls. Results expressed as median and interquartile range. Levels of VEGF in women with premenopausal breast cancer are higher than premenopausal controls but a similar relationship was not detected between postmenopausal breast cancer and postmenopausal controls.*

between levels of PDP VEGF and microvessel density whether patients with early breast cancer were measured overall (r=0.033, Spearman) or as separate groups of pre- (r=0.019) or postmenopausal (r=0.174) women (Figure 5). No difference in serum VEGF was detectable between different tumour grades (Bloom and Richardson), tumour size and lymph node status in primary tumours. Although levels of immunohistochemically detected intratumoral VEGF (median score 6, IQR 4-7) correlated with microvessel density (r=0.39, $p=0.0015$), there was no correlation between intratumoral VEGF and PDP VEGF (r=0.13, $p=0.09$).

Platelet depleted plasma VEGF in advanced breast cancer (ABC) (Table I). The highest PDP VEGF levels were observed in the women with advanced breast cancer (M=274.2 pg/ml, IQR=85.8-472.1). However differences in serum VEGF between women with advanced and early breast cancer were not significant ($p=0.3791$). Although median levels in the advanced breast cancer group were high, there was a wide variation in value and consequently a wide interquartile range.

Discussion

This study demonstrates that circulating levels of VEGF cannot be accurately assessed in platelet-rich plasma samples because of platelet contamination following routine venepuncture. Moreover, although levels of VEGF in platelet-depleted plasma are raised in women with early and advanced breast cancer when compared to normal controls, there is no direct correlation between these raised levels and

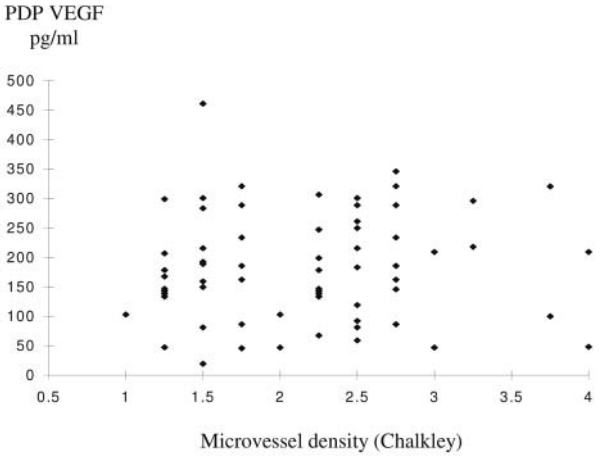

Figure 5. *Platelet-depleted plasma VEGF compared to microvessel density in all early breast cancers. There was no relationship between serum levels and angiogenesis. ($r=0.033$, $p=0.29$, Spearman).*

Table I. *Platelet-depleted plasma VEGF in advanced breast cancer compared to early breast cancer and normal controls: Although there is an increasing median value from normal to advanced, the wide range of values makes any differences statistically insignificant.*

		Postmenopausal women	
	Normal	EBC	ABC
VEGF (pg/ml)	80.4 [47.7-197.1]	142.4 [100.1-210.2]	274.2 [85.8-472.1]

EBC=early breast cancer; ABC=advanced breast cancer.

established clinicopathological prognostic parameters, microvessel density or intratumoral VEGF expression. Thus the measurement of circulating VEGF has limited clinical value for women with breast cancer.

VEGF has an intimate role in breast cancer angiogenesis (11-17) and tumour growth (34, 35). Expression of VEGF measured quantitatively by ELISA of breast tumour cytosols (17) and semi-quantitatively by immunohistochemistry (14) in breast cancer sections is up-regulated in breast cancer when compared to normal breast tissue. VEGF levels in tumour tissue correlate with lymph node status (14) and survival (14, 15, 17) and our data confirm the findings of others that intratumoral VEGF correlates with microvessel density, the current standard measure of tumoral angiogenesis (11, 17, 18). Based on this evidence, we hypothesised that serum levels of circulating VEGF might reflect levels in the tumour tissue of women with early and advanced breast cancer. Thus we sought to define a possible relationship between the soluble forms of vascular endothelial growth factor and established clinico-pathological prognostic parameters, angiogenesis and intratumoral levels, as assessed by immunocytochemistry of VEGF in breast cancer.

Before embarking on a comparative study of humoral levels of VEGF in breast cancer, it was essential to establish that we were accurately measuring true levels of VEGF. Most studies of circulating VEGF levels in normal and diseased states have measured VEGF in platelet-rich plasma or serum by ELISA (7, 19, 20, 22, 28 31-39). Our study confirms the findings of Banks *et al.* (27) and Mohle *et al.* (25) that VEGF is released in large quantities by activated platelets following routine phlebotomy and assay of serum. We have demonstrated that platelet contamination of serum can be prevented by rapidly depleting plasma of platelets following routine venepuncture. Moreover, VEGF levels in platelet-depleted plasma are some five times lower than in platelet-rich plasma and we have observed no correlation between levels of VEGF in platelet-rich and -poor plasma. In this study, therefore, we have attempted to eliminate platelets as a source of VEGF in serum samples and thus levels of VEGF measured in platelet-depleted plasma are more likely to reflect true circulating levels of VEGF in women with and without breast cancer.

Levels of platelet-depleted plasma VEGF in this study were higher in postmenopausal women than in pre-menopausal women. Thus, we compared levels of VEGF women with premenopausal breast cancer with premenopausal controls and women with postmenopausal breast cancer with postmenopausal controls.

Contrary to studies using platelet-rich serum (23, 24), no difference was detected in serum VEGF levels between follicular and luteal phases of the menstrual cycle although a peak of VEGF, coincident with the mid-cycle surge in luteinising hormone, was observed suggesting that circulating VEGF may be influenced by pituitary gonadotrophins. Median levels of VEGF in premenopausal women with early breast cancer were five times higher than premenopausal controls regardless of the stage of menstrual cycle indicating that large amounts of VEGF are derived from the tumour itself. However, levels of VEGF in premenopausal women with EBC did not correlate with histopathological prognostic parameters or microvessel density suggesting that serum levels of VEGF were not reflecting intratumoral angiogenesis. Perhaps more suprisingly, plasma VEGF in premenopausal EBC did not correlate with intratumoral levels of VEGF measured by immunochemistry. VEGF has at least four isoforms (40), three of which, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, are overexpressed in breast cancer tissue compared to normal breast tissue (11). Only one of these isoforms, $VEGF_{121}$, is soluble. Thus, it may be that one of the insoluble variants is the principle angiogenic variant in breast cancer whilst serum levels of VEGF reflect intratumoral levels of $VEGF_{121}$ in isolation. Several studies have claimed an improved survival for premenopausal women with EBC who undergo primary surgery in the luteal phase of the menstrual cycle compared to those who undergo surgery in the follicular phase (41-45), whilst others have disputed this finding (46-48). Observed differences may be influenced by the pro-angiogenic effect of high circulating levels of VEGF in the proliferative phase of the cycle. Heer *et al.* (23) found higher levels of VEGF, when measured in platelet-rich plasma, in premenopausal women with early breast cancer when compared to normal premenopausal women regardless of the phase of the menstrual cycle. In our study, median levels in premenopausal women were four times higher in the breast cancer group than in corresponding controls but levels did not correlate with clinicopathological parameters or microvessel density. It is therefore unclear if this increase has any pathophysiological or prognostic relevance. Moreover, because the levels of circulating VEGF in premenopausal women with EBC are higher than normal controls, one must conclude that the majority of circulating VEGF in these women is derived from the tumour effectively swamping physiologically derived VEGF. Thus, the physiological variation of VEGF levels across the menstrual cycle is unlikely to influence the outcome of surgery.

Levels of VEGF in normal postmenopausal women, in this study, were higher than levels in normal premenopausal women. However, levels of VEGF in normal women who had undergone hysterectomy, with or without oopherectomy, were similar to those seen in normal premenopausal women. Although we have not defined the source of increased levels of VEGF in non-hysterectomised women, low levels seen in normal

hysterectomised women suggest that the uterus has a role in the maintenance of high circulating VEGF levels found in postmenopausal women. In contrast to premenopausal women, circulating VEGF levels in postmenopausal women with breast cancer are not raised when compared to normal postmenopausal women. The reason for this observed difference between pre- and postmenopausal women with breast cancer remains obscure but this study suggests that the measurement of VEGF in postmenopausal women is unlikely to have any clinical prognostic relevance.

The highest median levels of VEGF in all groups studied was amongst women with advanced breast cancer. This study did not attempt to sub-classify women with advanced breast cancer into subgroups dependent on tumour load or response but the wide variation of levels (23% of patients having VEGF levels below median levels for normal postmenopausal women) suggests that VEGF is likely to have little value as a marker of advanced disease.

The role of the female reproductive tract in the maintenance of physiological levels of circulating VEGF requires clarification and high levels of VEGF found in circulating platelets suggests that the adherence of tumour cells to platelets and endothelial cells may result in platelet degranulation and release of VEGF, leading to increased vascular permeability and allowing cellular extravasation.

For any test to be useful as a monitor of a pathological process it must be easily measurable, it must reflect accurately the pathological process it is designed to measure and it must provide the clinician with an answer that can be easily interpreted. When taken in combination, the lack of correlation between circulating levels of VEGF with established prognostic parameters, angiogenesis and intratumoral VEGF levels together with high background levels of VEGF in postmenopausal women with early breast cancer and the wide variation of levels observed in women with advanced breast cancer, routine measurement of serum VEGF is unlikely to be of benefit to the oncologist.

References

1 Fidler IJ and Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis [comment]. Cell *79*: 185-188, 1984.

2 Bicknell R and Harris AL: Novel growth regulatory factors and tumour angiogenesis. Eur J Cancer *27*: 781-785, 1991.

3 Folkman J: Tumor angiogenesis. Adv Cancer Res *43*: 175-203, 1985.

4 Diaz-Flores L, Gutierrez R and Varela H: Angiogenesis: an update. Histol Histopathol *9*: 807-843, 1994.

5 Ferrara N and Davis-Smyth T: The biology of vascular endothelial growth factor. Endocr Rev *18*: 4-25, 1997.

6 Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS and Dvorak HF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science *219*: 983-985, 1983.

7 Dirix LY, Vermeulen PB, Pawinski A *et al*: Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. Br J Cancer *76*: 238-243, 1997.

8 Kim KJ, Li B, Winer J *et al*: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature *362*: 841-844, 1993.

9 Asano M, Yukita A, Matsumoto T, Kondo S and Suzuki H: Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor121. Cancer Res *55*: 5296-5301, 1995.

10 Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246*: 1306-1309, 1989.

11 Relf M, LeJeune S, Scott PA *et al*: Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res *57*: 963-969, 1997.

12 Brown LF, Berse B, Jackman RW *et al*: Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. Hum Pathol *26*: 86-91, 1995.

13 Yoshiji H, Gomez DE, Shibuya M and Thorgeirsson UP: Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res *56*: 2013-2016, 1996.

14 Obermair A, Kucera E, Mayerhofer K *et al*: Vascular endothelial growth factor (VEGF) in human breast cancer: correlation with disease-free survival. Int J Cancer *74*: 455-458, 1997.

15 Gasparini G, Toi M, Gion M *et al*: Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst *89*: 139-147, 1997.

16 Guidi AJ, Schnitt SJ, Fischer L *et al*: Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma *in situ* of the breast. Cancer *80*: 1945-1953, 1997.

17 Toi M, Kondo S, Suzuki H *et al*: Quantitative analysis of vascular endothelial growth factor in primary breast cancer. Cancer *77*: 1101-1106, 1996.

18 Kondo S, Asano M, Matsuo K, Ohmori I and Suzuki H: Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients. Biochim Biophys Acta *1221*: 211-214, 1994.

19 Ferrari G and Scagliotti GV: Serum and urinary vascular endothelial growth factor levels in non-small cell lung cancer patients [letter]. Eur J Cancer *32A*: 2368-2369, 1996.

20 Takano S, Yoshii Y, Kondo S *et al*: Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. Cancer Res *56*: 2185-2190, 1996.

21 Gadducci A, Ferdeghini M, Fanucchi A *et al*: Serum preoperative vascular endothelial growth factor (VEGF) in epithelial ovarian cancer: relationship with prognostic variables and clinical outcome. Anticancer Res *19*: 1401-1405, 1999.

22 Lee A, Christenson LK, Stouffer RL, Burry KA and Patton PE: Vascular endothelial growth factor levels in serum and follicular fluid of patients undergoing *in vitro* fertilization. Fertil Steril *68*: 305-311, 1997.

23 Heer K, Kumar H, Speirs V *et al*: Vascular endothelial growth factor in premenopausal women – indicator of the best time for breast cancer surgery? Br J Cancer *78*: 1203-1207, 1998.
24 Agrawal R, Conway GS, Sladkevicius P *et al*: Serum vascular endothelial growth factor (VEGF) in the normal menstrual cycle: association with changes in ovarian and uterine Doppler blood flow. Clin Endocrinol (Oxf) *50*: 101-106, 1999.
25 Mohle R, Green D, Moore MA, Nachman RL and Rafii S: Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc Natl Acad Sci USA *94*: 663-668, 1997.
26 Salgado R, Vermeulen PB, Benoy I *et al*: Platelet number and interleukin-6 correlate with VEGF but not with bFGF serum levels of advanced cancer patients. Br J Cancer *80*: 892-897, 1999.
27 Banks RE, Forbes MA, Kinsey SE *et al*: Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer. Br J Cancer *77*: 956-964, 1998.
28 Downey SE, Hoyland J, Freemont AJ, Knox F, Walls J and Bundred NJ: Expression of the receptor for parathyroid hormone-related protein in normal and malignant breast tissue. J Pathol *183*: 212-217, 1997.
29 Ding S, Li C, Lin S *et al*: Comparative evaluation of microvessel density determined by CD34 or CD105 in benign and malignant gastric lesions. Human Pathology *37*: 861-866, 2006.
30 Chalkley H: Method for quantitation morphological analysis of tissues. J Natl Cancer Inst *4*: 47-53, 1943.
31 Horak ER, Leek R, Klenk N *et al*: Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. Lancet *340*: 1120-1124, 1992.
32 Dickinson AJ, Fox SB, Persad RA, Hollyer J, Sibley GN and Harris AL: Quantification of angiogenesis as an independent predictor of prognosis in invasive bladder carcinomas. Br J Urol *74*: 762-766, 1994.
33 Toi M, Kashitani J and Tominaga T: Tumor angiogenesis is an independent prognostic indicator in primary breast carcinoma. Int J Cancer *55*: 371-374, 1993.
34 Toi M, Hoshina S, Takayanagi T and Tominaga T: Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. Jpn J Cancer Res *85*: 1045-1049, 1994.
35 Senger DR, Van de Water L, Brown LF *et al*: Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metastasis Rev *12*: 303-324, 1993.
36 Vermeulen PB, Dirix LY, Martin M, Lemmens J and Van Oosterom AT: Serum basic fibroblast growth factor and vascular endothelial growth factor in metastatic renal cell carcinoma treated with interferon alfa- 2b [letter]. J Natl Cancer Inst *89*: 1316-1317, 1997.
37 Akiyoshi F, Sata M, Suzuki H *et al*: Serum vascular endothelial growth factor levels in various liver diseases. Dig Dis Sci *43*: 41-45, 1998.
38 Baker PN, Krasnow J, Roberts JM and Yeo KT: Elevated serum levels of vascular endothelial growth factor in patients with preeclampsia. Obstet Gynecol *86*: 815-821, 1995.
39 Yamamoto S, Konishi I, Mandai M *et al*: Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. Br J Cancer *76*: 1221-1227, 1997.
40 Houck KA, Ferrara N, Winer J, Cachianes G, Li B and Leung DW: The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol *5*: 1806-1814, 1991.
41 Milella M, Nistico C, Ferraresi V *et al*: Breast cancer and timing of surgery during menstrual cycle: a 5-year analysis of 248 premenopausal women. Breast Cancer Res Treat *55*: 259-266, 1999.
42 Cooper LS, Gillett CE, Patel NK, Barnes DM and Fentiman IS: Survival of premenopausal breast carcinoma patients in relation to menstrual cycle timing of surgery and estrogen receptor/progesterone receptor status of the primary tumor. Cancer *86*: 2053-2058, 1999.
43 Saad Z, Vincent M, Bramwell V *et al*: Timing of surgery influences survival in receptor-negative as well as receptor-positive breast cancer. Eur J Cancer *30A*: 1348-1352, 1994.
44 Veronesi U, Luini A, Mariani L *et al*: Effect of menstrual phase on surgical treatment of breast cancer. Lancet *343*: 1545-1547, 1994.
45 Goldhirsch A, Gelber RD, Castiglione M *et al*: Menstrual cycle and timing of breast surgery in premenopausal node-positive breast cancer: results of the International Breast Cancer Study Group (IBCSG) Trial VI. Ann Oncol *8*: 751-756, 1997.
46 Nomura Y, Kataoka A, Tsutsui S, Murakami S and Takenaka Y: Lack of correlation between timing of surgery in relation to the menstrual cycle and prognosis of premenopausal patients with early breast cancer. Eur J Cancer *35*: 1326-1330, 1999.
47 Tsuchiya A, Furukawa H, Kanno M, Kimijima I and Abe R: Lack of the relationship between menstrual status and timing of surgery in survival of premenopausal patients with breast cancer. Fukushima J Med Sci *42*: 11-16, 1996.
48 Kroman N, Hojgaard A, Andersen KW *et al*: Timing of surgery in relation to menstrual cycle does not predict the prognosis in primary breast cancer. Danish Breast Cancer Cooperative Group. Eur J Surg Oncol *20*: 430-435, 1994.

Received June 22, 2007
Accepted July 4, 2007