INTERNATIONAL BREAST CANCER STUDY GROUP
Trial 22-00

Maintenance Chemotherapy in Hormone Non-Responsive Breast Cancer

SERUM SUBSTUDY
AMENDMENT 2

Assessment of Vascular Endothelial Growth Factor (VEGF), Soluble Her2 Protein (NRP, HER2-ECD) and Vascular Cellular Adhesion Molecule-1 (VCAM-1) in Serum Samples

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IBCSG Trial 22-00 Serum Substudy
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Approved by:
CEO, International Breast Cancer Study Group
Prof. M. Castiglione

(Signature on file) 01 November 2005

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Date

Approved by:
Group Statistician, International Breast Cancer Study Group
Prof. R.D. Gelber

(Signature on file) 01 November 2005

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Protocol Signature Page

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(Signature on file) 26 Nov 2002

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Principal Investigator and Co-investigator Protocol Signature Page

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I have read this protocol and agree to conduct this trial in accordance with all stipulations of the protocol and in accordance with the Declaration of Helsinki.

Name of Principal Investigator:

Signature:

_____ _____

Date

Name of Co-investigator:

Signature:

_____ _____

Date

Name of Co-investigator:

Signature:

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Date

Name of Co-investigator:

Signature:

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Date

Name of Co-investigator:

Signature:

_____ _____

Date

1 Introduction

1.1 VEGF

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in tumor progression of solid neoplasia. The switch from the avascular to the vascular phase is generally accompanied by rapid primary tumor growth and local invasiveness (1,2). Furthermore, angiogenesis is also necessary both at the beginning and end of the development of distant metastasis and is implicated in the phenomenon of dormant micrometastases (3).

Tumor cells may induce angiogenesis via release of numerous growth factors and by their attraction of inflammatory cells, which in turn release multiple angiogenic stimuli. The angiogenic response can be blocked by inhibitory modulation of individual steps of capillary growth following an angiogenic stimulus. (2). Antiangiogenic peptides may be altered in the serum or urine of cancer patients. In a study of 144 breast cancer patients, angiogenic protein basic fibroblast growth factor was abnormally elevated in the urine in 29% of cases and in the serum in 10%. Another angiogenic protein, vascular endothelial factor (VEGF), was abnormally elevated in the serum in over 70% of these breast cancer patients (4). Since platelets bind VEGF, platelet values will also be assayed.

1.2 HER2-ECD

The c-erbB2 oncogene (HER2/neu) encodes a type I trans-membrane tyrosine kinase family of receptors. Amplification and overexpression of this gene, leading to an abnormal activation of HER2 signalling pathways, has been linked to higher transforming activity, increased metastatic potential, angiogenesis and drug resistance in breast tumor experimental models (5). Moreover its over-expression is thought to be associated with a poorer prognosis in breast cancer and to correlate with different responsiveness to chemotherapy and hormonal treatment. Immunohistochemical detection of the oncogene HER/neu (protein p185) product is the standard method of evaluating the amplification and/or overexpression of the gene, present in 20-40% of breast cancers. A soluble form of the extracellular domain of HER/neu can be shed from the surface of tumor cells (6). No data are available regarding the presence of serum HER2-ECD (NRP) levels in c-erbB2 negative tumors, but the extracellular domain of the c-erbB2 oncogene product (NRP) is detectable in sera of 30-60% of patients with c-erbB2 positive tumors. Many reports have correlated the elevated serum levels of the c-erbB2 with gene amplification and c-erbB2 overexpression in tumor. These data support the hypothesis that the level of NRP protein can reflect the presence of c-erbB2 positive cells and that modification of the factor can predict a decrease of c-erbB2 positive cells during standard adjuvant chemotherapy. Moreover, change in the detectable NRP during the maintenance phase can suggest a possible modification in the biology and/or behaviour of hypothetic micrometastasis.

1.3 VCAM-1

The activation of endothelial adhesion molecules facilitates the local entry of inflammatory cells, which in turn promotes tissue neovascularization during tumor growth and wound repair. Soluble forms of several adhesion molecules are known to be shed from the cell surface. In tumors, endothelial VCAM-1 play a major role in the adhesion of leukocytes to the

endothelium, suggesting a relationship between cellular adhesion and angiogenesis. Soluble VCAM-1 has been implicated in the mediation of angiogenesis and some studies support the hypothesis that VCAM-1 provides surrogate markers for endothelial activation and angiogenesis occurring during cancers. Recently, VCAM-1 serum levels have been associated with microvessel density and response to endocrine therapy (7)

2 Objectives

2.1 To evaluate differences from baseline (after completion of induction chemotherapy but before commencement of CM Maintenance for patients randomized to CM Maintenance) to 18 months after the start of induction chemotherapy in serum VEGF, VCAM-1 and NRP protein values between patients in the Observation Group and those in the low dose Cyclophosphamide Methotrexate (CM) Maintenance Group.

2.2 Secondary objectives

2.2.1 To compare the differences from baseline to 12 and 36 months after the start of induction chemotherapy of serum VEGF, VCAM-1 and NRP values in the two randomized groups.

2.2.2 To compare VEGF, VCAM-1 and NRP values before and after progression in the two randomized groups.

3 Patient selection

3.1 Inclusion criteria

3.1.1. Patient must be randomized to the core protocol. Written informed consent for the serum substudy must be signed and dated by the patient and investigator.

3.1.2 Patient must not have begun CM maintenance (if randomized to CM maintenance).

4 Clinical laboratory evaluation

4.1 Design

Blood samples will be collected at:

1. Baseline, (after the completion of induction chemotherapy and, if randomized to CM maintenance, before start of CM maintenance.)
2. Months 12, 18 and 36 after start of induction chemotherapy
3. At time of confirmatory evidence of progression

4.2 Sample shipping

Serum and plasma samples will be stored at -80 °C and sent with dry ice. The delivery to the IEO laboratory must be organized when three cryogenic boxes are filled and at a minimum of once a year. To plan the delivery, Dr. L. Zorzino should be informed in advance by email (laura.zorzino@ieo.it) or by fax (+390257489417). The samples may be shipped Monday through Thursday to: Laboratory Medicine Unit

European Institute of Oncology
Via Ripamonti 435, 20141 – Milan – Italy

4.3 Sample collection and evaluation

4.3.1 Sample Collection.
Fifteen milliliters of native blood will be collected and divided into 3 tubes (five milliliters per tube); two plain tubes and one containing citrate solution, all of them labeled with protocol number (22-00), IBCSG patient identification number, patient's initials and date of collection. A Transporting Form 2002-TP (Section 12) must be filled out and submitted with each set of samples.

The tubes must be treated as follows:
1. The two PLAIN TUBES should be allowed to clot for two hours at room temperature, centrifuged at 3000 rpm for 10 min, and then separated.
2. The CITRATATED TUBE should be immediately centrifuged at 3000 rpm for 10 min and then separated.
3. The serum and the plasma must be separated in 500 µl aliquots (at least 6 for serum and 4 for plasma), and put into cryogenic tubes on which the following information must be reported:
   a. Protocol number: 22-00
   b. IBCSG patient's identification number (randomization number) and the patient's initials.
   c. Type of sample: S= serum or P= citrated plasma
   d. Date of collection

The cryogenic vials should be put into the appropriate cryogenic box, and a precise box map must be completed (Form 2002-BM, Section 12). Each box must be identified by a progressive letter (the first box will be box "A", the second box, box "B", and so on), and the same letter must be reported on the Box Map form, (Form 2002-BM, Section 12).

The cryogenic box must be stored in a – 80 °C freeze.

4.3.2 Sample evaluation
1. **VEGF** will be assayed with the use of commercial microplate ELISA kit.
2. **VCAM** will be assayed with the use of commercial microplate ELISA kit.
3. **HER2 ECD**. A magnetic particle separation immunoassay designed for the automated analyzer "Immuno 1" and provided by Bayer Diagnostics will be utilized.

4.3.3 The platelet count from the same day as the submitted blood draws (described above) should be recorded on the Transporting Form.

5 Patient registration and data management

Patients must register for the Serum Substudy at the time of randomization to the core protocol, IBCSG 22-00. The Confirmation of Registration Form for eligible patients on the core protocol (Form A) must indicate that the patient will be enrolled into the Serum Substudy. In addition, the serum substudy informed consent form must be signed and dated by the patient and the investigator. One copy must be given to the patient and the original retained in the investigator's trial records and available for data audits.

The Transporting Form must be submitted along with each submission of drawn blood described in Section 4.3.

6 Treatment details

Detailed information on the treatment regimens can be found in Section 5 of the core protocol.

7 Study parameters

7.1 Table of study parameters

7.1.1 Observation Group and CM Maintenance Group

	Baseline*		Months 12, 18, 36**	at Progression***
VEGF	X		X	X
VCAM-1	X		X	X
NRP	X		X	X
Platelet count	X		X	X

* After completion of the induction chemotherapy and, if randomized to CMM, before the start of CMM.
** Day 0 is defined as the day induction chemotherapy begins.
***Blood to be drawn at time of confirmatory evidence of progression

8 Statistical methods

8.1 Endpoints

The primary objective of this biological study is to evaluate differences from baseline to 18 months in serum VEGF, VCAM-1 and NRP values between the Observation and CM Maintenance groups.

The secondary objectives are:

- To compare the differences from baseline to 12 and 36 months in serum VEGF, VCAM-1 and NRP values in the two randomized groups.
- To compare VEGF, VCAM-1 and NRP values before and after progression in the two randomized groups.

8.2 Data analyses

For each analysis time point (12, 18, 36 months and at progression) the following analyses will be performed:
1. The 95% confidence interval for the percent change from baseline will be presented for the two randomized groups for each serum level.

2. The Wilcoxon two-sample rank sum test will be used to compare the percentage change from baseline in the serum levels between the two groups.

8.3 Sample size considerations

Previous data in the metastatic setting with similar cytotoxic agents (cyclophosphamide and methotrexate) yielded a mean decrease of 20% in VEGF levels with standard deviation of 38% (8). Based on a two-sided test with a type I error of 5% and a power of 80% this study is designed to detect a 20% difference from baseline to 18 months post randomization in the mean percentage decrease of VEGF, VCAM-1, and NRP and for the CM Maintenance Group compared with the Observation Group. We assume that the patients not receiving maintenance CM will have no change in their VEGF, VCAM-1, and NRP serum levels. 59 patients per group are required to detect this 20% difference, but to account for a 30% non accessible rate at 18 months due to either disease recurrence or patient drop out, 85 patients per randomized arm will be enrolled into the substudy, for a total accrual of 170 patients.

8.4 Data and Safety Monitoring Committee (DSMC)

The core study will be presented for review by the IBCSG Data and Safety Monitoring Committee (DSMC) at each of their semi-annual meetings. The DSMC may also request to review data from this serum evaluation substudy.

9 Ethics committee review and patient informed consent

9.1 Ethical Review Board/Ethics

All protocols and the patient informed consent forms must have the approval of a properly constituted committee or committees responsible for approving clinical trials. The ERB/IRB written, signed approval letter/form must contain approval of the designated investigator, the protocol substudy (identifying protocol title and version number), and of the patient informed consent. Documentation of ethical committee approval must be sent to the IBCSG Coordinating Center prior to each participating center's first patient's serum evaluation.

9.2 Informed consent

A separate informed consent form for each patient must be obtained prior to initiating any serum investigational procedures (see Appendix Ia, revised). The informed consent form is signed in duplicate by the Investigator and the Patient. The Investigator must retain an original signed informed consent form for Trial records. The Patient also retains one of the signed informed consent forms for her personal records.

The "Declaration of Helsinki" recommends that consent be obtained from each potential patient in biomedical research trials after the aims, methods, anticipated benefits, and potential hazards of the trial, and discomfort it may entail, are explained to the individual by the physician. The potential patient should also be informed of her right to not participate or to withdraw from the substudy at any time.

If the patient is in a dependent relationship to the physician or gives consent under duress, the informed consent should be obtained by an independent physician. If the patient is legally incompetent (i.e., mentally incompetent), informed consent must be obtained from the legal guardian, or legal representative in accordance with the law of the country in which the trial is to take place. By signing this protocol, the investigator agrees to conduct the trial in accordance with the "Declaration of Helsinki."

10 Administrative considerations

10.1 Insurance

IBCSG as the Sponsor of the Study contracts adequate Clinical Trial Insurance, in accordance with all relevant legal requirements, mandated by local regulations where the Study takes place. This insurance provides compensation to participants of the study.

Patients who suffer injuries due to the trial should report them immediately to their doctor.

The local group must report all alleged claims immediately to IBCSG.

11 References

1. Folkman J. The influence of angiogenesis research on management of patients with breast cancer. Breast Cancer Res Treat 36(2): 109-18, 1995
2. Kern FG, Lippman ME. The role of angiogenic growth factors in breast cancer progression. Cancer Metastasis Rev 15: 213-9, 1996
3. Gasparini G. Clinical significance of the determination of angiogenesis in human breast cancer: update of the biological background and overview of the Vicenza studies. Eur J Cancer 32A: 2485-93, 1996
4. Salven P, Maenpaa H. Serum vascular endothelial growth factor is often elevated in disseminated cancer. Clin Cancer Res 3: 647-651, 1997
5. Hynes N. The biology of erbB2/neu/HER2 and its role in cancer. Biochim Biophis Acta 1198:165-184, 1994
6. Revillion F. Plasma c-erbB2 concentrations in relation to chemotherapy in breast cancer patients Eur J Cancer 32A:231-234, 1996
7. Byrne GJ, Ghellal A, Iddon J, et al. Serum soluble vascular cell adhesion molecule-1: role as a surrogate marker of angiogenesis. J Natl Cancer Inst 92:1329-36, 2000
8. Colleoni M, Rocca A, Sandri MT, et al. Low-dose oral methotrexate and cyclophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. Ann of Oncol 13: 73-80, 2002

12 Study Forms

IBCSG TRIAL 22-00 SERUM SUBSTUDY
Transporting FORM (Form 2002-TP)

Patient's Date of Birth

D M Y

Patient's Initials (f, m, fl, sl) | | | | |

Participating Center Name _____ Affiliate _____

Center Code | | | | | |

IBCSG Patient Randomization Number | 2 | 2 | | | | | |

Three Tubes of Blood must be submitted:
Fifteen milliliters of native blood will be collected and divided into 3 tubes (five milliliters per tube); two plain tubes and one containing citrate solution, all of them labeled with protocol number (22-00), IBCSG patient identification number, patient's initials and date of collection.

Express Mailing:

Serum and plasma samples must be stored at -80 °C and sent on dry ice **with this form** by express mail to Laboratory Medicine Unit, European Institute of Oncology, Via Ripamonti 435, Milano, Italy.

DATE OF BLOOD DRAW

D M Y

PLATELET COUNT ($x10^9$/l)

IBCSG TRIAL 22-00 SERUM SUBSTUDY
Box Map Form - Form 2002-BM

Participating Center: _____ **Affiliate:** _____

Center Code | | | | | | **Box:** _____ **(page 1)**

Note: All 2002-TP forms must be included with this shipment

Position in box	Date of Blood Draw	Patient ID	Patient Initials	Patient Date of Birth	Sample Type (S or P)*	Notes
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*** S = serum, P = citrated plasma**

IBCSG TRIAL 22-00 SERUM SUBSTUDY
Box Map Form - Form 2002-BM

Participating Center: _____ **Affiliate:** _____

Center Code | | | | | | **Box:** _____ **(page 2)**

Note: All 2002-TP forms must be included with this shipment

Position in box	Date	Patient ID	Patient Initials	Patient Date of Birth	Sample Type (S or P)*	Notes
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*** S = serum, P = citrated plasma**