UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE

Centre Pluridisciplinaire d'Oncologie

Monitoring multiple angiogenesis-related molecules in the blood of cancer patients shows a correlation between VEGF-A and MMP-9 levels before treatment and divergent changes after surgical vs. conservative therapy

THESE

préparée sous la direction du Professeur Curzio RUEGG

et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

Khalil ZAMAN

Médecin diplômé de la Confédération Suisse
Originaire de Savigny (Vaud)

Lausanne

2011

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale
Doctorat en médecine

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Directeur de thèse	*Monsieur le Professeur* **Curzio Rüegg**
Co-Directeur de thèse	
Expert	
Directrice de l'Ecole doctorale	*Madame le Professeur* **Stephanie Clarke**

la Commission MD de l'Ecole doctorale autorise l'impression de la thèse de

Monsieur Khalil Zaman

intitulée

Monitoring multiple angiogenesis-related molecules in the blood of cancer patients show a correlation between VEGF-A and MMP-9 levels before treatment and divergent changes after surgical vs. Conservative therapy

Lausanne, le 30 novembre 2011

pour Le Doyen
de la Faculté de Biologie et de Médecine

Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale

Int. J. Cancer: **118,** 755–764 (2006)

Monitoring multiple angiogenesis-related molecules in the blood of cancer patients shows a correlation between VEGF-A and MMP-9 levels before treatment and divergent changes after surgical *vs.* conservative therapy

Khalil Zaman[1,2], Robert Driscoll[1,2], Diane Hahn[3], Patricia Werffeli[1,2], Simon L. Goodman[3], Jean Bauer[1], Serge Leyvraz[1], Ferdy Lejeune[1], Roger Stupp[1] and Curzio Rüegg[1,2]*

[1]*Centre Pluridisciplianire d'Oncologie (CePO), Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland*
[2]*Swiss Institute for Experimental Cancer Research (ISREC), NCCR Molecular Oncology, Epalinges s/Lausanne, Switzerland*
[3]*Merck, KGaA, Oncology Research, Darmstadt, Germany*

Anti-angiogenic therapies are currently in cancer clinical trials, but to date there are no established tests for evaluating the angiogenic status of a patient. We measured 11 circulating angiogenesis-associated molecules in cancer patients before and after local treatment. The purpose of our study was to screen for possible relationships among the different molecules and between individual molecules and tumor burden. We measured VEGF-A, PlGF, SCF, MMP-9, EDB$^+$-fibronectin, sVEGFR-2, sVEGFR-1, sαVβ3, sTie-2, IL-8 and CRP in the blood of 22 healthy volunteers, 17 early breast, 17 early colorectal, and 8 advanced sarcoma/melanoma cancer patients. Breast cancer patients had elevated levels of VEGF-A and sTie-2, colorectal cancer patients of VEGF-A, MMP-9, sTie-2, IL-8 and CRP, and melanoma/sarcoma patients of sVEGFR-1. sαVβ3 was decreased in colorectal cancer patients. A correlation between VEGF-A and MMP-9 was found. After tumor removal, MMP-9 and sαVβ3 significantly decreased in breast and CRP in colorectal cancer, whereas sVEGFR-1 increased in colorectal cancer patients. In sarcoma/melanoma patients treated regionally with TNF and chemotherapy we observed a rise in VEGF-A, SCF, VEGFR-2, MMP-9, Tie-2 and CRP, a correlation between CRP and IL-8, and a decreased in sVEGFR-1 levels. In conclusion, among all factors measured, only VEGF-A and MMP-9 consistently correlated to each other, elevated CRP levels were associated with tumor burden, whereas sVEGF-R1 increased after tumor removal in colorectal cancer. Treatment with chemotherapy and TNF induced changes consistent with an angiogenic switch. These results warrant a prospective study to compare the effect of surgical tumor removal *vs.* chemotherapy on some of these markers and to evaluate their prognostic/predictive value.

Key words: surrogate markers; angiogenesis; predictive; TNF; chemotherapy

Angiogenesis promotes local tumor progression and invasion and enables tumor cell dissemination and metastasis formation.[1] Many preclinical studies show that the inhibition of angiogenesis can suppress tumor growth and metastatic dissemination. Bevacizumab (Avastin®), a neutralizing antibody specific for vascular endothelial growth factor (VEGF)-A in combination with standard chemotherapy (*i.e.*, CPT-11, 5-FU and Leucovorin) extends survival, and this combination was approved for patients with advanced colorectal cancer.[2] This result represents a proof-of-concept for the role of angiogenesis in human cancer progression and demonstrates that VEGF-A is a valid molecular target for the management of colon cancer.

The inhibition of the formation of new vessels, or the disruption of established vessels is not easily seen using the standard response criteria of clinical oncology because these are based mainly on measurement of tumor size. The ability to monitor tumor angiogenesis in patients would greatly facilitate the clinical development of anti-angiogenic treatments. Some imaging techniques, such as angiography, contrast-enhanced dynamic nuclear magnetic resonance, computer tomography and position emission tomography allow perfusion measurements at tumor sites, but they are not routinely used in the clinic as they are expensive and require sophisticated equipment and expertise. No validated biochemical marker currently allows the reliable monitoring of tumor angiogenesis in patients.[3,4]

Several molecules implicated in angiogenesis have been identified, some detectable in the systemic circulation. VEGF-A, matrix metalloproteinase (MMP) -2[5] and MMP-9,[6] soluble VEGF receptor-1 (sVEGFR-1),[7] soluble VEGF receptor-2 (sVEGFR-2)[8] and soluble Tie-2 (sTie-2)[7] have been studied in a wide variety of cancers, including breast, ovary, brain, kidney, bladder, prostate, lung, gastro-intestinal cancers, hematological malignancies, hepatocarcinoma, melanoma and sarcomas.[3,9–11] Correlations between elevated levels of some of these molecules, in particular VEGF-A and MMP, and the presence of a tumor, its stage or grade, progression and clinical outcome have been reported.[12,13] In most studies, only one or a few markers were evaluated simultaneously. The possibility of improving sensitivity or specificity by the simultaneous measurement and analysis of multiple candidate markers has not yet been evaluated.

We measured in parallel 11 different angiogenesis-associated molecules in the blood of patients before and after curative-intent loco-regional treatment and of healthy volunteers. We selected these markers from previous reports of their altered levels in tissue or blood of cancer patients or because preclinical and pathological studies have indicated an association with tumor angiogenesis. Placenta growth factor (PlGF) and VEGF-A are vascular growth factors that bind to and activate VEGFR-1 and VEGFR-1/-2, respectively. PlGF has been reported recently to play a role in pathological angiogenesis,[14] but its biological and clinical relevance in cancer are unclear. Stem Cell factor (SCF) promotes release of hematopoietic and vascular progenitor cells from the bone marrow.[15] VEGFR-1 and VEGFR-2 are receptors for the VEGF family members, which can induce angiogenesis.[16] Integrin αVβ3 is a cell adhesion receptor highly upregulated on angiogenic endothelial cells.[17] Tie-2 is a receptor for the angiopoietins, which promotes endothelial cell survival and vascular remodeling.[18] MMP-9 is a matrix-degrading enzyme upregulated in inflammation and cancer that was found to induce the angiogenic switch in preclinical cancer models.[19] EDB$^+$-fibronectin (EDB$^+$-Fn) is a fibronectin-splice variant absent from most normal tissues, but

Abbreviations: BC, breast cancer; CRC, colorectal cancer; CRP, C-reactive protein; EDB-Fn, extra-domain B positive fibronectin; HV, healthy volunteers; IL-8, interleukin-8; ILP, isolation limb perfusion; MMP-9, matrix metalloproteinase-9; PLGF, placenta growth factor; SCF, stem cell factor; sVEGF-R1, soluble VEGF-receptor1; sVEGF-R2, soluble VEGF-receptor2; TNF, tumor necrosis factor; VEGF-A, vascular endothelial growth factor A.

Grant sponsor: Swiss National Science Foundation; Grant number: 31-63752; Grant sponsor: Molecular Oncology Program of the NCCR.

***Correspondence to:** Centre Pluridisciplinaire d'Oncologie, Swiss Institute for Experimental Cancer Research (ISREC), 155 Chemin des Boveresses, CH1066 Epalinges, Switzerland. Fax: +41-21-692-5872.
E-mail: curzio.ruegg@isrec.unil.ch

Received 28 March 2005; Accepted after revision 31 May 2005
DOI 10.1002/ijc.21408
Published online 19 August 2005 in Wiley InterScience (www.interscience.wiley.com).

UICC Publication of the International Union Against Cancer
global cancer control

expressed in tumors in close association with angiogenic vessels,[20] although the EDB domain itself is dispensable for tumor angiogenesis and tumor progression.[21] Interleukin (IL)-8 has been associated with tumor progression and angiogenesis in several cancers, including breast, prostate and colorectal.[22,23] It was reported recently that Ras-dependent IL-8 secretion is critically involved in initiating tumor-associated inflammation and neovascularization.[24] Elevated serum C-reactive protein (CRP) levels were reported to be associated with elevated risk of developing colorectal cancer,[25] a more aggressive progression and unfavorable prognosis.[26] More recently elevated CRP levels were found associated with angiogenic factors in multiple myeloma.[27,28]

The aim of our study was to identify possible trends and relationships between these factors, which could subsequently be validated in prospective trials for their prognostic or predictive value. Our study was carried out on patients with localized and previously untreated breast or colorectal cancers scheduled to undergo primary curative-intent resection. We also analyzed patients undergoing local treatment by isolated limb perfusion with high dose TNF and chemotherapy for locally advanced/relapsed melanoma or sarcoma.[29] This modality of treatment permitted us to explore the difference of angiogenic response between loco-regional surgery compared to chemotherapy.

Material and methods

Study population

Eligible patients were 18 years of age or older, presenting non-metastatic breast (BC) or colorectal cancer (CRC) and scheduled for tumor resection, and loco-regional refractory or relapsed melanoma or sarcoma scheduled for isolated limb perfusion (ILP). Exclusion criteria included patients with <10 g/dl hemoglobin, leucopenia, thrombocytopenia, deep venous thrombosis during the last 3 months, long-term vascular catheter, chronic vascular or inflammatory disease, surgery and chemotherapy during the last 4 and 6 months respectively.

ILP is a technique of locoregional treatment[30] where tumor necrosis factor alpha (TNFα, Beromun®; dose = 1–4 mg) and high dose melphalan (Alkeran®; 10 mg/l of the limb volume) are perfused through an extracorporeal circulation into a limb isolated from the systemic circulation by a tourniquet. For melanoma, gamma interferon (IFNγ, Imukin®; 0.2 mg) is also added in the perfusion.

Healthy volunteers were at least 18 years old, without history of cancer, chronic disease or medication other than hormonal contraception. Ethical and scientific approval were given by the local Ethics Committee. Written informed consent was obtained from all the patients and healthy volunteers.

Study design

Blood was obtained before and more than 4 weeks after treatment by peripheral venous puncture. Serum and plasma were extracted during the following 2 hr.

Plasma isolation

Blood was collected in heparin containing tubes. After 30 min centrifugation at 400*g* at 20°C, the plasma was extracted and frozen at −80°C.

Serum isolation

Blood was collected in tubes without anticoagulant. After 10 min centrifugation at 1,000*g*, the serum was extracted and frozen at −80°C.

Samples analyses

The samples were thawed just before use. All molecules were measured in serum, except for VEGF-A, MMP-9, sαvβ3, sVEGFR-1, sVEGFR-2, which were measured in heparin-plasma to avoid potential interfering effects due to platelets activation during clotting. Measurements of VEGF-A, PlGF, SCF, sVEGFR-1, sVEGFR-2, SCF, MMP-9 and sTie-2 were carried out using the Quantikine ELISA systems (R&D Systems, Abingdon, UK). The sVEGFR-1 Quantikine ELISA does not differentiate between sVEGFR-1 generated by alternative splicing and sVEGFR-1 generated by proteolytic cleavage at the cell surface. IL-8 was measured using and ELISA system from Amersham Bioscience (Buckinghamshire, UK). CRP was measured using an ELISA system from Hyphen BioMed (Neuville-sur-Oise, France). EDB^+-Fn was measured with an ELISA by Adeza Biomedical (Sunnyvale, CA). sαVβ3 was measured using an ELISA developed for our study (see below). Each data point was measured in duplicate. Optical density of each well was measured at 450 nm in a multiwell plate reader (Packard Spectra Count, Meriden, CT). For practical reasons, only means are described below, as there was very little divergence between means and medians. To identify putative correlations between the levels of individual factors they were compared to each other.

Capture ELISA for soluble αvβ3

Anti-integrin αV (clone 272-17E6, Calbiochem, San Diego, CA) was adsorbed on 96-well plates (MaxiSorp, Nunc, Roskilde, Denmark) from a coating solution of 2 μg/ml in PBS (1 hr at 4°C). Plates were blocked with 5% BSA in PBS. The assay was carried out in PBS containing 0.1% BSA and 0.01% Tween 20 (dilution buffer). Plasma samples were diluted and incubated on the plates for 60 min at 37°C. After washing, biotinylated anti-β3 antibody at 2 μg/ml (clone 20H9)[31] was incubated on the plates for 60 min at 37°C. Plates were washed with dilution buffer, and anti-biotin peroxidase conjugate (Sigma, Deisenhofen, Germany) was added for 60 min at 37°C. Results were quantified by comparison to a reference curve produced using purified recombinant sαvβ3.[32]

Statistical analysis

Analysis was descriptive and exploratory. Mann-Whitney *u*-test was used in the comparison between healthy volunteers and cancer patients. The changes in the levels of factors before and after treatment were estimated by Wilcoxon signed-rank test. Results were considered statistically significant for $p \leq 0.05$.

Results

Study population

Forty-six patients were recruited, 4 patients were excluded from the analysis because of the absence of an invasive cancer (3 BC patients) or the presence of metastatic disease (1 CRC patient). Of the 42 eligible patients there were 17 female BC patients, 17 CRC patients and 8 cases of loco-regional relapsing/refractory sarcoma or melanoma (ILP patients). Patient characteristics are described in Table I.

Breast cancer (BC). Thirteen patients with invasive cancer were treated by tumorectomy and 4 patients by mastectomy. Axillary lymph node and sentinel node dissection were carried out in 11 and 6 patients, respectively. Post-surgery blood samples were obtained at a median time of 6.9 weeks after treatment.

Colorectal cancer (CRC). Tumor localizations were in rectum ($n = 2$), rectosigmoid ($n = 3$) and colon ($n = 12$). Patients were treated by hemicolectomy ($n = 14$) and low anterior resection ($n = 3$). Post-surgery blood samples were obtained at a median time of 8.9 weeks after treatment.

Isolated limb perfusion (ILP) patients. Four sarcoma patients were included (1 liposarcoma Grade 2, 1 angiosarcoma Grade 3 and 2 synovial sarcomas, Grade 2 and 3). The indications for ILP in sarcoma patients were progression after first line surgery, bulky initial tumors and first local relapse. No lymph node or distant metastases were present before ILP. Melanoma patients were treated by ILP for in transit metastasis. Post-surgery blood sam-

TABLE I – STUDY POPULATION CHARACTERISTICS

	No. of patients[1]
Breast cancer	
Histology	Ductal invasive carcinoma = 15 Lobular invasive carcinoma = 1 Medullar invasive carcinoma = 1
Tumor size	T1 = 10; T2 = 7
Lymph node invasion	7
Stages	St1 = 7; St2 = 7; St3 = 2; local relapse = 1
Positive hormonal receptor	14
Grade	G1 = 2; G2 = 9; G3 = 6
Colorectal cancer	
Gender	Female = 5; Male = 12
Tumor size	T1 = 1; T2 = 4; T3 = 7; T4 = 5
Lymph node metastasis	8
Stage	St1 = 3; St2 = 6; St3 = 8
Grade	G1 = 2; G2 = 13; G3 = 1; ND = 1
ILP	
Histology	Melanoma 4 Sarcoma 4

[1]Forty-two cancer patients were analyzed in this study: 17 female breast cancer patients, 17 colorectal cancer patients and 8 cases of loco-regional relapsing/refractory sarcoma or melanoma, ND, not done.

ples for ILP patients were obtained at a median time of 7.4 weeks after treatment.

Measured concentrations of the individual factors

VEGF-A. The differences of the mean values between volunteers and cancer patients (Table II, Fig. 1*a*) were statistically significant for BC ($p = 0.036$) and CRC ($p = 0.001$). After surgical tumor resection a trend toward a decrease of the mean levels of VEGF-A was observed for BC and CRC. In 6 CRC patients, however, VEGF-A levels slightly increased after surgery (Table III, Fig. 1*b*). In ILP patients, an increase in VEGF-A concentration after therapy was observed in all patients, except one who had elevated level of VEGF-A at baseline (Table III, Fig. 1*c*). Of note is that this patient had in transit metastases of melanoma and subsequently developed a disseminated disease within 1 month. There was no correlation between platelets count and VEGF-A in the patients, thus excluding that we were measuring VEGF-A released from platelets.[33]

PlGF and SCF. No significant differences were observed for PlGF and SCF between healthy volunteers and BC, CRC or patients undergoing ILP (Table II). Treatment had no significant effect on PlGF levels. SCF levels did not change after treatment in BC and CRC whereas it rose by approximately 20% in ILP patients (Table III).

sVEGFR-1. The mean values of sVEGFR-1 were only slightly higher in BC and CRC patients compared to healthy volunteers whereas it was significantly higher in ILP patients compared to controls (Table II, Figure 2a). After treatment we observed a tendency of increasing values in BC patients, a significant increase in CRC patients and a significant decrease in ILP patients (Table III, Figure 2b and 2c).

sVEGFR-2. Only minor differences in sVEGFR-2 mean values were observed between healthy volunteers and cancer patients. CRC patients had significantly lower levels of sVEGFR-2 compared to healthy volunteers, but the difference was small (13%) (Table II). No relevant modifications were observed after treatment in BC and CRC patients, whereas the sVEGFR-2 mean level rose significantly in ILP patient (Table III).

sTie-2. The mean values of Tie-2 were significantly higher in BC and CRC patients, compared to healthy volunteers, although the differences were small (Table II). After treatment no relevant changes were observed in BC and CRC patients, whereas in ILP patients sTie-2 levels rose significantly (Table III).

TABLE II – MEAN LEVELS OF THE MEASURED FACTORS BEFORE TREATMENT IN COMPARISON TO HEALTHY VOLUNTEERS

	VEGF-A plasma pg/ml ± SD	PlGF serum pg/ml ± SD	SCF serum pg/ml ± SD	sVEGFR-1 plasma pg/ml ± SD	sVEGFR-2 plasma pg/ml ± SD	sTie-2 serum ng/ml ± SD	$\alpha V\beta 3$ plasma ng/ml ± SD	MMP-9 plasma ng/ml ± SD	EDB-Fn serum µg/ml ± SD	IL-8 serum pg/ml ± SD	CRP serum µg/ml ± SD
HV	37.6 ± 25.5	8.6 ± 4.3	746.9 ± 112	21.1 ± 8.2	9260 ± 1780	18.8 ± 3.3	26.4 ± 14.2	169 ± 114	12.7 ± 7.3	2.1 ± 1.4	1.4 ± 1.8
BC	**52.9** ± 29.2	10 ± 4.2	744.5 ± 155	29.3 ± 25.0	8824 ± 1088	**25.2** ± 4.8	24.3 ± 6.6	237.8 ± 165.8	**2.6** ± 1.7	3.3 ± 3.2	1.4 ± 1.4
	$p = 0.04$	$p = 0.37$	$p = 0.84$	$p = 0.97$	$p = 0.22$	$p = 0.001$	$p = 0.99$	$p = 0.19$	$p = 0.001$	$p = 0.34$	$p = 0.99$
CRC	**109.6** ± 128.1	10.1 ± 3.1	726.5 ± 158	23.5 ± 24.0	**8104** ± 1153	**25.5** ± 7.7	**12.7** ± 6.6	**370.1** ± 338.8	**1.9** ± 1.4	**7.4** ± 12.0	**6.7** ± 5.0
	$p = 0.001$	$p = 0.29$	$p = 0.59$	$p = 0.66$	$p = 0.02$	$p = 0.006$	$p = 0.001$	$p = 0.02$	$p = 0.001$	$p = 0.001$	$p = 0.001$
ILP	51.8 ± 60.6	11.8 ± 4.0	803.3 ± 164	**122.9** ± 92.0	8258 ± 2238	17.9 ± 1.3	**18.2** ± 4.3	243.1 ± 342.5	**6.3** ± 5.6	4.4 ± 5.6	**4.7** ± 5.3
	$p = 0.87$	$p = 0.08$	$p = 0.57$	$p = 0.001$	$p = 0.16$	$p = 0.629$	$p = 0.01$	$p = 0.84$	$p = 0.03$	$p = 0.46$	$p = 0.09$

Bold typing denotes values that are significantly different in cancer patients in comparison to healthy volunteers. HV, healthy volunteers; BC, breast cancer; CRC, colorectal cancer; ILP, sarcoma or melanoma. Results represent mean values ± SD. $p \leq 0.05$ was considered statistically significant.

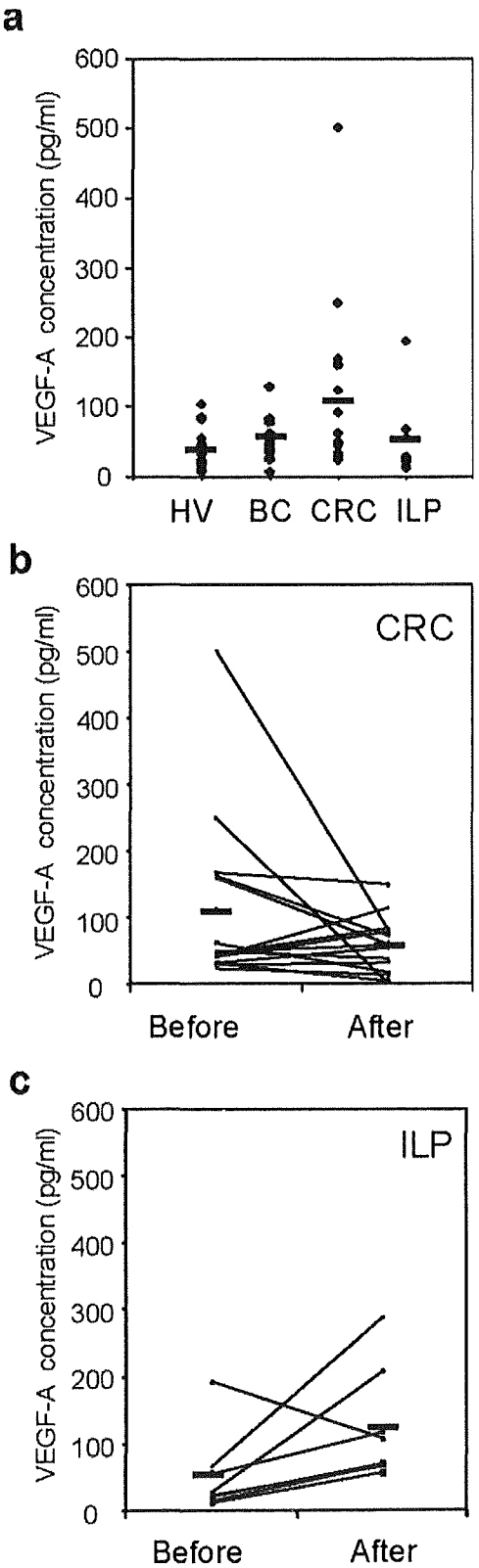


FIGURE 1 – VEGF-A levels. (*a*) VEGF-A values before treatment: breast cancer (BC) and colorectal cancer (CRC) patients had significantly higher VEGF-A levels compared to healthy volunteers (HV) and melanoma/sarcoma patents (ILP). (*b*) VEGF-A values before and after treatment in colorectal cancer patients. (*c*) VEGF-A values before and after treatment in ILP patients. The short horizontal lines indicate mean values.

TABLE III – MEAN LEVELS OF THE MEASURED FACTORS BEFORE AND AFTER TREATMENT

	VEGF pg/ml ± SD	PIFG pg/ml ± SD	SCF pg/ml ± SD	sVEGFR-1 pg/ml ± SD	sVEGFR-2 pg/ml ± SD	sTie-2 µg/ml ± SD	scVβ3 ng/ml ± SD	MMP-9 ng/ml ± SD	EDB-Fn ng/ml ± SD	IL-8 pg/ml ± SD	CRP µg/ml ± SD
BC											
Before	52.9 ± 29.2	10 ± 4.2	744.5 ± 155	29.3 ± 25.0	8824 ± 1088	25.2 ± 4.8	24.3 ± 6.6	237.8 ± 165.8	2.6 ± 1.7	3.3 ± 3.2	1.4 ± 1.4
	$p = 0.12$	$p = 0.93$	$p = 0.12$	$p = 0.49$	$p = 0.25$	$p = 0.34$	$p = 0.04$	$p = 0.02$	$p = 0.76$	$p = 0.10$	$p = 0.22$
After	43.8 ± 24.5	10.2 ± 5.0	729.2 ± 162	64.5 ± 157.6	8672 ± 1542	24.4 ± 4.9	**21.7** ± 5.2	**109.6** ± 64.8	2.7 ± 1.5	1.8 ± 0.9	1.8 ± 1.7
CRC											
Before	109.6 ± 128.1	10.1 ± 3.1	726.5 ± 158	23.5 ± 24.0	8104 ± 1153	25.5 ± 7.7	12.7 ± 6.6	370.1 ± 338.8	1.9 ± 1.4	7.4 ± 12.0	6.7 ± 5.0
	$p = 0.19$	$p = 0.24$	$p = 0.43$	$p = 0.01$	$p = 0.26$	$p = 0.51$	$p = 0.55$	$p = 0.12$	$p = 0.12$	$p = 0.09$	$p = 0.04$
After	57.6 ± 41.8	10.8 ± 4.7	776 ± 196	**310.7** ± 880	8626 ± 1764	24.6 ± 6.3	12.1 ± 7.5	190.3 ± 127.8	2.3 ± 1.7	3.3 ± 1.3	**3.4** ± 3.9
ILP											
Before	51.8 ± 60.6	11.8 ± 4.0	803.3 ± 164	122.9 ± 92.0	8258 ± 2238	17.9 ± 1.3	18.2 ± 4.3	243.1 ± 342.5	6.3 ± 5.6	4.4 ± 5.6	4.7 ± 5.3
	$p = 0.09$	$p = 0.58$	$p = 0.01$	$p = 0.02$	$p = 0.02$	$p = 0.01$	$p = 0.78$	$p = 0.16$	$p = 0.21$	$p = 0.77$	$p = 0.02$
After	123.4 ± 83.2	10.7 ± 4.7	**949.4** ± 160	**18.2** ± 11.6	**10135** ± 1924	**25.1** ± 4.0	17.8 ± 5.7	332.6 ± 242.1	7.3 ± 5.7	3.1 ± 5.8	**7.3** ± 7.1

Breast and colorectal cancer patients were treated by surgery whereas melanoma and sarcoma patients were treated by isolated limb perfusion with TNFα, IFNγ and Melphalan. Bold typing denotes values that are significantly different in cancer patients after therapy compared to pre-treatment values. HV, healthy volunteers; BC, breast cancer; CRC, colorectal cancer; ILP, sarcoma or melanoma. Results represent mean values ± SD. $p \leq 0.05$ was considered statistically significant.

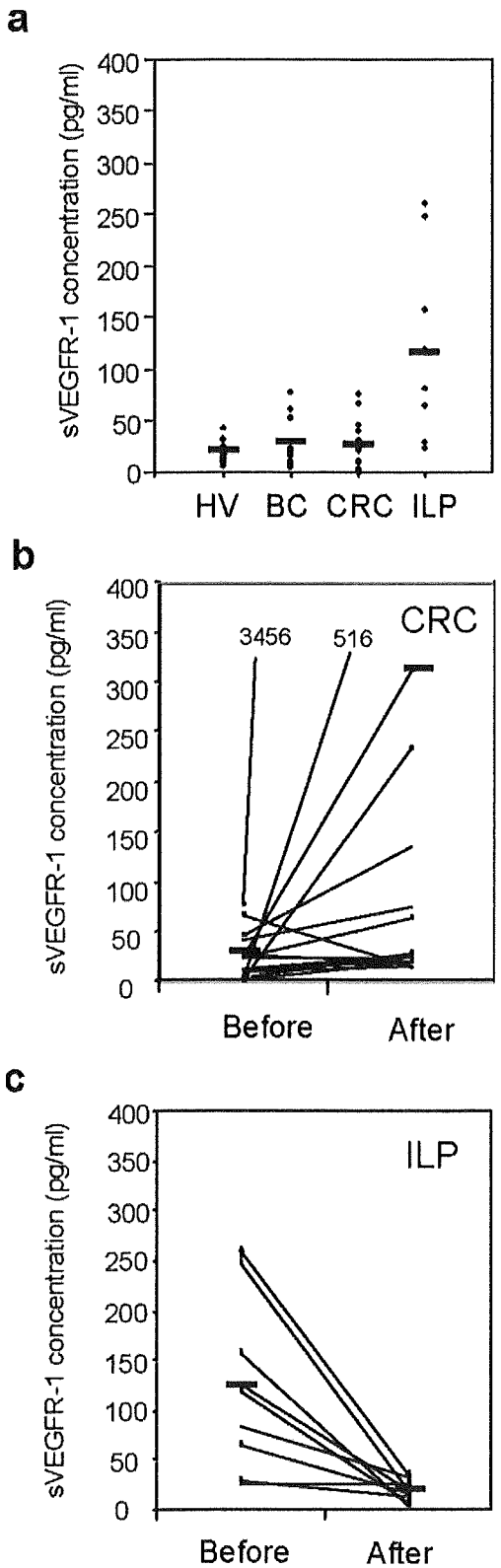


FIGURE 2 – sVEGFR-1 levels. (*a*) sVEGFR-1 values before treatment: sarcoma and melanoma patients had significantly higher levels compared to healthy volunteers. (*b*) sVEGFR-1 values before and after therapy in colorectal cancer patients: values were higher after surgical tumor removal. (*c*) sVEGFR-1 values before and after therapy in sarcoma and melanoma patients before and after ILP: sVEGFR-1 decreased after therapy. The short horizontal lines indicate mean values.

sαVβ3. The mean values of sαVβ3 were significantly lower in CRC and ILP patients compared to healthy volunteers, whereas in BC patients sαVβ3 concentrations were comparable to healthy donors (Table II). A minor, but statistically significant decrease in sαVβ3 was observed in BC patients after therapy (Table III). In CRC and ILP patients sαVβ3 levels were not affected by the treatment. There was no correlation between the sαVβ3 level and number of circulating platelets.

MMP-9. The mean concentrations of MMP-9 were higher in all patient populations, compared to healthy donors, although the difference was statistically significant only in CRC patients (Table II, Fig. 3*a*). After treatment we observed a significant drop in the mean levels of MMP-9 in BC patients, a trend toward decrease concentrations in CRC patients and a trend toward increased levels in ILP patients (Table III, Fig. 3*b,c*).

EDB^+-Fn. The mean values of EDB^+-Fn were significantly lower in BC, CRC and ILP patients than in healthy volunteers (Table II). EDB^+-Fn levels were slightly higher after treatment than before therapy, but the modifications did not reach significance in any of the cancer types (Table III).

IL-8. The mean values of IL-8 were significantly higher in CRC patients compared to healthy volunteers (Table II). These elevated levels decreased upon tumor removal, but did not reach statistical significance (Table III).

CRP. Mean CRP levels were elevated in CRC and ILP patients, but reached statistical significance only in CRC patients (Table II, Fig. 4*a*). CRP levels returned within the normal range in CRC patient upon surgical tumor removal, whereas they increased significantly in ILP patients after treatment (Table III, Fig. 3*b,c*).

Correlation between different factors

The analyses of the results showed that subjects with higher pre-treatment levels of VEGF-A tended to have higher levels of MMP-9 and the converse. This correlation was observed in both BC and CRC patients, although it was most relevant for the latter ($R^2 = 0.58$ and $R^2 = 0.77$, respectively) (Fig. 5*a* and not shown). In ILP patients we also observed a strong correlation, which, however, was largely influenced by one single patient ($R^2 = 0.95$; if highest value omitted, $R^2 = 0.55$) (Fig. 5*b*). There was a trend for a correlation between elevated IL-8 levels and VEGF-A and MMP-9 concentrations ($R^2 = 0.604$ and $R^2 = 0.402$, respectively, data not shown). Similarly CRP and MMP-9 levels and CRP and VEGF-A levels tended to correlate ($R^2 = 0.43$ and $R^2 = 0.36$, respectively), although to a lesser extent. There was a correlation between post-treatment levels of IL-8 and CRP in ILP patients ($R^2 = 0.69$) (Fig. 5*c*). No other relevant positive or negative correlations were observed between any of the other factors.

Correlation with clinical parameters

Given the small number of patients these correlations are only indicative of trends based on median values.

VEGF-A. In BC we observed a trend between increased VEGF-A levels and advanced tumor stage (Stage I < Stage II < Stage III), size (T1 < T2), grade (G1 < G2 < G3), absence of estrogen receptor expression (negative > positive) and lymph node invasion (negative < positive). In colorectal cancer, VEGF-A levels were higher in patients with larger tumors, higher tumor stages (Stage I < Stage II–III), tumor infiltration (T1 < T2 < T3) and lymph node invasion (N1 < N2).

sVEGFR-1. In BC patients lower sVEGFR-1 levels were associated with higher stages (Stage I > Stage II > Stage III), larger tumors (T1 > T2) and lymph node infiltration (negative > positive). In CRC patients, however, the trend was the opposite: sVEGFR-1 levels were higher for higher stages (Stage I < Stage II–III), tumor infiltration (T1 < T2–T3) and lymph node invasion (N1 < N2).

MMP-9. In BC, MMP-9 levels were higher in higher stages (Stage I < Stage II < Stage III) and higher tumor size (T1 < T2).

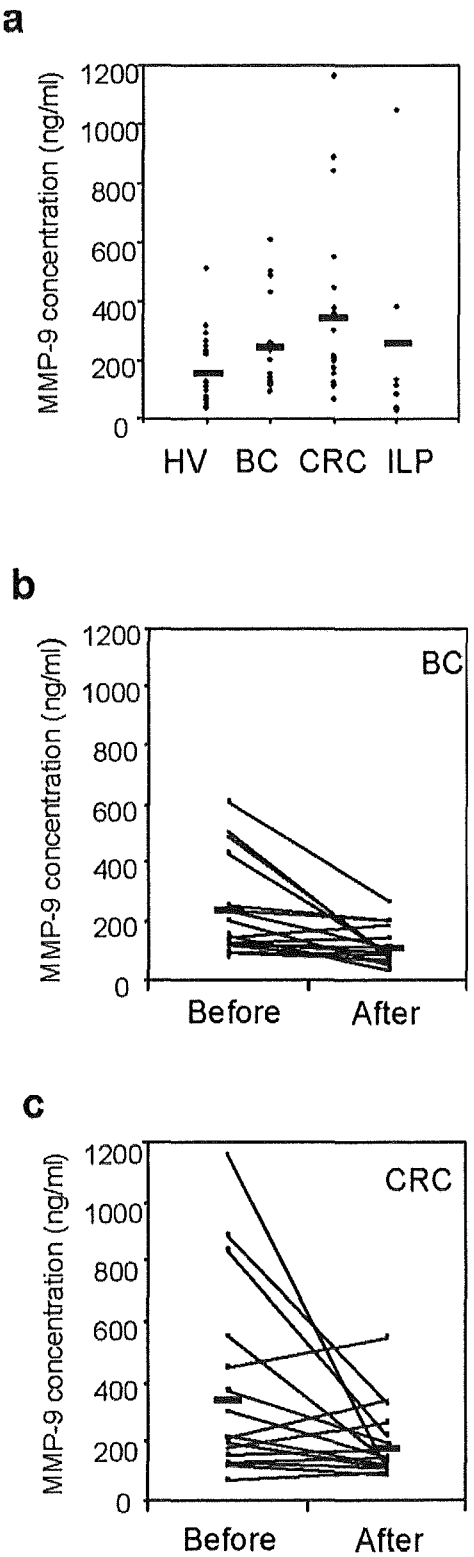


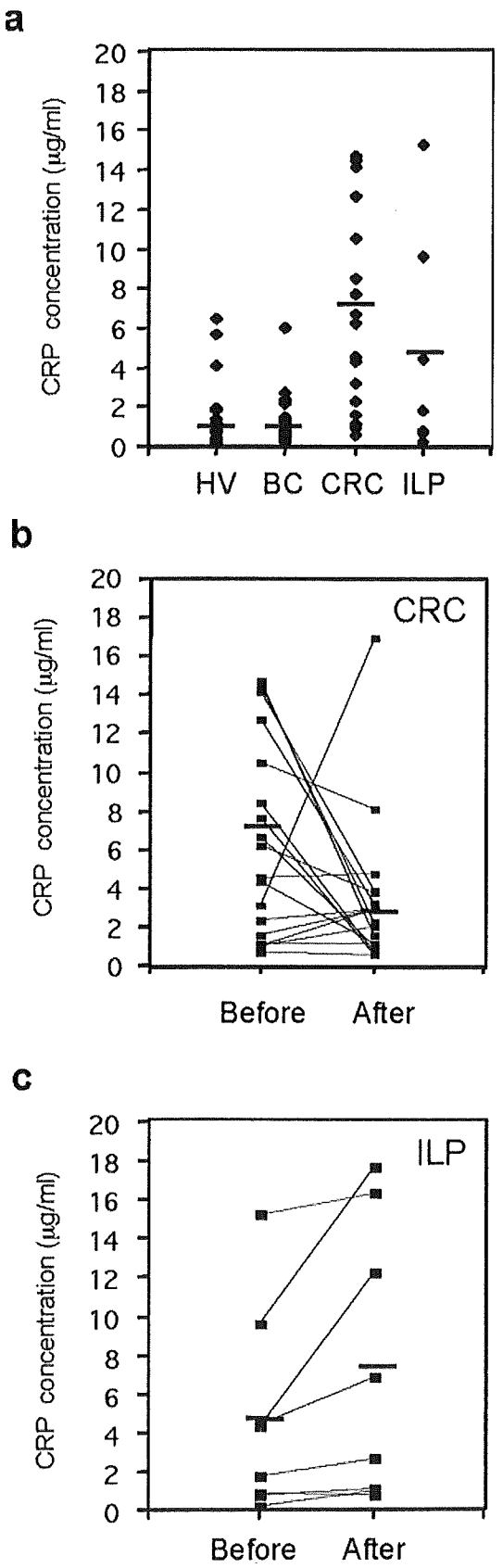
FIGURE 3 – MMP-9 levels (*a*) MMP-9 values before treatment. Colorectal cancer patients had significantly higher levels compared to healthy volunteers. (*b*) Values before and after treatment in breast cancer patients. (*c*) Values before and after treatment in colorectal cancer patients. HV, healthy volunteers; BC, breast cancer; CRC, colorectal cancer; ILP, sarcoma and melanoma patients. The short horizontal lines indicate mean values.

In CRC, higher MMP-9 correlated with the stage (Stage I < Stages II–III) tumor infiltration (T1 < T2 < T3) and lymph node invasion (N1 < N2).

Discussion

Our work was initiated as an exploratory study aimed at measuring multiple angiogenesis-associated molecules in the blood of cancer patients, analyzing putative correlations and identifying molecules as candidate markers for further investigation in prospective clinical studies. Each factor was measured within the same patient before and after resection of the primary tumor (BC and CRC). The measurements were also carried out in a small group of patients undergoing curative-intent regional chemotherapy with TNF for loco-regionally advanced sarcoma or melanoma of the limb, and in healthy individuals.

In breast and colorectal cancer patients, only few of the tested factors were altered significantly compared to healthy volunteers. There was an important inter-patient variation leading to extensive overlap with the healthy subjects. If the level of a given factor was directly related to the presence of the tumor, one would expect altered levels for it in a majority of the patients. This simple expectation was not confirmed. SCF, PlGF and sVEGFR-1 were not increased in patients with tumors, whereas VEGF-A, sVEGFR-2, sTie-2, sαVβ3, EDB$^+$-Fn and MMP-9 showed some significant differences compared to healthy donors, but with an important heterogeneity within patients. In breast and colorectal cancer patients, VEGF-A and MMP-9 were the 2 markers that best fulfilled the characteristics expected for surrogate marker of angiogenesis: they were elevated in patients before treatment (although only in about a third of the breast cancers and a half of the colorectal cancers), and their values dropped after tumor removal. Fourteen colorectal cancer patients and one breast cancer patient responded to tumor removal by a clear increase in sVEGFR-1 levels (Fig. 2*b*). The reason for this change is not clear. VEGFR-1 is produced in 2 isoforms with contrasting functions in angiogenesis. The full-length transmembrane form stimulates angiogenesis by transactivating VEGFR-2,[14] promoting the release of hematopoietic and angiogenic precursor cells from bone marrow,[15] stimulating the migration of monocytes[34] and activating lung endothelial cells at premetastatic sites.[35] In contrast, the soluble form (sVEGFR-1), which consists of the extracellular domain as a result of alternative mRNA splicing,[16] binds and inactivates circulating VEGF-A[36] and is considered to be an endogenous negative regulator of angiogenesis. In breast cancer, a low VEGFR-1/VEGF-A ratio in the tumor tissue itself was reported to be a poor prognostic factor.[37] One could hypothesize that in our patients, the tumor itself may inhibit sVEGFR-1 production, and that this inhibition is abrogated upon tumor removal. The implication of the increase in sVEGFR-1 observed in some of the operated patients is unknown at this point. It should also be stressed that sVEGFR-1 can also be generated by proteolytic cleavage of the transmembrane form at the cell surface. The assay used in our study does not allow to differentiate between the 2 forms. It is therefore possible that the sVEGFR-1 measured in these patients may consist of a mixture of the alternatively spliced variant, and of the proteolytically cleaved form. Because these 2 forms may reflect different biological events or cellular sources, it may be worth to address this question in future studies. Compared to healthy volunteers, sVEGFR-2 and sTie-2 were slightly but significantly increased (approximately 15–50 % of normal values). In contrast, the mean values of sαVβ3 and EDB$^+$-Fn were several folds lower in cancer patients *vs.* healthy volunteers. The absence of correlation between the number of circulating platelets and the level of sαVβ3 suggests that platelets are unlikely to be the source of the sαVβ3 measured here. Although on a per platelet basis, αVβ3 is much less abundant than αiibβ3 (about 1,000 *vs.* 50,000 copies),[38] platelet-derived αVβ3 could be of potential concern considering the large number of circulating platelets. The biological significance of these molecules as surrogate markers of angiogenesis is questionable because their levels did not change after tumor removal. This



observation, however, raises the intriguing questions of whether these patients may have constitutively altered levels of these molecules and whether altered levels may correlate with an enhanced angiogenic response or susceptibility to tumor progression ('predisposition factors'). This hypothesis may be of particular relevance for $\alpha V\beta 3$, because mice lacking $\alpha V\beta 3$ expression were reported to have enhanced tumor angiogenesis and tumor growth.[39]

CRP behaved as a tumor marker for colorectal cancer patients: it was significantly elevated before treatment and it returned within normal levels after surgery. The trend toward a positive association with VEGF-A and MMP-9 levels, although not as strong and between VEGF-A and MMP-9, hints at a possible association with angiogenesis. Apparently contrasting observations about a possible role of CRP in controlling angiogenesis exist in the literature. In multiple myeloma CRP positively correlated with blood levels of VEGF[27,28] and with increased bone marrow angiogenesis.[40,41] Elevated levels of CRP were reported associated with a reduced number of circulating endothelial cell precursors in cardiac patients,[42] and CRP was shown to decrease survival, differentiation and function of endothelial cell precursors[43] and to inhibit angiogenesis by suppressing nitric oxide production.[44] Although our results clearly associate elevated levels of CRP with the presence of colorectal cancer, more studies are necessary CRP levels can be linked to tumor angiogenesis.

Among the 9 factors investigated we could only find a strong correlation between VEGF-A and MMP-9 levels, most notably in CRC patients (Fig. 3). To our knowledge, this correlation has not been described previously in the blood of patients with solid tumors, but it was seen in pleural effusions in patients with tuberculosis and lung cancer[45] and in the plasma of patients with adult T cell leukemia.[46] This correlation is consistent with preclinical data reporting functional interdependence between VEGF-A and MMP-9. VEGF-A can induce MMP-9 expression in different cells, whereas MMP-9 can promote the release of ECM- and cell surface-bound VEGF-A, thereby increasing its bioavailability. VEGF-A was reported to enhance production of MMP-9 by human vascular smooth muscle cells[47] and to induce MMP-9 in pre-metastatic lung endothelial cells and macrophages, thereby promoting lung metastasis formation.[35] In the Rip1Tag2 model of multistep carcinogenesis, increased MMP-9 activity promoted the release of matrix-bound VEGF-A resulting in the angiogenic switch and enhanced tumor progression.[19] In ovarian cancer cells MMP-9 activity promoted angiogenesis[48] and dose-dependent VEGF-A release.[49] A correlation trend was found between increased levels of CRP and VEGF-A, and CRP and MMP-9 ($R^2 = 0.43$ and $R^2 = 0.36$, respectively) and between IL-8 levels and VEGF-A and MMP-9 ($R^2 = 0.604$ and $R^2 = 0.402$, respectively).

Although our study was not designed to demonstrate correlations between the measured factors and clinical parameters, some interesting trends between VEGF-A or MMP-9 and clinical-pathological characteristics, were nevertheless observed. In breast cancer, VEGF-A levels tended to be higher in patients with larger tumors, advanced tumor stages and more aggressive histology (higher grade, negative hormone receptor status). For MMP-9, a trend between elevated levels and larger tumor size and more advanced stages was also observed. In colorectal cancer patients, VEGF-A and MMP-9 values tended to be higher in patients with more advanced tumor stages, local infiltration and lymph node metastases. These observations are in line with published studies

FIGURE 4 – (*a*) CRP values before treatment. Colorectal cancer patients had significantly higher levels compared to healthy volunteers. ILP patients also had increased levels but the difference was not significant (*b*) CRP values before and after treatment in colorectal cancer patients. (*c*) Values before and after treatment in ILP patients. HV, healthy volunteers; BC, breast cancer; CRC, colorectal cancer; ILP, sarcoma and melanoma patients. The short horizontal lines indicate mean values.

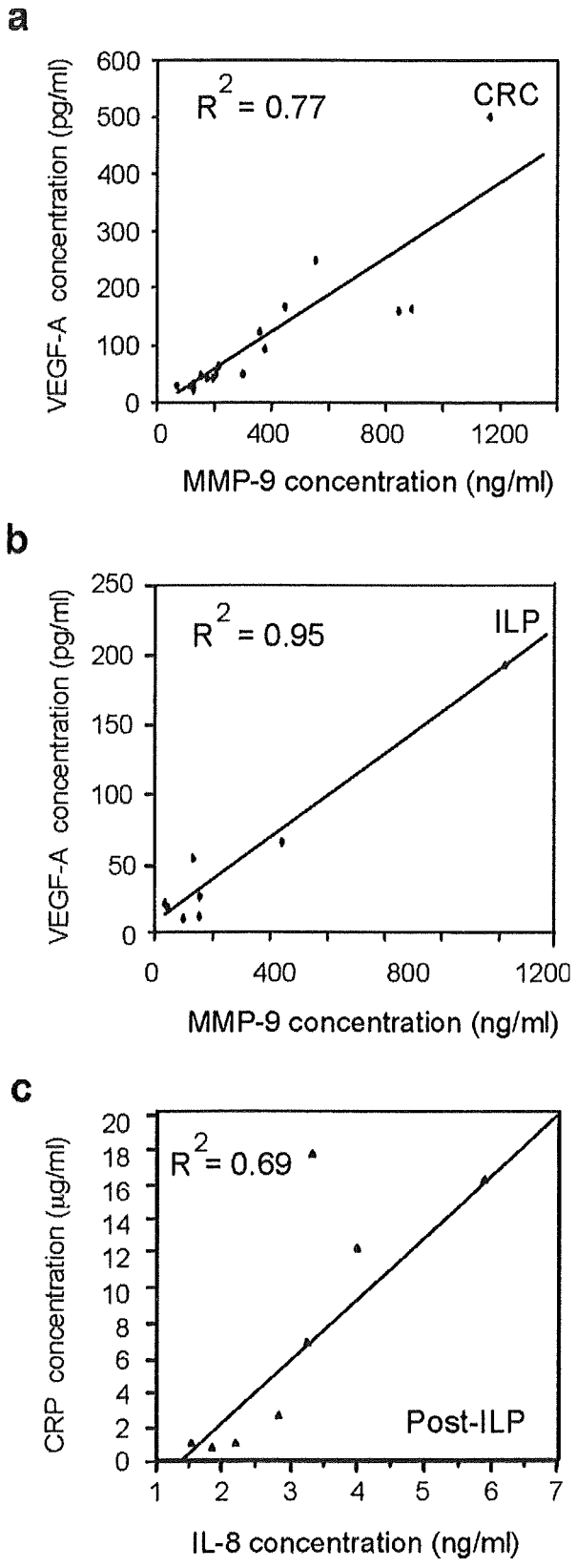


FIGURE 5 – Correlations. (*a*) Correlation between pre-treatment levels of VEGF-A and MMP-9 in colorectal cancer patients and (*b*) in ILP patients (*c*). Correlation between post-treatment levels of CRP and IL-8 in ILP patients. The correlation coefficient (R^2) is given within each individual panel. RC, colorectal cancer; ILP, sarcoma and melanoma patients.

reporting correlations between circulating levels of VEGF-A and tumor stage,[50,51] disease progression[52,53] and patient survival[54–56] in different cancers. Elevated systemic levels of MMP-9 were reported to be associated to tumor infiltration, tumor stage, node metastasis and poorer survival[57,58] in various cancers.

In comparison to breast and colorectal cancer patients who were treated by curative surgical resection, we included 8 patients with advanced but still local tumors (*i.e.*, limited to a limb) who received regional chemotherapeutic treatment by ILP. ILP allows local administration of high dose chemotherapy without affecting other organs, leading to a high proportion of complete tumor regressions.[59] The pattern of the measured factors before therapy in ILP patients was strikingly different compared to BC and CRC patients and is, in part related to the differences in the type, stage and size of the tumors. The systemic changes observed in ILP patients after therapy were nearly the opposite of those seen in surgically treated patients: significant increase in VEGF-A, SCF, sVEGFR-2, MMP-9, sTie-2 and CRP, and decrease in sVEGFR-1 levels. The rise in SCF level, although modest, may reflect an involvement of the bone marrow in mobilizing angiogenic endothelial cell precursors or mast cells.[15] These changes are consistent with enhanced angiogenesis, whereas changes observed after surgical tumor removal are consistent with decreased angiogenesis. TNF causes a selective and acute vascular damage to the tumor vasculature resulting in increased accumulation of melphalan in the tumor tissue, followed by a vascular collapse, and a massive, long lasting tumor necrosis.[30] TNF also elicits a systemic inflammatory response, as demonstrated by the increased systemic levels of sTNF-R2, IL-6, CRP and Tenascin-C.[60,61] The increase in CRP concentration after ILP was confirmed here and its association with IL-8 levels suggests the possibility that a sustained inflammatory reaction may persists in or around the treated tumors. Such a condition would be consistent with the reported upregulation of IL-8 in response to Ras activation, which is a paradigm of a signaling pathway of tissue repair.[24] One can therefore postulate that sustained inflammation and tissue hypoxia may persist in and around necrotic tissues after ILP, thereby contributing to an 'angiogenic switch' in these patients. If confirmed, this 'angiogenic switch' is likely to have potential clinical implications because it may favor re-growth of angiogenic vessels thereby promoting survival and growth of tumor cells not killed by the treatment. Among the patients analyzed, 5 experienced a loco-regional relapse and one developed distant metastases on both. It was, however, difficult to correlate progression with the degree of increase in the levels of these factors because of the small sample size. Two patients were treated by surgery after ILP (sarcoma) and tumor tissue was analyzed histologically. The patient with the highest degree of increase in VEGF and MMP-9 showed residual viable tumor tissue, whereas the patient who had only minor modifications in the levels of VEGF, MMP-9 had complete histological remission. These 2 observations are consistent with the hypothesis that a putative angiogenic switch may occur in patients with incomplete anti-tumor response and residual tumor tissue persists after ILP. Because of the potential clinical relevance of this observation, we plan to prospectively compare VEGF-A, MMP-9 and sVEGF-R1 levels in patients with locally advanced sarcoma undergoing surgical tumor removal or ILP, respectively. In parallel we will evaluate the presence of hypoxic regions in and around the tumors by position emission tomography.[62] If this hypothesis is confirmed, the administration of an anti-angiogenic agent after ILP to suppress reactive angiogenesis may improve outcome.

In conclusion, among all factors measured, only VEGF-A and MMP-9 consistently correlated to each other, elevated CRP levels were associated with tumor burden, whereas sVEGF-R1 increased after tumor removal in colorectal cancer. Treatment with chemotherapy and TNF induced caused changes consistent with an 'angiogenic switch.' These results warrant a prospective study to compare the effect of surgical tumor removal *vs.* chemotherapy on some of these markers and to evaluate their prognostic/predictive value. If the 'angiogenic switch' after ILP will be confirmed, we

will consider the addition of an adjuvant anti-angiogenic therapy to counteract this potentially pro-angiogenic effect of ILP.

Acknowledgements

The authors wish to thank all clinical colleagues and nurses, in particular Pr. M. Gillet, Dr. F. Delaloye, Dr. V. Gribinsky, Dr. O. Michielin and Mrs. S. Willcox, for their invaluable help in obtaining patient samples. We thank Dr. G. Van Melle for expert advise in the statistical analysis of the results. We also thank Dr. F. Mitjans, Merck LBI, for cloning mAb 20H9. Our study was supported by a grant from the Swiss National Science Foundation (31-63752) and by the Molecular Oncology Program of the National Center for Competence in Research (NCCR), a research instrument of the Swiss National Science Foundation.

References

1. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Cancer Biol 1992;3:65–71.
2. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, et al. Bevacizumab plus Irinotecan, Fluorouracil, and Leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–42.
3. Ruegg C, Meuwly JY, Driscoll R, Werffeli P, Zaman K, Stupp R. The quest for surrogate markers of angiogenesis: a paradigm for translational research in tumor angiogenesis and anti-angiogenesis trials. Curr Mol Med 2003;3:673–91.
4. Libutti SK, Choyke P, Carrasquillo JA, Bacharach S, Neumann RD. Monitoring responses to antiangiogenic agents using noninvasive imaging tests. Cancer J Sci Am 1999;5:252–6.
5. Sheen-Chen S, Chen H, Eng H, Sheen C, Chen W. Serum levels of matrix metalloproteinase 2 in patients with breast cancer. Cancer Lett 2001;173:79–82.
6. Zucker S, Lysik R, Zarrabi M, Moll U. M(r) 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. Cancer Res 1993;53:140–6.
7. Harris A, Reusch P, Barleon B, Hang C, Dobbs N, Marme D. Soluble Tie2 and Flt1 extracellular domains in serum of patients with renal cancer and response to antiangiogenic therapy. Clin Cancer Res 2001;7:1992–7.
8. Hu Q, Dey A, Yang Y, Shen Y, Jilani I, Estey E, Kantarjian H, Giles F, Albitar M. Soluble vascular endothelial growth factor receptor 1, and not receptor 2, is an independent prognostic factor in acute myeloid leukemia and myelodysplastic syndromes. Cancer 2004;100: 1884–91.
9. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002;2:727–39.
10. Poon RT, Fan ST, Wong J. Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol 2001;19:1207–25.
11. Ziche M, Sorriso C, Parenti A, Brogelli L, Tricarico C, Villari D, Pazzagli M. Biological parameters for the choice of antiangiogenic therapy and efficacy monitoring. Int J Biol Markers 1999;14:214–7.
12. George D, Halabi S, Shepard T, Vogelzang N, Hayes D, Small EJ, Kantoff PW, Cancer and Leukemia Group B 9480. Prognostic significance of vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B9480. Clin Cancer Res 2001;7:1932–6.
13. Ranuncolo S, Armanasco E, Cresta C, Bal De Kier Joffe E, Puricelli L. Plasma MMP-9 (92 kDa-MMP) activity is useful in the follow-up and in the assessment of prognosis in breast cancer patients. Int J Cancer 2003;106:745–51.
14. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001;7:575–83.
15. Rafii S, Lyden D, Benezra R, Hattori K, Heissig B. Vascular and hematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2002;2:826–35.
16. Sato Y, Kanno S, Oda N, Abe M, Ito M, Shitara K, Shibuya M. Properties of two VEGF receptors, Flt-1 and KDR, in signal transduction. Ann N Y Acad Sci 2000;902:201–5.
17. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 1994;264:569–71.
18. Jones N, Dumont DJ. Tek/Tie2 signaling: new and old partners. Cancer Metastasis Rev 2000;19:13–7.
19. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737–44.
20. Castellani P, Viale G, Dorcaratto A, Nicolo G, Kaczmarek J, Querze G, Zardi L. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. Int J Cancer 1994;59:612–8.
21. Astrof S, Crowley D, George EL, Fukuda T, Sekiguchi K, Hanahan D, Hynes RO. Direct test of potential roles of EIIIA and EIIIB alternatively spliced segments of fibronectin in physiological and tumor angiogenesis. Mol Cell Biol 2004;24:8662–70.
22. Lin Y, Huang R, Chen L, Li S, Shi Q, Jordan C, Huang RP. Identification of interleukin-8 as estrogen receptor-regulated factor involved in breast cancer invasion and angiogenesis by protein arrays. Int J Cancer 2004;109:507–15.
23. Haraguchi M, Komuta K, Akashi A, Matsuzaki S, Furui J, Kanematsu T. Elevated IL-8 levels in the drainage vein of resectable Dukes' C colorectal cancer indicate high risk for developing hepatic metastasis. Oncol Rep 2002;9:159–65.
24. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell 2004; 6:447–58.
25. Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C-reactive protein and the risk of incident colorectal cancer. JAMA 2004;291:585–90.
26. Nozoe T, Matsumata T, Kitamura M, Sugimachi K. Significance of preoperative elevation of serum C-reactive protein as an indicator for prognosis in colorectal cancer. Am J Surg 1998;176:335–8.
27. Alexandrakis MG, Passam FH, Boula A, Christophoridou A, Aloizos G, Roussou P, Kyriakou DS. Relationship between circulating serum soluble interleukin-6 receptor and the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in multiple myeloma. Ann Hematol 2003;82:19–23.
28. Alexandrakis MG, Passam FH, Sfiridaki K, Moschandrea J, Pappa C, Liapi D, Petreli E, Roussou P, Kyriakou DS. Interleukin-18 in multiple myeloma patients: serum levels in relation to response to treatment and survival. Leuk Res 2004;28:259–66.
29. Lejeune FJ, Ruegg C, Lienard D. Clinical applications of TNF-alpha in cancer. Curr Opin Immunol 1998;10:573–80.
30. Lejeune F, Kroon B, Di Filippo F, Hoekstra H, Santinami M, Lienard D, Eggermont A. Isolated limb perfusion: the European experience. Surg Oncol Clin N Am 2001;10:821–32.
31. Sipos B, Hahn D, Carceller A, Piulats J, Hedderich J, Kalthoff H, Goodman SL, Kosmahl M, Kloppel G. Immunohistochemical screening for beta-integrin subunit expression in adenocarcinomas using a novel monoclonal antibody reveals strong upregulation in pancreatic ductal adenocarcinomas in vivo and in vitro. Histopathology 2004; 45:226–36.
32. Mehta RJ, Diefenbach B, Brown A, Cullen E, Jonczyk A, Gussow D, Luckenbach GA, Goodman SL. Transmembrane-truncated alphav-beta3 integrin retains high affinity for ligand binding: evidence for an 'inside-out' suppressor? Biochem J 1998;330:861–9.
33. Gunsilius E, Petzer A, Stockhammer G, Nussbaumer W, Schumacher P, Clausen J, Gastl G. Thrombocytes are the major source for soluble vascular endothelial growth factor in peripheral blood. *Oncology* 2000;58:169–74.
34. Barleon B, Sozzani S, Zhou D, Weich H, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor Flt-1. Blood 1996;87:3336–43.
35. Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley J, Senior R, Shibuya M. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2002;2:289–300.
36. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci USA 1998;95:9349–54.
37. Toi M, Bando H, Ogawa T, Muta M, Hornig C, Weich H. Significance of vascular endothelial growth factor (VEGF)/soluble VEGF receptor-1 relationship in breast cancer. Int J Cancer 2002;98:14–8.
38. Quinn MJ, Cox D, Foley JB, Fitzgerald DJ. Glycoprotein IIb/IIIa receptor number and occupancy during chronic administration of an oral antagonist. J Pharmacol Exp Ther 2000;295:670–6.
39. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, Hodivala-Dilke KM. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 2002;8:27–34.
40. Munshi NC, Wilson C. Increased bone marrow microvessel density in newly diagnosed multiple myeloma carries a poor prognosis. Semin Oncol 2001;28:565–9.
41. Alexandrakis MG, Passam FH, Dambaki C, Pappa CA, Stathopoulos EN. The relation between bone marrow angiogenesis and the prolifer-

ation index Ki-67 in multiple myeloma. J Clin Pathol 2004;57: 856–60.
42. George J, Goldstein E, Abashidze S, Deutsch V, Shmilovich H, Finkelstein A, Herz I, Miller H, Keren G. Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation. Eur Heart J 2004;25:1003–8.
43. Verma S, Kuliszewski MA, Li SH, Szmitko PE, Zucco L, Wang CH, Badiwala MV, Mickle DA, Weisel RD, Fedak PW, Stewart DJ, Kutryk MJ. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. Circulation 2004;109:2058–67.
44. Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. Circulation 2002;106:913–9.
45. Jin HY, Lee KS, Jin SM, Lee YC. Vascular endothelial growth factor correlates with matrix metalloproteinase-9 in the pleural effusion. Respir Med 2004;98:115–22.
46. Hayashibara T, Yamada Y, Onimaru Y, Tsutsumi C, Nakayama S, Mori N, Miyanishi T, Kamihira S, Tomonaga M, Maita T. Matrix metalloproteinase-9 and vascular endothelial growth factor: a possible link in adult T-cell leukaemia cell invasion. Br J Haematol 2002;116: 94–102.
47. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of Flt-1. Circ Res 1998;83:832–40.
48. Huang S, Van Arsdall M, Tedjarati S, McCarty M, Wu W, Langley R, Fidler I. Contribution of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. J Natl Cancer Inst 2002;94:1134–42.
49. Belotti D, Paganoni P, Manenti L, Garofalo A, Marchini S, Taraboletti G, Giavazzi R. Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation. Cancer Res 2003;63:5224–9.
50. George M, Eccles S, Tutton M, Abulafi A, Swift R. Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelets scavenging. Clin Cancer Res 2000;6:3147–52.
51. Chin K, Greenman J, E. G, Kumar H, Topping K, Monson J. Preoperative serum vascular endothelial growth factor can predict stage in colorectal cancer. Clin Cancer Res 1998;4:1279–85.
52. Dirix L, Vermeulen P, Hubens G, I. B, Martin M, C. DP, Van Oosterom A. Serum basic fibroblast growth factor and vascular endothelial growth factor and tumor growth kinetics in advanced colorectal cancer. Ann Oncol 1996;7:843–8.
53. Wu Y, Saldana L, Chillar R, Vadgama J. Plasma vascular endothelial growth factor is useful in assessing progression of breast cancer post surgery and during adjuvant treatment. Int J Oncol 2002;20:509–16.
54. Karayiannakis A, Syrigos K, Zbar A, Baibas N, Polychronidis A, Simopoulos C, Karatzas G. Clinical significance of preoperative serum vascular endothelial growth factor levels in patients with colorectal cancer and the effect of tumor surgery. Surgery 2002;131:548–55.
55. Salven P. High pre-treatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small cell lung cancer. Int J Cancer 1998;79:144–6.
56. Chin K, Greenman J, Gardiner E, Kumar H, Topping K, Monson J. Pre-operative serum vascular endothelial growth factor can select patients for adjuvant treatment after curative resection in colorectal cancer. Br J Cancer 2000;83:1425–31.
57. Ruokolainen H, Paakko P, Turpeenniemi-Hujanen T. Expression of matrix metalloproteinase-9 in head and neck squamous cell carcinoma: a potential marker for prognosis. Clin Cancer Res 2004;10: 3110–6.
58. Lin S, Wang Y, Sheu W. Preoperative plasma concentrations of vascular endothelial growth factor and matrix metalloproteinase 9 are associated with stage progression in papillary thyroid cancer. Clin Endocrinol (Oxf) 2003;58:513–8.
59. Lejeune F, Pujol N, Lienard D, Mosimann F, Raffoul W, Genton A, Guillou L, Landry M, Chassot P, Chiolero R, Bischof-Delaloye A, Leyvraz S, et al. Limb salvage by neoadjuvant isolated perfusion with TNFalpha and melphalan for non-resectable soft tissue sarcoma of the extremities. Eur J Surg Oncol 2000:669–78.
60. Gerain J, Lienard D, Pampallona S, Baumgartner M, Ruegg C, Buurman WA, Eggermont A, Lejeune F. Systemic release of soluble TNF receptors after high-dose TNF in isolated limb perfusion. Cytokine 1997;9:1034–42.
61. Schienk S, Lienard D, Gerain J, Baumgartner M, Lejeune FJ, Chiquet-Ehrismann R, Ruegg C. Rapid increase in plasma tenascin-C concentration after isolated limb perfusion with high-dose tumor necrosis factor (TNF), interferon gamma (IFN gamma) and melphalan for regionally advanced tumors. Int J Cancer 1995;63:665–72.
62. Barthel H, Wilson H, Collingridge DR, Brown G, Osman S, Luthra SK, Brady F, Workman P, Price PM, Aboagye EO. In vivo evaluation of [18F]fluoroetanidazole as a new marker for imaging tumour hypoxia with positron emission tomography. Br J Cancer 2004;90: 2232–42.