# Anemia and Elevated Systemic Levels of Vascular Endothelial Growth Factor (VEGF)

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**Background:** Tissue hypoxia is a major stimulus for the up-regulation of vascular endothelial growth factor (VEGF). Anemia might theoretically impact on angiogenesis via impairment of tissue oxygenation. We have investigated this hypothesis in patients with solid cancers and benign diseases.

**Patients and Methods:** 49 patients with untreated locoregionally confined solid cancers of the head and neck, cervix, rectum and lung and 59 additional patients with non-malignant diseases (36 normemic patients without serious diseases and 23 patients with renal anemia) were enrolled and the impact of anemia on plasma VEGF levels were determined. VEGF was measured with a commercially available sandwich enzyme immunoassay technique.

**Results:** Plasma levels of VEGF were 16.2 ± 12.7 pg/ml in 36 normemic patients without malignant disease, 49.2 ± 34.5 pg/ml in 49 patients with cancers ($p < 0.001$), and 89.9 ± 67.8 pg/ml in 23 patients with renal anemia ($p = 0.001$). VEGF levels in cancer patients were significantly correlated with hemoglobin (hb) levels and platelet counts (each $p = 0.001$), but not with type of tumor, stage, histology or age. Patients with cancers had higher plasma levels of VEGF than patients with non-malignant diseases in case of hb ≥ 12 g/dl (33.1 ± 17.5 vs 16.6 ± 13.0 pg/ml, $p < 0.001$) and in case of hb between 11.0 and 11.9 g/dl (56.1 ± 26.4 vs 18.5 ± 14.5 pg/ml, $p = 0.038$). In case of a hb < 11 g/dl, plasma VEGF levels were significantly elevated in patients with and without cancers (67.0 ± 47.5 vs 88.9 ± 68.8 pg/ml, n.s.). In a multivariate model, a significant association between low hb levels and increased plasma levels of VEGF was confirmed. In 16 patients with renal anemia, changes in hb under erythropoietin treatment were inversely correlated with changes in plasma VEGF levels with decreasing VEGF after increase in hb ($p = 0.01$).

**Conclusions:** Anemic patients have elevated levels of VEGF. The data suggest that anemia might impact on the progression of angiogenesis in malignant and benign diseases.

**Key Words:** Anemia · Angiogenesis · Hypoxia · Cancer

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**Anämie und erhöhte systemische Spiegel des Vascular Endothelial Growth Factor (VEGF)**

**Hintergund:** Gewebehypoxie ist ein wichtiger Stimulus für die Hochregulierung des Vascular Endothelial Growth Factor (VEGF), einem Schlüsselzytokin der Angiogenese. Eine Anämie kann die Tumorhypoxie verstärken. Wir haben deshalb die Hypothese geprüft, ob eine Anämie (über die Verstärkung von Hypoxie) mit verstärkter Angiogenese assoziiert ist.

**Patienten und Methodik:** 49 Patienten mit unbehandelten, lokoregionär begrenzten Tumoren der Kopf-Hals-Region, der Zervix, des Rektums und der Lunge gingen in die Untersuchung ein, außerdem als Kontrolle 59 Patienten ohne maligne Erkrankungen (36 normämische Patienten aus einer Allgemeinarztpraxis und 23 Patienten mit renaler Anämie). Bei diesen Patienten wurde der Plasmaspiegel von VEGF mittels eines kommerziell erhältlichen Sandwich-Enzym-Immunoassays gemessen.

**Ergebnisse:** Die VEGF-Plasmaspiegel betrugen 16,2 ± 12,7 pg/ml bei 36 normämischen Patienten ohne Tumoren, 49,2 ± 34,5 pg/ml bei 49 Patienten mit Tumoren ($p < 0,001$) und 89,9 ± 67,8 pg/ml bei 23 Patienten mit renaler Anämie ($p = 0,001$). Es fand sich eine signifikante Korrelation der VEGF-Spiegel mit den Hämoglobin-(Hb-) und den Thrombozytenwerten (jeweils $p = 0,001$). Dagegen bestand kein Zusammenhang zwischen VEGF und Tumorstadium, Alter oder Tumorlokalisation. Tumorpatienten hatten im Vergleich zu Patienten ohne Tumoren signifikant erhöhte VEGF-Spiegel, wenn der Hb-Wert ≥ 12 g/dl war (33,1 ± 17,5 vs. 16,6 ± 13,0 pg/ml, $p < 0,001$) und bei einem Hb-Wert zwischen 11,0 und 11,9 g/dl (56,1 ± 26,4 vs. 18,5 ± 14,5 pg/ml, $p = 0,038$). Bei anämischen Patienten mit einem Hb < 11 g/dl waren die Plasma-VEGF-Spiegel in beiden Gruppen signifikant erhöht und nicht verschieden voneinander (67,0 ± 47,5 vs. 88,9 ± 68,8 pg/ml, n.s.). Der Zusammenhang zwischen Hb-Wert und VEGF-Spiegel wurde

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in einem multivariaten Modell bestätigt. Bei 16 Patienten mit renaler Anämie wurden Verlaufsmessungen des VEGF-Spiegels unter Behandlung mit Erythropoietin durchgeführt; dabei fand sich ein signifikanter Zusammenhang mit einem Abfall des erhöhten VEGF bei Anstieg des Hb-Wertes (p = 0,01).

**Schlussfolgerungen:** Anämische Patienten haben erhöhte systemische Konzentrationen von VEGF. Dies deutet auf einen eigenständigen Einfluss der Anämie auf Angiogenese hin.

**Schlüsselwörter:** Anämie · Angiogenese · Hypoxie · Tumoren

## Introduction

Angiogenesis is a major prognostic factor in human tumors [8]. It is regulated by different cytokines of which the most important is vascular endothelial growth factor (VEGF) [24, 29]. The VEGF protein can be detected immunohistologically in tumors and can quantitatively be measured in sera and cell culture supernatants. Elevated levels of soluble VEGF have been detected in sera of tumor-bearing animals and patients with cancer suggesting that tumors may release relevant amounts of VEGF in the blood circulation [20, 33]. Cancer patients with elevated systemic VEGF levels have a worse prognosis as compared to patients with normal VEGF levels [17, 21, 22, 27]. A significant increase of measurable serum levels of VEGF has recently also been described in patients with non-malignant disorders, such as acute myocardial infarction, rheumatoid arthritis or burns [8, 12].

Anemia is well-known as a major prognostic factor in various solid tumors and hematological malignancies. Several recent experimental and clinical investigations have demonstrated that anemia, even if mild, worsens tumor tissue oxygenation [2, 18, 19]. Hypoxia, however, is a major stimulus for the up-regulation of VEGF [4, 5, 29]. We have recently demonstrated an association between tumor hypoxia and elevated systemic levels of VEGF in head and neck cancers [3, 7]. If anemia impacts on tumor hypoxia and if hypoxia stimulates angiogenesis, one might assume an indirect association between anemia and angiogenesis. Preliminary results of our lab have supported an association between low hemoglobin (hb) levels and elevated levels of serum VEGF in cancer patients [6]. In this article, we therefore propose the hypothesis that anemia may act as a cofactor for angiogenesis via an indirect effect on tissue hypoxia.

## Patients and Methods

Plasma levels of VEGF were measured in 49 patients with locoregionally confined head and neck, cervical, gastrointestinal or lung cancers who were treated in our department with curative intent in the period from 1998 through 1999. Patients' characteristics with regard to tumor type are listed in Table 1. The hb levels in cancer patients ranged from 7.9 through 16.0 g/dl. 22/49 (45%) patients had hb levels in the normal range (> 12 g/dl), 19 (39%) had mild to moderate anemia and 8 (16%) had severe anemia with a hb below 9 g/dl.

59 patients with non-malignant diseases were also investigated and served as control group without cancers. 36 of them presented in a general practice for various, mainly not serious diseases, e.g. hypertension, diabetes, low back pain. These patients had hb levels in the normal range and represented a non-anemic control group without cancers. A further number of 23 patients with renal anemia served as an anemic control group without cancers (see Table 1).

### Measurement of Plasma VEGF

Measurement of soluble plasma VEGF ($VEGF_{165}$) was done with a commercially assay (Quantikine, R&D Systems, Europe) according to the manufacturer's instructions with slight modifications. Plasma samples were prepared by centrifugation with 2 400 × g for 30 minutes using citrate as anticoagulant and aliquots of 500 µl were stored at –20 °C until performing the measurement. Samples were only thawed once for the assay. The quantitative sandwich enzyme immunoaasay uses a murine monoclonal antibody against VEGF which is precoated onto microplates. In brief, 100 µl of assay diluent and 100 µl of samples were added to the wells and incubated

**Table 1.** Main characteristics of patients with tumors and non-malignant disease.

**Tabelle 1.** Patientencharakteristika.

| | Tumor patients | Control patients without tumors |
|---|---|---|
| n | 49 | 59 |
| Age | • Mean: 60 ± 11 years<br>• Range: 38–87 years | • Mean: 50 ± 17 years<br>• Range: 10–84 years |
| Type of disease | • H&N cancers (n = 28)<br>• Cervical cancers (n = 14)<br>• Lung cancers (n = 4)<br>• Gastrointestinal cancers (n = 3) | • Unserious non-malignant diseases (n = 36)<br>• Renal anemia (n = 23) |
| Stages | • Localized disease<br>• No distant metastases | – |
| Hemoglobin levels | • Mean: 12.0 ± 1.9 g/dl<br>• Range: 7.9–16.0 g/dl | • Mean: 12.1 ± 2.3 g/dl<br>• Range: 8.3–15.6 g/dl |

for 2 hours, aspirated and washed three times. After adding 200 µl of conjugate (containing a polyclonal antibody against VEGF conjugated to horseradish peroxidase), incubation for 2 hours, aspiration and washing, 200 µl of substrate solution were added and incubated for 25 minutes. After stopping the reaction, the optical density was determined at 450 nm. All samples were measured in duplicate together with standards and controls and the VEGF concentration was determined by regression analysis.

Plasma levels were in general about 15 times lower than serum levels but we found a close correlation between both parameters (data not shown). In our control population, the normal range (mean ± 2 standard deviations) was 0–42 pg/ml. Therefore, values over 42 pg/ml were considered as elevated for this analysis. The upper border of our normal range is lower than the upper border according to the manufacturer's information, probably because of the preparation technique (own results, data not shown).

### Statistical Procedures

All statistical calculations were performed with a commercially available software package (SPSS 10.0). Comparisons of means were done with a two-sided t-test, and differences were considered significant in case of $p < 0.05$. Multivariate analysis was performed using a generalized linear model.

## Results

### Plasma Levels of VEGF

Plasma levels of VEGF were 16.2 ± 12.7 pg/ml (range: 2.8–77.2 pg/ml) in 36 patients without malignant disease or anemia, 49.2 ± 34.5 pg/ml (range: 10.0–186.0 pg/ml) in 49 patients with tumors ($p < 0.001$), and 89.9 ± 67.8 pg/ml (range: 10.2–240.0 pg/ml) in 23 patients with renal anemia ($p = 0.001$ vs patients with tumors).

In patients with cancers, VEGF levels were correlated neither with tumor specific parameters (site of cancer, stage, or histology) nor sex or age (Table 2), although VEGF levels in patients with T4 cancers were slightly (not significantly) higher than in the group with T1–3 tumors. There was a significant positive correlation with platelet counts. The association between platelets and systemic VEGF levels was, however, only found in cancer patients and not in patients with benign diseases.

### Anemia and VEGF Levels

The VEGF levels in tumor patients with normal hb levels (≥ 12 g/dl) were in the normal range in 17/22 patients (67%) and elevated in 5/22 (23%) patients. In contrast, 21/27 (78%) anemic tumor patients (hb < 12 g/dl) had elevated plasma VEGF levels (Table 3). In patients without tumors, only 1/36 had an elevated plasma VEGF in case of an hb above 11 g/dl. In patients with lower hb levels, however, a significant number of patients presented with elevated VEGF levels (see Table 3).

For to determine the impact of anemia on systemic VEGF levels, plasma levels were calculated for the groups of patients with tumors and without tumors depending on their hb levels. The results are demonstrated in Table 3. Tumor patients had significantly higher VEGF levels than patients with benign diseases if they were normemic. In case of moderate to severe anemia (hb < 11 g/dl), plasma concentrations of VEGF were significantly elevated in both groups of patients and the difference between patients with and without cancers was no longer significant. Figure 1 summarizes the data and further demonstrates the association between decreasing hb levels and plasma concentrations of VEGF. The different slopes for patients with cancers as compared to patients with benign diseases underline that the impact of anemia on systemic concentrations of VEGF differs between patients with and without cancers.

For to further demonstrate an association between anemia and elevated VEGF levels, a multivariate analysis was performed, including T category (T1–2 tumors were excluded due to small numbers), age and platelet count as cofactors. The model supported a significant association between plasma VEGF and hb levels.

### Impact of Changes in hb on Plasma VEGF Levels

A possible impact of changes in hb (e.g. by anti-anemic treatment) on systemic VEGF levels is difficult to assess in cancer patients because changes in plasma VEGF may occur as a re-

**Table 2.** No significant association between various prognostic factors and plasma levels of VEGF. There was, however, a significant correlation with platelet counts.

**Tabelle 2.** Einfluss verschiedener Variablen auf Plasma-VEGF-Spiegel.

| Parameter | n | Plasma VEGF | Significance |
|---|---|---|---|
| Type of disease | | | |
| – Tumor | 49 | 49.2 ± 34.5 | |
| – Non-malignant disease, no anemia | 36 | 16.2 ± 12.7 | p = 0.001 |
| – Renal anemia | 23 | 89.9 ± 87.8 | |
| Age groups | | | |
| – < 45 years | 26 | 33.3 ± 40.0 | |
| – 45–60 years | 39 | 47.7 ± 49.3 | n.s. |
| – > 60 years | 43 | 54.2 ± 50.0 | |
| Tumor site | | | |
| – Cervix | 28 | 46.8 ± 37.2 | |
| – Head and neck | 14 | 44.7 ± 19.7 | n.s. |
| – Gastrointestinal | 3 | 44.7 ± 37.4 | |
| – Lung | 4 | 85.0 ± 45.1 | |
| Tumor stage (T-category) | | | |
| – T1–2 | 2 | 36.0 ± 18.4 | |
| – T3 | 20 | 40.6 ± 20.1 | n.s. |
| – T4 | 21 | 59.2 ± 46.6 | |
| – Local recurrence | 6 | 47.3 ± 18.1 | |
| Platelet counts | | | |
| – < 200 Gpt/l | 32 | 39.6 ± 40.1 | |
| – 200–350 Gpt/l | 56 | 41.6 ± 46.6 | p = 0.02 |
| – > 350 Gpt/l | 20 | 73.1 ± 53.3 | |

sult of tumor progression or after therapy. However, we undertook repeated VEGF measurements in 16 patients with renal anemia 3–4 weeks after the first measurement. Some of these patients had received erythropoietin. The hb had increased in 11/16 patients and further decreased in 5/16. There was a significant correlation between the changes in hb and the corresponding changes in VEGF levels. Patients with an increasing hb level showed a significant decrease in plasma VEGF levels and vice versa (Figure 2).

## Discussion

Hypoxia occurs frequently in solid tumors as a result of an imbalance between $O_2$ consumption rate and $O_2$ supply [16, 31]. It seems to represent a general pathophysiological phenomenon. Radiobiological hypoxia (that means a $pO_2$ of 2.5–5 mm Hg or less) decreases the radiosensitivity and decreases survival [25]. Low oxygen pressure in this range has also been demonstrated to induce up-regulation of certain hypoxia-inducible genes including VEGF in various tissues [4, 29].

We have found higher systemic levels of VEGF in patients with cancers as compared to normemic patients with benign diseases. This finding is in accordance with data from the literature and was to be expected because of the well-known angiogenic properties of malignant tumors. We have recently demonstrated a strong correlation between the hypoxic tumor volume and serum concentrations of VEGF in head and neck cancers [7]. Tumor hypoxia therefore probably represents the main stimulus for angiogenesis in tumors. This would support the hypothesis that the microenvironment of a tumor represents an independent prognostic factor and a possible target for therapeutic interventions.

We found a strong correlation between anemia and systemic VEGF levels in cancer patients in our investigation. Several clinical and experimental findings have demonstrated an association between hb levels and tumor hypoxia. Tumors in anemic animals are poorer oxygenated than tumors in normemic animals [18, 19]. The association between hb levels and tissue oxygenation of human tumors was recently investigated in 133 patients with head and neck cancers [2]. This analysis represents the largest sample size in the literature with regard to this question. Decreased hb levels had no impact on normal tissue oxygenation but were in fact associated with decreased tumor oxygenation; in a multivariate analysis, anemia was the most important prognostic parameter for the occurrence of tumor hypoxia. According to these findings, the most likely explanation for the association of anemia with elevated systemic levels of VEGF is an indirect effect of low hb levels on angiogenesis via impaired tissue oxygenation.

Unexpectedly, we have found significantly elevated levels of plasma VEGF in severely anemic patients with non-malignant diseases. As these patients had no malignant tumors, one must conclude that the plasma VEGF in these patients origins from normal tissue which, as a result of severe anemia, is probably insufficiently oxygenated so that up-regulation of VEGF in response to tissue hypoxia occurs. This process, however, requires a more pronounced decrease in hb levels below about 10–11 g/dl as demonstrated in Figure 1. The different slopes in Figure 1 suggest that low hb levels can be compensated with regard to tissue oxygenation over a broader range in normal tissue as compared to tumors. Our current explanation is that a moderate decrease in hb provokes tissue hypoxia in tumors (with subsequent up-regulation of VEGF) but not in normal tissues.

The impact of anemia was further supported by a significant inverse correlation between changes in hb levels and

**Table 3.** Anemic patients have significantly higher levels of VEGF, irrespective whether or not they have cancers. Patients with solid cancers have higher levels of plasma VEGF as compared to patients without tumors in case of a hemoglobin (hb) value of > 11 g/dl, but there are no significant differences between patients with and without cancers in case of an hb < 11 g/dl. Number of patients in each group in parentheses.

**Tabelle 3.** Anämische Patienten haben signifikant höhere Plasmaspiegel von VEGF, auch wenn sie keinen Tumor haben. Patienten mit Tumoren haben im Vergleich zu Patienten ohne Tumoren höhere VEGF-Spiegel bei Hb-Werten > 11 g/dl. Bei Hb < 11 g/dl besteht kein signifikanter Unterschied. Patientenzahl in Klammern.

| Hemoglobin | Plasma VEGF levels (pg/ml) in patients with non-malignant diseases (n = 59) | Plasma VEGF levels (pg/ml) in patients with solid cancers (n = 49) | |
|---|---|---|---|
| ≥ 12 g/dl | 16.6 ± 13.0 (33) | 33.1 ± 17.5 (22) | p = 0.001 |
| 11.0–11.9 g/dl | 18.5 ± 14.5 ( 3) | 56.1 ± 26.4 (12) | p = 0.038 |
| 10.0–10.9 g/dl | 49.7 ± 47.1 ( 5) | 73.3 ± 56.0 (7) | p = 0.461 |
| < 10 g/dl | 99.8 ± 70.9 (15) | 62.0 ± 41.9 (8) | p = 0.171 |
| | p < 0.001 | p = 0.015 | |
| | **Elevated plasma VEGF (> 42 pg/ml) in patients with non-malignant diseases (n = 59)** | **Elevated plasma VEGF (> 42 pg/ml) in patients with solid cancers (n = 49)** | |
| ≥ 12 g/dl | 1/33 (3%) | 5/22 (23%) | |
| 11.0–11.9 g/dl | 0/3 (0%) | 9/12 (75%) | |
| 10.0–10.9 g/dl | 2/5 (40%) | 5/7 (71%) | |
| < 10 g/dl | 14/18 (78%) | 7/8 (88%) | |
| Total | 17/59 (29%) | 24/49 (49%) | |
| | p < 0.001 | p = 0.01 | |

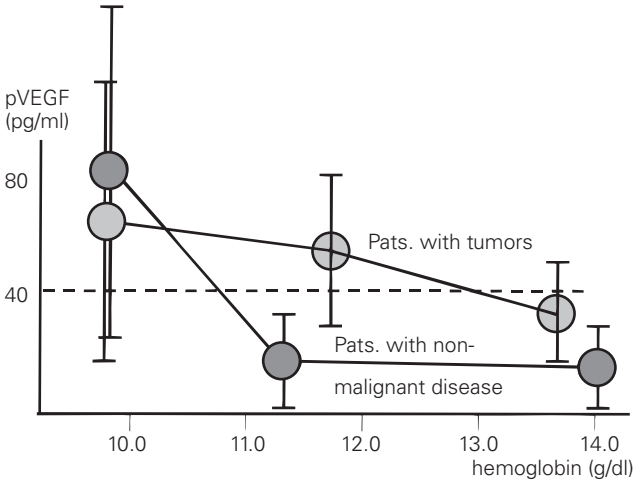


**Figure 1.** Plasma levels of VEGF in patients with cancers (n = 49) or non-malignant diseases (n = 59) depending on the hemoglobin (hb) levels. Significant differences between patients with tumors and without tumors in the hb range > 11 g/dl. Anemic patients (hb < 11 g/dl) had elevated VEGF levels irrespective whether or not they had tumors.

**Abbildung 1.** VEGF-Plasmaspiegel bei Patienten mit soliden Tumoren (n = 49) oder nicht malignen Erkrankungen (n = 59).

changes in plasma VEGF levels (see Figure 2). These findings support a causal relationship between anemia and systemic VEGF levels. From these data, treatment of anemia might have an indirect impact on angiogenesis and should be investigated in future clinical trials.

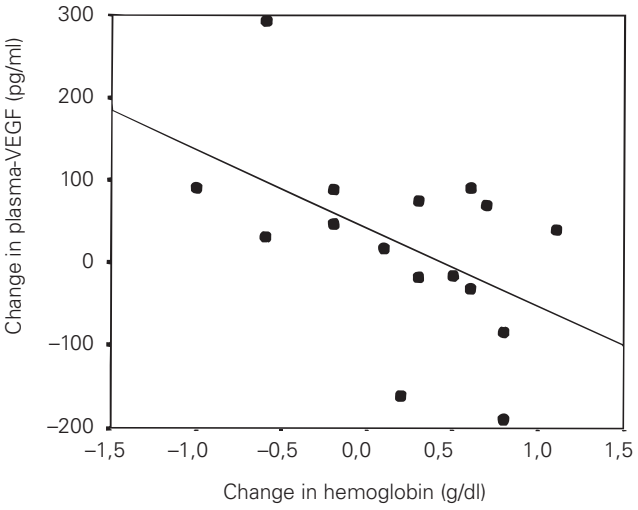


**Figure 2.** Significant inverse correlation between changes in hemoglobin (hb) and changes in plasma VEGF in 16 patients with renal anemia and two measurements of plasma VEGF during the course of disease (p = 0.024, Pearson correlation coefficient –0.50). An increase in hb was associated with a decrease in plasma VEGF.

**Abbildung 2.** Signifikante invase Korrelation zwischen Änderungen des Hb-Wertes und Änderungen des VEGF-Spiegels bei zwei Verlaufsmessungen.

If the high concentrations of plasma VEGF in anemic patients without cancers origin from normal tissue, it is reasonable to assume that high systemic VEGF levels in cancer patients may also arise from normal and not only from tumor tissue if these patients have severe anemia. Some recent investigations have described significantly elevated VEGF levels in cancer patients and have found a correlation with stage and metastases [17, 26, 27]. Anemia, either present prior to treatment or therapy-induced, is a frequent phenomenon in cancer patients but was not specifically addressed in these investigations. Our findings challenge the hypothesis that the elevated systemic VEGF levels always reflect large tumor burden.

The impact of platelets on systemic levels of VEGF has been recently described [1, 12, 32] and was supported in this investigation in tumor patients but not in subjects without tumors. Thus, interpretation of elevated levels of VEGF in plasma or sera must also take into account the platelet count. The storage of VEGF in and its release from platelets can be explained by the physiological role of VEGF during wound healing. In tumors, a correlation of high platelet counts with poor prognosis has been described in various cancers [11, 15, 30]. It is currently not clear whether this is only an epiphenomenon or whether platelets may play a specific role in angiogenesis in tumors.

In summary, our results suggest that anemia might be an independent factor for the progression of angiogenesis. The induction of angiogenic (and maybe other) cytokines by anemia offers an explanation for the independent prognostic impact of anemia in surgically treated patients or lymphomas [10, 14, 23]. Our findings might therefore result in some important conclusions with regard to current clinical practice. Firstly, elevated systemic levels of VEGF in cancer patients must not necessarily reflect an aggressive angiogenic tumor or large tumor burden, but may also be considered as a result of spontaneous or therapy-induced anemia and should therefore be interpreted only with regard to the hb levels. Secondly, severe anemia probably provokes the secretion of VEGF from normal tissues and the impact of elevated systemic levels of angiogenic cytokines on the promotion of a malignant disease has to be determined. Further investigations on the impact of anemia on angiogenesis in benign diseases are also warranted for to determine whether prolonged severe anemia via increased systemic levels of angiogenic cytokines may act independently on the pathological neovascularization in certain diseases, e.g. in chronic inflammatory processes such as rheumatoid arthritis. If these hypotheses are supported by further data, the current recommendations for the treatment of anemia in malignant and benign diseases might be questioned.

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