An *in vitro* study on the effect of bevacizumab on endothelial cell proliferation and VEGF concentration level in patients with hereditary hemorrhagic telangiectasia

HANEEN SADICK[1], ELENA SCHÄFER[1], CHRISTEL WEISS[2], NICOLE ROTTER[1],
CORNELIA EMIKA MÜLLER[3], RICHARD BIRK[3], MALIHA SADICK[4] and DANIEL HÄUSSLER[1]

Departments of [1]Otorhinolaryngology, Head and Neck Surgery and [2]Medical Statistics, University Hospital Mannheim,
Medical Faculty Mannheim of The University of Heidelberg, D-68135 Mannheim; [3]Department of Otorhinolaryngology,
University Hospital Marburg, Philipps-Universität Marburg, D-35043 Marburg; [4]Clinic for Radiology and Nuclear Medicine,
University Hospital Mannheim, Medical Faculty Mannheim of The University of Heidelberg, D-68135 Mannheim, Germany

Abstract. Previous studies have demonstrated that vascular endothelial growth factor (VEGF) is upregulated in patients with hereditary hemorrhagic telangiectasia (HHT). The use of Bevacizumab as an anti-angiogenic treatment agent seems promising. The purpose of the present *in vitro* study was to determine the efficacy and potential toxicity levels of bevacizumab on cell proliferation and VEGF concentrations in endothelial cells of HHT patients. In this *in vitro* study, endothelial cells from patients with HHT and HUVECs (control) were incubated with different concentration levels of bevacizumab (2, 4, 6, 8 or 10 mg/ml). After 24, 48 or 72 h, the cell proliferation was assessed by Alamar Blue® Assay and the VEGF levels in the cell culture supernatants were measured by VEGF-ELISA. All endothelial cells incubated with bevacizumab showed an initial decrease in cell proliferation. Cell proliferation recovered within 72 h in cell cultures incubated with concentration levels of up to 4 mg/ml bevacizumab, whereas those incubated with higher concentration levels showed a continuous decline in cell proliferation. VEGF expression decreased after 24 h in cell cultures incubated with bevacizumab concentration levels of 2 and 4 mg/ml but increased again after 48 h. Cell cultures incubated with bevacizumab concentration levels of 10 mg/ml showed a constant decline in VEGF expression without any tendency for recovery. Translating these results into daily clinical practice, the present study suggests that the intranasal submucosal injection of bevacizumab in HHT patients should not exceed a concentration level of 4 mg/ml. Overall, higher bevacizumab concentration levels not only reduce VEGF expression but pose a higher risk of toxic effects on endothelial cells as they jeopardize cell proliferation.

Introduction

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Rendu-Weber disease, is an autosomal dominant vascular disorder with an estimated prevalence of 1 in 5.000-8.000 (1,2). The disease is characterized by mucocutaneous telangiectasias and visceral arteriovenous malformations (AVMs). The aberrant vessels lack a capillary bed with direct shunting between arteries and veins (1-3). Pulmonary manifestations can cause severe cardiac output failures and pulmonary hypertension (1-3). However, the most common clinical symptoms are recurrent epistaxis, which affects up to 95% of all HHT patients (3) Extreme cases can necessitate hospitalization for blood transfusions (4-6).

Genetically, HHT is caused by mutations encoding the *ENG*, *ACVRL1* and *Smad4* proteins. These proteins are part of the TGF-ß1/BMP signaling pathway (7,8), which regulates cell differentiation and angiogenesis in endothelial cells (9).

In HHT, the TGF-ß1 signaling pathway is impaired and the activation phase of angiogenesis tends to persist with enhanced production of angiogenetic factors. Vascular endothelial growth factor (VEGF) is a well-characterized pro-angiogenic cytokine that plays a key role in angiogenesis (10,11). Previous studies have shown significantly increased plasma concentrations and high tissue expression levels of VEGF in HHT patients compared with healthy controls (12), thus indicating its important role in HHT angiodysplasia (13). Based on these findings, Han *et al* (14) showed in an animal study that blocking VEGF in ACVRL1-deficient mice with a VEGF antibody (G6-31) prevented AVMs and even normalized already-established AVMs.

Bevacizumab is a selective recombinant human antibody against VEGF that was designed to inhibit tumor-induced

---

Correspondence to: Professor Haneen Sadick, Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Mannheim, Medical Faculty Mannheim of The University of Heidelberg, Theodor-Kutzer-Ufer 1-3, D-68135 Mannheim, Germany
E-mail: haneen.sadick@umm.de

neo-angiogenesis. It has been approved by the FDA for the treatment of metastatic colorectal, breast, renal cell, and NSC lung cancers, as well as glioblastomas (www.accessdata.fda. gov/drugsatfda_docs/label/2011/125085s225lbl.pdf) (15,16). Its first off-label use was described in Ophthalmology for the treatment of intraocular neovascular disorders (17).

Recently, an increasing number of studies have focused on the clinical use of Bevacizumab in HHT patients. Its intravenous application has shown a positive effect on HHT symptoms by reducing gastrointestinal bleeding, hepatic complications, and epistaxis (18-20). However, the majority of the study protocols used oncological dosing parameters with severe systemic side effects, e.g. hypertension, proteinuria, thromboembolic events, intestinal perforation, and impaired wound healing (21).

To avoid these systemic side effects, current studies have applied Bevacizumab locally in the form of nasal sprays or submucosal injections within the nose (22,23). Regarding the efficacy of topically applied Bevacizumab, submucosal intranasal Bevacizumab injections seemed to have a more beneficial effect on HHT-related epistaxis than when it was applied as a nasal spray (24-30). However, the main drawbacks in most of these studies are the different dosing levels of Bevacizumab. They are very inconsistent and vary considerably between 3.75 and 25 mg/ml, without providing any details concerning their efficacy or even toxicity to the nasal mucosa. Additionally, the majority of the studies do not suggest optimal dosing guidelines for the safe and effective application of submucosal Bevacizumab injections. Ongoing clinical and research studies are required to define these treatment details.

The aim of this *in vitro* study was to determine the efficacy and the potential toxicity level of intranasally applied submucosal Bevacizumab injections on endothelial cell proliferation and VEGF concentration levels in HHT patients and healthy controls, thus allowing possible conclusions on the optimal dosage for submucosal nasal Bevacizumab injections in clinical practice.

Materials and methods

Tissue collection, and culturing of HHT endothelial cells and HUVECs. Tissue samples were obtained intraoperatively from 3 HHT patients (2 female patients aged 62 and 63 years, 1 male patient aged 67 years, altogether with an average age of 64 years) undergoing treatment for recurrent epistaxis at the Department of ORL-HNS of the University Hospital Mannheim (Heidelberg, Germany). Prior to surgery, written consent to take a small tissue sample from their telangiectatic nasal mucosa of the inferior turbinate was obtained from all patients. This study was approved by the Ethics Committee of the University Hospital of Mannheim (approval no. 0238.2). The ethics approval included the use of tissue samples taken endonasally from HHT patients to culture endothelial cells. A commercially available HUVEC cell line served as a control.

Endothelial cells from a HUVEC cell line (PromoCell; cat. no. C-12250) were used as the control. According to the manufacturer's protocol, all collected nasal tissue samples were cleaned in PBS. The samples were then cut into 0.5x0.5 cm small pieces and incubated with 1.5 ml fibroblast growth medium (DMEM high glucose, 200 mmol L-Glutamine, 10%

fetal calf serum, 1% Penicillin-Streptomycin solution) for 1-2 weeks. Once per week, the medium was replaced. As soon as the T25 tissue culture flasks were filled with endothelial cells, the cells were passaged with a trypsin solution and 0.02% EDTA. On reaching 80% subconfluency, the endothelial cells were once again passaged with a trypsin solution and 0.02% EDTA. All endothelial cell cultures were used at passage 3.

Alamar Blue® Assay. Following incubation with different concentrations of Bevacizumab (0, 2, 4, 6, 8 or 10 mg/ml), cell proliferation was measured using an AlamarBlue® Assay (BIOZOL Diagnostica) after 24, 48 or 72 h in fluorescence units (FU). The assay used the cell-permeable fluorescent indicator dye resazurin as an oxidation-reduction indicator. As the color and the intensity of fluorescence changed proportional to the metabolic activity, the assay indicated the degree of cell proliferation. The measurements were repeated three times and the mean value was subsequently calculated.

VEGF-ELISA. The exact concentration of VEGF was evaluated using the VEGF-Immunoassay from Bio-Techne (R&D Systems, cat. no DVE 00). The assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific to VEGF was pre-coated onto a microplate. According to the manufacturer's instructions, standards and samples were pipetted into wells and the present VEGF was bound by the immobilized antibody. The unbound substances were then washed out, and an enzyme-linked polyclonal antibody specific to VEGF was added. Again, unbound substances were washed out. Finally, a substrate solution was added to the wells, inducing color development in proportion to the amount of VEGF bound in the initial step. The color development was stopped, and the intensity of the color was measured. The exact concentration of VEGF was determined using a standard curve.

After 24, 48 or 72 h of incubation with 0, 2, 4, 6, 8 or 10 mg/ml Bevacizumab (kindly provided by the Pharmacy supplying the Oncological Department, University Hospital Mannheim, Medical Faculty of the University of Heidelberg, Germany), the expression of VEGF was analyzed in the supernatants of the HHT cell cultures and the HUVECs (Fig. 1).

In this study, experiments were performed with even concentration levels of Bevacizumab of 2, 4, 6, 8 or 10 mg/ml as these concentration levels were obtained directly from the pharmaceutical and cytostatic laboratory of the hospital already in ready to be used 'pre-filled syringes' same as they were used in the clinic for patient treatment in the Hematooncology department. With these provided concentration levels of Bevacizumab we were wanting to evaluate the concentration levels and the means how the effects differed on endothelial cell proliferation and VEGF expression in HHT cell cultures.

Statistical analysis. Quantitative variables have been presented by their mean values and standard deviations. For qualitative factors, absolute and relative frequencies are given.

To compare the mean values of the three groups, a one-way ANOVA was used. In the case of a significant test result, a post-hoc Scheffé test was performed. $P<0.05$ was considered to indicate a statistically significant difference. For statistical evaluation, the SAS software version 9.4 (SAS Institute Inc.) was used.
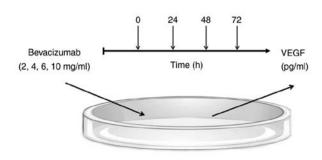
Figure 1. Experimental overview. Incubation of the HUVEC cell line (control) and the HHT cell cultures with different concentration levels of Bevacizumab (2, 4, 6 and 10 mg/ml) and measurement of the VEGF levels in their supernatants after 24, 48 and 72 h.

Results

Cell proliferation following incubation with different Bevacizumab concentrations.
HUVECs. In the HUVECs, untreated endothelial cells showed an increase in cell proliferation throughout the entire 72 h period. The proliferation rate after 24 h increased from 18.14 to 26.13 FU. After 48 h the proliferation rate was 29.46 FU and after 72 h it was 41.54 FU.

A positive trend in cell proliferation could also be seen in endothelial cells incubated with 2 and 4 mg/ml Bevacizumab. After 24 h, cell proliferation increased from 18.14 to 26.67 and 25.7 FU, respectively, and was nearly identical to that of untreated cells. Cell proliferation then almost plateaued until 48 h and started to increase again after 72 h to 30.7 and 27.38 FU, respectively.

In general, incubation with 6, 8 and 10 mg/ml Bevacizumab led to a decrease in cell proliferation with a significant decline in cell numbers after 72 h. This negative trend was already obvious 24 h after incubation with 10 mg/ml Bevacizumab. The considerable decline in cell numbers reached values <10 FU and continued to decrease after 48 and 72 h. Values <10 FU were considered as full apoptosis of the cell culture and therefore considered toxic in this study. A similar trend, though more prolonged in the decrease of cell proliferation, was seen after incubation with 8 mg/ml Bevacizumab. A toxicity level of <10 FU was reached after 72 h without any recovery of cell proliferation. Incubation with 6 mg/ml Bevacizumab first increased after 24 h from 18.14 to 25.12 FU, but after 48 and 72 h, the cell number decreased to 21.38 and 19.98 FU, respectively. However, the 6 mg/ml Bevacizumab concentration level did not reach the toxic cut-off level of 10 FU (Fig. 2A).

HHT cell cultures. The cell proliferation of the HHT 1 cell culture was similar to that of the HUVECs. Whereas concentration levels of 0, 2 and 4 mg/ml Bevacizumab had a positive impact on cell proliferation, concentration levels of 8 and 10 mg/ml Bevacizumab induced a toxic effect leading to cell apoptosis after 48 and 72 h. The number of endothelial cells incubated with 6 mg/ml Bevacizumab almost stagnated for 24 h at a rate between 13.74 and 14.32 FU before they started to increase again to a value of 16 FU (Fig. 2B).

The findings for endothelial cell proliferation in the HHT 2 and HHT 3 cell cultures were similar to those of the HHT 1 group. The proliferation rate at 24 and 48 h after incubation with

2 and 4 mg/ml Bevacizumab first decreased slightly before it increased again at 72 h. Endothelial cells incubated with 10 mg/ml Bevacizumab exhibited negative proliferation after only 24 h of incubation, with a massive decrease in cell numbers reaching values below the toxic cut-off level of 10 FU. After 48 and 72 h, a further decrease in cell numbers was observed without any signs of recovery. Apoptosis was also observed in cell cultures incubated with 8 mg/ml Bevacizumab even though the toxic level of 10 FU was not reached until after 48 h. Endothelial cells of the HHT 2 and 3 cell cultures which were incubated with 6 mg/ml Bevacizumab showed a slight decrease in cell proliferation after a period of 72 h to 17.29 and 14.57 FU, respectively (Fig. 2C and D).

A detailed overview of the data regarding the cell proliferation rates 24, 48 and 72 h after incubation of the HUVECs and the HHT cell cultures with different concentration levels of Bevacizumab are provided in Table I.

The mean values and P-values for the cell proliferation rate of the HUVEC, HHT 1, HHT 2 and HHT 3 endothelial cells incubated with different concentration levels of Bevacizumab are given in Table II.

VEGF tissue expression after incubation with different Bevacizumab concentrations.
HUVECs. VEGF tissue expression in the HUVECs initially showed a slight increase after 24 h of incubation with 2 mg/ml concentration. However, it then decreased after 48 h and increased again after 72 h. The same effect was observed with 4 mg/ml Bevacizumab, although the suppression in VEGF tissue expression was considerably stronger than with 2 mg/ml Bevacizumab. However, this effect did not seem to be long-lasting, as the VEGF tissue expression increased again after 72 h of incubation.

Higher concentration levels of 6 and 10 mg/ml Bevacizumab showed an immediate suppression of VEGF tissue expression after only 24 h of incubation, which continued even after 72 h (Fig. 3A).

HHT cell cultures. In all three HHT cell cultures, Bevacizumab had a suppressive effect on VEGF expression in endothelial cells throughout the entire 72 h period of incubation. However, lower concentration levels of Bevacizumab (0, 2 and 4 mg/ml) had a more delayed effect and appeared to exert its suppressive action after 24 h of incubation. Higher concentration levels of 6 and 10 mg/ml Bevacizumab had an immediate effect after only 24 h of incubation and continued to decrease the VEGF expression levels consistently over the entire 72 h period of incubation without any recovery (Fig. 3B-D).

A detailed overview of the data regarding the VEGF expression levels 24, 48 and 72 h after incubation of the HUVECs and the HHT cell cultures with different concentration levels of Bevacizumab are given in Table III.

Discussion

The monoclonal VEGF-antibody Bevacizumab appears to be a promising therapy for patients with HHT (12,22,23). Its systemic intravenous application in HHT patients has been abandoned in favor of topical applications of Bevacizumab. Very few studies describe the topical use of Bevacizumab in HHT patients (22,23). Most studies that have assessed this are small heterogeneous case
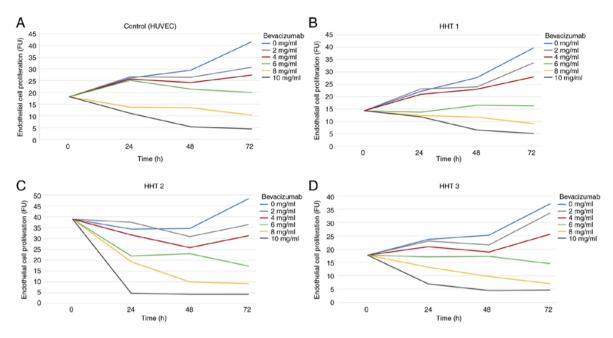
Figure 2. Endothelial cell proliferation after incubation with different concentration levels of bevacizumab. (A) HUVEC cell line serving as a control, (B) HHT cell culture 1, (C) HHT cell culture 2 (D) and HHT cell culture 3. Incubation of all endothelial cells with concentration levels of 2, 4, 6, 8 and 10 mg/ml of bevacizumab with measurement of the cell proliferation rate after 24, 48 and 72 h.

series with notable differences in the dosing regimens, which makes the analysis of outcomes extremely difficult.

Endonasal submucosal injections of Bevacizumab seem to be more effective regarding the epistaxis severity score (ESS) and the hemoglobin level than when it is used as a topical nose spray (22). They seem to be well tolerated by HHT patients without major signs of severe systemic side effects (26-30). However, nasal septal perforations are a potential side effect of the intranasal delivery of Bevacizumab, especially when injected bilateral to the cartilaginous septum. Therefore, injections should favor non-cartilaginous areas within the nose to avoid septal perforations (22-24).

In the literature, the dosing levels of Bevacizumab for endonasal injections in HHT patients vary considerably between 3.75 and 25 mg/ml (24-30). To date, no Otorhinolaryngological studies have provided ideal dosing guidelines for Bevacizumab administration. For the practicing clinician, details on the efficacy and damaging potency of different Bevacizumab concentration levels to the nasal mucosa are missing. An inconsiderate, reckless application of high concentration levels of Bevacizumab should be avoided.

In Ophthalmology, studies have described the well-established use of 1.25 and 2.5 mg Bevacizumab in patients with retinal neovascularization for local intravitreal injections every 4-12 weeks (31-33). The results indicate a very low complication rate of ≤0.21% (31). These complications include blood pressure elevation, corneal abrasion, mild discomfort, and inflammation uveitis.

Studies are needed to show the efficacy of lower doses of Bevacizumab on the severity of epistaxis and visceral AVM involvement and their potentially lower profile of side effects (34).

To the best of our knowledge, the results presented in this paper are the first *in vitro* data assessing the effects of different concentration levels of Bevacizumab on endothelial cell cultures from HHT patients and the VEGF concentration in their supernatant with the aim of identifying a safe dose. The proliferation rate of endothelial cells was affected by different concentration levels of Bevacizumab. The higher the concentration level of Bevacizumab, the greater the decrease in the proliferation rate was. Concentration levels of 8 mg/ml Bevacizumab and higher were shown to harm cell proliferation to the point of induction of cell apoptosis in all specimens as an indicator for cell toxicity (values <10 FU).

The results of the present study showed the inhibitory effect of Bevacizumab on endothelial cell proliferation. Even though we did not undertake any specific TUNEL staining of cells, it was assumed that the cytotoxic effects of Bevacizumab enhanced endogenous apoptosis in primary endothelial cell lines as well as established HHT cell cultures as already described in other studies for different cell lines and tumor entities in the literature (35,36).

These results stand in contrast to the doses used in submucosal injections in previous studies, which mainly ranged between 10-25 mg/ml with an overall application of 50-100 mg Bevacizumab to each nasal cavity (24,26,27,29). After Simonds *et al* (24) and Chen *et al* (22) described the risk of septal perforations associated with submucosal injections of Bevacizumab, subsequent studies avoided injections in the cartilaginous septum.

The results of the present study suggest that the risk of systemic adverse events and septal perforations may be reduced by using considerably lower doses. Lower concentration levels of up to 4 mg/ml Bevacizumab initially decelerated endothelial cell proliferation within the first 48 h but had a tendency towards recovery after 72 h of incubation.

Concentration levels between 4 and 6 mg/ml Bevacizumab should be investigated more carefully. Concentration levels of 4 mg/ml Bevacizumab seem to be effective in slowing down endonasal cell proliferation but allow the cells to recover after 48 h of incubation. Concentration levels of 6 mg/ml Bevacizumab impair cell recovery by inducing stagnation

Table I. Detailed overview of cell proliferation rates 24, 48 and 72 h after incubation of the HUVEC cell line (control) and the HHT cell cultures with different concentration levels of Bevacizumab.

Incubation time (h)	Bevacizumab					
	0 (mg/ml)	2 (mg/ml)	4 (mg/ml)	6 (mg/ml)	8 (mg/ml)	10 (mg/ml)
HUVEC (Control)						
0	18.14	18.14	18.14	18.14	18.14	18.14
24	26.13	26.67	25.7	25.12	13.69	11.06
48	29.46	26.42	24.15	21.38	13.45	5.31
72	41.54	30.7	27.38	19.98	10.29	4.43
HHT 1						
0	14.32	14.32	14.32	14.32	14.32	14.32
24	22.11	23.08	20.95	13.74	12.38	11.85
48	27.7	23.97	23.01	16.55	11.47	6.54
72	39.64	33.63	27.97	16.28	9.12	5.12
HHT 2						
0	38.88	38.88	38.88	38.88	38.88	38.88
24	34.3	37.43	31.61	21.93	19.27	4.76
48	34.55	30.86	25.78	23.01	9.99	4.31
72	48.29	36.37	31.26	17.29	9.24	4.29
HHT 3						
0	17.74	17.74	17.74	17.74	17.74	17.74
24	23.66	23.05	20.94	17.09	13.24	6.96
48	25.27	21.59	18.93	17.32	9.76	4.48
72	36.96	33.48	25.58	14.57	7.1	4.69

Data are mean values. Experiments were performed in triplicate.

or even deceleration of endonasal cell proliferation. These findings highlight 4 mg/ml Bevacizumab as a cut-off concentration level that should not be exceeded when applied as a submucosal injection to the nose, merely to be on the safe side and not destroy endothelial cell function.

A potential drawback of this study was the one-to-one translation of the described *in vitro* data to the endonasal *in vivo* injections of Bevacizumab in HHT patients. The local delivery of lower doses of Bevacizumab may still result in a high tissue load. Potential damage to the nasal endothelial cells after the application of ≥6 mg/ml Bevacizumab need to be more seriously considered (34). However, conclusions drawn from these results should be treated with care due to the small sample size.

To the best of our knowledge, this *in vitro* study is the first Rhinological study that aims to suggest a dosing guideline for the safe and effective application of Bevacizumab when applied intranasally as a submucosal injection in HHT patients. This data may provide valuable information to assess the risk/benefit ratio of Bevacizumab on the endothelial cells of the nasal mucosa. This study also clearly demonstrates that oncological dosing parameters are not indicated to improve medically refractory epistaxis in patients with HHT (37).

However, further clinical research is required to define these treatment details and optimize the dosing and delivery routes for Bevacizumab in the treatment of epistaxis caused by HHT (29,30,34).

Regarding the efficacy of Bevacizumab on endonasal endothelial cells, low concentration levels of 2 and 4 mg/ml effected a considerable decline in VEGF expression within the first 48 h. After 48 h, VEGF concentration increased again when compared to higher concentration levels of 6 and 10 mg/ml, which indicated no signs of recovery. These results indicate that incubation of endonasal endothelial cells with low concentration levels does have an effect, even though it might be short-lasting. Our hypothesis of a sufficient efficacy of low-dose Bevacizumab is also supported by the observations of Rohrmeier *et al*, who showed a benefit of combined treatment with submucosal injections of 3.75 mg/ml (in total, 3.75 mg per nasal cavity) and YAC-Laser vs. laser therapy alone (28). However, conclusions drawn from these results should be treated with care due to the small sample size.

Other than in the control group, the results of the HHT endothelial cell cultures indicated that incubation up to 72 h with Bevacizumab concentration levels of 4 mg/ml or less seemed to be effective enough to suppress the VEGF level without jeopardizing cell proliferation. However, in this study, we could not see a significant correlation between cell proliferation and a decreased VEGF concentration level after 24 h of incubation of the HHT cell lines with Bevacizumab.

Further research studies with odd concentration levels of Bevacizumab are necessary to determine even more accurately the safety concentration level of Bevacizumab in the treatment

Table II. Mean values and P-values for the cell proliferation rate of the HUVEC, HHT 1, HHT 2 and HHT 3 endothelial cells incubated with different concentration levels of bevacizumab.

| | Concentration levels | | | | |
Incubation time (h)	Low (2 mg/ml, 4 mg/ml)	Medium (6 mg/ml)	High (8 mg/ml, 10 mg/ml)	Comparisons	P-value[a]
HUVEC (Control)					
0	18.14	18.14	18.14		
24	26.19	25.12	12.38	Low vs. High	<0.0001
48	25.29	21.38	9.38	Low vs. Medium	0.0265
72	29.04	19.98	7.36	Medium vs. High	0.1413
HHT 1					
0	14.32	14.32	14.32		
24	22.01	13.74	12.11	Low vs. High	<0.0001
48	23.49	16.55	9.14	Low vs. Medium	0.0002
72	30.8	16.28	7.12	Medium vs. High	<0.0001
HHT 2					
0	38.88	38.88	38.88		
24	34.52	21.93	12.02	Low vs. High	<0.0001
48	28.32	23.01	7.15	Low vs. Medium	<0.0001
72	33.99	17.29	6.77	Medium vs. High	0.0163
HHT 3					
0	17.74	17.74	17.74		
24	22	17.09	10.1	Low vs. High	<0.0001
48	20.26	17.32	7.12	Low vs. Medium	<0.0001
72	29.53	14.57	5.9	Medium vs. High	0.045

[a]Changes in cell proliferation over time in association with the concentration level. Data are mean values of cell proliferation rate (FU).
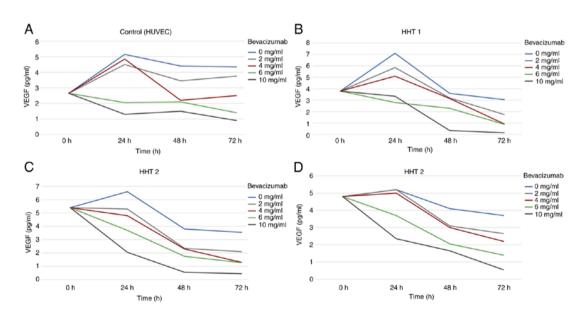

Figure 3. VEGF levels in the supernatants of the HHT cell cultures and the HUVEC cell line. (A) VEGF expression in the HHT cell line serving as a control, (B) HHT cell culture 1, (C) HHT cell culture 2 and (D) HHT cell culture 3 after 24, 48 and 72 h of incubation with different concentration levels of bevacizumab (2, 4, 6 and 10 mg/ml).

of epistaxis in HHT patients. In addition, further investigations would help to get a better insight into the pathophysiology and inhibitory mechanism of Bevacizumab. Of special relevance would be a more precise answer to the question whether Bevacizumab inhibits cell proliferation or induces endogenous apoptosis.

Table III. Detailed overview of VEGF expression levels 24, 48 and 72 h after incubation of the HUVEC cell line (control) and the HHT cell cultures with different concentration levels of Bevacizumab.

	Bevacizumab				
Incubation time (h)	0 (mg/ml)	2 (mg/ml)	4 (mg/ml)	6 (mg/ml)	10 (mg/ml)
HUVEC (Control)					
0	2.65	2.65	2.65	2.65	2.65
24	5.15	4.5	4.85	2.05	1.3
48	4.4	3.45	2.2	2.1	1.5
72	4.35	3.75	2.5	1.4	0.9
HHT 1					
0	3.8	3.8	3.8	3.8	3.8
24	7.1	5.85	5.1	2.8	3.35
48	3.6	3.2	3.15	2.3	0.35
72	3.05	1.75	0.93	0.9	0.155
HHT 2					
0	5.4	5.4	5.4	5.4	5.4
24	6.6	5.3	4.8	3.7	2.05
48	3.8	2.35	2.3	1.75	0.55
72	3.55	2.1	1.3	1.28	0.45
HHT 3					
0	4.8	4.8	4.8	4.8	4.8
24	5.2	5.2	5	3.7	2.35
48	4.1	3.1	3	2.05	1.65
72	3.7	2.65	2.2	1.4	0.55

Data are mean values. Experiments were performed in triplicate.

In summary, this study suggests that incubation of endonasal endothelial cells with Bevacizumab in HHT patients should not exceed the concentration level of 4 mg/ml. Higher concentration levels risk a more toxic effect on endothelial cells, as they jeopardize cell proliferation. Further studies are now necessary to investigate the toxicity of 6 mg/ml Bevacizumab over a longer period, as well as *in vivo* studies addressing the efficacy of intranasal submucosal injections of low-dose Bevacizumab between 2 and 6 mg/ml.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS, ES and DH made substantial contributions to concept, design and data acquisition and were major contributors in writing the manuscript. CW performed the statistical analysis and helped with data analysis and interpretation. MS, NR, CEM and RB helped drafting the manuscript and revising it critically after initial analysis and interpretation of the data. HS and ES confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Prior to surgery, written consent to take a small tissue sample from their telangiectatic nasal mucosa of the inferior turbinate was obtained from all patients. This study was approved by the Ethics Committee of the University Hospital of Mannheim (approval no. 0238.2).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Donaldson JW, McKeever TM, Hall IP, Hubbard RB and Fogarty AW: The UK prevalence of hereditary haemorrhagic telangiectasia and its association with sex, socioeconomic status and region of residence: A population-based study. Thorax 69: 161-167, 2014.
2. Kjeldsen AD, Vase P and Green A: Hereditary haemorrhagic telangiectasia: A population-based study of prevalence and mortality in Danish patients. J Intern Med 245: 31-39, 1999.
3. Sautter NB and Smith TL: Hereditary hemorrhagic telangiectasia-related epistaxis: Innovations in understanding and management. Int Forum Allergy Rhinol 2: 422-431, 2012.
4. Plauchu H, de Chadarévian JP, Bideau A and Robert JM: Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. Am J Med Genet 32: 291-297, 1989.
5. Ingrand I, Ingrand P, Gilbert-Dussardier B, Defossez G, Jouhet V, Migeot V, Dufour X and Klossek JM: Altered quality of life in Rendu-Osler-Weber disease related to recurrent epistaxis. Rhinology 49: 155-162, 2011.
6. Kritharis A, Al-Samkari H and Kuter DJ: Hereditary hemorrhagic telangiectasia: diagnosis and management from the hematologist's perspective. Haematologica 103: 1433-1443, 2018.
7. Jakobsson L and van Meeteren LA: Transforming growth factor β family members in regulation of vascular function: in the light of vascular conditional knockouts. Exp Cell Res 319: 1264-1270, 2013.
8. Steineger J, Geirdal AØ, Osnes T, Heimdal KR and Dheyauldeen S: Intranasal bevacizumab injections improve quality of life in HHT patients. Laryngoscope 130: E284-E288, 2020.
9. Prigoda NL, Savas S, Abdalla SA, Piovesan B, Rushlow D, Vandezande K, Zhang E, Ozcelik H, Gallie BL and Letarte M: Hereditary haemorrhagic telangiectasia: Mutation detection, test sensitivity and novel mutations. J Med Genet 43: 722-728, 2006.
10. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S and Li E: Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci USA 97: 2626-2631, 2000.
11. Sadick H, Hage J, Goessler U, Bran G, Riedel F, Bugert P and Hoermann K: Does the genotype of HHT patients with mutations of the ENG and ACVRL1 gene correlate to different expression levels of the angiogenic factor VEGF? Int J Mol Med 22: 575-580, 2008.
12. Sadick H, Riedel F, Naim R, Goessler U, Hörmann K, Hafner M and Lux A: Patients with hereditary hemorrhagic telangiectasia have increased plasma levels of vascular endothelial growth factor and transforming growth factor-beta1 as well as high ALK1 tissue expression. Haematologica 90: 818-828, 2005.
13. Sadick H, Naim R, Sadick M, Hörmann K and Riedel F: Plasma level and tissue expression of angiogenic factors in patients with hereditary hemorrhagic telangiectasia. Int J Mol Med 15: 591-596, 2005.
14. Han C, Choe SW, Kim YH, Acharya AP, Keselowsky BG, Sorg BS, Lee YJ and Oh SP: VEGF neutralization can prevent and normalize arteriovenous malformations in an animal model for hereditary hemorrhagic telangiectasia 2. Angiogenesis 17: 823-830, 2014.
15. Ferrara N, Hillan KJ and Novotny W: Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 333: 328-335, 2005.
16. Shih T and Lindley C: Bevacizumab: An angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther 28: 1779-1802, 2006.
17. Amarakoon S, Martinez-Ciriano JP, van den Born LI, Baarsma S and Missotten T: Bevacizumab in age-related macular degeneration: A randomized controlled trial on the effect of on-demand therapy every 4 or 8 weeks. Acta Ophthalmol 97: 107-112, 2019.
18. Iyer VN, Apala DR, Pannu BS, Kotecha A, Brinjikji W, Leise MD, Kamath PS, Misra S, Begna KH, Cartin-Ceba R, et al: Intravenous Bevacizumab for refractory hereditary hemorrhagic telangiectasia-related epistaxis and gastrointestinal bleeding. Mayo Clin Proc 93: 155-166, 2018.
19. Andrejecsk JW, Hosman AE, Botella LM, Shovlin CL, Arthur HM, Dupuis-Girod S, Buscarini E, Hughes CCW, Lebrin F, Mummery CL, et al: Executive summary of the 12th HHT international scientific conference. Angiogenesis 21: 169-181, 2018.
20. Dupuis-Girod S: Anti-VEGF: A new therapeutic option in hereditary hemorrhagic telangiectasia. Presse Med 42: 385-387, 2013 (In French).
21. Cohen MH, Gootenberg J, Keegan P and Pazdur R: FDA drug approval summary: Bevacizumab (Avastin) plus Carboplatin and Paclitaxel as first-line treatment of advanced/metastatic recurrent nonsquamous non-small cell lung cancer. Oncologist 12: 713-718, 2007.
22. Chen S IV, Karnezis T and Davidson TM: Safety of intranasal Bevacizumab (Avastin) treatment in patients with hereditary hemorrhagic telangiectasia-associated epistaxis. Laryngoscope 121: 644-646, 2011.
23. Stokes P and Rimmer J: Intranasal bevacizumab in the treatment of HHT -related epistaxis: A systematic review. Rhinology 56: 3-10, 2018.
24. Simonds J, Miller F, Mandel J and Davidson TM: The effect of bevacizumab (Avastin) treatment on epistaxis in hereditary hemorrhagic telangiectasia. Laryngoscope 119: 988-992, 2009.
25. Dupuis-Girod S, Ambrun A, Decullier E, Fargeton AE, Roux A, Bréant V, Colombet B, Rivière S, Cartier C, Lacombe P, et al: Effect of bevacizumab nasal spray on epistaxis duration in hereditary hemorrhagic telangectasia: A randomized clinical trial. JAMA 316: 934-942, 2016.
26. Steineger J, Osnes T, Heimdal K and Dheyauldeen S: Long-term experience with intranasal bevacizumab therapy. Laryngoscope 128: 2237-2244, 2018.
27. Riss D, Burian M, Wolf A, Kranebitter V, Kaider A and Arnoldner C: Intranasal submucosal bevacizumab for epistaxis in hereditary hemorrhagic telangiectasia: A double-blind, randomized, placebo-controlled trial. Head Neck 37: 783-787, 2015.
28. Rohrmeier C, Sachs HG and Kuehnel TS. A retrospective analysis of low dose, intranasal injected bevacizumab (Avastin) in hereditary haemorrhagic telangiectasia. Eur Arch Otorhinolaryngol 269: 531-536, 2012.
29. Karnezis TT and Davidson TM: Efficacy of intranasal Bevacizumab (Avastin) treatment in patients with hereditary hemorrhagic telangiectasia-associated epistaxis. Laryngoscope 121: 636-638, 2011.
30. Karnezis TT and Davidson TM: Treatment of hereditary hemorrhagic telangiectasia with submucosal and topical bevacizumab therapy. Laryngoscope 122: 495-497, 2012.
31. Lynch SS and Cheng CM: Bevacizumab for neovascular ocular diseases. Ann Pharmacother 41: 614-625, 2007.
32. Gordon MS, Margolin K, Talpaz M, Sledge GW Jr, Holmgren E, Benjamin R, Stalter S, Shak S and Adelman D: Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. J Clin Oncol 19: 843-850, 2001.
33. Michels S: Is intravitreal bevacizumab (Avastin) safe? Br J Ophthalmol 90: 1333-1334, 2006.
34. Thompson AB, Ross DA, Berard P, Figueroa-Bodine J, Livada N and Richer SL: Very low dose bevacizumab for the treatment of epistaxis in patients with hereditary hemorrhagic telangiectasia. Allergy Rhinol 5: 91-95, 2014.
35. Färkkilä A, Pihlajoki M, Tauriala H, Bützow R, Leminen A, Unkila-Kallio L, Heikinheimo M and Anttonen M: Serum vascular endothelial growth factor A (VEGF) is elevated in patients with ovarian granulosa cell tumor (GCT), and VEGF inhibition by Bevacizumab induces apoptosis in GCT in vitro. J Clin Endocrinol Metab 96: E1973-E1981, 2011.
36. Ramezani S, Vousooghi N, Kapourchali FR, Hadjighasem M, Hayat P, Amini N and Joghataei MT: Rolipram potentiates bevacizumab-induced cell death in human glioblastoma stem-like cells. Life Sci 173: 11-19, 20217.
37. Guilhem A, Fargeton AE, Simon AC, Duffau P, Harle JR, Lavigne C, Carette MF, Bletry O, Kaminsky P, Leguy V, et al: Intra-venous bevacizumab in hereditary hemorrhagic telangiectasia (HHT): A retrospective study of 46 patients. PLoS One 12: e0188943, 2017.