

ORIGINAL ARTICLE

AIRWAY DISEASES

Vascular endothelial growth factor receptor 1 expression in nasal polyp tissue

S. Bobic¹, V. Hox¹, I. Callebaut¹, S. Vinckier^{2,3}, B. Jonckx⁴, J.-M. Stassen⁴, M. Jorissen⁵, P. Gevaert⁶, P. Carmeliet^{2,3}, C. Bachert⁶, J. L. Ceuppens¹ & P. W. Hellings^{1,5}

¹Laboratory of Clinical Immunology, Department of Microbiology and Immunology, Catholic University of Leuven; ²Laboratory of Angiogenesis and Neurovascular Link, Vesalius Research Center (VRC), VIB; ³Laboratory of Angiogenesis and Neurovascular Link, Vesalius Research Center (VRC), Catholic University of Leuven; ⁴ThromboGenics NV; ⁵Clinical Department of Otorhinolaryngology, Head and Neck Surgery, Catholic University Hospitals, Leuven; ⁶Department of Oto-Rhino-Laryngology, Upper Airway Research Laboratory (URL), Ghent University Hospital, Ghent, Belgium

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Keywords

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Correspondence

Dr. Peter Hellings, Clinical Department of Otorhinolaryngology, Head and Neck Surgery, Catholic University Hospitals, Kapucijnenvoer 33, 3000 Leuven, Belgium.
Tel.: +32-16-332342
Fax: +32-16-343835
E-mail: Peter.Hellings@uzleuven.be

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Abstract

Background: Edema represents a key feature of nasal polyp (NP) disease. Members of the vascular endothelial growth factor (VEGF) family may be involved, but the precise role of VEGF-A, VEGF-B, placental growth factor (PlGF), and their receptors VEGFR1 and VEGFR2 in NP edema formation remains elusive.

Objective: Exploring the expression of VEGF family members and their receptors and their correlation with clinical, radiological, and edema markers in NP.

Methods: The expression of VEGF-A, VEGF-B, PlGF, VEGFR1, and VEGFR2 was measured in NP ($n = 23$) and control tissue ($n = 22$) at mRNA and protein level. Edema was evaluated by measuring albumin levels and wet/dry ratios. Computed tomography (CT) scans were scored using the Lund-Mackay scoring system. IL-5 mRNA expression was determined by real-time RT-PCR. Cell suspensions from NP ($n = 10$) and control tissue ($n = 12$) were stimulated *in vitro* with IL-1 β or TNF α .

Results: mRNA expression of VEGFR1 and VEGF-B was significantly higher in NP compared with control tissue. Expression levels of VEGF-B and VEGFR1 significantly correlated with NP albumin content (VEGF-B: $P = 0.0208$; VEGFR1: $P = 0.0293$), CT scan scores (VEGF-B: $P = 0.0075$; VEGFR1: $P = 0.0068$), and IL-5 mRNA (VEGF-B: $P = 0.0027$; VEGFR1: $P = 0.0001$). *In vitro* stimulation of control and NP tissue cell suspensions with IL-1 β or TNF α significantly reduced the expression of VEGFR2 in control tissue, without altering VEGFR1 and VEGF-B expression. hVEGF-B induced nitric oxide production in NP macrophages ($P < 0.05$).

Conclusion: Expression levels of VEGFR1 and VEGF-B correlate with edema and clinical markers of NP disease and therefore represent potential therapeutic targets.

Chronic rhinosinusitis with nasal polyps (CRSwNP) represents a chronic disorder of the upper airways of unknown origin, being reported in up to 5% of the Western population and severely impairing the quality of life of affected patients (1). Inflammation, remodeling, and edema constitute the key histological features of CRSwNP (1). Despite the fact that edema in CRSwNP is considered important for the size of the nasal polyps (NP), it has not been properly studied so far. Edematous swelling of the airway

mucosa is also found in allergic rhinitis (2) and asthma (3), where members of the VEGF family have been suggested to be involved (3–5).

Vascular endothelial growth factor-A (VEGF-A) is a growth factor for endothelial cells, capable of inducing their proliferation, migration, differentiation, and survival (6). Vascular endothelial growth factor-A is indispensable in angiogenesis and is a strong inducer of vascular permeability (6). Vascular endothelial growth factor-B (VEGF-B), another

member of the VEGF family abundantly expressed in the heart and skeletal muscle, is able to directly stimulate endothelial cell growth and migration, but it is less potent than VEGF-A (7). Placental growth factor (PIGF) shares high structural homology with VEGF-A, and it is up-regulated in pathological conditions associated with angiogenesis (8).

Vascular endothelial growth factor-A binds two tyrosine kinase receptors: VEGFR1 and VEGFR2, which exist also in soluble forms as decoy receptors (9). VEGFR1 has 10–100 times higher affinity toward VEGF-A compared with VEGFR2, but its tyrosine kinase activity is very weak. On the other hand, VEGFR2, main angiogenic receptor, has a strong tyrosine kinase activity, but the affinity for binding VEGF-A is low (10). Vascular endothelial growth factor-B and PIGF bind and activate only VEGFR1 (10).

A limited number of studies have been performed on the role of VEGF family members in airway edema. In the lower airways, increased levels of VEGF-A have been detected in tissues and biological samples from patients with asthma (3, 11) and VEGF-A has been implicated in edema formation (3), which was not confirmed by others (12). Placental growth factor is reported to be up-regulated in chronic obstructive pulmonary disease (COPD) (13) and asthma (4), where PIGF has been suggested to play a role in edema formation (4). In the upper airways, VEGF-A has also been reported to be up-regulated in diseases such as allergic rhinitis (AR) (14) and CRSwNP (15, 16), without insight into the precise function. So far, the expression of VEGFR1 and VEGFR2 in relation to upper airway edema remains unknown.

The aims of this study were to explore the expression levels of VEGF-A, VEGF-B, and PIGF and their receptors VEGFR1 and VEGFR2 in NP disease and under pro-inflammatory condition and correlate their expression levels with markers of edema, local inflammation, and CT abnormalities.

Methods

Patients and controls

Nasal polyps tissue was harvested during endonasal sinus surgery from 30 patients with CRSwNP, who met the EP³OS criteria for CRSwNP (17). All patients had bilateral NP disease undergoing endoscopic sinus surgery for persisting symptoms despite guideline-based (1) treatment for rhinosinusitis symptoms. Patients with unilateral NP disease, cystic fibrosis, primary ciliary dyskinesia, or nasal malignancies were excluded. Biopsies of nasal turbinate were taken from 32 subjects who underwent septal or turbinate surgery for functional and/or esthetic reasons. Smoking was an exclusion criterion. Patients' characteristics are listed in Table 1. Patients' samples were used for 1) expression studies (23 NP, 22 control) and 2) *in vitro* stimulation experiment (10 NP, 12 control) with some samples overlapping (Table 1).

Atopy was defined as having at least one positive skin prick test in a panel of 18 most common inhalant allergens (HAL Allergy, Leiden, the Netherlands). Asthma was

Table 1 Patients' characteristics

	Control		Nasal polyp	
	Expression	Stimulation	Expression	Stimulation
Gender (female)	13/32		9/30	
Nasal corticosteroids	14/32		25/30	
Atopy	12/22	4/12	20/23	8/10
	9/32		11/30	
Asthma	7/22	3/12	8/23	3/10
	3/32		11/30	
AIA	3/22	0/12	10/23	2/10
	0/32		8/30	
	0/22	0/12	7/23	1/10

diagnosed based on previous or current proof of reversibility of FEV1 $\geq$ 12% after inhalation of salbutamol or positive histamine provocation test. Aspirin-induced asthma (AIA) syndrome was defined as a patient having NP with a doctor-based diagnosis of asthma and history of aspirin intolerance. Computed tomography (CT) scanning was performed before the surgery. The study was approved by the ethical committee of the Catholic University Hospital Leuven.

Evaluation of edema

Edema was evaluated by measuring albumin levels and wet/dry ration of control vs NP tissue (Data S1).

Radiological evaluation of NP size

Sinus opacity was obtained after objectively scoring CT scans using the Lund–Mackay system (Data S1).

Real-time RT-PCR

Quantification of human VEGF-A, VEGF-B, PIGF, VEGFR1, VEGFR2, and IL-5 mRNA expression by real-time RT-PCR was performed as previously (18) (Data S1).

Immunohistochemical staining

VEGFR1 and VEGF-B were stained on nasal mucosa and NP tissue paraffin sections (Data S1).

Differential cell count

Nasal polyps tissue paraffin sections were stained with H&E. Differential cell count was performed by counting number of eosinophils, neutrophils, macrophages, and lymphocytes in five different fields of the slide. Cell count was determined as mean value of cells/field.

Evaluation of protein levels of VEGFs

Pieces of nasal turbinate/polyp tissue were homogenized, and protein levels of VEGF-A, VEGF-B, PIGF, VEGFR1, and

VEGFR2 were determined in the supernatants by ELISA (Data S1).

In vitro stimulation of cells from nasal turbinate and NP tissue

Total cell suspensions were made from biopsies of nasal turbinates of 12 control donors and from polyp tissue of 10 NP patients (Table 1). 10^6 cells/ml were stimulated with/without IL-1 β or TNF α , and mRNA expression of VEGF-A, VEGF-B, PlGF, and receptors was determined in the cell pellet (Data S1).

Nitric oxide (NO) production in NP macrophages and epithelial cells

Nitric oxide production was evaluated in NP tissue macrophages and epithelial cells and stimulated with hPlGF-2 or hVEGF-B₁₆₇ (Data S1).

Data analyses

Statistical analysis was performed with GraphPad software Prism 4 (<http://www.graphpad.com/scientific-software/prism/>). All outcome variables were compared using non-parametrical tests. The Mann–Whitney *U*-test was used for between-group comparison. The Wilcoxon signed-rank test was performed for within-group comparison in stimulation experiments. A difference was significant when $P < 0.05$. Data are shown in a scattered dot plot and expressed as mean values.

Results

Enhanced expression of VEGFR1 and VEGF-B in NP tissue

The expression of mRNA for membrane VEGFR1 was significantly up-regulated in NP (7.9 ± 1.9) compared with control tissue (1.1 ± 0.2 , $P < 0.001$; Fig. 1A). Interestingly, the expression of VEGF-B, that is, the ligand of VEGFR1, was also up-regulated in NP tissue (4.4 ± 1.0) compared with control (1.9 ± 0.4 , $P < 0.05$; Fig. 1B) and correlated with the expression of VEGFR1 (Spearman $r = 0.8022$, $P = 0.001$). The expression of PlGF was not different between the tissues (control: 2.4 ± 0.5 , NP: 2.8 ± 0.7 , $P > 0.05$). The expression of membrane VEGFR2, the main signaling receptor for VEGF-A, was not different between the control (3.1 ± 0.5) and NP tissue (3.0 ± 0.6 , $P > 0.05$; Fig. 1C). Vascular endothelial growth factor-A expression on mRNA level was down-regulated in the NP tissue (5.0 ± 1.1) compared with control (10.9 ± 2.0 , $P < 0.05$; Fig. 1D).

Subanalysis of samples in relation to nasal corticosteroid use ($\pm$ ns) showed that intranasal corticosteroid treatment did not affect the mRNA expression levels of VEGFs in control tissue (VEGFR1 -ns: 1.1 ± 0.5 , +ns: 1.1 ± 0.3 , $P > 0.5$; VEGF-B -ns: 2.0 ± 0.7 , +ns: 1.8 ± 0.6 , $P > 0.5$; VEGFR2 -ns: 3.6 ± 0.8 , +ns: 2.7 ± 0.6 , $P > 0.5$; VEGF-A -ns: 7.7 ± 2.1 , +ns: 13.5 ± 3.0 , $P > 0.5$). Presence of allergy or asthma also did not significantly affect the expression of VEGFs (data not shown).

Protein expression of VEGF-B was significantly up-regulated in NP tissue (19 ± 6 pg/mg) compared with control (6 ± 2 pg/mg, $P < 0.05$). Protein levels of total VEGFR1 (membrane and soluble) was down-regulated in NP

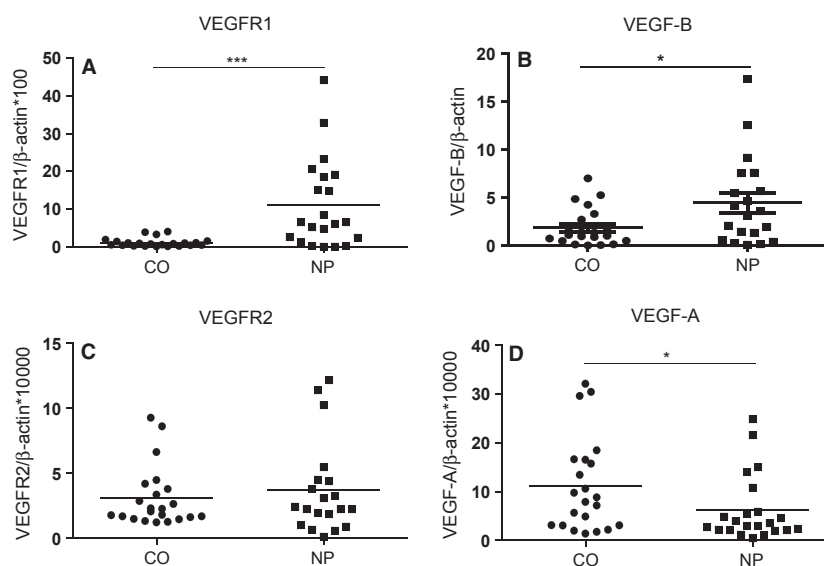


Figure 1 (A) VEGFR1, (B) VEGF-B, (C) VEGFR2, and (D) VEGF-A mRNA expression in control nasal turbinates (CO) vs nasal polyp tissue (NP). VEGF-B and VEGFR1 had significantly higher mRNA expression in nasal polyp tissue compared with control nasal turbin-

ates. Expression levels of corresponding genes were corrected for expression of housekeeping gene β -actin. CO = 22, NP = 23, * $P < 0.05$ and *** $P < 0.001$.

(532 ± 78 pg/mg) compared with control tissue (848 ± 143 pg/mg, $P > 0.05$). Vascular endothelial growth factor-A was significantly lower in NP tissue (56 ± 10 pg/mg) compared with control (87 ± 11 pg/mg, $P < 0.05$) as shown by PCR. Protein expression of PlGF (control: 40 ± 5 pg/mg, NP: 32 ± 6 pg/mg, $P > 0.05$) and total VEGFR2 (control: 305 ± 51 pg/mg, NP: 245 ± 42 pg/mg, $P > 0.05$) did not differ between the groups as shown by PCR. Sub-analysis in relation to nasal corticosteroid use showed that there was no effect on VEGFs protein levels in control tissue (data not shown).

Immunohistochemical staining of VEGFR1 and VEGF-B

In nasal turbinate tissue, VEGFR1 was weakly expressed in the epithelial cells and on resident macrophages (Fig. 2A). VEGFR1 was also stained in epithelial cells of NP tissue with additional strong expression in NP inflammatory cells (Fig. 2B). Vascular endothelial growth factor-B expression was hardly detected in the control tissue (Fig. 2C), whereas inflammatory cells positive for VEGF-B were found in the subepithelium of NP tissue (Fig. 2D).

Edema markers correlate with VEGFR1 and VEGF-B expression

Edema markers albumin (control: 274.8 ± 33.4 μ g/mg, NP: 398.2 ± 50.7 μ g/mg, $P < 0.01$; Fig. 3A) and wet/dry ratio (control: 8.5 ± 0.3 , NP: 11.3 ± 0.6 , $P < 0.001$) were significantly higher in NP tissue compared with control nasal turbinate. There was a significant correlation between albumin

concentration in the NP tissues and mRNA levels of VEGFR1 (Spearman $r = 0.5441$, $P = 0.0293$; Fig. 3B), mRNA levels of VEGF-B (Spearman $r = 0.5893$, $P = 0.0208$) and protein levels of VEGF-B (Spearman $r = 0.5429$, $P = 0.0365$; Fig. 3C). There was no correlation between albumin and protein or mRNA levels of VEGF-A, PlGF, and VEGFR2.

VEGFR1 and VEGF-B levels correlate with total CT scan scores

Nasal polyps size was assessed by scoring CT scans. Total CT scan scores correlated significantly with protein levels of VEGFR1 (Spearman $r = 0.5984$, $P = 0.0068$; Fig. 3D), VEGF-B (Spearman $r = 0.6592$, $P = 0.0075$; Fig. 3E), and PlGF (Spearman $r = 0.5675$, $P = 0.0091$), but not with VEGF-A (Spearman $r = 0.087$, $P = 0.7315$) nor VEGFR2 (Spearman $r = 0.1615$, $P = 0.5812$). Of note, albumin levels in the nasal tissue significantly correlated with total CT scan score (Spearman $r = 0.5023$, $P = 0.024$).

VEGFR1 and VEGF-B correlate with inflammatory parameters

Expression of IL-5 mRNA was significantly up-regulated in NP tissue (13.7 ± 2.8) compared with control (1.8 ± 0.6 , $P < 0.01$). IL-5 levels in the NP tissue significantly correlated only with mRNA levels of VEGFR1 (Spearman $r = 0.9597$, $P = 0.0001$; Fig. 4A) and VEGF-B (Spearman $r = 0.6331$, $P = 0.0027$; Fig. 4B).

Differential cell count was performed on the NP tissue sections, and cells were differentiated into macrophages,

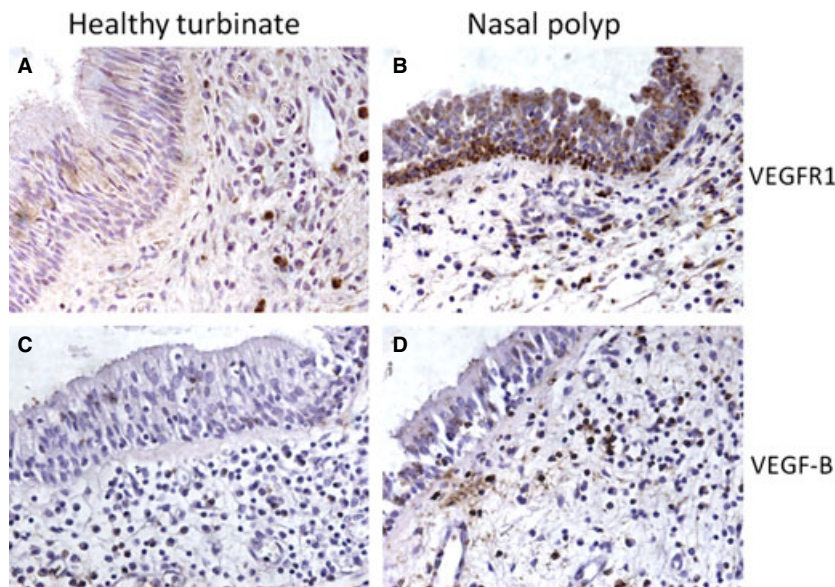


Figure 2 Expression of VEGFR1 (A and B) and VEGF-B (C and D) in control nasal turbinate vs nasal polyp tissue analyzed by immunohistochemistry. VEGFR1 was constitutively expressed in epithelial cells and resident macrophages in control nasal turbinate,

whereas in NP tissue, VEGFR1 expression was stronger also detected in infiltrating inflammatory cells. VEGF-B was expressed by inflammatory cells of NP tissue. Magnification is 40 $\times$.

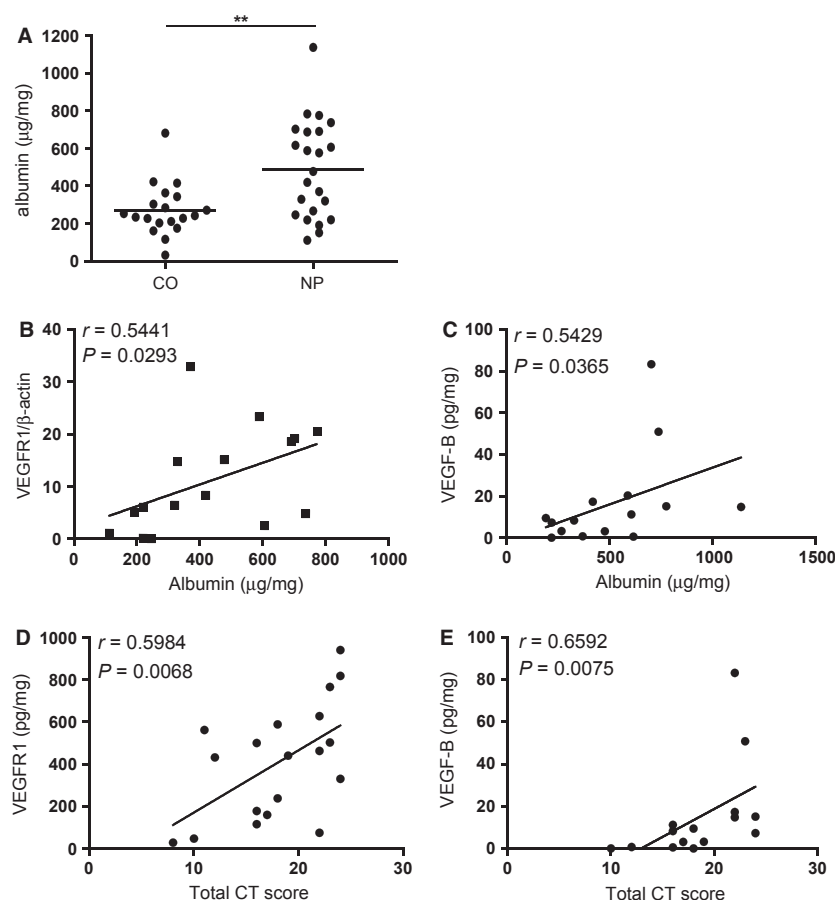


Figure 3 (A) Protein levels of albumin in homogenized control nasal turbinate vs nasal polyp tissue. Albumin levels were significantly higher in NP tissue compared with control nasal turbinate. CO = 19, NP = 23, $**P < 0.01$. Correlation between albumin levels and (B) VEGFR1 mRNA and (C) VEGF-B protein levels in nasal

polyp tissue was statistically significant. Correlation between total Computed tomography (CT) scan score and protein levels of (D) VEGFR1 and (E) VEGF-B. Protein levels of both VEGFR1 and VEGF-B significantly correlate with the degree of opacification of the sino-nasal cavities expressed by the total CT scan score.

neutrophils, lymphocytes, and eosinophils. Only number of tissue macrophages per field correlated with mRNA expression of VEGFR1 (Spearman $r = 0.6209$, $P = 0.0235$; Fig. 4C) and total CT scan score (Spearman $r = 0.632$, $P = 0.0153$). Vascular endothelial growth factor-B did not significantly correlate with any cell type. Surprisingly, number of eosinophils did not correlate with any of the VEGFs studied. Number of neutrophils per field correlated with PlGF mRNA expression (Spearman $r = 0.7084$, $P = 0.0067$).

Pro-inflammatory cytokines down-regulate VEGFR2 expression *in vitro*

Culturing of mucosal cell suspensions revealed higher baseline VEGF-B (NP: 11.5 ± 4.4 , control: 1.2 ± 0.9 , $P < 0.05$) and VEGFR1 expression in cells derived from NP tissue compared with control, but the difference was not statistically significant for VEGFR1 (Fig. 5A,B). Levels of VEGFR2 were significantly lower in NP tissue (14.6 ± 2.8) compared with control (42.1 ± 7.7 , $P < 0.05$; Fig. 5C).

Baseline mRNA expression of VEGF-A (Fig. 5D) and PlGF (Fig. 5E) did not differ between groups.

In vitro stimulation with pro-inflammatory cytokines IL-1 β and TNF- α did not influence the expression of VEGFR1 and VEGF-B (Fig. 5A,B). *In vitro* stimulation of cell suspensions from control tissue showed that the expression of VEGFR2 was significantly down-regulated by pro-inflammatory cytokines (Fig. 5C), but this effect was not observed in NP tissue. IL-1 β induced significantly higher production of VEGF-A (Fig. 5D) and PlGF (Fig. 5E) only in control tissue.

hVEGF-B stimulates NO production in NP macrophages

In vitro stimulation of NP macrophages with hVEGF-B₁₆₇ induced significantly higher nitrite production compared with the medium condition (hVEGF-B₁₆₇: 175.8 ± 54.5 ng/ml, medium: 51.3 ± 17.8 ng/ml, $P < 0.05$), but the effect was not seen in NP epithelial cells (hVEGF-B₁₆₇: 89.0 ± 43.3 ng/ml, medium: 135.8 ± 50.6 ng/ml, $P > 0.05$). In contrast to

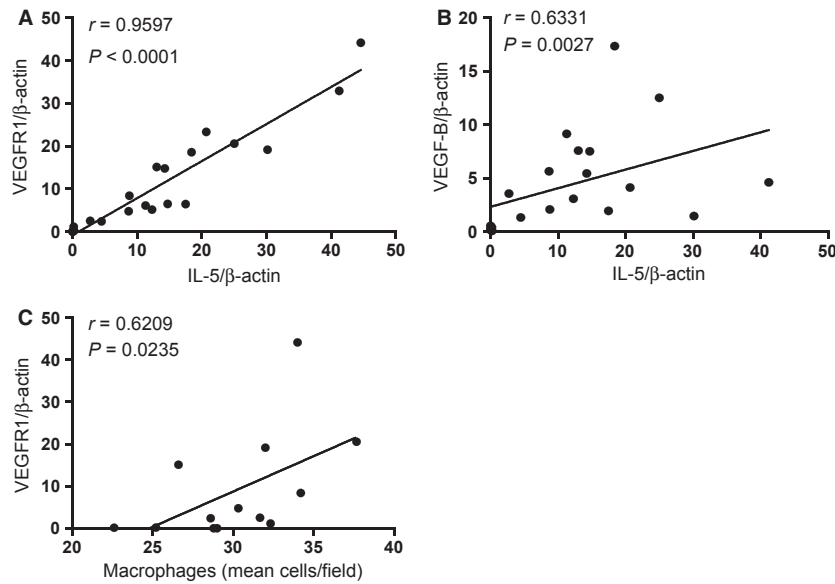


Figure 4 IL-5 mRNA expression in correlation with mRNA levels of (A) VEGFR1 and (B) VEGF-B. There is a strong correlation between mRNA expression of both VEGFR1 and VEGF-B with IL-5 mRNA

expression. (C) Correlation of macrophage numbers with mRNA levels of VEGFR1. VEGFR1 mRNA expression significantly correlates with macrophage numbers.

hVEGF-B₁₆₇, hPIGF-2 did not affect nitrite production neither in NP macrophages (hPIGF-2: 78.5 ± 48.8 ng/ml, medium: 51.3 ± 17.8 ng/ml, $P > 0.05$) nor in NP epithelial cells (hPIGF-2: 204.4 ± 119.9 ng/ml, medium: 135.8 ± 50.6 ng/ml, $P > 0.05$).

Discussion

In the present study, we report for the first time on the expression of VEGFR1 and its ligand VEGF-B in inflammation and edema in CRSwNP. Both VEGFR1 and VEGF-B were up-regulated in NP tissue compared with controls, and their expression correlated with important disease parameters such as the edema marker albumin, total CT scan score and with the typical Th2 cytokine IL-5. However, when interpreting data, it should be taken into account that inferior turbinate tissue originates from control subjects, but not from NP patients who donated polyp tissue.

One of the most striking observations was the enhanced mRNA expression of membrane VEGFR1 in NP. IHC staining revealed that VEGFR1 is constitutively expressed in epithelial cells and on resident macrophages in control tissue with enhanced expression by inflammatory cells, epithelial cells, and endothelial cells within NP. This is in line with previous studies where VEGFR1 was up-regulated in NP tissue compared with control (16). Interestingly, the expression of VEGF-B was also up-regulated in the NP tissue compared with controls on mRNA and protein level, localized in sub-epithelial inflammatory cells of NP tissue.

We found lower expression of VEGF-A mRNA and protein in NP compared with control tissue which is in contrast to other studies (15, 16, 19, 20). Similarly, expression of

VEGFR2 was not different between the groups even though VEGFR2 was reported to be up-regulated in NP compared with control tissue (16, 20). To explain these data, we have performed immunohistochemistry for VEGF-A and VEGFR2 (data not shown). In the control tissue, VEGF-A was expressed in epithelial cells, in resident macrophages, and in the glands. In the NP tissue, VEGF-A was mainly expressed in the inflammatory cells, while epithelium was weakly positive. Gland density is known to be low in edematous portion of NP tissue (21), which might explain VEGF-A down-regulation in NP. VEGFR2 was expressed mostly on the endothelial cells. Blood vessels in NP tissue are sparse (22), which might explain why VEGFR2 was not up-regulated in NP tissue compared with control.

VEGFR1 and VEGF-B were not only up-regulated in NP compared with control tissue, but also found to correlate with total CT scan score, edema marker albumin, and IL-5 in the NP tissue. Total CT scan score takes into account the extent of opacification of the sinonasal cavities (1), and in addition, it correlated with albumin, providing further evidence for a close interaction between the degree of edema and CT scan abnormalities. Typical Th2 cytokine IL-5 is known to be up-regulated in NP (23), and it enhances the secretion of VEGF-A in human eosinophils *in vitro* (24), but the link between IL-5 and VEGFR1 or VEGF-B has not yet been shown. Furthermore, VEGFR1 correlated with infiltrating macrophages, but no correlation was found with eosinophil counts, despite the fact that ECP correlates with the severity of edema in NP tissue (25). VEGFR1 is expressed by macrophages (26), but the role of macrophages in edema formation is not well established, although there are reports suggesting that some macrophage products can induce edema

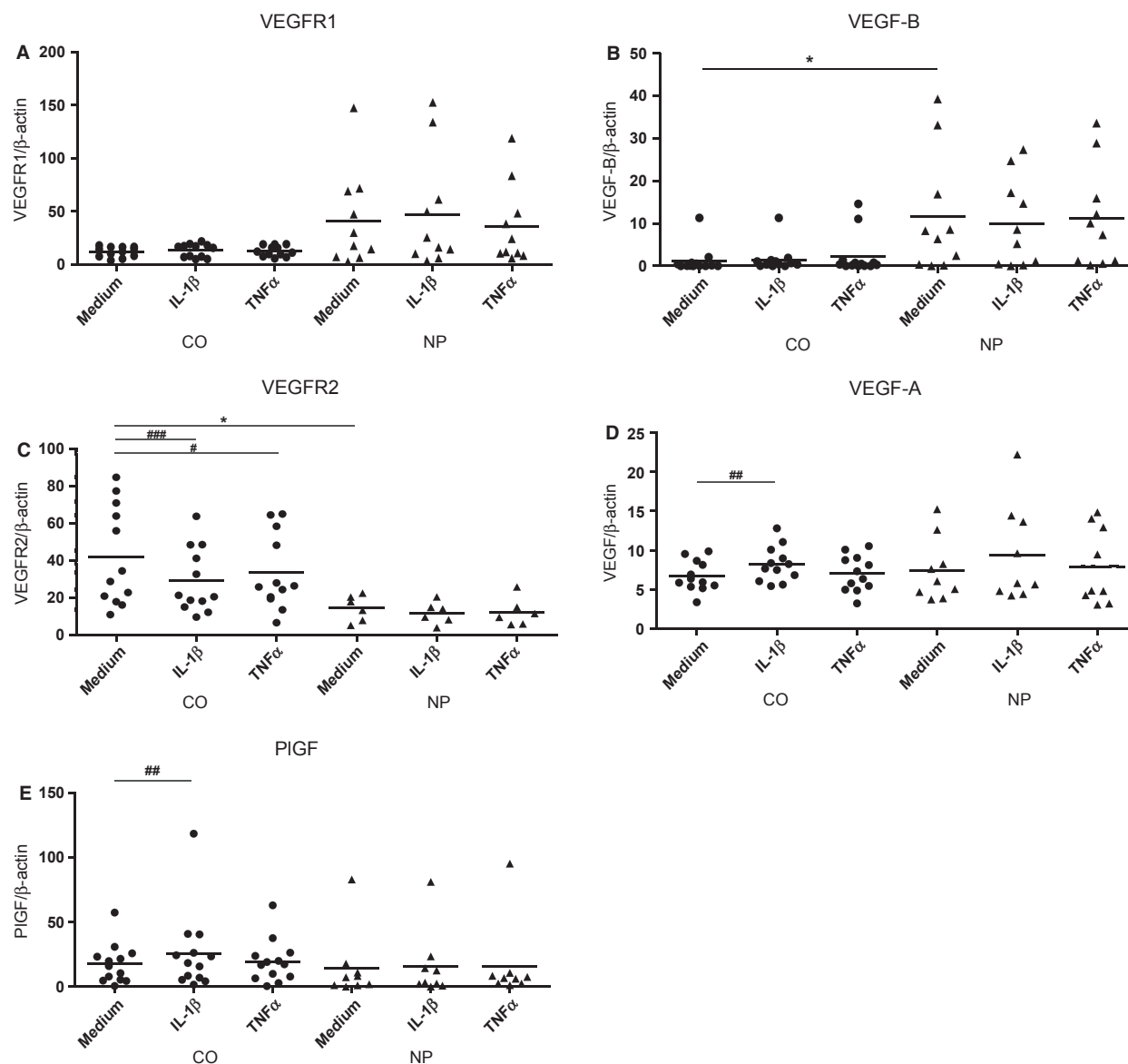


Figure 5 (A) VEGFR1, (B) VEGF-B, (C) VEGFR2, (D) VEGF-A, and (E) PlGF mRNA expression in control nasal turbinate and NP cell suspensions in response to IL-1 β or TNF α (10 ng/ml) stimulation for 24 h. Expression levels of corresponding genes were corrected

for expression of housekeeping gene β -actin. CO = 12, NP = 10; comparison between CO and NP * P < 0.05; comparing stimulation within the group # P < 0.05, ## P < 0.01, ### P < 0.001.

(27, 28). Finally, we have demonstrated that VEGF-B, but not PlGF, was able to induce NO production in NP macrophages. Nitric oxide (NO) is known to induce microvascular leakage, which can lead to the tissue edema (29), but the effect of VEGF-B on NO production has not yet been shown.

Our *in vitro* study explored the effects of pro-inflammatory cytokines IL-1 β and TNF- α on the expression of VEGFs. Expression of VEGFR1 and VEGF-B was not affected by pro-inflammatory stimulation, although their basal expression was higher in the NP tissue cells compared with nasal turbinate cells. Surprisingly, expression of VEGFR2 was

strongly down-regulated by IL-1 β and TNF α in cells from nasal turbinate. This is in contrast to HUVECs where IL-1 β and TNF α were described as inducers of VEGFR2 expression (30, 31). Basal expression of VEGFR2 was lower in NP cells compared with nasal turbinate cells, which suggests that the main angiogenic receptor signaling is diminished in the inflammatory environment and the role of VEGFR1 is predominant. VEGFR2 angiogenic pathway might be skewed by VEGFR1 pro-inflammatory signaling, thus explaining less capillaries and blood vessels in the NP tissue compared with controls (22). It has been shown that VEGFR1 negatively regulates VEGFR2-mediated proliferation of HUVECs via

NO production (32). VEGFR1 and inflammation are also linked in rheumatoid arthritis (RA), where treatment with anti-VEGFR1 antibodies, but not anti-VEGFR2 antibodies, significantly reduced synovial inflammation and joint destruction (33). Similar reduction in synovial inflammation was seen in VEGF-B^{-/-} mice (34), further supporting the role for VEGFR1 signaling in RA.

In conclusion, our study highlights the importance of VEGFR1 and its ligand VEGF-B in NP edema and size. These findings open new perspectives for therapeutic strategies in NP.

Author contributions

Most of the experimental work described in this article has been conducted in the Laboratory of Clinical Immunology by the first author under the supervision of Professors P. Hellings and J. Ceuppens who designed the research. Valerie Hox and Ina Callebaut actively contributed in the experimental part of the study. Immunohistochemistry staining was performed in the laboratory of Professor P. Carmeliet with the experimental help of S. Vinckier. Nasal polyp tissue and nasal turbinate tissue were harvested by P. Hellings, V. Hox, M. Jorissen, P. Gevaert, and C. Bachert. B. Jonckx provided experimental help and together with Professor J.-M. Stassen donated hPIGF-2 and gave practical advice for the study. Professors P. Carmeliet, C. Bachert, and P. Gevaert helped with comments and advice on the final shape of the article. The first author analyzed the data and wrote the article.

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Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Vascular endothelial growth factor receptor 1 expression in nasal polyp tissue.

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Data Supplement**Vascular Endothelial Growth Factor Receptor 1 in Edema of Nasal Polyp Tissue**

Sonja Bobic¹, Valerie Hox¹, Ina Callebaut¹, Stefan Vinckier^{2,3}, Bart Jonckx⁴, Jean-Marie Stassen⁴, Mark Jorissen⁵, Philippe Gevaert⁶, Peter Carmeliet^{2,3}, Claus Bachert⁶, Jan L. Ceuppens¹ and Peter W. Hellings^{1,5}

Methods

Evaluation of edema

Mucosa of the inferior nasal turbinate or nasal polyp tissue was taken for the estimation of the degree of edema by measuring the wet/dry ratio. The weight of the biopsy was measured before and after 6h of drying at 65°C.

Another piece of frozen nasal turbinate/polyp tissue was homogenized and supernatants were used for determining protein levels of albumin (E1) by ELISA (AssayMax Human Albumin ELISA Kit EA3201-1, AssayPro).

Radiological evaluation of NP size

Measure of sinus opacity was obtained after scoring CT scans for all sinuses on both sides using the Lund-Mackay system. Lund-Mackay system is based on points given for degree of opacification: 0 = normal, 1 = partial opacification, 2 = total opacification. These points are applied to the maxillary, anterior ethmoid, posterior ethmoid, sphenoid and frontal sinus on each side. The osteomeatal complex is graded as 0 = not occluded, or 2 = occluded deriving a maximum score of 12 per side, or 24 for the total CT scan score (E2).

Real-time RT-PCR

Piece of frozen nasal turbinate/polyp tissue was used for determination of VEGF-A, VEGF-B, PlGF, VEGFR1, VEGFR2 and IL-5 mRNA expression by real-time RT-PCR. Total mRNA was extracted using RNeasy mini protocol. A fix amount of target RNA (1 µg) was reverse transcribed using 200U Superscript II RT at 42°C for 60 min. Real-time RT-PCR was performed for human *VEGF-A* (all isoforms), *VEGF-B* (all isoforms), *PlGF* (all isoforms), *VEGFR1* (only membrane receptor) and *VEGFR2* (only membrane receptor) mRNA in ABI prism 7700 Sequence detector (E3). Human β -actin was used as a housekeeping gene. Two

additional housekeeping genes were tested as controls (human *GNB2L1* and human *PPIA*). PCR amplification for each sample was performed in duplicate wells, using the following conditions: 10 min at 94°C, followed by a total of 40 two temperature cycles (15 s at 94°C and 1min at 60°C).

Primers and TaqMan probes used are listed in Table EI. They were designed using PrimerExpress software with one of the primers/probes overlapping two exons in order to exclude possible amplification of contaminating DNA.

Immunohistochemical staining

Nasal mucosa from healthy donors and polyp tissue from CRSwNP patients were fixed in 6% formol and subsequently embedded in paraffin. Six-micrometer sections of embedded tissues were cut, placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (H&E). For immunohistochemistry detection of VEGF-B and VEGFR1 deparaffinized 6 µm sections were incubated with antigen retrieval solution (Dakocytomation ref. S1699) for 20 min on 95°C. After cooling down, sections were washed and endogenous peroxidase was quenched with 0.3 % H₂O₂ in methanol for 20 min. Slides were subsequently blocked and incubated with primary antibody (goat anti-VEGF-B (C-19, sc-1876), rabbit anti-Flt-1 (C-17, sc-316), Santa Cruz Biotechnology overnight on 4°C. After washing slides were incubated with adequate secondary antibody (goat anti-rabbit IgG (E0432) and rabbit anti-goat IgG (E0466), Dakocytomation). Slides were subsequently incubated with streptavidine-HRP and signal was amplified with biotinyI-tyramide (NEL700 kit, Perkin Elmer) following manufacturer's instructions. In the end, slides were stained by adding DAB reagent with H₂O₂ and counterstained with hematoxyline.

Evaluation of protein levels of VEGFs

Pieces of frozen nasal turbinate/polyp tissue were homogenized and supernatants were used for determining protein levels of VEGF-A, VEGF-B, PlGF, VEGFR1 and VEGFR2 by ELISA. Tissue was homogenized in 300 µl of homogenizing buffer (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 11 mM Glc, 2 mM PMSF (phenyl methyl sulphonyl fluoride), 1 tablet per 10 ml buffer of cocktail protease inhibitors (Roche, Complete Mini, EDTA free, LOT 13840300), 1 mM EDTA, pH=7.2) on ice. After centrifuging for 20 min on 5000 rpm, 4°C, supernatants were collected and protein concentration was determined by bicinchoninic acid assay (BCA) method. Human VEGF-A (DuoSet DY293B, R&D), hVEGF-B (E90144Hu, Uscn Life Science Inc.), huPlGF-1/2 (DuoSet DY264, R&D), total VEGFR1 (DYC4347, R&D) and total VEGFR2 (DYC1780, R&D) were measured in supernatants of nasal biopsy/polyp tissue homogenates. Values were corrected for the total protein content measured by BCA method.

In vitro stimulation of cells from nasal turbinate and NP tissue

Tissues were dissected, cut into small pieces and digested with 2.5 mg/ml collagenase A (Roche, Mannheim, Germany) for 30 min at 37°C. Cells were passed through a cell strainer to obtain a single cell suspension. Cells were centrifuged (1500 rpm, 10 min) and washed twice in culture medium (DMEM, Lonza BioWhittaker supplemented with L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 % (v/v) ultrosor G). Cells were counted in Bürker's chamber and 1×10^6 cells/ml were incubated in 1 ml medium with/without IL-1 β (10 ng/ml) or TNF- α (10 ng/ml) for 24h. mRNA expression of VEGF-A, -B, PlGF and receptors was determined by real-time RT-PCR in the cell pellet.

Nitric-oxide (NO) production in NP macrophages and epithelial cells

Nasal polyp tissue from 6 NP patients was cut into small pieces and lavaged with RPMI 1640 medium containing 5 mM EDTA, 1% L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Tissue was digested with 2.5 mg/ml collagenase A (Roche, Mannheim, Germany) in the culture medium for 30 min at 37°C. Digested tissue was passed through a cell strainer to obtain a single cell suspension. Cells were centrifuged and washed two times in PBS and resuspended in 5 ml culture medium (RPMI 1640, 10% FCS, supplemented with 1% L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin). Cells were counted and plated so that 10^6 macrophages/ml were plated in each well. After 2 h of incubation in 24-well cell culture plates, the non-adherent cells were washed away and fresh medium was added. The adherent purified macrophages were incubated overnight. Medium was changed and macrophages were incubated with/without hPIGF-2 (50 ng/ml) and hVEGF-B₁₆₇ (50 ng/ml) for 24h at 37°C.

Nasal epithelial cells were isolated from NP tissue as described previously (E4). Nasal epithelial cells were incubated at the concentration of 10^6 cells/ml with/without hPIGF-2 (50 ng/ml) and hVEGF-B₁₆₇ (50 ng/ml) for 24h at 37°C.

NO production in the form of nitrite was measured in supernatants of macrophages and epithelial cells with Griess reagent (E5).

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Table EI. Sequences of primers and TaqMan probes used for real-time RT-PCR

Gene	Forward primer	Reverse primer	TaqMan Probe (5'Fam 3'Tamra)
hVEGF-A	agccttgcccttgctgctctac	tcctccttctgcatgggt	accatgccaagtgggtcccaggct
hVEGF-B	cctgacgatggcctggagt	ttaggtctgcattcacactggc	cagatcctcatgatccggtacccgag
hPIGF	gccaatgtcaccatgcagc	cctcctttccggcttcac	ctgcgaatgccggcctctgc
hVEGFR1	gcaaattgtacaacaggatggtaaaga	cctcagagaaggcaggagtga	ccaatcaatgccatactgacaggaaatagtg
hVEGFR2	cttccaagtggctaaggcat	gataagaggatatttcgtgccgc	tcttggcatcgcgaaagtgtatccaca
hIL-5	cccacaagtgcattggtgaa	cctcagagtctcattggctatcag	agaccttggcactgctttctactcatcgaa
hβ-actin	ggacatccgcaaagacctgt	ctcaggaggagcaatgatcttgat	ctggcggcaccacctgtaccct
hPPIA	cgcgctctccttgagctgtt	ctgacacataaacctggaataattc	cagacaagggtcccaaagacagcagaaaaattt
hGNB2L1	cactgtccaggatgagagcca	cataccttgaccagcttgccc	tccgcttctcgccaacagcag