JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 55 (6): 203–211, 2022
doi: 10.1267/ahc.22-00098

Regular Article

Cathepsin D Inhibits Angiogenesis in Pituitary Neuroendocrine Tumors

Ren Fujiwara[1,2], Hirotomo Ten[3], Hui Chen[4], Chuan-lu Jiang[4], Ken-ichi Oyama[5], Keisuke Onoda[1,2] and Akira Matsuno[1,2]

[1]Graduate School of Medicine, International University of Health and Welfare, 4–3 Kozunomori, Narita, Chiba 286–8686, Japan, [2]Department of Neurosurgery, International University of Health and Welfare, Narita Hospital, 852 Hatakeda, Narita, Chiba 286–8520, Japan, [3]Department of Judo Physical Therapy, Faculty of Health Care, Teikyo Heisei University, 2–51–4 Higashiikebukuro, Toshima, Tokyo 170–8445, Japan, [4]Department of Neurosurgery, 2nd Affiliated Hospital, Harbin Medical University, 246 Xuefu Road, Nan'gang District, Harbin 150081, China and [5]Department of Neurosurgery, International University of Health and Welfare, Mita Hospital, 1–4–3, Mita, Minato-ku, Tokyo 108–8329, Japan

Received October 28, 2022; accepted November 29, 2022; published online December 20, 2022

Prolactin and growth hormone can acquire anti-angiogenic properties after undergoing proteolytic cleavage by Cathepsin D and bone morphogenetic protein 1 (BMP-1) into fragments known as vasoinhibins. Little is known about the effect of vasoinhibins on angiogenesis through the involvement of key cleavage enzymes Cathepsin D and BMP-1 in pituitary neuroendocrine tumors (PitNETs, formerly pituitary adenomas). The purpose of this study was to investigate the mechanism of action of Cathepsin D and BMP-1 on angiogenesis in PitNETs compared with that of pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-2 (FGF2). A total of 43 patients were enrolled in a retrospective analysis and 22 samples were suitable for RNA extraction, including 16 non-functional PitNETs and six somatotroph tumors. The mRNA and protein levels of Cathepsin D, BMP-1, VEGF, and FGF2 were compared with those of von Willebrand factor, which was assessed to determine the vascularization of PitNETs. Cathepsin D and FGF2 were significantly correlated with vascularization in PitNETs. Both Cathepsin D and FGF2 are highly involved in angiogenesis in PitNETs, although the effect of Cathepsin D as an anti-angiogenic factor is dominant over that of FGF2 as a pro-angiogenic factor.

Key words: Cathepsin D, angiogenesis, pituitary neuroendocrine tumor

I. Introduction

Pituitary neuroendocrine tumors (PitNETs, formerly pituitary adenomas) account for approximately 15% of intracranial tumors [10]. In most human tumors, angiogenesis is correlated with a series of tumor behaviors [17]; however, PitNETs have been reported to be less vascularized than normal pituitary tissue [34]. Although the role of angiogenesis in PitNETs is controversial, a significantly higher degree of vasculature has been noted in invasive and macro PitNETs [32].

Correspondence to: Akira Matsuno, MD, PhD, Department of Neurosurgery, International University of Health and Welfare, Narita Hospital, 852 Hatakeda, Narita, Chiba 286–8520, Japan.
E-mail: akira.dr.ruby@gmail.com

Pituitary hormones are versatile; they can affect angiogenesis either directly through actions on endothelial cells or indirectly by regulating pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [13, 31]. Prolactin (PRL) and growth hormone (GH) are remarkably unique in their regulation of angiogenesis; these molecules can function as pro-angiogenic factors by direct or indirect actions on endothelial cells, and can also acquire anti-angiogenic properties after undergoing proteolytic cleavage to vasoinhibins, a family of N-terminal PRL fragments ranging from 14 to 18 kDa in size that have a totally opposite effect on angiogenesis compared with their parental molecules [4, 6, 14, 29].

Cathepsin D is the key enzyme that proteolytically cleaves PRL into vasoinhibins, which subsequently exert an anti-angiogenetic effect in colon and breast cancers [3, 9].

Piwnica *et al.* reported that Cathepsin D also cleaves PRL into an anti-angiogenic 17-kDa fragment in PitNETs [29]. In other cancers, Cathepsin D is a negative prognostic marker associated with an increased risk of relapse and metastasis [19, 20], particularly in breast cancer [12]. However, there are no reports on the effect of Cathepsin D in PitNETs.

Another key enzyme that cleaves PRL and GH into vasoinhibins is BMP-1. It has been reported that placental BMP-1 gene expression is increased in women and rats with diabetes and leads to vascular degeneration of the placenta as a result of increased PRL proteolysis in the placenta [28]. However, little is known about the angiogenic effect imposed by BMP-1 in PitNETs.

Several molecules have been investigated concerning vasculature in pituitary cells and other organs [2, 3, 18]. von Willebrand factor has been used extensively to quantify angiogenesis in a variety of tumors [16, 35]. Vasculature formation of tumors relies on the balance between stimulators and inhibitors. Among pro-angiogenetic factors, VEGF is the central mediator of angiogenesis in endocrine glands [7]. Another potent angiogenic factor is basic fibroblast growth factor-2 (FGF-2), which has multiple activities affecting both the vasculature and parenchyma cell proliferation through its upregulation during pituitary tumorigenesis and growth [23].

Considering the unique effect imposed on angiogenesis by PRL, GH, and the fragments resulting from their cleavage by the key enzymes Cathepsin D and BMP-1, we aimed to investigate the involvement of Cathepsin D and BMP-1 in angiogenesis of PitNETs, with a focus on the balance between stimulators and inhibitors of angiogenesis.

II. Materials and Methods

Patients and tumor samples

Patients who underwent surgical operation for resection of PitNETs at Teikyo University Hospital between April 2015 and July 2016 were retrospectively analyzed. A total of 43 samples were initially enrolled but samples that were not suitable for RNA extraction and staining were excluded. Finally, 22 PitNET samples from 22 patients (12 men, 10 women; mean age 53.5 years; range 30–78 years) were examined, including 16 nonfunctional PitNETs and six somatotroph tumors (Table 1). Seven patients (Table 1: No. 1, 2, 6, 8, 11, 12, 16) showed hyperprolactinemia but the specimens were negative for PRL staining and were classified as nonfunctional PitNETs due to the stalk effect [27]. Patients with acromegaly were not treated with octreotide preoperatively. This study was approved by the ethics committee of Teikyo University Hospital. Data were analyzed anonymously and informed consent was obtained from each patient.

Immunohistochemistry

PitNET tissues were removed during surgery and fixed overnight in 4% paraformaldehyde dissolved in 0.01 M phosphate buffered saline, pH 7.4 (PBS). The tissues were embedded in paraffin blocks using routine techniques. Immunohistochemical (IHC) assays were performed on 4-μm thick sections of paraffin-embedded specimens. Slides were deparaffinized, rehydrated, subjected to antigen retrieval, and incubated overnight with the following primary antibodies: rabbit anti-human Cathepsin D antibody (ab75852 at 1:250 dilution, Abcam, USA), rabbit anti-human BMP-1 antibody (ab118520 at 1:1,000 dilution, Abcam), rabbit anti-human VEGF antibody (sc-152 at 1:200 dilution, Santa Cruz Biotechnology, USA), rabbit anti-human FGF2 antibody (sc-79 at 1:200 dilution, Santa Cruz Biotechnology), mouse anti-human von Willebrand factor antibody (M0616 at 1:50 dilution, Dako, Japan). After incubation for 60 min with biotinylated secondary antibody the sections were processed with avidin–biotin complex (Vectastain Elite ABC kit, Vector Laboratories, USA), stained using diaminobenzidine as chromogen, counterstained with Mayer's hematoxylin, and mounted.

Staining for pituitary hormone was performed to establish the histological hormone phenotype of the tumor samples using commercially available antibodies for GH (A0570, Dako, USA), ACTH (M3501, Dako, USA), PRL (A0569, Dako, USA), TSH (M3503, Dako, USA), LH (0374, MBL, Japan), FSH (0373, MBL, Japan), αSU (5953689, NIH, USA).

Negative control studies included substituting normal sera for the primary antibody.

The representative immunohistochemical stainings are shown in Figure 1.

Semiquantitative analysis of immunohistochemical staining

The tumor cell area of each section was viewed in three different fields at ×400 magnification. The number of cells positive for Cathepsin D, BMP-1, FGF2, or VEGF was counted and the percentage of immunoreactive positive cells was calculated using ImageJ software (National Institutes of Health, USA). To assess the vascularization of PitNETs, both the immunoreactive positive areas for von Willebrand Factor and the whole tissue area in each field were measured manually by Image J and the percentage of the total area positive for von Willebrand factor was calculated. Finally, the average percentage of positive staining of each molecule was determined.

For hormone staining, specimens with positive staining in less than 1% of cells were assessed as negative, 1–25% as +, 25–50% as 2+, 50–75% as 3+, and 75–100% as 4+ (Table 1).

Evaluation of tumor proliferation

Staining for Ki-67 (M7240, Dako, USA) was performed to evaluate proliferative potential and the percentage of positive cells was calculated using e-Count software (e-Path, Japan).

Table 1. *Characteristics of patients*

Patient	Gender	Age (yr)	Diagnosis	Pre-operational Abnormal Hormone (reference range)	Ki-67	Hormone staining
1	F	53	NF	PRL: 148.8 (4.9–29.3 ng/ml) LH: 0.1 (2.4–12.6 mIU/ml) FSH: 0.8 (3.5–12.5 mIU/ml)	1.3% P53: weak	negative
2	M	73	NF	PRL: 21.3 (4.3–13.7 ng/ml) FSH: 80.8 (1.7–12.4 mIU/ml)	<1%	FSH(3+) αSU(+)
3	F	48	NF	Normal	<1%	negative
4	M	66	NF	Normal	1%	αSU(+)
5	M	46	NF	Cortisol: 19.9 (4–18.3 ug/dL) PRL: 3.7 (4.3–13.7 ng/ml)	2.8%	negative
6	M	78	NF	T3: 1.31 (2.3–4.0 pg/ml) PRL: 70.7 (4.3–13.7 ng/ml)	1.8%	LH(+) αSU(+)
7	M	50	NF	FSH: 13.1 (1.8–12 mIU/ml)	<1%	FSH(4+) αSU(2+)
8	F	35	NF	PRL: 59.6 (4.9–29.3 ng/ml) LH: 0.1 (2.4–12.6 mIU/ml) FSH: 1.2 (3.5–12.5 mIU/ml)	1.5%	FSH(4+)
9	F	61	NF	Normal	1.4%	negative
10	F	74	NF	PRL: 0.6 (4.9–29.3 ng/ml) TSH: 0.048 (0.34–4.5 uIU/ml) FSH: 20.0 (3.5–12.5 mIU/ml) IGF1: 178 (53–165 ng/ml)	1.8%	FSH(+) αSU(+)
11	F	60	NF	T4: 0.58 (0.9–1.7 ng/dL) Cortisol: 2.6 (6.2–19.4 ug/dL) GH: 0.05 (0.13–9.88 ng/ml) PRL: 45.4 (4.9–29.3 ng/ml) LH: 0.1 (1.4–15.0 mIU/ml) IGF1: 43 (70–201 ng/ml)	1.5%	FSH (most negative, partly positive)
12	M	54	NF	PRL: 15.7 (4.3–13.7 ng/ml) FSH: 13.2 (3.5–12.5 mIU/ml)	<1%	FSH(+) αSU(+)
13	M	38	NF	Normal	3% P53 (partly positive)	FSH(4+)
14	F	63	NF	FSH: 21.0 (3.5–12.5 mIU/ml)	1.7% P53 (weak)	FSH(2+)
15	M	51	NF	Cortisol: 3.5 (4–18.3 ug/dL) PRL: 2.2 (4.3–13.7 ng/ml)	<1% P53 (partly positive)	PRL(±)
16	F	40	NF	T4: 0.82 (0.9–1.7 ng/dL) PRL: 145.7 (4.9–29.3 ng/ml)	3% P53 < 1%	FSH(±) αSU(4+)
17	F	61	GH	GH: 44.8 (0.13–9.88 ng/ml) PRL: 58.3 (4.9–29.3 ng/ml) LH: 0.1 (2.4–12.6 mIU/ml) FSH: 0.5 (3.5–12.5 mIU/ml) IGF1: 521 (69–198 ng/ml)	2.20%	GH(4+) PRL(2+) αSU(+)
18	M	53	GH	GH: 5.65 (0–2.47 ng/ml) IGF1: 580 (85–240 ng/ml)	1%	GH(4+) FSH(+) αSU(4+)
19	M	62	GH	GH: 8.83 (0–2.47 ng/ml) TSH: 0.215 (0.5–5.0 uIU/ml) ACTH: 84.5 (7.2–63.3 pg/ml) IGF1: 635 (76–228 ng/ml)	1.5%	GH(3+) PRL(+)
20	M	30	GH	GH: 7.84 (0–2.47 ng/ml) IGF1: 902 (109–303 ng/ml)	2.3%	GH(2+)
21	M	39	GH	GH: 16.2 (0–2.47 ng/ml) IGF1: 718 (95–266 ng/ml)	1.9%	GH(4+)
22	F	41	GH	GH: 11.6 (0.13–9.88 ng/ml) PRL: 36.7 (4.9–29.3 ng/ml)	2.6%	GH(3+) PRL(−)

M: Male, F: Female, NF: Clinically non-functioning PitNET, GH: Somatotropinoma.

Table 2. *Primer and probe sequence for qRT-PCR*

	Primer	Probe
Cathepsin D	F:5'CATCTTCTCCTTCTACCTGAGCA3' R:5'GTCTGTGCCACCCAGCAT3'	#64 probe from Roche company (cat.no.04688635001)
BMP-1	F:5'CAGTCCTTTGAGATTGAGCGC3' R:5'TGCTGCTCTCACTGTGCCC3'	5'ACGACAGCTGTGCCTACGACTATCTGGAGGT3'
vWF	F:5'ACCTGGAGGTGATTCTCCATAA3' R:5'CCCATTCACCGTCACCTC3'	#1 probe from Roche company (cat.no.04684974001)
VEGF	F:5'TGCTCTACCTCCACCATGCCAA3' R:5'TGATGATTCTGCCCTCCTCCTTC3'	5'TGGTCCCAGGCTGCACCCATGGC3'
FGF2	F:5'CGACCCTCACATCAAGCTACAA3' R:5'CCAGGTAACGGTTAGCACACACT3'	5'CGACCCTCACATCAAGCTACAA3'

Gene expression analyses

Extraction of RNA

Total RNA was isolated from the formalin-fixed paraffin-embedded slides using the High Pure RNA Paraffin Kit (Roche Applied Science, USA) according to the instructions. The quantity and quality of RNA were assessed by spectrophotometer (e-spect, BM equipment, Japan). A total of 18 samples qualified for RNA extraction, including 13 nonfunctional PitNETs and five somatotroph tumors.

Quantitative RT-PCR (qRT-PCR) gene expression analysis

Total RNA from each sample was reverse-transcribed to cDNA using a cDNA synthesis kit (First-Strand cDNA Synthesis, Roche Applied Science). qRT-PCR was performed in a LightCycler 480 System using the LightCycler 480 Probes Master kit (Roche Applied Science). Each assay was performed in duplicate and the mean value of mRNA expression was used. Gene expression was normalized to that of β-actin. Primers and probes for each molecule are shown in Table 2.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7.0 (GraphPad Software Inc., CA, USA). Linear regression with 95% confidence intervals was graphed using GraphPad Prism. Correlations were calculated using the Pearson test and shown as R-values. Significance was taken as $p < 0.05$. Receiver operating characteristic (ROC) curves were generated to measure the sensitivity, and the area under the curve (AUC) was calculated.

III. Results

Cathepsin D and angiogenesis

There was a significant inverse relationship between Cathepsin D and von Willebrand factor expression by qRT-PCR in nonfunctional adenomas, with higher Cathepsin D expression tending to indicate less vascularization (Fig. 2A, R = −0.70, $p = 0.0082$). The same trend was also observed in somatotroph tumors but the inverse correlation was not as significant as that for nonfunctional PitNETs (Fig. 2B, R = −0.22, $p = 0.7259$).

A significant inverse correlation was confirmed using IHC in both the nonfunctional group (Fig. 2C, R = −0.73, $p = 0.0015$) and the somatotroph group (Fig. 2D, R = −0.42, $p = 0.0346$). Consistent with mRNA levels, the inverse trend for protein expression was more significant in the nonfunctional PitNETs than in somatotroph tumors.

BMP-1 and angiogenesis

No significant correlation was found between mRNA and protein levels of BMP-1 and von Willebrand factor in both nonfunctional PitNETs (Fig. 3A, R = −0.013, $p = 0.9671$; Fig. 3C, R = −0.34, $p = 0.2041$) and somatotroph tumors (Fig. 3B, R = 0.62, $p = 0.2616$; Fig. 3D, R = 0.51, $p = 0.3029$).

VEGF and angiogenesis

No significant correlation was found between mRNA and protein levels of VEGF and von Willebrand factor in nonfunctional PitNETs (Fig. 4A, R = 0.26, $p = 0.3846$; Fig. 4C, R = −0.05, $p = 0.8094$) and somatotroph tumors (Fig. 4B, R = −0.08, $p = 0.8958$; Fig. 4D, R = 0.47, $p = 0.3479$).

FGF2 and angiogenesis

A significant positive correlation between FGF2 and von Willebrand factor was observed only for protein levels in nonfunctional PitNETs (Fig. 5C, R = 0.57, $p = 0.0192$), with higher FGF2 expression tending to indicate more vascularization. No significant correlation was found for mRNA expression (Fig. 5A, R = 0.23, $p = 0.4572$).

No significant correlation was found between mRNA and protein levels of FGF2 and von Willebrand factor in somatotroph tumors (Fig. 5B, R = 0.36, $p = 0.5540$; Fig. 5D, R = −0.07, $p = 0.8825$).

Ki-67 index and angiogenesis

A mild but non-significant positive correlation was found between von Willebrand factor protein level and Ki-67 staining index in both nonfunctional PitNETs (Fig. 6A, R = 0.31, $p = 0.2439$) and somatotroph tumors (Fig. 6B, R = 0.35, $p = 0.4908$).

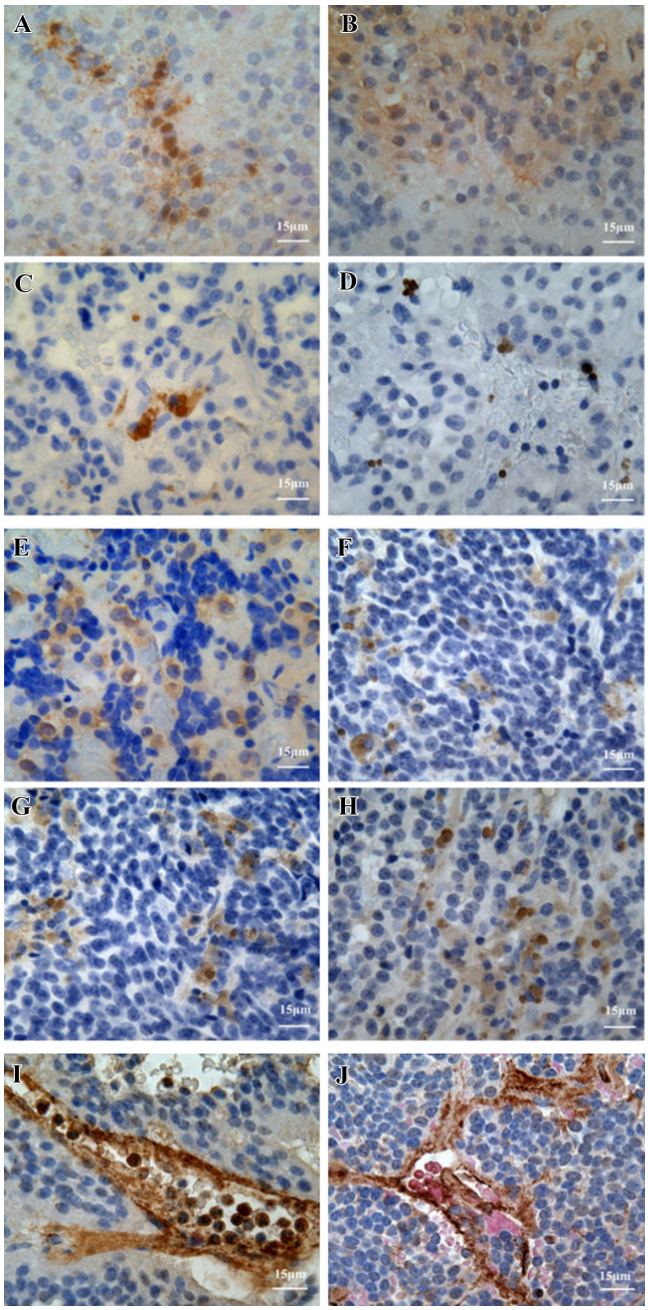

Fig. 1. Immunohistochemical staining for: Cathepsin D (**A**: NF; **B**: somatotroph tumors; 600×), bone morphogenetic protein 1 (BMP-1) (**C**: NF; **D**: somatotroph tumors; 600×), basic fibroblast growth factor (FGF2) (**E**: NF; **F**: somatotroph tumors; 600×), vascular endothelial growth factor (VEGF) (**G**: NF; **H**: somatotroph tumors; 600×) and von Willebrand factor (**I**: NF; **J**: somatotroph tumors; 600×). NF: nonfunctional PitNETs.

Comparison of the effect of Cathepsin D and FGF2 on angiogenesis

Receiver operating characteristic curves (Fig. 7) showed that Cathepsin D had a stronger effect on angiogenesis in nonfunctional PitNETs than FGF2 (AUC = 0.9492 versus 0.8828, respectively).

IV. Discussion

Angiogenesis plays a key role in tumor growth and is closely associated with an increased risk of relapse and metastasis. In contrast, PitNETs seem to be less vascularized than the normal gland [8], although there are some discordant results [24, 26].

Several studies have shown the involvement of Cathepsin D in regulation of blood vessel formation, especially in solid tumors. It has been reported that Cathepsin D positively correlates with angiogenesis in ovarian and breast cancer [15, 21] and is considered a negative prognostic marker [12, 19, 20]. Moreover, Cathepsin D can also impose anti-angiogenic effects by activating anti-angiogenic factors including angiostatin, vasoinhibin, and endostatin in prostate carcinoma cells and endothelial cells [9, 11, 25].

To our knowledge, our study is the first to investigate the relationship between Cathepsin D and angiogenesis in PitNETs. We found a significant inverse relationship between Cathepsin D and vascularization, showing that higher Cathepsin D expression notably indicates less vascularization in PitNETs. This effect was more significant in nonfunctional PitNETs than in somatotroph tumors and was confirmed at both mRNA and protein levels. This finding is totally opposite to a previous report that Cathepsin D acts as a pro-angiogenic factor in solid tumors [19]. However, it is consistent with findings of Piwnica *et al.* that Cathepsin D cleaves PRL into vasoinhibins and thus exerts an anti-angiogenic effect [29], although we could not detect the level of vasoinhibins directly because this was a retrospective analysis using only paraffin-embedded samples.

Another key enzyme in the production of vasoinhibins is BMP-1; however, our study showed no significant correlation between BMP-1 and angiogenesis. Compared with BMP-1, Cathepsin D was the dominant anti-angiogenic factor controlling angiogenesis in PitNETs.

Tumor vascularization depends on the balance between pro-angiogenic and anti-angiogenic factors. Several studies showed that PitNETs demonstrated lower VEGF expression at the protein level than normal pituitary tissue [30], although there are some discordant results [18]. In our study, no significant correlation was found between VEGF expression level and angiogenesis. This was consistent with a previous report that VEGF does not play a significant role in the angiogenesis of pituitary adenomas [36].

As a key pro-angiogenic factor, FGF2 showed a significant positive relationship with vascularization, but only in nonfunctional PitNETs. This was consistent with previous studies showing that FGF2 is closely associated with high vascularization in PitNETs [22, 23].

Since only Cathepsin D and FGF2 showed a significant correlation with angiogenesis in this study, a ROC curve analysis was performed to compare their effects. The data showed that the anti-angiogenic effect imposed by Cathepsin D overwhelmed the pro-angiogenic effect

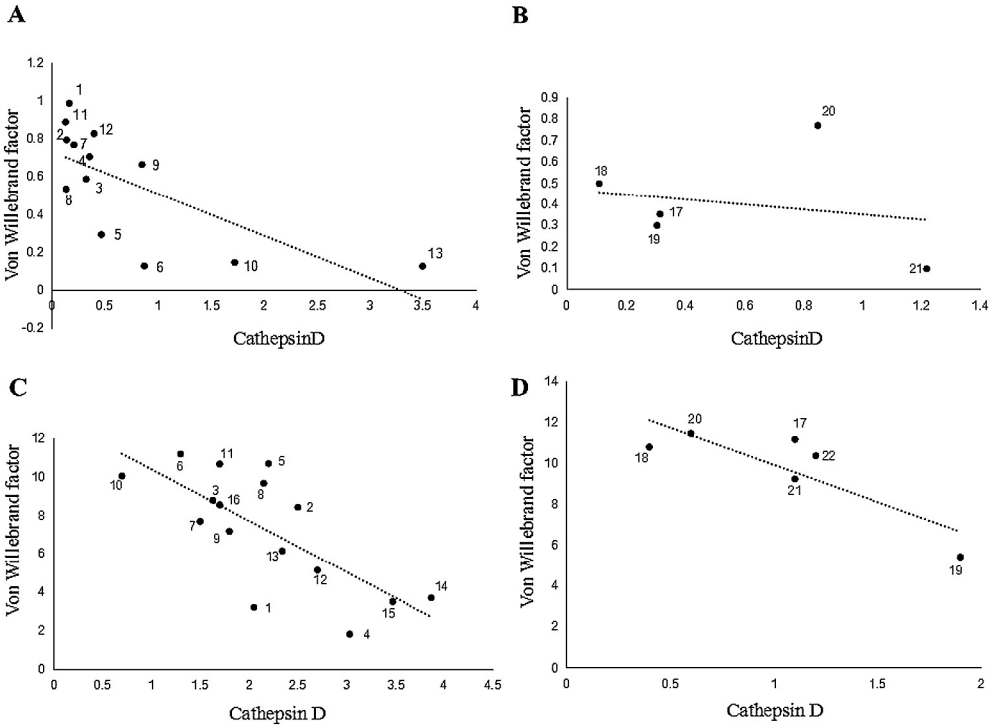

Fig. 2. Correlation between the mRNA expression of Cathepsin D and von Willebrand factor in nonfunctional group (**A**, R = −0.70, p = 0.0082) and somatotroph group (**B**, R = −0.22, p = 0.7259) and between protein levels in nonfunctional group (**C**, R = −0.73, p = 0.0015) and somatotroph group (**D**, R = −0.42, p = 0.0346).

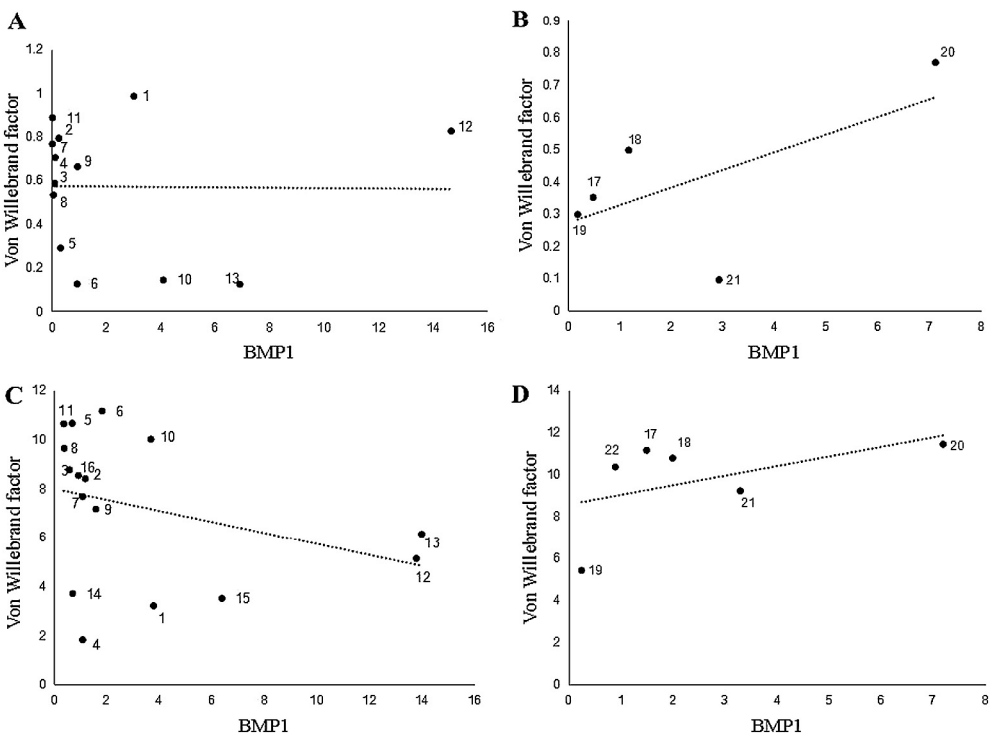

Fig. 3. Correlation between the expression of bone morphogenetic protein 1 (BMP-1) and von Willebrand factor at the mRNA level in nonfunctional group (**A**, R = −0.013, p = 0.9671) and somatotroph group (**B**, R = 0.62, p = 0.2616) and at the protein level in nonfunctional group (**C**, R = −0.34, p = 0.2041) and somatotroph group (**D**, R = 0.51, p = 0.3029).

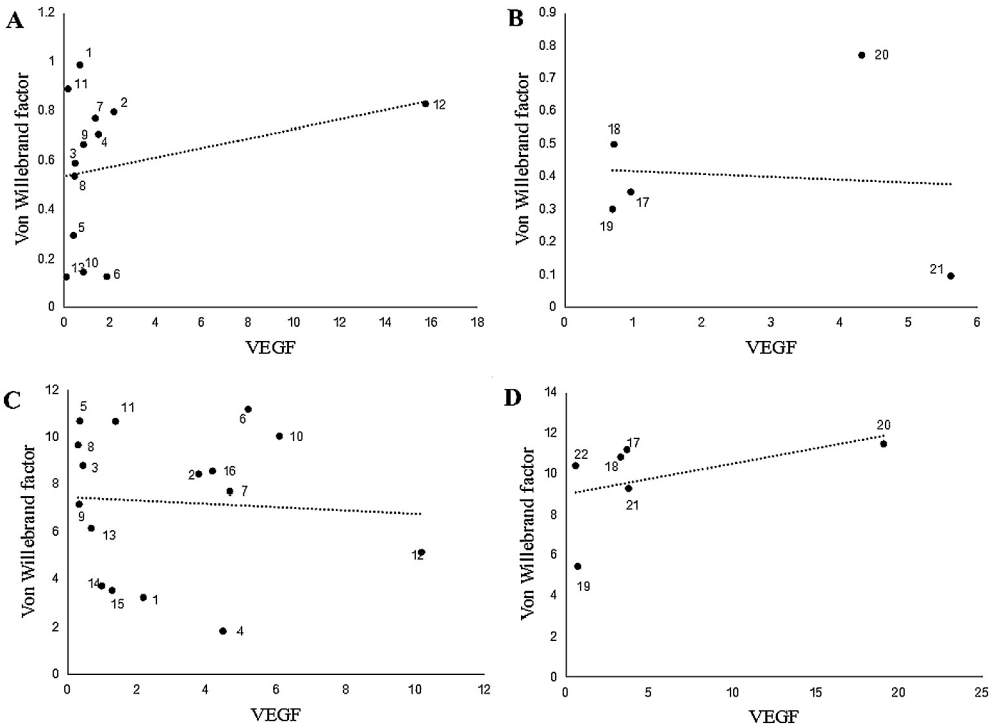

Fig. 4. Correlation between the expression of vascular endothelial growth factor (VEGF) and von Willebrand factor at mRNA level in nonfunctional group (**A**, R = 0.26, $p = 0.3846$) and somatotroph group (**B**, R = −0.08, $p = 0.8958$), and at the protein level in nonfunctional group (**C**, R = −0.05, $p = 0.8094$) and somatotroph group (**D**, R = 0.4, $p = 0.3479$).

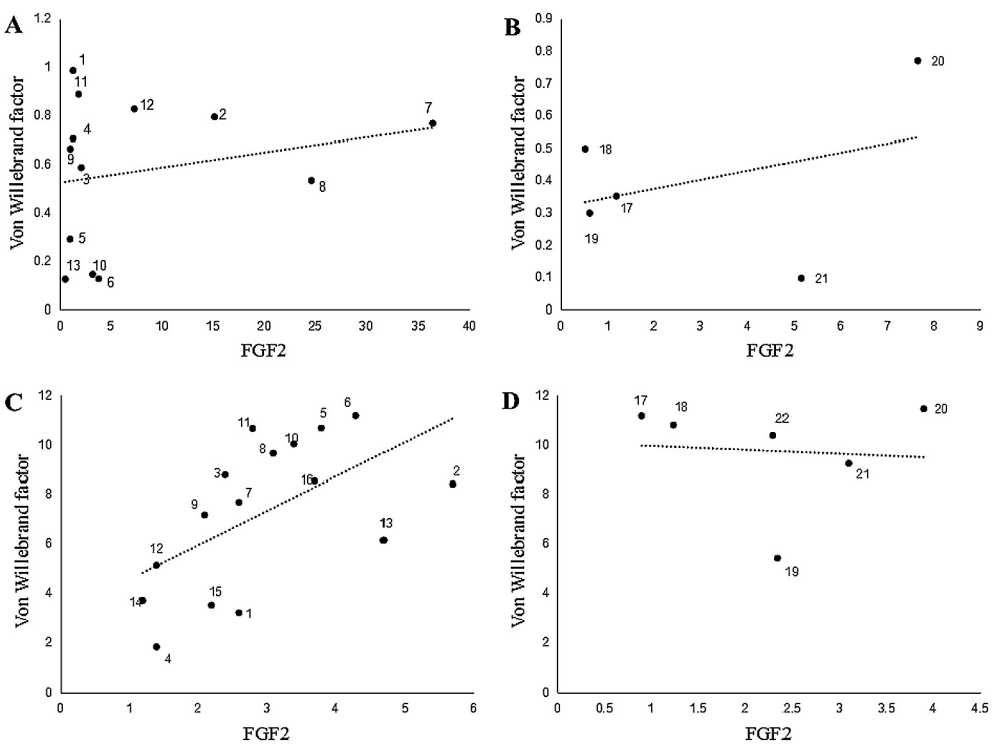

Fig. 5. Correlation between the expression of basic fibroblast growth factor-2 (FGF2) and von Willebrand factor at the mRNA level in nonfunctional group (**A**, R = 0.23, $p = 0.4572$) and somatotroph group (**B**, R = 0.36, $p = 0.5540$), and at the protein level in nonfunctional group (**C**, R = 0.57, $p = 0.0192$) and somatotroph group (**D**, R = −0.07, $p = 0.8825$).

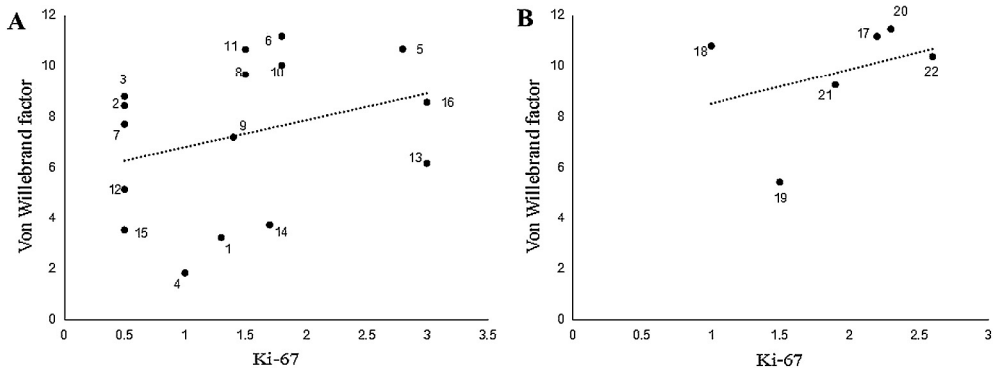

Fig. 6. Correlation between the Ki-67 staining index and von Willebrand factor staining index in nonfunctional group (**A**, R = 0.31, p = 0.2439) and somatotroph group (**B**, R = 0.35, p = 0.4908).

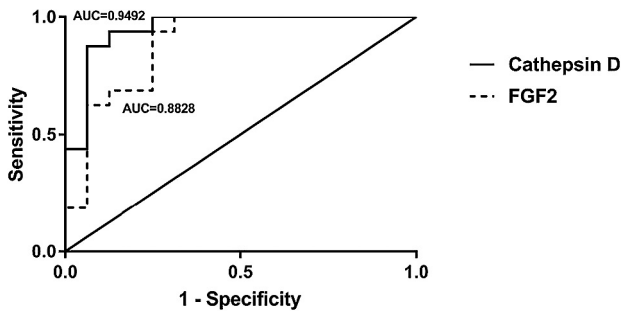

Fig. 7. Receiver operating characteristic curves showing the effect of Cathepsin D (AUC = 0.9492) and basic fibroblast growth factor-2 (FGF2) (AUC = 0.8828) on angiogenesis. AUC: area under the curve.

induced by FGF2 in nonfunctional PitNETs. It has been reported that Cathepsin D facilitates the release of FGF2 in breast cancer cells [5, 33]. Moreover, other authors observed that Cathepsin D is responsible for the generation of angiostatin, which inhibits FGF2-induced angiogenesis in human prostate carcinoma cells [25]. Although the mechanism for the regulation of FGF2 by Cathepsin D in PitNETs is not clear, facilitation of FGF2-induced angiogenesis by Cathepsin D was not observed in our study.

Angiogenesis is also associated with the proliferation of tumor cells and it has been reported that Ki-67 is associated with angiogenesis in multiple myeloma [1]. In our study, a positive but non-significant correlation was found between von Willebrand factor and Ki-67 in both nonfunctional PitNETs and somatotroph tumors.

Some limitations of this study should be noted. First, the volume of samples was limited. Moreover, angiogenesis is a complex procedure because in addition to Cathepsin D and BMP-1, the matrix metalloproteinase (MMP) family can also cleave PRL into vasoinhibins. Whether vasoinhibins directly participate in the inhibition of angiogenesis should be examined in a prospective analysis using western blotting to confirm the existence of vasoinhibins. It is also necessary to confirm the involvement of other enzymes, including the MMP family, in the production of vasoinhibins.

In conclusion, the present study demonstrates that expression of Cathepsin D and FGF2 is significantly correlated with angiogenesis in PitNETs, and particularly in nonfunctional PitNETs. Moreover, the anti-angiogenic effect imposed by Cathepsin D is more significant than the pro-angiogenic effect of FGF2.

V. Conflicts of Interest

The authors declare that they have no conflict of interest.

VI. Disclosure

The author report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

VII. References

1. Alexandrakis, M. G., Passam, F. H., Dambaki, C., Pappa, C. A. and Stathopoulos, E. N. (2004) The relation between bone marrow angiogenesis and the proliferation index Ki-67 in multiple myeloma. *J. Clin. Pathol.* 57; 856–860.
2. Azuma, M., Tsukada, T., Inagaki, T., Casmad, F., Jindatip, D., Tofrizal, A., *et al.* (2018) Immunohistochemical Study of the Laminin α5 Chain and Its Specific Receptor, Basal Cell Adhesion Molecule (BCAM), in both Fetal and Adult Rat Pituitary Glands. *Acta Histochem. Cytochem.* 51; 145–152.
3. Batchuluun, K., Azuma, M., Fujiwara, K., Yashiro, T. and Kikuchi, M. (2017) Notch Signaling and Maintenance of SOX2 Expression in Rat Anterior Pituitary Cells. *Acta Histochem. Cytochem.* 50; 63–69.
4. Bentzien, F., Struman, I., Martini, J. F., Martial, J. and Weiner, R. (2001) Expression of the antiangiogenic factor 16K hPRL in human HCT116 colon cancer cells inhibits tumor growth in Rag1(−/−) mice. *Cancer Res.* 61; 7356–7362.
5. Briozzo, P., Badet, J., Capony, F., Pieri, I., Montcourrier, P., Barritault, D., *et al.* (1991) MCF7 mammary cancer cells respond to bFGF and internalize it following its release from extracellular matrix: a permissive role of cathepsin D. *Exp. Cell Res.* 194; 252–259.
6. Clapp, C., Aranda, J., Gonzalez, C., Jeziorski, M. C. and

Martinez, de la Escalera, G. (2006) Vasoinhibins: endogenous regulators of angiogenesis and vascular function. *Trends Endocrinol. Metab.* 17; 301–307.
7. Cristina, C., Perez-Millan, M. I., Luque, G., Dulce, R. A., Sevlever, G., Berner, S. I., *et al.* (2010) VEGF and CD31 association in pituitary adenomas. *Endocr. Pathol.* 21; 154–160.
8. Cristina, C., Luque, G. M., Demarchi, G., Lopez, Vicchi, F., Zubeldia-Brenner, L., *et al.* (2014) Angiogenesis in pituitary adenomas: human studies and new mutant mouse models. *Int. J. Endocrinol.* 2014; 608497.
9. Faupel-Badger, J. M., Ginsburg, E., Fleming, J. M., Susser, L., Doucet, T. and Vonderhaar, B. K. (2010) 16 kDa prolactin reduces angiogenesis, but not growth of human breast cancer tumors in vivo. *Horm. Cancer* 1; 71–79.
10. Fernandez, A., Karavitaki, N. and Wass, J. A. (2010) Prevalence of pituitary adenomas: a community-based, cross-sectional study in Banbury (Oxfordshire, UK). *Clin. Endocrinol. (Oxf)* 72; 377–382.
11. Ferreras, M., Felbor, U., Lenhard, T., Olsen, B. R. and Delaissé, J. (2000) Generation and degradation of human endostatin proteins by various proteinases. *FEBS Lett.* 486; 247–251.
12. Foekens, J. A., Look, M. P., Bolt-de, Vries. J., Meijer-van, Gelder, M. E., van Putten, W. L. and Klijn, J. G. (1999) Cathepsin-D in primary breast cancer: prognostic evaluation involving 2810 patients. *Br. J. Cancer* 79; 300–307.
13. Gaytan, F., Morales, C., Bellido, C., Aguilar, E. and Sanchez-Criado, J. E. (1997) Role of prolactin in the regulation of macrophages and in the proliferative activity of vascular cells in newly formed and regressing rat corpora lutea. *Biol. Reprod.* 57; 478–486.
14. Ge, G., Fernández, C. A., Moses, M. A. and Greenspan, D. S. (2007) Bone morphogenetic protein 1 processes prolactin to a 17-kDa antiangiogenic factor. *Proc. Natl. Acad. Sci. U S A* 104; 10010–10015.
15. González-Vela, M. C., Garijo, M. F., Fernández, F., Buelta, L. and Val-Bernal, J. F. (1999) Cathepsin D in host stromal cells is associated with more highly vascular and aggressive invasive breast carcinoma. *Histopathology* 34; 35–42.
16. Ito, T. K., Ishii, G., Chiba, H. and Ochiai, A. (2007) The VEGF angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells. *Oncogene* 26; 7194–7203.
17. Izawa, Y., Tanihata, M., Imanishi, M., Onishi, M., Sakiyama, M., Inoue, S., *et al.* (2018) Stem-like Human Breast Cancer Cells Initiate Vasculogenic Mimicry on Matrigel. *Acta Histochem. Cytochem.* 51; 173–183.
18. Kurosaki, M., Saeger, W., Abe, T. and Ludecke, D. K. (2008) Expression of vascular endothelial growth factor in growth hormone-secreting pituitary adenomas: special reference to the octreotide treatment. *Neurol. Res.* 30; 518–522.
19. Leto, G., Gebbia, N., Rausa, L. and Tumminello, F. M. (1992) Cathepsin D in the malignant progression of neoplastic diseases (review). *Anticancer Res.* 12; 235–240.
20. Leto, G., Tumminello, F. M., Crescimanno, M., Flandina, C. and Gebbia, N. (2004) Cathepsin D expression levels in nongynecological solid tumors: clinical and therapeutic implications. *Clin. Exp. Metastasis* 21; 91–106.
21. Lösch, A., Schindl, M., Kohlberger, P., Lahodny, J., Breitenecker, G., Horvat, R., *et al.* (2004) Cathepsin D in ovarian cancer: prognostic value and correlation with p53 expression and microvessel density. *Gynecol. Oncol.* 92; 545–552.
22. Mallea-Gil, M. S., Cristina, C., Perez-Millan, M. I., Villafañe, A. M., Ballarino, C., Stalldecker, G., *et al.* (2009) Invasive Giant Prolactinoma with Loss of Therapeutic Response to Cabergoline: Expression of Angiogenic Markers. *Endocr. Pathol.* 20; 35–40.
23. McCabe, C. J., Khaira, J. S., Boelaert, K., Heaney, A. P., Tannahill, L. A., Hussain, S., *et al.* (2003) E xpression of pituitary tumor transforming gene (PTTG) and fibroblast growth factor-2 (FGF-2) in human pituitary adenomas: relationships to clinical tumour behaviour. *Clin. Endocrinol. (Oxf)* 58; 141–150.
24. McCabe, C. J., Boelaert, K., Tannahill, L. A., Heaney, A. P., Stratford, A. L., Khaira, J. S., *et al.* (2002) Vascular endothelial growth factor, its receptor KDR/Flk-1, and pituitary tumor transforming gene in pituitary tumors. *J. Clin. Endocrinol. Metab.* 87; 4238–4244.
25. Morikawa, W., Yamamoto, K., Ishikawa, S., Takemoto, S., Ono, M., Fukushi, J. I., *et al.* (2000) Angiostatin generation by cathepsin D secreted by human prostate carcinoma cells. *J. Biol. Chem.* 275; 38912–38920.
26. Niveiro, M., Aranda, F. I., Peiro, G., Alenda, C. and Pico, A. (2005) Immunohistochemical analysis of tumor angiogenic factors in human pituitary adenomas. *Hum. Pathol.* 36; 1090–1095.
27. Nomikos, P., Buchfelder, M. and Fahlbusch, R. (2001) Current management of prolactinomas. *J. Neurooncol.* 54; 139–150.
28. Perimenis, P., Bouckenooghe, T., Delplanque, J., Moitrot, E., Eury, E., Lobbens, S., *et al.* (2014) Placental antiangiogenic prolactin fragments are increased in human and rat maternal diabetes. *Biochim. Biophys. Acta* 1842; 1783–1793.
29. Piwnica, D., Touraine, P., Struman, I., Tabruyn, S., Bolbach, G., Clapp, C., *et al.* (2004) Cathepsin D processes human prolactin into multiple 16 K-like N-terminal fragments: study of their antiangiogenic properties and physiological relevance. *Mol. Endocrinol.* 18; 2522–2542.
30. Raica, M., Coculescu, M., Cimpean, A. M. and Ribatti, D. (2010) Endocrine gland derived-VEGF is down-regulated in human pituitary adenoma. *Anticancer Res.* 30; 3981–3986.
31. Rymaszewski, Z., Cohen, R. M. and Chomczynski, P. (1991) Human growth hormone stimulates proliferation of human retinal microvascular endothelial cells in vitro. *Proc. Natl. Acad. Sci. U S A* 88; 617–621.
32. Sánchez-Ortiga, R., Sánchez-Tejada, L., Moreno-Perez, O., Riesgo, P., Niveiro, M. and Picó Alfonso, A. M. (2013) Over-expression of vascular endothelial growth factor in pituitary adenomas is associated with extrasellar growth and recurrence. *Pituitary* 16; 370–377.
33. Takei, Y., Higashira, H., Yamamoto, T. and Hayashi, K. (1997) Mitogenic activity toward human breast cancer cell line MCF-7 of two bFGFs purified from sera of breast cancer patients: co-operative role of cathepsin D. *Breast Cancer Res. Treat.* 43; 53–63.
34. Turner, H. E., Nagy, Z., Gatter, K. C., Esiri, M. M. and Harris, A. L. (2000) Angiogenesis in pituitary adenomas and the normal pituitary gland. *J. Clin. Endocrinol. Metab.* 85; 1159–1162.
35. Vermeulen, P. B., Gasparini, G., Fox, S. B., Toi, M., Martin, L., McCulloch, P., *et al.* (1996) Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. *Eur. J. Cancer* 32; 2474–2484.
36. Viacava, P., Gasperi, M., Acerbi, G., Manetti, L., Cecconi, E., Bonadio, A. G., *et al*, (2003) Microvascular density and vascular endothelial growth factor expression in normal pituitary tissue and pituitary adenomas. *J. Endocrinol. Invest.* 26; 23–28