Endocr Pathol (2010) 21:154–160
DOI 10.1007/s12022-010-9119-6

VEGF and CD31 Association in Pituitary Adenomas

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Published online: 15 May 2010

Abstract Pituitary tumors are usually less vascularized than the normal pituitary, and the role of angiogenesis in these adenomas is contentious. Appraisal of microvascular density and expression of the potent angiogenic vascular endothelial growth factor (VEGF) by immunohistochemistry has yielded controversial results, as a broad spectrum of immunostaining can be found. We determined the protein expression of VEGF and CD31, an endothelial marker, in a series of 56 surgically removed pituitary adenomas using Western blot assay. Prolactinomas had higher VEGF protein expression compared to nonfunctioning or ACTH- and GH-secreting adenomas, while CD31 was similar in the different adenoma histotypes. VEGF and CD31 were not affected by sex, age, years of adenoma evolution, or proliferation rate (Ki67 and PCNA) for all adenoma types. Only in nonfunctioning adenomas CD31 concentration increased significantly with age. There was a positive correlation between CD31 and VEGF expression when all adenoma histotypes were considered, or when prolactinomas and nonfunctioning adenomas were evaluated separately. The positive association of VEGF and CD31 expression suggests the participation of angiogenesis in adenoma development, while epithelial cell proliferation in pituitary tumors is not directly related to VEGF or CD31 expression, and other factors, such as primary genetic alterations may be involved.

Keywords Pituitary adenoma · CD31 · VEGF · Pelioisis · Angiogenesis · Proliferation

CC and MIPM shared the work equally.

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Introduction

An increase in tumor size necessarily requires a corresponding increase in vascularization that is assured by means of the complex dynamic process of angiogenesis [1]. In most human tumors, including breast, bladder, and stomach, angiogenesis has been shown to be correlated with tumor behavior. On the other hand, pituitary tumors are usually less vascularized than the normal pituitary tissue, as suggested by Schechter [2], and later confirmed by other authors [3–5]. Differences in the angiogenic pattern of pituitary adenomas have yielded highly controversial results concerning hormonal phenotypes, size, or invasion [6–8]. In most studies, immunohistochemistry evaluation of different markers of microvascular density (MVD) such as endothelial cluster differentiation molecules CD31 and CD34, factor eight-related antigen, and ulex europaeus agglutinin I have been used. Nevertheless, the appraisal of MVD by immunohistochemistry has a number of substantial limitations, which are mainly due to the complex biology of tumor vasculature and the irregular geometry of the vascular system.

Vascular endothelial growth factor (VEGF) plays a pivotal role as an angiogenic promoter by stimulating endothelial cells proliferation and migration and enhancing vascular permeability. VEGF expression has been described in all cell types in the normal pituitary, with greater expression in somatotroph and follicle-stellate cells [9, 10]. In a group of pituitary adenomas, ACTH- and GH-secreting adenomas and pituitary carcinomas had the strongest VEGF immunoreactivity [11]. Elevated serum VEGF concentrations have been demonstrated in patients harboring pituitary tumors [12, 13], and approximately 90% of human pituitary tumors cultured in vitro show measurable VEGF secretion [14].

These data indicate that even though the role of angiogenesis in pituitary adenomas is controversial, VEGF might contribute to adequate temporal vascular supply with mechanisms other than endothelial cell proliferation. Tumor angiogenesis in the pituitary, as well as in other endocrine neoplasms, probably reflects the basic observation that tumors require neovascularization to grow; however, the changes that occur may be somewhat different from some other tissues that are less highly vascularized in the nonneoplastic state.

Most of the above studies on VEGF protein expression and MVD evaluation have been performed using immunohistochemistry. There are only two reports reporting VEGF expression pituitary adenomas using Western blot, one in which a small selection of human pituitary adenomas were evaluated [15] and a second one performed in 24 adenomas, most of which were nonfunctioning [16]. As VEGF immunostaining is highly heterogeneous between adenoma samples [17] and MVD evaluation using immunohistochemistry has some limitations, we wished to study the expression of VEGF and CD31 measured by Western blot in a series of pituitary adenomas in order to add to the comprehension of angiogenic markers in these tumors.

Materials and Methods

Patients

Fifty-six surgically removed pituitary adenoma samples were investigated: 21 males with mean age of 51.0±

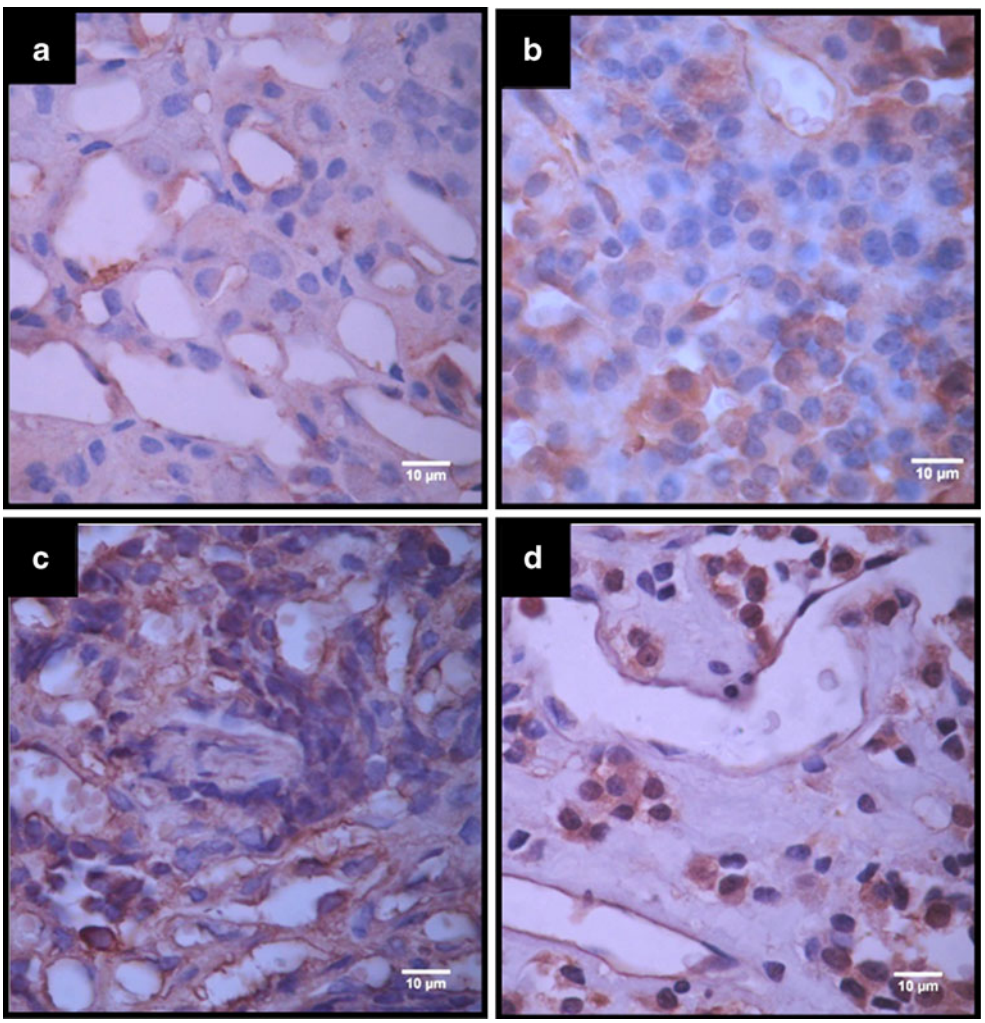

Fig. 1 VEGF in pituitary adenomas. Immunohistochemical study. Representative immunohistochemistries of VEGF (*brown staining*) in different adenoma types: **a** ACTH-secreting adenoma, **b** somatotropinoma, **c** nonfunctioning adenoma, and **d** prolactinoma. Staining can be visualized in endothelial cells lining vessels, in cytoplasm, cell nuclei, or the extracellular matrix

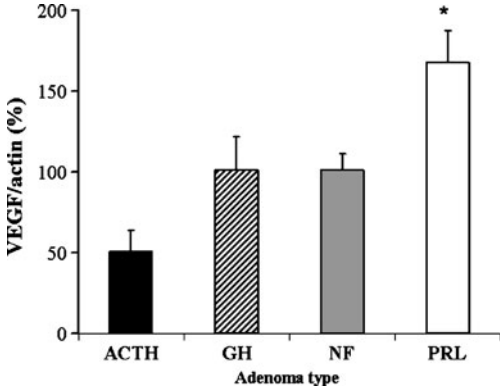

Fig. 2 VEGF in pituitary adenomas (Western blot). VEGF in pituitary adenomas, measured by Western blot and normalized to actin content of the same sample. *NF* nonfunctioning adenoma, *PRL* prolactinoma. Average and standard errors are depicted, N=4, 7, 27, and 10; *$P<$ 0.05 vs NF, ACTH-, and GH-secreting adenomas. In each membrane, each VEGF/actin band intensity was divided by the average of NF/actin band intensities which was considered 100%

3.4 years and 35 females with mean age of 43.2±1.9 years. There were eight GH-secreting tumors, 12 prolactinomas (1 micro-, the rest macroprolactinomas, all resistant to dopamine therapy), four ACTH secreting tumors, one thyrotropinoma, one mixed GH-prolactin secreting adenoma, and 30 nonfunctioning pituitary adenomas. Histological examination and immunohistochemistry for anterior pituitary hormones was performed, and together with the clinical, endocrine and radiological data were used to fully characterize each tumor type.

The project was approved by the Research Ethical Committees of the Instituto de Biología y Medicina Experimental-CONICET and Santa Lucía Hospital, Buenos Aires. Patients signed an approved informed consent.

Western Blots

Pituitary adenoma samples were homogenized in 80–300 μl ice-cold buffer containing 60 mM Tris–HCl, 1 mM EDTA (pH6.8), and a mix of protease inhibitors (phenyl-methyl-sulfonyl, TPCK, TAME, ZPCK, and TLCK) in a handheld microtissue homogenizer. The homogenate was then centrifuged at 800×g for 5 min at 4°C. An aliquot of supernatant was taken to quantify proteins by the Qubit™ Quant-iT protein Assay Kit (Invitrogen, Buenos Aires). Proteins (30 μg) in 10 μl of homogenization buffer were mixed with 10 μl 2× sample buffer (60 mM Tris–HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.02% bromophenol blue, and 50 mM dithiotreitol (pH6.8)). Samples were sonicated during 20 s, heated 5 min at 95°C, and subjected to 12% SDS-polyacrylamide gel electrophoresis. The gel was then blotted onto a nitrocellulose membrane (Bio-Rad) and probed with the corresponding primary antibody followed by a secondary antibody conjugated with horseradish peroxidase (1:1,000, Santa Cruz Biotechnologies Inc. Santa Cruz, CA, USA). Polyclonal rabbit antibodies (VEGF sc-5846, 1:1,000 and PCNA FL-261, 1:1,000, Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) and polyclonal goat antibody PECAM for CD31 detection (sc-1506, 1:800 Santa Cruz Biotechnologies) were used. Actin expression was evaluated to confirm equivalent total protein loading (mouse anti-actin 6276, 1:5,000, Abcam, Cambridge, MA, USA). Endothelial cell lysates or purified VEGF protein were included in the electrophoresis as positive controls for CD31 and VEGF, respectively. Immunoreactive proteins were detected by enhanced chemoluminiscence (Amersham, Aylesbury, UK). For repeated immunoblotting, membranes were incubated in stripping buffer (62.5 mM Tris, 2% SDS, and 100 mM mercaptoethanol, pH6.7) for 40 min at 50°C and reprobed. Band intensities were quantified using the ImageQuant software. Each band intensity was normalized to the correspondent actin band intensity.

Ki67 and VEGF Immunohistochemistry

Immunohistochemistry of paraffin embedded samples was performed as previously described [18]. Antibodies used were rabbit polyclonal Ki67 sc-15402, 1:200, Santa Cruz Biotechnologies and rabbit polyclonal VEGF sc-5846,

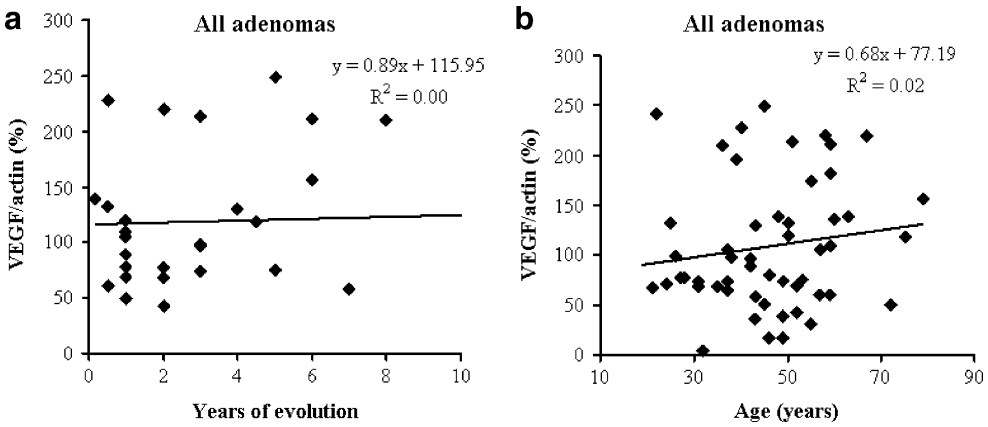

Fig. 3 VEGF correlations. Association of VEGF content and **a** age of the patient and **b** years of adenoma evolution for all adenoma subtypes. *Inset*, equation of linear regression, R^2, and p from Spearman correlation test

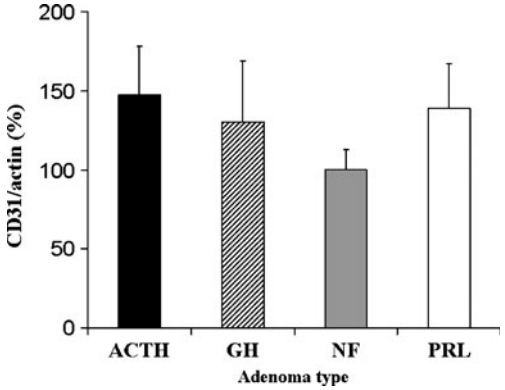

Fig. 4 CD31 in pituitary adenomas. CD31 in pituitary adenomas, measured by Western blot and normalized to actin content of the same sample. *NF* nonfunctioning adenoma, *PRL* prolactinoma. Average and standard errors are depicted, N=4, 7, 25, and 10

1:200, Santa Cruz Biotechnologies. The Ki67 labeling index was manually determined by counting brown stained nuclei and expressed as "percentage of positive nuclei" in selected fields counterstained with hematoxylin dye. A mean of 30 fields, each containing 100 cells was assessed. Cells considered positive showed unequivocal nuclear staining.

Statistical Analysis

Since assumptions for a parametric test were not valid (Kolmogorov–Smirnov $P<0.05$), the Kruskal–Wallis analysis of variance was used for between-group comparisons of more than two groups. Correlations were performed using the Spearman test. Significance was taken as $P<0.05$.

Results

We first evaluated VEGF protein expression by immunohistochemistry. VEGF was present in all adenoma samples studied (Fig. 1). VEGF staining patterns were highly heterogeneous among samples, including endothelial cell staining in many cases and also clusters/groups of tumor cells positive for this angiogenic factor that made it difficult to quantify.

We decided to evaluate VEGF content in pituitary adenomas using Western blot analysis. VEGF was also detected in all samples studied. Prolactinomas had higher VEGF protein expression compared to nonfunctioning and ACTH- or GH-secreting adenomas (Fig. 2., $P<0.02$). VEGF was similar in both sexes ($P=0.37$ for all adenoma types, not shown) and did not correlate with years of adenoma evolution ($P=0.27$, Fig. 3a) or age ($P=0.78$, Fig. 3b).

CD31 was similar in the different immunohistochemical phenotypes ($P=0.52$, Fig. 4), and there were no differences between sexes ($P=0.69$, not shown) or correlation with age ($P=0.27$) or years of adenoma evolution ($P=0.49$) when all adenomas were considered (Figs. 5a, b). In nonfunctioning adenomas, CD31 increased with age ($P<0.005$, Fig. 5c).

We next evaluated the correlation between VEGF and CD31. There was a positive correlation between both markers, when all adenoma types were considered ($P<0.001$, Fig. 6a). The positive correlation was also observed if prolactinomas or nonfunctioning adenomas were considered separately ($P<0.02$ and 0.010, respectively), and not in GH-secreting adenomas ($P=0.48$, Figs. 6b–d).

Neither VEGF nor CD31 correlated with proliferating cell nuclear antigen (PCNA) measured by Western blot or Ki67 index assessed by immunohistochemistry (Figs. 7a, b, $P=0.81$ and 0.22 for VEGF vs Ki67 and PCNA, respectively, similar results for CD31), indicating that both angiogenic markers did not associate, at least in a direct way, to proliferation of pituitary adenomas.

Discussion

Antiangiogenesis is a therapeutic strategy to lower tumor burden in some cancers before surgery. But, pituitary

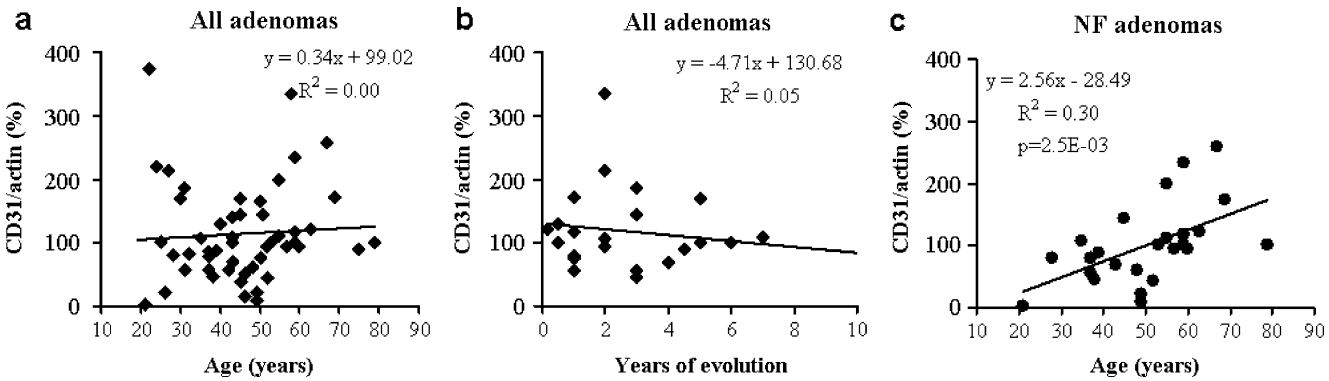

Fig. 5 CD31 correlations. Association of CD31 content and **a** age of the patient, **b** years of adenoma evolution for all adenoma subtypes, and **c** age in nonfunctioning adenoma samples. *Inset*, equation of linear regression, R^2, and p from Spearman correlation test

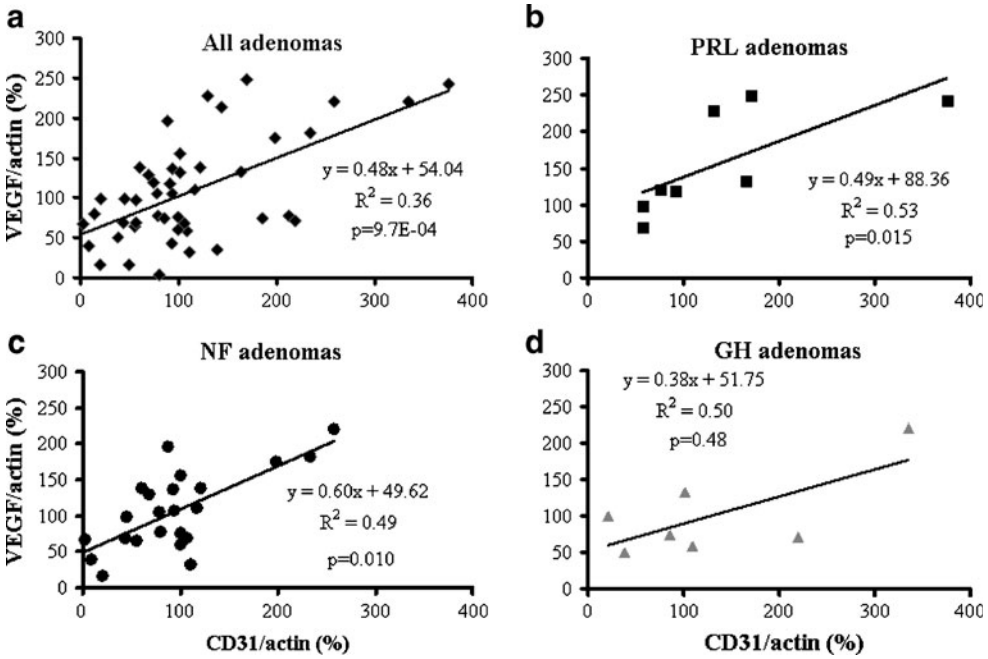

Fig. 6 VEGF and CD31 correlation. Association of VEGF and CD31 content (samples in which both markers could be measured) in **a** all adenomas, **b** prolactinomas, **c** nonfunctioning, and **d** GH-secreting adenomas. *Inset*, equation of linear regression, R^2, and p from Spearman correlation test

tumors are usually less vascularized than the normal pituitary tissue, and the role of angiogenesis in these adenomas is contentious. Many studies do not demonstrate uniformity in the estimation of microvessels in terms of evaluation methods and classification of the results. Furthermore, pituitary adenomas are highly heterogeneous in their presentations. This has led to contradictory results with regard of MVD and VEGF appraisal in different studies.

We found VEGF protein expression was unevenly distributed in the different cellular components of pituitary adenomas, as others have described. This may have led to contradictory data reported in the literature. Using immunohistochemistry, a broad spectrum of immunoreactivity for VEGF has been described in various types of adenomas [11, 19–21]. Viacava et al. [21] found no differences in VEGF expression among tumors of different histotype using immunohistochemistry, while Lloyd et al. found lower VEGF staining in normal glands compared to adenomas, but higher expression in pituitary carcinomas [11]. McCabe et al. comparing VEGF mRNA in a series of adenomas composed of 77% nonfunctioning adenomas, and only 4% of prolactinomas [16], found highest expression in nonfunctioning adenomas and GH-producing adenomas; results which were confirmed by Western blot using only 20% of the samples.

In our present study, Western blot analysis of 56 pituitary adenomas revealed that VEGF protein expression was higher in prolactinomas compared to NF, GH-, and ACTH-secreting adenomas. This finding may be related to the high percentage of macroprolactinomas in this series (11/12). To this respect, using angiogenic markers, it has been described that macroprolactinomas are significantly more vascularized than microprolactinomas [4, 22]. Furthermore, lower VEGF in ACTH-producing adenomas may be consistent with the finding that VEGF production can be suppressed by glucocorticoids which are potent inhibitors of VEGF production in vitro [14].

On the other hand, with regard to MVD, some authors have found more prominent vasculature in prolactin-

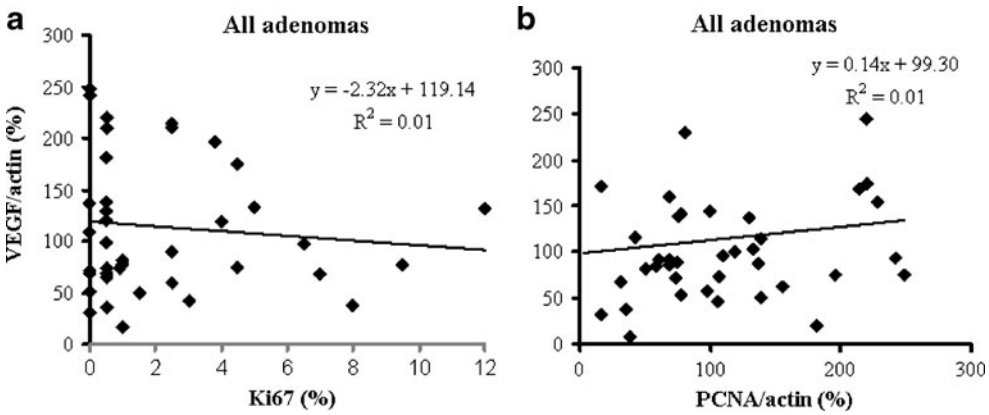

Fig. 7 VEGF and proliferation markers. Association of VEGF and **a** Ki67 labeling index or **b** PCNA content in pituitary adenomas. *Inset*, equation of linear regression, R^2

secreting tumors [4, 23], and others found that these tumors had the lowest while TSH-secreting adenomas had the highest MVD [20]. It has also been reported that ACTH-secreting tumors had the lowest MVD [4, 24], while other authors found that GH-secreting adenomas had the lowest [3, 25, 26], or the highest MVD [4]. Finally, some authors did not find any significant difference in MVD between the hormonal subtypes [5, 21], adding to the complex panorama of MVD analysis in pituitary tumors.

In our series, we found a high correlation of VEGF and CD31 expression for all adenoma types, and for prolactinomas and nonfunctioning adenomas, in particular. This is in contrast to results published by Viacava et al. in which MVD did not correlate with VEGF expression [21]. Differences in methodology may account for the discrepancy.

Two proliferation markers previously used to study pituitary adenomas were evaluated: Ki67, a nuclear antigen expressed in G1, G2, and synthesis phases of the cell cycle but not in the quiescent G0 phase [27], and PCNA, a nuclear protein identified as the auxiliary protein of deoxyribonucleic acid polymerase delta, whose gene expression correlates with cell proliferation [28], and which we measured by Western blot analysis. Neither proliferation marker correlated with the angiogenic markers CD31 and VEGF, as described by others [6, 8, 20, 26, 29, 30]. Taken together, these results might reflect the contribution of VEGF to adequate tumoral vascular supply through complex mechanisms, other than tumor cell proliferation. Some data suggest that VEGF may prolong cell survival by inducing expression of the anti-apoptotic protein bcl-2 in pituitary adenomas, suggesting that part of its angiogenic activity is related to protection of endothelial cells from apoptosis [30, 31]. VEGF has been associated to intratumoral hemorrhage [32], and might also participate in the occurrence of peliosis, a form of vasculogenic mimicry [29, 33]. Peliosis may be linked to the permeabilizing function of this growth factor and to the increased fenestration induced in blood vessels stimulated by VEGF overexpression. Peliosis occurrence has been related to high VEGF expression in hepatocarcinogenesis, spleen damage, and in a lethal hepatic syndrome in mice [34–36]. This process may be seen in prolactinomas [37] and other pituitary adenomas [38], though it usually goes unrecognized. In dopamine D2 receptor knockout mice which develop lactotroph hyperplasia and eventually prolactinomas [39], we have described increased peliosis occurrence in these pituitary tumors in association with increased VEGF expression [18].

VEGF was similar in both sexes and was not influenced by age or years of adenoma evolution when all adenomas were considered. This is in agreement with most studies which reveal that sex, age, or even rate of recurrence did not influence VEGF expression [21] [40].

With regard to the relation between MVD and sex or age of the patients, contradictory findings have also been reported. Jugenburg [3] reported no significant correlations, whereas Turner et al. [22] found tumor MVD clearly decreased with age in GH-producing adenomas, and there was a trend in other tumor types from older patients to have lower MVD. In contrast, a positive correlation between age and MVD has also been reported [20, 26]. In our present series, CD31 was not different between sexes and did not correlate with patients' age when all adenomas were considered. Nevertheless, if only nonfunctioning adenomas were analyzed, there was a positive correlation of CD31 with increasing age, in agreement with other authors [26, 41], and therefore age may have an influence on the extent of neovascularization of nonfunctioning adenomas.

The role of angiogenesis in adenomas of the highly vascularized pituitary gland remains intriguing. Our study reveals that VEGF is widely expressed in pituitary tumors, with higher levels in macroprolactinomas. The rate of epithelial cell proliferation in pituitary tumors is not directly related to neovascularization, and other factors, such as primary genetic alterations, may directly affect the proliferation rate, invasiveness, and behavior of tumors. Nevertheless, the strong positive association of VEGF and CD31 expression found in pituitary adenomas suggests the participation of tumor vascularization in adenoma development.

Acknowledgments This work was supported by grants from CONICET (PIP 640 to DBV), Fundación Alberto J. Roemmers (DBV), Fundación Fiorini (DBV), and Agencia Nacional de Promoción Científica y Técnica, Buenos Aires, Argentina (PICT 2006, N206, to DBV).

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

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