



Endothelial cells of oral pyogenic granulomas express eNOS and CD105/endoglin: an immunohistochemical study

Spyridon I. Vassilopoulos¹, Konstantinos I. Tosios², Vassilios G. Panis¹, John A. Vrotsos¹

¹Department of Periodontology, Dental School, National and Kapodestrian University of Athens, Athens, Greece; ²Department of Oral Pathology and Oral Surgery, Dental School, National and Kapodestrian University of Athens, Athens, Greece

BACKGROUND: The pyogenic granuloma (PG) is a common localized hyperplastic lesion of the oral cavity. The purpose of the present study was to investigate the immunohistochemical expression of endothelial nitric oxide synthases (eNOS) and CD105/endoglin in oral PGs, to evaluate their involvement in the angiogenetic pathways of the lesion.

MATERIALS AND METHODS: Ninety-three PGs were included in the study. Sixteen tumors were further subclassified as pregnancy tumors (PT) and seventeen as pyogenic granulomas with fibrosis (PGFM). Immunohistochemical expression of eNOS and CD105/endoglin was quantified by computerized image analysis with a semi-automated system. Percentage of staining and number of objects (positive vessels) were recorded for each case.

RESULTS: Intense eNOS expression was seen in 92 of 93 lesions. A statistically significant association was found between eNOS percentage of staining/eNOS positive vascular spaces (objects) and age of the patients (9% increase per decade of life). Approximately 40% less eNOS positive objects were recorded in PGFM compared with PGs. Intense membranous CD105/endoglin expression was seen in all cases. The percentage of CD105/endoglin staining was statistically increased in PGs compared with PT. Approximately 40% less CD105/endoglin objects were found in PGFM compared with PGs; 56% more CD105/endoglin objects were found in tongue lesions, compared with gingival lesions. There was no statistically significant correlation considering percentage of staining and number of objects between CD105/endoglin and eNOS.

CONCLUSIONS: It is suggested that eNOS and CD105/endoglin are involved in the angiogenetic pathways of PG.

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Introduction

The pyogenic granuloma (PG) is a common tumor of the oral mucosa that is usually considered as a reaction to local trauma or hormonal factors. It shows a predilection for the gingiva of females and when localized on the gingiva of pregnant women, it is usually described as pregnancy tumor (PT) or *epulis gravidarum*. Microscopically, PG is characterized by intense vascular proliferation with formation of numerous small- and medium-sized vessels lined by discrete endothelial cells, in an edematous stroma heavily infiltrated by inflammatory cells. Over time, some tumor may undergo fibrosis or *fibrous maturation* (PGFM).

PG is an example of inflammatory angiogenesis (1). Angiogenesis, defined as the formation of new vessels by proliferation and migration of endothelial cells of pre-existing vessels, is abundant in embryonic development, but in adult organism, it is normally confined to the female reproductive track (2, 3). It is also reactivated in wound healing and pathologic conditions such as cancer, rheumatoid arthritis, retinopathies, and psoriasis (4).

Angiogenesis in PGs involves an imbalance between proangiogenic and antiangiogenic factors, with most prominent being the overexpression of vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFGF) (1, 5, 6). *In vitro*, the release and function of VEGF (7, 8) and the angiogenic effects of bFGF (9, 10) are mediated by nitric oxide (NO). NO is important for maintaining normal vascular function, in particular, for regulating vascular tone and homeostasis (11), and its biosynthesis and release are stimulated by a family of heme-containing flavoprotein enzymes termed nitric oxide synthases (NOS) that includes neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) NOS. NO is active in endothelial cells where it has a

multitude of angiogenic actions, including enhancement of endothelial proliferation, survival and inhibition of apoptosis, and migration, as well as interaction with the extracellular matrix (12). eNOS activity is regulated by VEGF through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (11), whereas eNOS antagonists block VEGF- or bFGF-induced angiogenesis (10, 13).

The expression and activity of eNOS are also regulated by CD105 protein or endoglin (14, 15). This is a homodimeric, transmembrane glycoprotein of 180 kD (16, 17), mainly expressed by endothelial cells and placental syncytiotrophoblasts, as well as other cell types (18, 19). CD105/endoglin binds with great affinity the TGF- β superfamily members, in particular, TGF- β 1 and TGF- β 3. It may also function independently of TGF- β (20), while it interacts with other growth factors, such as activin-A, and the bone morphogenetic proteins BMP-7 and BMP-2 (21). CD105/endoglin has a critical role in angiogenesis, as it promotes proliferation and suppresses apoptosis of endothelial cells by antagonizing the effects of TGF- β (17, 22).

The aim of the present study was to investigate the immunohistochemical expression of eNOS and CD105/endoglin in oral PGs to evaluate their involvement in the angiogenic pathways of the lesion.

Materials and methods

Specimens

Ninety-three PGs were retrospectively collected among a series of 1140 cases diagnosed from 1974 to 2002, using standard microscopic criteria (23). All cases represented excisional biopsies, fixed in 10% buffered formalin and embedded in paraffin wax (FFPE). Data on age, gender and site of the tumors were drawn from the patients' records and pathology reports. Seventeen cases, where the stroma consisted of dense fibrous connective tissue, were further sub-classified as PGFM and 16 cases, developing on the gingiva of pregnant women, as PT. In the PG subgroup, 40 patients were women and 20 were men. The average age was 42.9 ± 19.3 years and most lesions were located on the gingiva ($n = 48$), followed by the tongue ($n = 11$), and lip ($n = 1$). In the PGFM group, 12 patients were women and five were men. The average age was 43.1 ± 22.2 years and most lesions were located on the gingiva ($n = 11$), followed by the tongue ($n = 3$), lips ($n = 2$), and buccal mucosa ($n = 1$). The average age for women with PT was 26.9 ± 5.6 years.

Immunohistochemistry

Immunohistochemistry was performed with primary antibodies against eNOS (rabbit polyclonal, cat. no. 905-386; Assay Designs, Ann Arbor, MI, USA, dilution 1:50) and CD105/endoglin (mouse monoclonal, clone 8E11; Innovex Biosciences, Middleton, WI, USA, dilution 1:50), with a standard avidin-biotin-peroxidase method. In summary, 3–4 μ m serial tissue sections were cut and dried overnight at 56–58°C. After deparaffinization, endogenous peroxidase was blocked with 37% H₂O₂ (10 ml in 90 ml demineralised water, 30 min in

dark place), and antigen retrieval was achieved with citric acid pH6.0 in a conventional steamer for 5 min. After application of the primary antibodies for 1–2 h at room temperature, immunostaining was developed with a two-step immunohistochemical staining technique (EnVision™ System: 5007; Dako, Glostrup, Denmark)

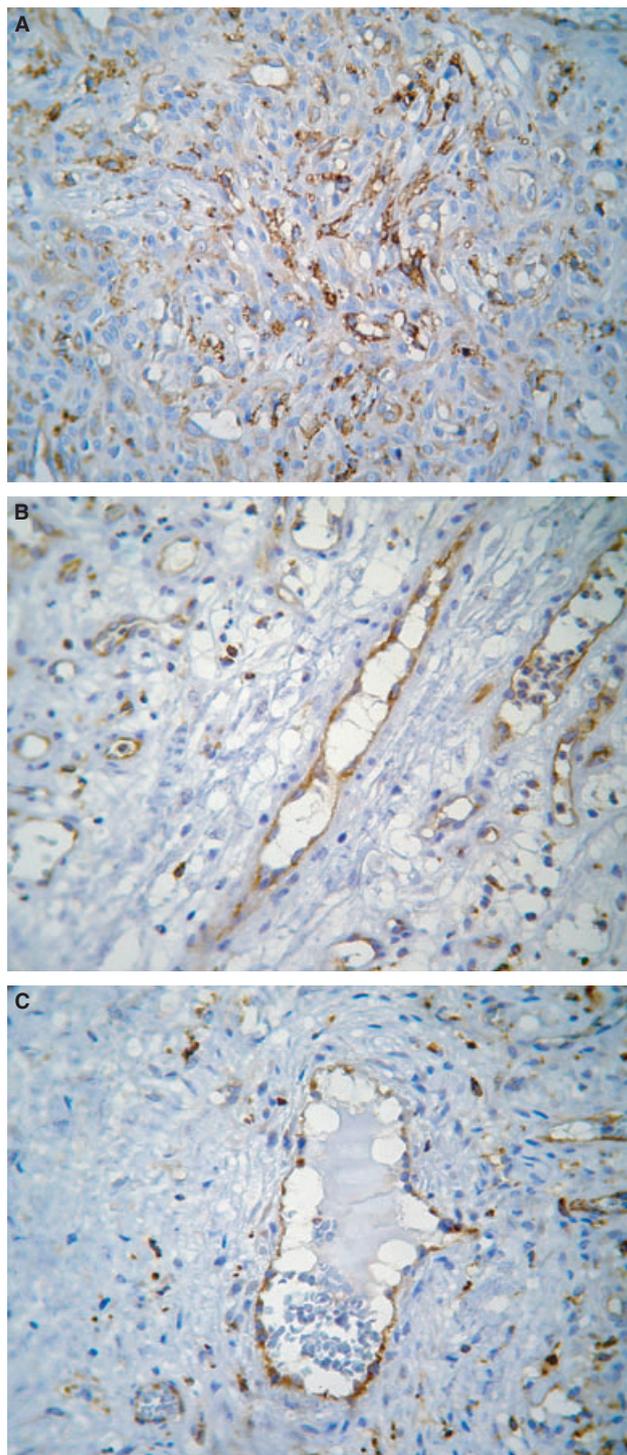


Figure 1 Endothelial nitric oxide synthases staining of endothelial cells. (A) Pyogenic granuloma. (B) Pyogenic granuloma with fibrous maturation. (C) Pregnancy tumor (DAB with Mayer's hematoxylin, original magnification $\times 400$).

Table 1 Correlation of endothelial nitric oxide synthases (eNOS) percentage of staining and objects (positive vessels) between pyogenic granuloma (PG) sub-groups and age

Covariate	eNOS percentage of staining			eNOS objects(positive vessels)		
	Exp.C ^a	95% CI ^b	P	IRR ^c	95% CI ^b	P
PG sub-groups						
PG ^d	1			1		
PGFM	0.483	(0.243, 0.959)	0.038*	0.599	(0.427, 0.840)	0.003**
Pregnancy tumors	1.924	(0.919, 4.027)	0.082	1.119	(0.789, 1.587)	0.528
Age (per 10 years)	1.156	(1.001, 1.334)	0.048***	1.090	(1.019, 1.167)	0.012 [†]

^aExponentiated regression coefficient. Values > 1 indicate higher eNOS percentage of staining. Values < 1 indicate lower eNOS percentage of staining.

^bConfidence Interval.

^cIncidence rate ratio.

^dBaseline category.

*Significant difference exists at $P < 0.05$ when compared with baseline (PG) after weighting for location. Simple linear regression after logit transformation.

**Significant difference exists at $P < 0.05$ when compared with baseline (PG) after weighting for location. Negative binomial regression model.

***Statistical significant increase of eNOS percentage of staining per decade exists at $P < 0.05$ after balancing for PG sub-groups. Simple linear regression after logit transformation.

[†]Statistical significant increase of eNOS objects per decade exists at $P < 0.05$ after balancing for PG-subgroups. Negative binomial regression model.

for 40', and 3,3' tetrahydrochloride diaminobezidine (DAB; Sigma, St. Louis, MO, USA). Sections were finally counterstained with Mayer's hematoxylin, cleared and mounted. Proper controls were utilized.

Evaluation of staining

Immunostaining was evaluated by a computerized semi automated image analysis system that allowed quantification of qualitative data in a rapid and accurate way, as has been previously shown in tumors' angiogenesis (24–26). In summary, 5 representative microscopic fields from each lesion, showing adequate cellularity and staining reaction, were digitized with a software program (Framegrabber Matrox II running on an Intel Pentium IV PC) through a videocamera (Microwave Systems, Tokyo, Japan, 800 × 600) mounted on a microscope with 20× magnification (Olympus BX-50, Tokyo, Japan). Percentage of staining and number of objects (positive vessels) were recorded [Image Pro Plus ver. 3.0; Media Cybernetics 1997 (Silver Spring, MD, USA)] for each digital image and the average value was calculated in each lesion. Values ranged between 0 (black) and 255 (white), with 0 representing strong expression and 255, no expression. Staining intensity of > 190 was characterized as negative (27).

Statistical analysis

For comparisons among the studied sub-groups (PG, PGFM, PT), parametric (t -test, one-way ANOVA) and non-parametric (Mann–Whitney U -test, Kruskal–Wallis test) methods were utilized for quantitative data, and chi-square and Fisher's exact test for qualitative data. Simple linear regression after logit transformation was applied for the investigation of parameters that affect for CD105/endothelin and eNOS percentages, and negative binomial regression for CD105/endothelin and eNOS objects. The logit transformation has been used to normalize the distribution of the CD105/endothelin and

eNOS percentages, whereas the negative binomial regression model was the most appropriate one for modeling the overdispersed counts of CD105/endothelin and eNOS objects. Association of CD105/endothelin and eNOS was assessed by Spearman's rank correlation. $P \leq 0.05$ was considered statistically significant. Statistical analysis was performed with a statistical software program (Stata 10.1; Stata Corp., College Station, TX, USA).

Results

Intense eNOS expression (values 111–155) was seen in 92 of 93 lesions (Fig. 1). Only cells lining discrete vascular structures were positive. A statistically significant correlation was found between age of patients and eNOS percentage of staining ($P = 0.048$), and number of eNOS positive vessels ($P = 0.012$, 9% increase per decade) after balancing for PG-subgroups. The percentage of eNOS staining was statistically increased in pyogenic granulomas compared with pyogenic granulomas with fibrous maturation ($P = 0.038$), but not with pregnancy tumors (PT, $P = 0.082$). Approximately 40% less eNOS positive vascular spaces (objects) were recorded in pyogenic granulomas with fibrous maturation compared with pyogenic granulomas ($P = 0.003$), after correcting for location (Table 1).

Intense CD105/endothelin expression (values 111–155) was seen in all cases (Fig. 2). Only cells lining discrete vascular structures were positive, while peripheral (periendothelial) cells were non-reactive. The percentage of CD105/endothelin staining was statistically increased in pyogenic granulomas compared with PT ($P = 0.047$), but not with pyogenic granulomas with fibrous maturation ($P = 0.183$). Approximately 40% less CD105/endothelin positive vascular spaces were found in pyogenic granulomas with fibrous maturation compared with pyogenic granulomas ($P = 0.007$), after

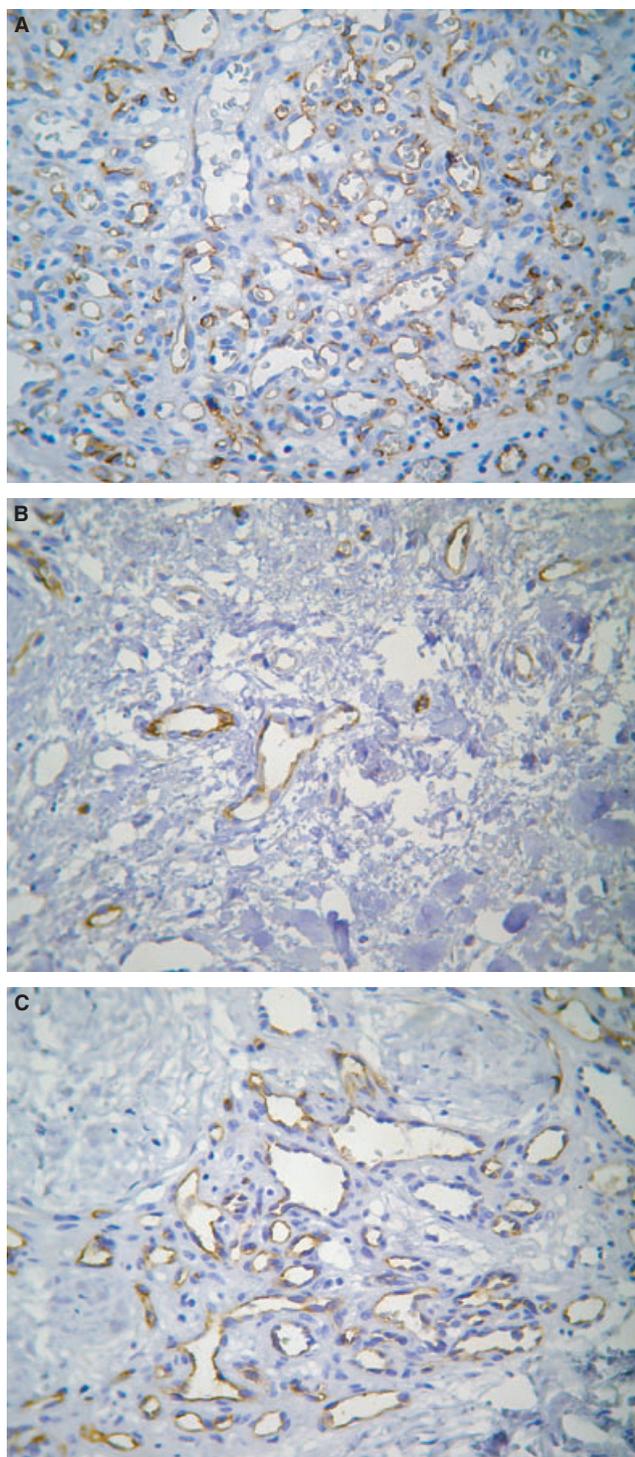


Figure 2 CD105/endoglin staining of endothelial cells. (A) Pyogenic granuloma. Notice lack of expression in peripheral (periendothelial) cells. (B) Pyogenic granuloma with fibrous maturation. (C) Pregnancy tumor (DAB with Mayer's hematoxylin, original magnification $\times 400$).

weighting for location. Furthermore, 56% more CD105/endoglin positive vascular spaces were found in tongue lesions, compared with gingival lesions ($P = 0.022$) after balancing for PG-subgroups (Table 2).

There was no statistically significant correlation in the percentage of staining and number of objects between CD105/endoglin and eNOS (Table 3).

Discussion

In the present study, it was shown that eNOS and CD105/endoglin are expressed in the endothelial cells of PGs, including PGFM and PT, indicating their involvement in the angiogenic pathways of the lesion. Our data add a piece of information to the puzzle of inflammatory angiogenesis in PG, where overexpression of proangiogenic VEGF and bFGF (1, 5, 6, 28), and vascular morphogenesis factors Tie-2, angiopoietins-1 and -2, ephrins-B2 and -B4 (29, 30), as well as decreased expression of the antiangiogenic factors angiostatin and thrombospondin-1 (1, 6) have been shown to be involved.

eNOS stimulates release of endothelium-derived NO that is a critical mediator of angiogenesis (12), while release of NO by bFGF (9, 10) and TGF-beta1 (31) is necessary for their function. VEGF is upregulated in PGs and is mainly expressed by macrophages and fibroblasts (1, 6). bFGF is abundantly expressed in the extracellular matrix of PG (1, 5) and is released by macrophages, mast cells, and endothelial cells early in the development of the lesion (5). Thus, it may be assumed that in PGs release of VEGF and bFGF by stromal cells stimulates expression of eNOS in endothelial cells, activating the eNOS-dependent angiogenic pathway.

A statistically significant association was found between eNOS percentage of staining/eNOS positive vessels and age of the patients (9% per decade of life). eNOS expression and activity and NO production in endothelial cells' cultures are down-regulated by ageing (32), but in humans (33) and experimental animals (34), eNOS expression does not differ significantly in young vs. aged. However, eNOS phosphorylation decreases by increasing age, resulting in a loss of PKI3/Akt activity (35). As the antibody used in the present study does not discriminate between activated and non-activated eNOS, we cannot deliberate on the association of eNOS expression with ageing.

eNOS is transcriptionally regulated by estrogen receptors (ERs) (11). In particular ER-alpha, an ER isoform actively involved in angiogenesis, controls activation and production of NOS in various environments that result in the induction of NO release by endothelial cells (36–40). As ERs have been immunohistochemically recognized in the endothelial cells of PGs in some studies (1, 41, 42), the association of eNOS expression with ER and in particular ER-alpha in PGs would be of interest to be evaluated. In a preliminary study in our material (data not shown), no nuclear ER-alpha expression was evident in PGs, but ERs involved in eNOS activation are usually membranous (43).

The angiogenic potential in PGs has been mainly supported by microvessel density assessed by immunohistochemistry for CD31 and von Willebrand factor (6). However, this is indirect evidence as those markers

Table 2 Correlation of CD105/endothelin percentage of staining and objects (positive vessels) between pyogenic granuloma (PG) sub-groups and tumor location

Covariate	CD105 percentage of staining			CD105 objects (positive vessels)		
	Exp.C ^a	95% CI ^b	P	IRR ^c	95% CI ^b	P
PG sub-groups						
PG ^d	1			1		
PGFM	0.659	(0.355, 1.223)	0.183	0.599	(0.413, 0.869)	0.007*
Pregnancy tumors	0.535	(0.289, 0.992)	0.047**	0.785	(0.540, 1.142)	0.205
Location						
Gingiva ^d	1			1		
Tongue	1.351	(0.713, 2.559)	0.352	1.556	(1.065, 2.274)	0.022***
Lip/Buccal mucosa	2.505	(0.782, 8.020)	0.120	1.032	(0.514, 2.071)	0.929

^aExponentiated regression coefficient. Values > 1 indicate higher CD105 percentage of staining. Values < 1 indicate lower CD105 percentage of staining.

^bConfidence Interval.

^cIncidence rate ratio.

^dBaseline category.

*Significant difference exists at $P < 0.05$ when compared with baseline (PG) after weighting for location. Negative binomial regression model.

**Significant difference exists at $P < 0.05$ when compared with baseline (PG) after weighting for location. Simple linear regression after logit transformation.

***Statistically significant increase of CD105/endothelin objects exists at $P < 0.05$ when compared with baseline (gingiva) after balancing for PG-subgroups. Negative binomial regression model.

Table 3 Association of CD105/endothelin and endothelial nitric oxide synthases (eNOS) percentage of staining and objects (positive vessels) overall, by pyogenic granuloma (PG)-subgroups and by location

	CD105/endothelin and eNOS correlation			
	Percentage of staining		Objects (positive vessels)	
	R*	P**	R*	P**
Overall	0.023	0.829	0.133	0.203
PG sub-groups				
PG	0.113	0.391	0.203	0.120
PGFM	-0.083	0.751	0.060	0.820
Pregnancy tumors	-0.199	0.461	-0.156	0.564
Location				
Gingiva	-0.002	0.988	0.116	0.321
Tongue	0.130	0.659	0.121	0.681
Lip/Buccal mucosa	0.400	0.600	-0.800	0.200

*Spearman's rank correlation coefficient; **Significant correlation exists at $P < 0.05$.

cannot discriminate between newly formed and pre-existing vessels, whereas other proangiogenic factors, such as VEGF or bFGF, are usually expressed by macrophages and fibroblasts, not by endothelial cells (1, 6). CD105/endothelin applied in the current study is expressed by proliferating endothelial cells during embryogenesis, inflammation, wound healing and neoplasia, but is not detectable in resting endothelial cells of normal tissues (17, 19). Although CD105/endothelin may also be expressed by macrophages that are abundant in PGs (1, 6), reaction in the present study only in cells lining vascular channels, is consistent with expression by endothelial cells.

CD105/endothelin expression is a strong indication that vessels in PGs are mature and functional, as it is associated with vascular maturation and maintenance of normal vascular architecture (16, 17, 44, 45). The presence of pericytes around the vascular channels of PGs (46) is also in favor of the presence of mature

vessels. It is noted that periendothelial cells in the present study were non-reactive for CD105/endothelin.

The statistically significant decrease in the percentage of CD105/endothelin positive vessels in PT may be associated with vessels' regression, as its excision is usually delayed until after parturition. Yuan and Lin (30) reported a decrease in endothelial cells and VEGF concentration in PT in postpartum period than during pregnancy. Tongue PGs showed statistically more CD105/endothelin positive vessels than gingival PG. It is possible that more tongue PGs are consistent with lobular capillary hemangiomas, where angiogenesis is more intense.

Less (40%) eNOS- and CD105-positive vessels were seen in PGFM compared with PG, but this was expected, as fibrous maturation is associated with a decreased density of vessels (47, 48). However, eNOS and CD105/endothelin expression by endothelial cells in PGFMs suggests that suppression of this pathway of

angiogenesis is not a key element in the maturation of PGs. In PT, percentages of CD105/endothelin and eNOS staining were more or less decreased, compared with PG. This decrease may reflect the expected decrease in the number of vessels in PT, as excision is usually delayed until after parturition, when the local VEGF concentration is reduced (6, 30).

In conclusion, the results of the present study imply that eNOS and CD105/endothelin are involved in the angiogenic pathways of PG that can be considered a unique *in vivo* example for the study of inflammatory angiogenesis. As both molecules are associated with ER- α , and PG is considered an example of hormone-dependent tumor, the role of ER- α should be further evaluated.

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None.

Conflict of interest

The authors declare that they have no conflict of interest.