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TEMPORAL CHANGES IN NEUTRAL ENDOPEPTIDASE/CD10 IMMUNOEXPRESSION IN THE CYCLIC AND EARLY PREGNANT CANINE ENDOMETRIUM

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Running Head: Neutral endopeptidase in dog endometrium

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Abstract

CD10 is a multifunctional transmembrane neutral endopeptidase (NEP), considered to be a reliable marker of ectopic human endometrial stroma. Available information in NEP/CD10 protein expression in animal endometria is scarce. This study focused on the immunolocalization of NEP/CD10 in the canine uterus and on its temporal changes during the oestrous cycle and early pregnancy (days 11 to 23 post-LH surge) in healthy females. NEP/CD10 expression was found in the canine endometrial stroma in all stages of the oestrous cycle, showing cyclic differences both in intensity and in distribution pattern. A small population of negative stromal cells in subsurface position was also observed. This population shared some morphological characteristics with the human predecidual cells, which became positive in progesterone-associated stages of the cycle. In addition, positive immunolabelling was also observed in canine myometrial stroma. In early pregnancy, the basal glandular epithelia and the syncytium cords, remained negative to this marker,, contrasting with the trophoblast and the lacunar epithelium. A weak to moderate intensity of immunolabelling was observed in the decidual cells, while stromal immunolabelling was more intense at the delimitation of the syncytium cords. In conclusion, CD10 is consistently expressed in the canine endometrial stroma and myometrium, but not in endometrial epithelia. The characteristic pattern seen in early pregnancy also suggests a role for this molecule in the process of embryo invasion at implantation.

Key words: Neutral endopeptidase; CD10; endometrial stroma; oestrous cycle; early pregnancy; immunohistochemistry; female dog.

1. Introduction

The mammalian endometrium is a highly complex tissue that undergoes accurately defined, cyclic morphological changes in response to sex steroids stimulation. The ultimate goal is to guarantee embryo survival, implantation and the success of pregnancy [1, 2].
Although under the control of sex steroids, endometrial cyclic changes are ultimately controlled by several autocrine and paracrine factors that include a multitude of local molecules that determine proliferation of the epithelial endometrial elements, epithelial-stromal cells interaction and invasiveness, angiogenesis, apoptosis, differentiation, as well as immune cells infiltration, among others [3-5]. A correct equilibrium of these molecules, both in sequential changes and in quantity is essential for fertility [6].
Endometrial stroma cell functions are not limited to maintaining the endometrial structure; the stroma is involved in epithelial development and proliferation [7], cell adhesion, tissue remodelling and organ immune competence [8]. These are notorious during the cyclic changes and at implantation, particularly in species with deciduae placenta.

CD10 protein is a membrane-associated neutral peptidase, also known as neprylisin, enkephalinase, common acute lymphoblastic leukemia antigen or neutral endopeptidase (NEP) [9, 10]. CD10 is a 90- to 110-kDa cell-surface zinc-dependent metalloprotease, shown to be expressed by a widely variety of cell types and tissues, including the uterus (Chu and Arber, 2000). CD10 functions as a cell surface enzyme, acting to reduce the cell response to some peptide factors, including oxytocin, endothelins and interleukin 1 [11]; through cleavage and inactivation of those peptides, NEP/CD10 reduces its local concentrations and decreases their effects [12-14].
NEP/CD10 has been implicated in the regulation of growth and differentiation in many cellular systems, in which it plays an important role in the maintenance of homeostasis [15-17], as well as in carcinogenesis and tumour progression [18-22], possibly mediated through its role on angiogenesis [23], in cell cycle activity [24] and apoptosis [25].

In human, NEP/CD10 is frequently used as a reliable immunohistochemical marker of normal endometrial stroma [26, 27], and is used for diagnosis of several neoplasic [27-29] and non-neoplasic [30, 31] gynaecological conditions. Yet, NEP/CD10 functions in the endometrium remain poorly understood.

Although clinical conditions such as endometriosis are not proven to exist in dogs, additional knowledge of the location and cyclic variation of this endopeptidase in the canine endometrium may be valuable, especially in pathological alterations such as cystic endometrial hyperplasia or when fertility may be compromised. In domestic animals, although previous work by Riley et al. [32] reported the presence of this enzyme in the sheep uterus, limited information is available on uterine pattern of the CD10/NEP protein expression.

The purpose for this study was to determine the pattern for NEP/CD10 protein expression in the normal canine endometrium by using an immunohistochemical technique and to investigate whether this pattern changes during the oestrous cycle and in early pregnancy (days 11 to 23 post-LH surge). By establishing the normal pattern of CD10 expression in canine endometrium, this study will further allow provide reference data that might be essential in the study of endometrial diseases, especially in angiogenesis and stromal-epithelial cross-talk.

2. Material and methods

Tissue collection and preparation

Forty-eight post-pubertal, healthy non-pregnant bitches, and 16 pregnant females of different breeds and ages ranging from 10 months to 6-year old, were used in this study. Endometrial tissue, collected at ovariohysterectomy (OVH), was used with the owners' informed consent, in accordance to the International Ethical standards.

For immunohistochemistry, samples from the uterus were fixed in 10% formalin immediately after the surgery. Transversal fragments were collected from each uterine horn, embedded in

paraffin wax, sectioned at 3 µm and stained with haematoxylin and eosin for histological staging of the oestrous cycle and for excluding uterine disease. Samples showing histological signs of delayed uterine involution (glandular dysplasia and increased number of macrophages in the presence of large vessels within the *stratum vasculare*) or endometrial disease (such as cystic endometrial hyperplasia or pyometra) were excluded from the study. For the pregnant group, transversal samples were collected from zonary invasion areas and interplacental areas (or paraplacenta). When those were not distinguishable, longitudinal sections were obtained. For western blotting (WB), adjacent 1cm thick uterine sections were collected from non-pregnant samples and immediately snap frozen in liquid nitrogen before being stored at -70ºC, until analysis.

Before surgery, a vaginal cytological specimen was obtained and a blood sample was collected from the jugular vein into a controlled vacuum tube (*Serum-gel*, S-Monovette®, Sarstedt, Nümbrecht, Germany), centrifuged and stored at -20ºC until analysis. Serum progesterone levels were determined by chemiluminescence immunoassay system (Immulite®; DPC-Diagnostic Products Corp., Los Angeles, CA, USA).

Oestrous cycle and pregnancy staging

Non-pregnant animals were initially selected on the basis of the vaginal cytology. At OVH, the stage of the oestrous cycle for each bitch was determined by ovaries inspection and later confirmed upon the histological examination of the ovaries and by progesterone levels, which were used in the fine tuning the histological staging [8]. Uterine samples for the prooestrus (n=9), oestrus (n=8), dioestrus (n=20) and anoestrus (n= 10) were used in this study. Considering that, in carnivores, implantation is not an early event [33], with dog embryos interacting with the endometrium around post-ovulatory day 16 [34], the dioestrus was further divided in two stages: an early dioestrus period (n=10), with rising progesterone levels and with young, cavitary corpora lutea in the ovaries; and a full dioestrus period (n=10), with high progesterone levels and mature, compact, active corpora lutea in the ovaries.

Pregnancy samples were obtained from females with unwanted pregnancies up to 3 weeks post-coitus, submitted to OVH. Pregnancy samples were then staged on the basis of cumulative information gathered from dioestrus-compatible cytology, known unwanted breeding plus high progesterone levels and the co-existence of small-sized (<3 cm) uterine swellings. When uterine swellings were not noticed, but knowledge of coitus existed, pregnancy estimated of less than 17 days was determined upon embryo collection by flushing each uterine tube and uterine horns separately, as described by Tsutsui et al. [35]. Pregnancies were further divided in two groups: Group 1 (PGr1; n= 5) corresponded to the period previous to embryo attachment to the maternal endometrium and mainly corresponded to pregnancies days 11 to 13, were morulae or young blastocysts were found in the oviducts or in the uterus; group 2 (PGr2; n= 12) corresponded to pregnancy days 15 to 23. Between pregnancy days 16 to 21, morphological changes in the endometrium are visible although embryos are still non-adherent. After day 22 embryos attach and invasion begins. The chronology of the pregnancy was confirmed according to the histological descriptions of canine early pregnancy events [33, 36] and aligned to the day from pre-ovulatory surge [37]. Briefly, by day 13 to 15 some changes in the superficial endometrium are observed, including increased interstitial oedema and depth of the endometrial crypts. Around day 17 the embryo is apposed to the endometrium; the trophoblast grows down and wedges the maternal surface epithelium (SE). Only small lacunae are visible. By day 20, the trophoblast continues to spread down, and the syncytial cells penetrate deeper in the endometrium appearing as strong, linear cords, frequently presenting mitotic figures. After day 22, the crypts at implantation sites elongate, and became tortuous and closely packed, with enlarged lacunae below. The deep endometrial glands start to grow [33, 36].

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 3 µm thick tissue sections on silane-coated slides were used for the immunohistochemistry study of CD10 expression in the bitch endometrium, using

a streptavidin-biotin-peroxidase technique (UltraVision Detection System, Fremont, CA, USA) with a monoclonal antibody to CD10 (clone 56C6, reference NCL-CD10-270; Novocastra®, New Castle Upon Tyne, UK). Sections were routinely deparaffinized in xylene and hydrated through graded alcohol and water. Antigen retrieval was performed in a steamer, with slides immersed in boiling citrate buffer (pH 6.0; *ca*. 94ºC) for 2 minutes. After blocking the endogenous peroxidases in 3% hydrogen peroxide/PBS for 30 minutes and non-specific binding by incubation with Ultra V-Block® for 5 min, the slides were incubated overnight with the primary antibody at a 1:50 dilution in PBS at 4ºC, in a humid chamber. Thereafter, samples were incubated with a biotin conjugated secondary antibody and then incubated using streptavidin-biotin system, for 10 minutes each, at room temperature. Reactions were visualized using DAB (3,3'-diaminobenzidine) as chromogen. Sections were then counterstained with Gill's haematoxylin, dehydrated and mounted.

Sections from canine ovaries were included as negative controls, since ovarian stroma is negative for CD10/NEP. Additional negative controls were used, whereby endometrial specimens were submitted to the same procedure, with the exception that the primary antibody was omitted and replaced by PBS or by a normal mouse IgG (sc-2025; Santa Cruz Biotechnology Inc., Europe, Heidelberg, Germany). In neither negative control was CD10-immunoreactivity observed.

Immunohistochemical scoring

Microscopic examination at low magnification (40x) was performed to examine the overall pattern of immunoreaction against NEP/CD10 in the canine uterus. Thereafter, staining results were evaluated at a higher magnification (200x and 400x) to define the intensity and the pattern of the immunoreaction. Positivity was indicated by the presence of a distinct golden-brown cytoplasmic labelling.

Two independent observers performed a blind semi-quantitative assessment of the intensity of staining, using a three-point score classification (weak, moderate and strong), operator-wise. The repeatability of the results from both observers, in all the 3-point scales used for scoring,

was assessed in selected samples from anoestrus, oestrus and dioestrus; only cases providing repeatable scores in immunostaining were used.

In cyclic endometrial samples, positive reaction was scored independently for each endometrial component (Stroma – S, Surface Epithelium – SE and Glandular Epithelia – GE). According to the pattern perceived under small magnification, the endometrial stroma in cyclic samples was further evaluated individually for the deep basal layer (DbS; equivalent to the *stratum basalis* of the human endometrium), the intermediate and superficial layers (equivalent to the *stratum functionalis* of the human endometrium and thereafter named as upper stromal layer - UpS) and a sub-surface, adluminal layer (SSL), located just beneath the endometrial surface epithelium, which could correspond to the endometrial *stratum compactum*.

The same stromal regions were used to analyse NEP/CD10 immunoreaction in samples from pre-attachment pregnancy (PGr1). In the samples from the attachment period (PGr2), individual scoring was performed for the trophoblast and the epithelium of the syncytium cords (or *lamellae*), the *lacunae* and the deep glands, as well as for the adluminal decidualized stroma, the syncytial and the intermediate and deep endometrial stromal layers..

Western Blot analysis

Western blotting analysis (WB) was used to test the specificity of the human CD10 antibody labelling in canine endometrium. For WB, fragments of five frozen unthawed tissues corresponding to different stages of the canine estrous cycle (anoestrus, early dioestrus and dioestrus) were homogenized separately in ice-cold phosphate buffer saline (PBS in mM: 1.76 KH2PO4, 10 NaH2PO4, 2.7 KCl, 137 NaCl; pH 7.0) supplemented with a cocktail of protease and phosphatase inhibitors (1 µl mL^{-1} Sigma P4830 protease inhibitor cocktail). Protein concentration was determined by the Bradford method.

After denaturation at 95 ºC for 5 min in a Laemmli buffer (in mM: 25 Tris, 182 glycine, 0.1% SDS; pH 8.3), equal protein (25 µg) of each sample were loaded in duplicate and electrophoresed on SDS–polyacrylamide gel for 90 min at a constant 100 V and transferred onto polyvinylidene difluoride membrane at 100 V for 90 min at 4ºC. Membranes were blocked

for 60 min at room temperature, in Tris buffered saline (in mM: 20 Tris–HCl; 137 NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk. Blots were then incubated for 1 h with the primary antibody (NCL-CD10-270; Novocastra®, New Castle Upon Tyne, UK) diluted at 1:1000, with gentle agitation. The solution of primary antibody was prepared in 1% fat free dry milk in TBS-T. After extensive washing with 0.5% fat free dry milk in TBS-T solution, immunodetection was performed with WesternDot 625 goat anti-mouse western blot kit. Membranes were imaged using a Versa Doc instrument (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The IBM SPSS Statistics Base 19.0 statistical software for Windows® was used to perform statistical comparisons. Statistical analysis of the differences in the intensity of immunoexpression for CD10/NEP between the stages of the oestrous cycle and the cell type were performed using the chi-square and Fisher exact tests. A P value ≤ 0.05 was regarded as statistically significant. The Z-test was performed for group comparisons (stage of the oestrous cycle; early dioestrus *vs.* pregnancy; distribution for the diverse stromal layers)..

3. Results

Specificity of the CD10/NEP antibody, developed in mouse for the human molecule, was assessed by Western blot analysis in canine endometrial cell lysates. On those blots, the antibody used recognised a molecular band of approximately 100 kDa, consistent with the reported molecular weight of CD10 protein (Figure 1).

CD10 immunoexpression in the canine uteri was present in all samples analysed. Data gathered for this study on the canine endometrial expression of CD10 molecule are summarized in Tables 1 and 2.

Assessment of endometrial samples at low magnification showed that during all stages of the oestrous cycle positive immunoreaction existed both at the endometrial stroma and the

myometrium, but not in the epithelial elements of the endometrium (Figure 2; Table 1; Graph 1). In the myometrium, smooth muscle cells were negative for NEP/CD10, the immunoreaction being restricted to the cells perimysium (Figure 2G). In general, the intensity of myometrial immuno labelling tended to match that of deep basal stroma, and did not vary significantly during the oestrous cycle (*P*=0.054; Fisher = 14.353).

Within the endometrium, cytoplasmic NEP/CD10 expression was consistently found in the endometrial stroma throughout all stages of the oestrous cycle of the bitch (Table 1). However, immunolabelling differences in intensity and distribution pattern were seen in different stages of the oestrous cycle (Graph 2; Figure 2).When comparing the relative overall intensity of immunostaining in the canine endometrial stroma (Table 1; Graph 1), the differences among the cycle stages were greater in the subsurface and the upper stromal layers (*P*<0,001; Fisher = 40.499 and 33.427, respectively), which also presented lower overall intensity scores than the deep basal layer (*P*=0,050; Fisher = 13.357). Furthermore, overall staining scores were higher in anoestrus and prooestrus, than in other stages, when suprabasal levels of progesterone exist (*P*<0.001; Fisher = 98.417).

In anoestrus, strong immunoreaction against NEP/CD10 was observed (Graph 2) in a rather uniform distribution pattern throughout the endometrial stroma (Figure 2.A), with the exception of a negative stromal population located beneath the surface epithelium. NEP/CD10 negative population was composed mainly composed of fusiform cells, while scant in cytoplasm, with poorly defined cell borders and ovoid or elongated, dense nuclei without visible nucleoli (Figure 2.D).

During prooestrus, a diffuse stromal immunoexpression was observed in lower magnifications, partly due to endometrial oedema and red blood cells infiltration. At higher magnifications, upper stromal cells showed a decrease in the intensity of immunolabelling in comparison to anoestrus (*P*= 0.020; Fisher = 6.798), with prevalence of a moderate intensity of immunostaining and a homogeneous pattern observed in the entire endometrium. In this stage, the subsurface stromal area beneath the surface epithelium remained negative for

NEP/DC10 (Figure 2.D). In general, overall immunoreaction against this neutral endopeptidase in prooestrus was not different from that found in anoestrus (P = 0.115; Fisher = 5.912).

In the oestrus stage, a relative, non-significant decrease on NEP/CD10 expression was observed in the upper and deep basal endometrial stroma, when compared to those of prooestrus (P > 0.104; Fisher = 3.310 and 4.930, respectively). Furthermore, an apparent increase in the endometrial stroma intensity of staining was perceived at lower magnifications (Table 1). The deep basal stromal layer showed a relatively stronger cytoplasmic expression than the upper layer, particularly around deep basal glands (Figure 2.B). A predominance of fusiform NEP/CD10 negative stromal cells was observed underneath the surface epithelium (Figure 2.E), although in some of the samples (n=2; 25%) a faint NEP/CD10 positivity was observed. Overall immunoreaction against this molecule in oestrus differed from that found in prooestrus (P = 0.005; Fisher = 12.465).

In early dioestrus, a decrease in NEP/CD10 expression was detected in the upper and deep basal endometrial stroma (Table 1; Figure 2.C). The upper stromal layer cells tend to show lower NEP/CD10 expression than the deep basal stromal layer (P= 0.05; Fisher = 17.084). All stromal cells in the subsurface layer are now positive to this protein, contrasting to the observed in previously described stages (P= 0.005; Fisher = 7.976) (Figure 2.F); the majority of these cells, with scanty cytoplasm, now present round to ovoid nuclei and rough chromatin, with the nucleoli seldom visible. The overall intensity of immunoreaction against NEP/CD10 was significantly different from the one evidenced in oestrus (P= 0.038; Fisher = 7.434).

In full dioestrus, apart from the subsurface stromal layer, NEP/CD10 immunoexpression observed in the endometrial stroma resembled that of the prooestrus and oestrus stages. Stromal cells adjacent to the surface epithelium showed morphological features similar to those described for early dioestrus, maintaining identical positive immunoreaction for NEP/CD10. No statistical differences were found in the intensity of immunolabelling between early dioestrus and full dioestrus for the subsurface and deep stromal layers (Figure 2.H), despite the slight increase in the overall staining for these layers; the upper layer stroma

showed a marked increase in the intensity of the immunoreaction when compared to the one observed in early dioestrus (*P*= 0.001; Fisher = 12.847). Nonetheless, the overall intensity of immunostaining was considered different between these two stages (*P*= 0.032; Fisher = 6.647).

NEP/CD10 immunoreaction was also found in all the samples from canine pregnancy days 11 to 13 (PGr1; Table 1 and Graph 3) and 16 to 23 (PGr2; Table 2 and Graph 4).
In the pre-attachment period (PGr1), no morphological changes in the endometrium developed in comparison to early dioestrus, despite the differences found in the overall NEP/CD10 immunolabelling (*P*= 0.002; Fisher = 11.415). There was a relative increase in the intensity of immunoreaction against NEP/CD10 in the subsurface and upper stromal layers compared to that of early dioestrus (respectively *P*= 0.004, Fisher = 8.282 and *P*= 0.031; Fisher = 7.166; Graph 3), but not to that of full dioestrus. In contrast, non-significant differences in immunostaining were observed in the deep basal stroma between groups. In contrast to the observed throughout the canine oestrous cycle, a moderate to strong immunoreaction against NEP/CD10 was observed in apical position in the epithelial cells of more than 75% of the endometrial glands, although no cytoplasmic immunostaining was visible.

In samples from the attachment period (PGr2), in the implantation area, the intermediate peri-lacunar stroma was found to be more compact than the basal stroma (Graph 4), and displayed a strong intensity of immunostaining when compared to equivalent endometrial layer in PGr1 (*P*= 0.09; Fisher = 8.055), thus giving to the overall pattern of the organ the appearance of a ring or a barrier (Figure 3.A to 3.D). In comparison to subsurface stromal layer pattern in PGr1, no differences were found between pre-attachment and attachment periods, although the decidual cells located closer to the foetal-maternal interface showed an increased intensity of immunolabelling. Furthermore, the scores for decidual cells were significantly higher than the recorded for stroma at the syncytium cords (*P*= 0.038; Fisher = 6.553) (Figure 3.D to 3.F). The embryo trophoblast showed a weak to moderate intensity of immunolabelling (Graph 4).

However, the cells from the cords or lamellae are negative to NEP/CD10 (Figure 3.F). A faint cytoplasmic immunoreaction for this molecule was observed in lacunar epithelial cells, which displayed an apical reinforcement (Figure 3.G), although the deep glandular epithelium remained negative for this molecule. This same fact was observed during the canine oestrous cycle. Sporadically, apical moderate to strong positivity was found in DGE cells.

In the interplacental areas, an increased intensity of immunostaining was observed in the upper stroma (Figure 3.H), similar in intensity to the one registered for the area adjacent to the syncytium cords in matched samples. Both surface and glandular epithelia were negative to the NEP/CD10.

3. Discussion

NEP, a neutral metalloendopeptidase, also named as CD 10 antigen, has been proposed as a marker for normal endometrial stroma in women [26, 27] and is considered a valuable marker for ectopic endometrial stroma identification [27, 30]. In this study, we localized for the first time NEP/CD10 expression in the normal bitch endometrial stroma where it showed a diffuse cytoplasmic staining, similar to that described in human normal endometrium [12, 38]. It was also possible to demonstrate the existence of a temporal variation in the expression pattern during the canine oestrous cycle. In opposition to the described in women, we found NEP/CD10 expression in the canine myometrium. Myometrial positivity for this molecule has also been reported in sheep [32] and in the uterus of pregnant mice [39]. In contrast to that described in sheep [32], a positive reaction for NEP/CD10 in the canine myometrium was found only in the perimysium of the smooth muscle cells, whatever of the myometrial layer considered. A possible explanation is that the differences in the immunostaining intensity observed in small magnifications are due to differences in the arrangement of bundles of smooth muscle fibres in myometrium layers. It has been proposed that NEP/CD10 functions in smooth muscle layers might include oxytocin cleavage and the regulation of uterine contractibility [32, 38]. However, in the present study no significant changes were found between the stages, thus impairing any inference.

The present work also shows that NEP/CD10 expression is observed in cyclic variations according to the phase of the canine oestrous cycle. In women, diffuse NEP/CD10 expression is observed in the functional and basal layer of the endometria, although more intense staining was detected in the deeper portion of the functional layer and in basal layer [12, 28, 38]. In the canine endometrium, the lowest variations in the intensity scores were found for the deep basal stromal layer, which is in accordance with that reported in women, where the basal endometrial stroma has been described as relatively independent of sex steroids influences [38]. In dogs, oestrogen associated stages and anoestrus were the stages with higher NEP/CD10 expression.

The current study also allowed the identification of a small population of NEP/CD10 negative stromal cells located beneath the surface epithelium. As far as we know, there are no previous reports on the existence of a consistently NEP/CD10 negative population of stromal cells in non-luteal stages, despite the report by Riley et al. [32] on the presence of a stromal layer of decreased intensity in sheep endometrium, in both the caruncular and intercaruncular areas of cyclic endometrium. However, the authors did not comment on putative cyclic changes in this pattern of immunostaining. Such a layer would correspond to the stromal compartment described by Johnson et al [40], proving morphological characteristics of decidualization during early pregnancy, which was also osteopontin (OTP) negative.

In the bitch, NEP/CD10 negative cells, located underneath the surface epithelium, are fusiform cells showing an ovoid nucleus of dense chromatin that tend to become round and rough during the progestagenic stages of the oestrous cycle. These NEP/CD10 negative cells in the endometrial stroma of the bitch then share some morphological resemblances with predecidual cells, which are detected in the women endometrium in middle to late secretory phase [41, 42]. The canine NEP/CD10 negative stromal cells express vimentin during the entire oestrous cycle and also desmin during the oestrus and dioestrus (Payan-Carreira, data not shown); furthermore, they are negative for OTP, except during the early dioestrus stage.

Vimentin and desmin expression was also found in predecidual and decidual stromal cells of women and mouse [43-45]. Considering that, in dogs, experimental deciduoma formation is possible in the progesterone-primed uterus [46,47], inducing placenta-like lesions, we consider the possibility of a spontaneous pre-decidualization differentiation of this particular population of stromal cells occurring in the canine endometrium during progesterone-associated stages, favouring decidualization during canine embryo implantation as a part of the endometrial receptivity mechanism in dogs. This pre-differentiation would further progress into decidualization in a process possibly mediated by the embryo.

In the endometrium of the bitch, major temporal differences in NEP/CD 10 expression included: 1) an increased intensity of immunostaining against NEP/CD10 in canine anoestrus, when basal oestrogen and progesterone levels exists in the canine oestrous cycle [48]. This result is similar to the reported by Riley et al. [32] for ovariectomized non-supplemented sheep; 2) a decrease in the overall expression for this molecule during early dioestrus, the phase corresponding to the implantation period for the species. Iwase et al. [49] reported a similar decrease in NEP/CD10 expression in the human endometrium using a semi-quantitative western blot technique. A decrease in NEP/CD10 expression has also been reported in sheep [32], in early dioestrus and again around luteolysis.

Riley et al. [32] report a different pattern for NEP/CD10 expression in the caruncular and intercaruncular areas of the sheep endometrium. In contrast, in canine pregnant endometrium no differences were found from scorings in matching longitudinal cuts, which correlates with the uniform distribution of the glandular elements across the canine endometrium and suggests the inexistence of pre-determined implantations sites in this species. Evidences obtained with different markers, such as integrin αvβ3 [50], TNF [8] and Interleukin 18 [51] further support this feature.

In early pregnant endometrium in human and sheep, a decrease in the NEP/CD10 overall expression has been reported [32]. However, available data is still insufficient to determine the regional pattern of immunoexpression of NEP/CD10 in peri-implantation endometria. In our study, a relative decrease in the intensity of immunolabelling was found in the endometrial stroma at the syncytial cords areas, adjacent to the faint to moderate positivity of trophoblastic cells, similar to human early pregnancies [43]. However, a new fact is the observation of a ring-like stronger positivity for NEP/CD10 at the intermediate peri-lacunar area, suggesting a possible barrier to the progress of embryo invasiveness.

Although useful as a marker of endometrial stroma in human endometriosis, the role of NEP/CD10 in endometrial function remains unclear and needs to be further studied. Initially, the role proposed for this molecule in the endometrial stroma was limited to its catalytic activity over small bioactive peptides, thus decreasing its concentration and inhibiting its local function. A broad range of peptides have been proposed to serve as substrates to NEP/CD 10, such as bradykinin, substance P, atrial natriuretic peptide, interleukin 1β, oxytocin and endothelin [12-14]. Endothelin, interleukin 1β and oxytocin are known to be present in the endometrium of different species [49]. However, additional roles have been advanced in past years for NEP/CD10 besides the regulation of peptide bioactivity, including its interplay with different signalling mechanisms related to cell growth and proliferation, apoptosis and angiogenesis [23, 52, 53]. Studies developed on distinct malignancies and on several immuno-mediated pathologies revealed that NEP/CD10 is involved in the regulation of inflammation [15, 17, 54], in angiogenesis [23], and also in the regulation of cell proliferation [24], migration [14, 18] and apoptosis [19-21].

The endometrial cycle, in women as in other mammal females, integrates a highly precise and orchestrate events that, drove by cyclic sex hormone changes, also include mechanisms of regeneration, cell growth, proliferation and migration, modulation of angiogenesis and neovascularization, as well as the control of invasiveness during embryo implantation. Furthermore, epithelial and stromal interactions in the uterus are major determinants in the

435 success of the endometrial cycle [55]. Moreover, several molecules known to interact with
436 NEP/CD10, such as endothelins, integrins, interleukin 1β and PTEN, have been located in the
437 endometrium of different species [4, 56].
438 The Transforming Growth Factor beta1 (TGF-β1) is an important stimulator of NEP/CD10
439 activity [57]. In dog endometrial stroma, TGF-β1 immunoexpression presents the temporal
440 changes described herein for NEP/CD10 [58]: its immunoexpression was higher in anoestrus
441 and prooestrus and the lowest in early dioestrus, while oestrus and dioestrus presented
442 intermediate scores. Considering the close association between the temporal pattern between
443 these two molecules, and the important role of TGF β family in endometrial remodelling in
444 human endometrium [59], the possibility that NEP/CD10 may also play an important role in
445 cyclic endometrial regeneration, cell proliferation and differentiation phenomena during the
446 endometrial cycle can not be excluded, thus exceeding the functions of a simple stromal
447 marker usually assigned to this molecule.
448 Many NEP/CD10 actions involve the interplay with signal transduction via AkT (also called as
449 protein kinase B) pathways [52], through catalytic-dependent and independent mechanisms.
450 AkT are important pathways for numerous cellular functions in the uterus, such as
451 proliferation, adhesion, migration, angiogenesis and apoptosis [60]. It is important to
452 acknowledge that modulation of AkT pathways in the stromal cell decidualization has been
453 previously demonstrated [61, 62]. Moreover, AkT has been located in human and mice
454 endometrium, in both stroma and epithelial cells, and the phosphorylation of this protein
455 presents temporal variations in the endometrium [63, 64]. Additionally, NEP/CD10 negatively
456 regulates angiogenesis via different signalling mechanisms [23, 52] and small increased
457 vascularization in the superficial stromal layer is observed in the canine endometrium in the
458 initial dioestrus, which becomes more evident when embryos are present [65]. Although not
459 assessed in the present study, it would be reasonable to conjecture that these two molecules
460 might present complementary functions in canine endometrium. Thus, when NEP/CD10
461 expression in endometrial stroma is strong, as we found in canine endometrium during
462 anoestrus, an important stage for endometrial remodelling, impairment of AkT pathways would

463 restrict cell proliferation, reduce cell protection against apoptosis and limited vascularization in
464 the endometrial stroma. Future studies will be endorsed to explore the involvement of AkT
465 pathways in the mechanisms of the canine endometrial cycle.
466 An interesting feature observed in samples from the attachment period concerns the existence
467 of a stronger immunoreaction against NEP/CD10 in the stromal area neighbouring the
468 *lacunae*, while in the syncytium crypts stromal cells are negative. The former feature assumes
469 the appearance of a barrier to embryo invasiveness and may be part of a strategy to limit the
470 superficial endometrial changes during the formation of placenta. The later coexists in an area
471 of intense morphological changes and trophoblast invasion. NEP/CD10 possess inhibitory
472 effects on cell migration that have been described in different organs and neoplasia, where a
473 decrease in NEP/CD10 is envisaged as a prognosis for malignancy and invasiveness [23, 52].
474 Surprisingly, in samples from days 22 and 23 of pregnancy, positive apical staining was
475 noticed in epithelial cells of lacunae. NEP/CD10 is expressed as a typical brush-border
476 enzyme in enterocytes and renal tubules and glomeruli [10]; it is also associated with human
477 trophoblast or chorion tumours [66]. Moreover, some soluble variants of NEP/CD10 have been
478 detected in human urine [67], although it remains unclear if they are produced in a soluble
479 form or are shed from a membrane-bound form. In contrast to the negative expression of
480 NEP/CD10 protein in normal endometrial epithelial cells during the canine cycle, in an early
481 report on endometrial adenocarcinoma, sporadic expression of this protein was found in
482 epithelial cells of the tumour [68]. The exact significance for this finding in canine placental
483 lacunae remains unclear and needs additional studies for clarification.
484
485 In this study, we characterized the distribution and the immunostaining pattern of NEP/CD10
486 expressing cells during the oestrous cycle of the bitch and described changes during early
487 pregnancy. Diffuse NEP/CD10 immunoexpression is consistently observed in the endometrial
488 stroma throughout the oestrous cycle, showing particular changes at different stages of the
489 cycle. In addition, the existence of a sub-surface layer of NEP/CD10 negative stromal cells
490 sharing some morphological characteristics with human predecidual cells was detected. The

results from this study raise interesting questions on the participation of NEP/CD10 in events characterizing the canine endometrial cycle and implantation, although additional studies are needed to highlight the possible down-stream mechanisms and pathways involved in NEP/CD10 function in the canine uterus.

5. Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Acknowledgements

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8. Figure Legends

Figure 1 – Western blot for anti-CD10 showing bands corresponding in size to the 100 kDa protein in extracts of canine endometrium.

Figure 2 – Immunohistochemical expression of NEP/CD10 in normal canine endometrium (counterstained with Gill´s Haematoxylin; bar: 100 µm). Overall, epithelial endometrial cells are negative for this protein. (A) In anoestrus, an uniform strong immunoreaction against NEP/CD10 was observed in the endometrium. (B) In prooestrus, though a homogeneously strong to moderate intensity of immunostaining was observed in the endometrium, the intense oedema difficult the visualization of the immunostaining. On the image are depicted the upper stromal (UpS) and the deep basal (DbS) stromal areas. (C) In early dioestrus and dioestrus, NEP/CD10 expression was decreased compared to non-progesterone stages of the cycle. (D) In higher magnifications, in anoestrus as in prooestrus it was clearly identified a subsurface layer negative for NEP/CD10. (E) The subsurface stromal layer (SSL) remains negative for most of the oestrus length; the surface epithelium (SE) and of the glandular epithelium (GE) are negative for NEP/CD10. (F) In early dioestrus and dioestrus all the stromal cells in the subsurface layer are positive to NEP/CD10. (G) Strong to moderate intensity of immunolabelling was found in myometrium (Myo), were positivity was identified in cells perimysium, while the myocytes remained negative. (H) Detail of the deep endometrial layer showing a moderate intensity of immunolabelling; it was also often found that an increased density of stromal cells around the deep glandular elements (GE – glandular epithelium) may confer to this particular area the appearance of increased intensity.

Figure 3 – Immunohistochemical expression for NEP/CD10 in the early stages of canine pregnancy (PGr2) (counterstained with Gill´s Haematoxylin; bar: 100 µm). In low magnifications (images A to C), the immunoreaction against NEP/CD10 showed a ring-like area of increased intensity of immunostaining (arrow) surrounding the invading trophoblast, compared to the faint intensity of labeling in the remainder of the endometrial stroma. (A) On

pregnancy day 15. (B) Around pregnancy day 17. (C) After pregnancy day 22, this ring-like area was reduced. (D) A detail of the superficial area in the implantation area on day 15, focusing the ring-like area, displaying increased intensity of immunolabelling along with an increased density of stromal cells. (E) Detail of the upper endometrial area on day 17 with the decidual cells showing strong intensity of immunolabelling, while the endometrial epithelia (SE – surface epithelium; GE – Glandular epithelium) remain negative for NEP/CD10; embryo membranes (EM), still unattached, show faint intensity of immunolabelling. (F) After day 22, cells from the cords or lamellae (LamE) are negative to NEP/CD10. A faint positive reaction was recorded in the invading trophoblast (TC), while the lamellar stroma (LamS) showed faint to moderate immunoreaction. (G) By day 22, the epithelial cells of lacunae displaying an epical reinforcement of immunostaining. (H) In the interplacental areas the upper endometrial stroma (UpS) showed a strong immunostaining similar to the observed surrounding the invading trophoblast in matched samples.

Graph 1 – Graphic representation of the overall intensity scores for NEP/CD10 in canine endometrium (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DbS – deep basal layer; SE - Surface Epithelium; GE - Glandular Epithelia).

Graph 2 – Graphic representation of the temporal variations in the relative intensity scores for NEP/CD10 in the different endometrial layers (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DBS – deep basal layer; SE - Surface Epithelium; GE - Glandular Epithelia) during the canine oestrous cycle.

Graph 3 – Graphic representation of the relative intensity scores for NEP/CD10 in the pre-attachment period of canine pregnancy (PGr1) and the non-pregnant endometrium in early and full dioestrus.

741 **Graph 4** – Graphic representation of the relative intensity scores for NEP/CD10 in the

742 attachment period of canine pregnancy (PGr2).
743

744

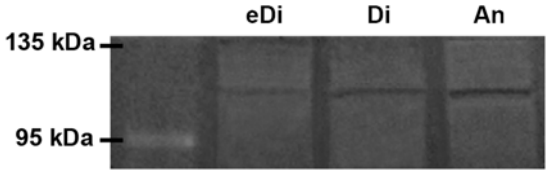
Table 1 – NEP/CD10 immunoreactivity scores in the endometrial samples of female dogs throughout the stages of the oestrous cycle and in pre-attachment PGr1.

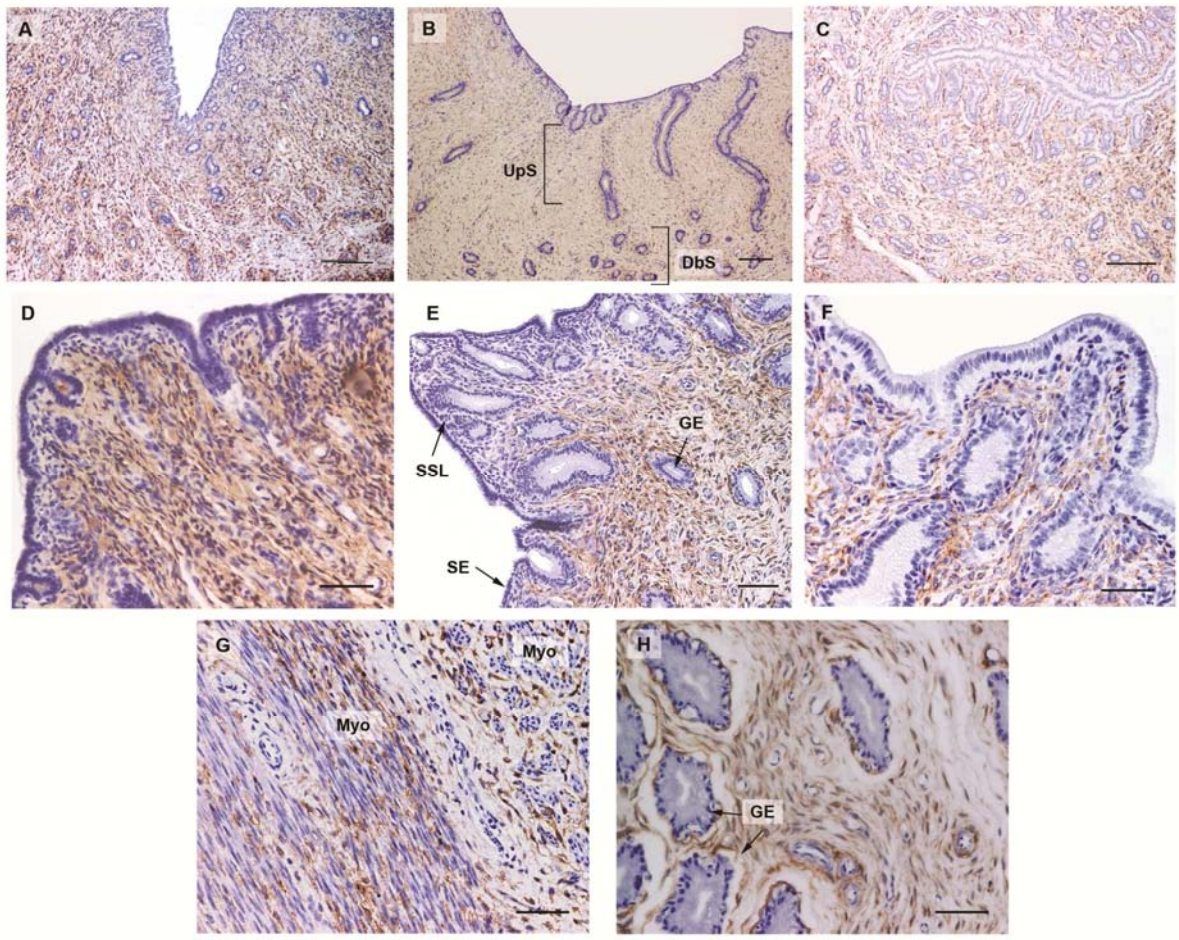
	Scores	Anoestrus (n=10)	Prooestrus (n=9)	Oestrus (n=8)	Early Dioestrus (n=10)	Dioestrus (n=10)	PGr1 (n=5)
SSL	neg	10	9	6	0	0	0
	1	0	0	2	10	8	2
	2	0	0	0	0	2	3
	3	0	0	0	0	0	0
UpS	neg	0	0	0	0	0	0
	1	0	1	4	9	1	2
	2	2	7	4	1	8	3
	3	8	1	0	0	1	0
DbS	neg	0	0	0	0	0	0
	1	2	1	3	3	3	0
	2	1	2	4	5	3	5
	3	7	6	1	2	4	0
SE	neg	10	9	8	10	10	5
	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
GE	neg	10	9	8	10	10	5
	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0

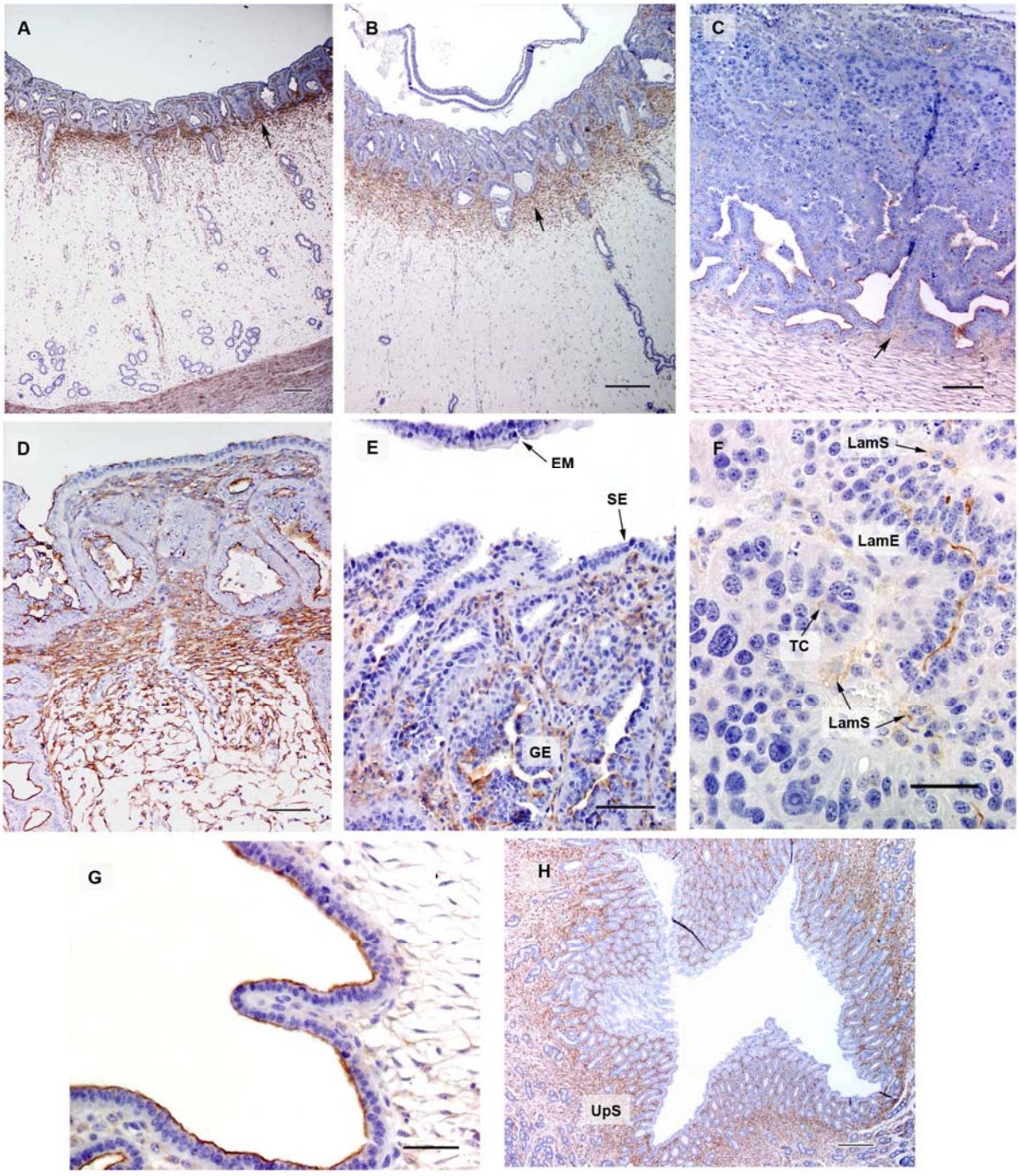
(SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DbS – deep basal layer; SE - Surface Epithelium; GE - Glandular Epithelia).

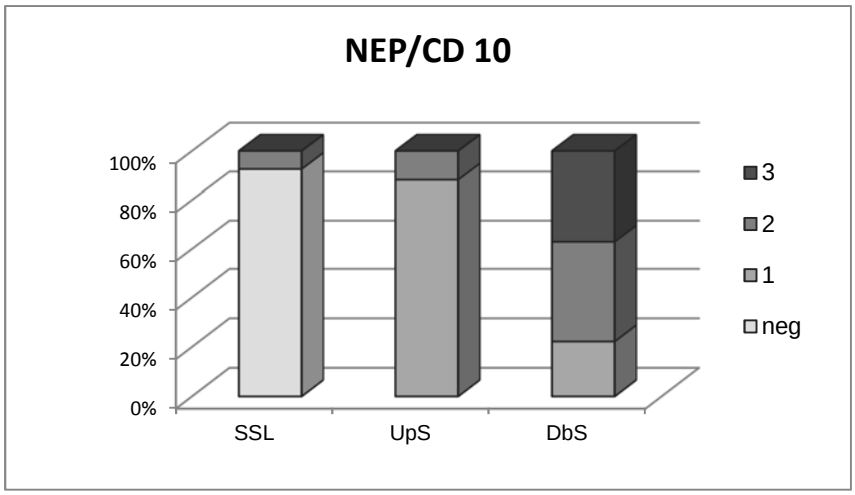
Table 2 – NEP/CD10 immunoreactivity scores in the implantation area in samples from canine **pregnancy days 16 to 23** (attachment period, PGr2).

Scores	**Trophoblast**	**Decidual cells**	**Lamellar stroma**	**Lamellar epithelium**	**Intermediate stroma**	**Lacunar epithelium**	**DGE**	**Deep stroma**
neg	0	0	2	12	0	0	12	0
1	5	4	8	0	1	12	0	9
2	7	8	2	0	2	0	0	3
3	0	0	0	0	9	0	0	0

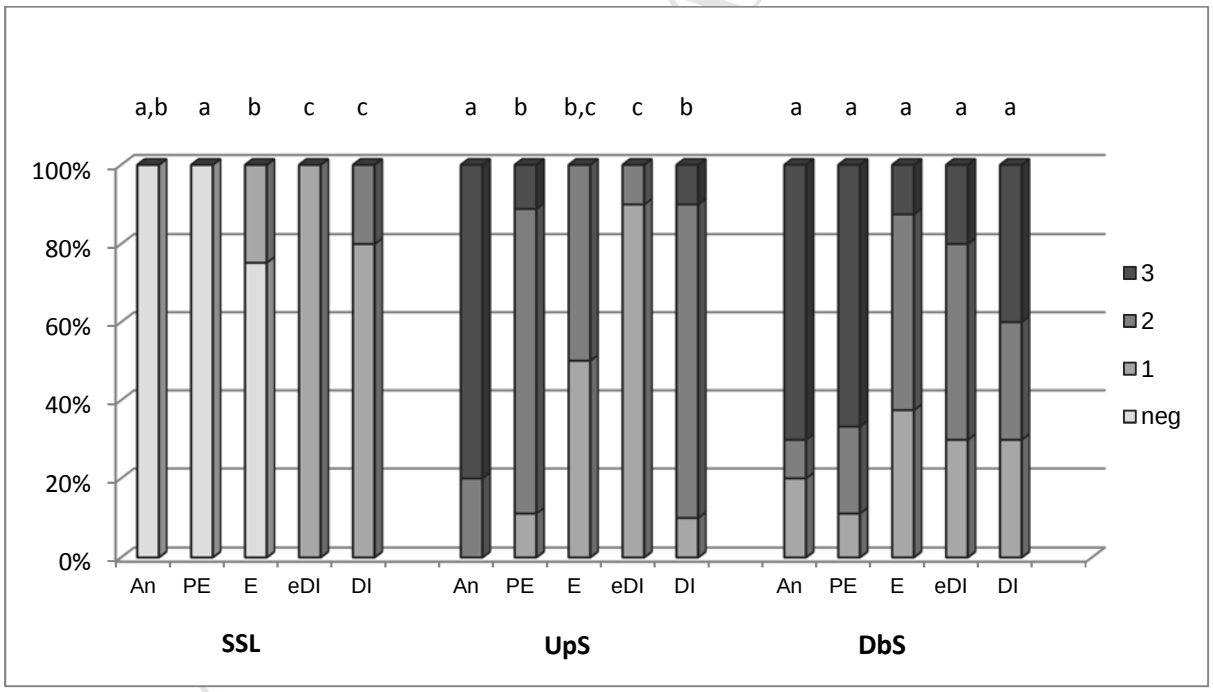




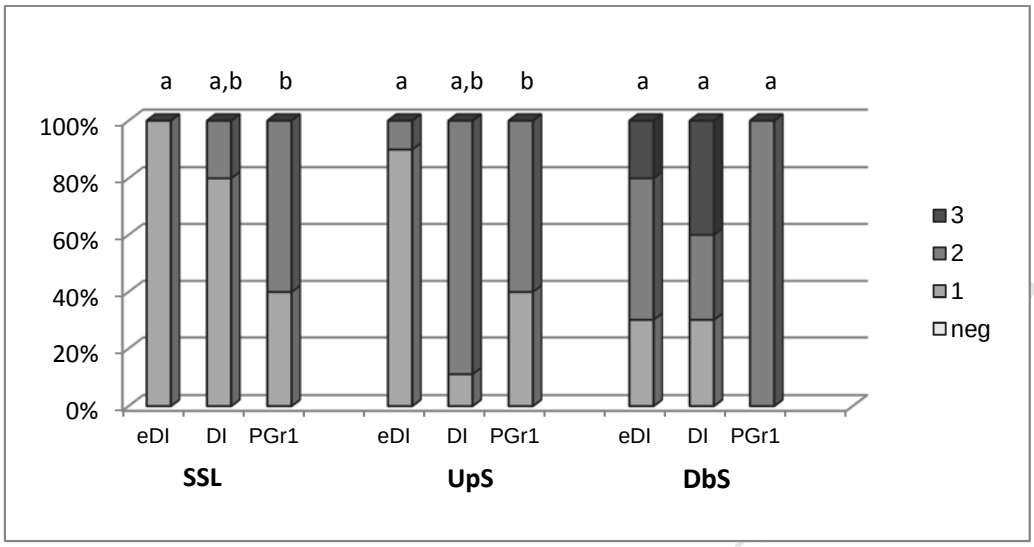




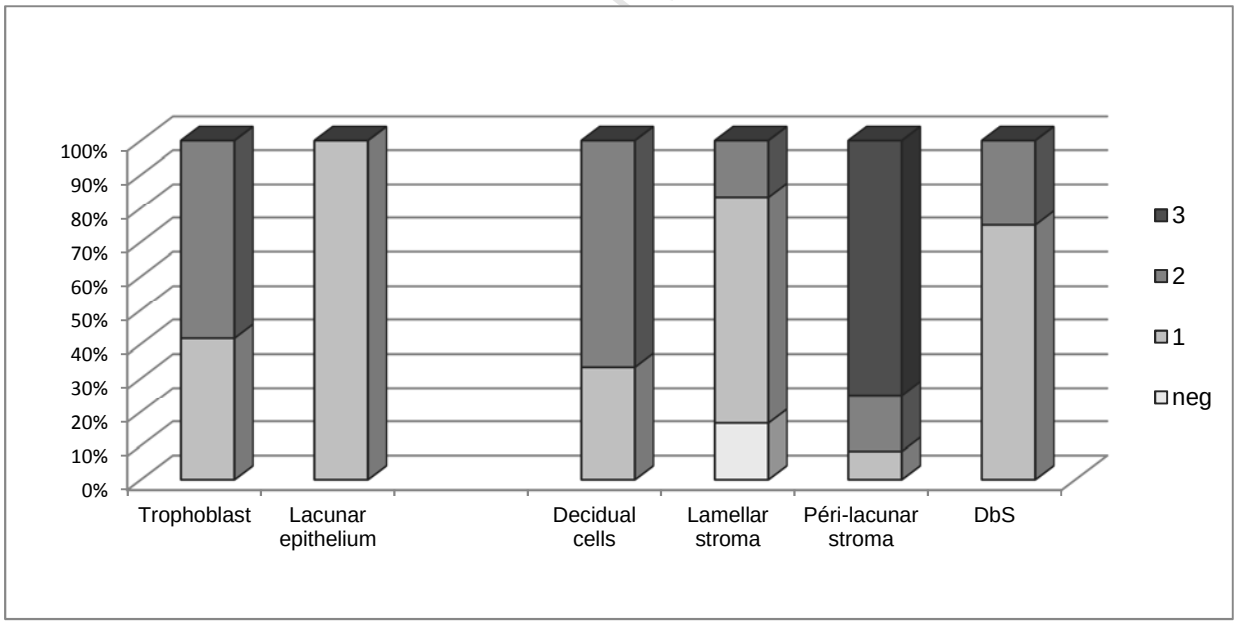
Graph 1 – Graphic representation of the overall intensity scores for NEP/CD10 in canine endometrium (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DbS – deep basal layer; SE - Surface Epithelium; GE - Glandular Epithelia).



Graph 2 – Graphic representation of the temporal variations in the relative intensity scores for NEP/CD10 in the different endometrial layers (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DBS – deep basal layer; SE - Surface Epithelium; GE - Glandular Epithelia) during the canine oestrous cycle.



Graph 3 – Graphic representation of the relative intensity scores for NEP/CD10 in the pre-attachment period of canine pregnancy (PGr1) and the non-pregnant endometrium in early and full dioestrus.



Graph 4 – Graphic representation of the relative intensity scores for NEP/CD10 in the attachment period of canine pregnancy (PGr2).

Highlights

We revised the staging of canine early pregnancy as requested.

We reformulated the sentences that were less clear, containing overinterpretation or that could be misinterpreted

Some concerns pointed on material and methods section were clarified

Western blot experiences were replicated and new images obtained. Also some labels for different endometrial structures were introduced in Figures 2 and 3

However, images with the Vimentin and Desmin staining were not shown in the manuscript. Pondered the benefits and the inconveniences of the need to add new descriptions for those markers (either in material and method as in the results sections), the authors decided not to provide this information in the current manuscript.

An English revision of the final manuscript form was performed.