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Membrane-bound progesterone receptors in the canine uterus and placenta; possible targets in the maintenance of pregnancy

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ABSTRACT

To date, the biological functions of P4 within the canine placenta have been attributed to maternal stroma-derived decidual cells as the only placental cells expressing the nuclear P4 receptor (PGR). However, P4 can also exert its effects via membrane-bound receptors. To test the hypothesis that membrane-bound P4 receptors are involved in regulating placental function in the dog, the expression of mPRα, -β, -γ, PGRMC1 and -2 was investigated in the uterine and placental compartments derived from different stages of pregnancy and from prepartum luteolysis. Further, to assess the PGR signaling-mediated effects upon membrane P4 receptors in canine decidual cells, *in vitro* decidualized dog uterine stromal (DUS) cells were treated with type II antigestagens (aglepristone or mifepristone). The expression of all membrane P4 receptors was detectable in reproductive tissues and in DUS cells. The main findings indicate their distinguishable placental spatio-temporal distribution; PGRMC2 was predominantly found in decidual cells, PGRMC1 was strong in maternal endothelial compartments, and syncytiotrophoblast showed abundant levels of mPRα and mPRβ. *In vitro* decidualization was associated with increased expression of PGRMC1 and -2, while their protein levels were diminished by antigestagen treatment. The involvement of membrane-bound P4 signaling in the regulation of canine placental function is implied, with P4 effects being directly exerted through maternal and fetal cellular compartments. The indirect effects of PGR might involve the modulation of membrane-bound receptors availability in decidual cells, implying a self-regulatory loop of P4 in regulating the availability of its own receptors in the canine placenta.

1. Introduction

Luteal progesterone (P4) is responsible for successful pregnancy in the dog, exerting morphofunctional effects within the utero-placental compartments required for the initiation, maintenance, and termination of pregnancy [1]. Interaction with its nuclear receptor (PGR) is the most investigated signaling P4-related pathway, shown to be responsible for a wide range of P4-mediated biological effects [2–4]. Binding of P4 to the PGR activates the genomic (canonical) pathway of P4 signaling, by employing the two main isoforms of PGR, PGR-A and -B, that are encoded by the same gene but transcribed by utilizing distinct promoters [5,6]. This combination dimerizes and translocates to the nucleus to activate the transcription of target genes [3]. The importance of this signaling pathway is highlighted in the species-specific regulatory mechanisms of canine pregnancy, with a placenta devoid of steroidogenic activity, and the maintenance of pregnancy being fully dependent on luteal P4 and placental PGR interaction [7]. This interaction requires the response of the

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canine decidual cells, as the only placental cell population expressing PGR [8,9], to circulatory P4. The preterm decrease in circulatory levels of P4 signals the onset of parturition, recruiting the fetal trophoblast cells to activate the cellular prostaglandin machinery leading to the prepartum output of luteolytic PGF2α [1,9,10]. Moreover, blockage of decidual cell-derived PGR via antigestagens (competitive P4 antagonists) results in the disruption of P4-PGR signaling and activation of the placenta-derived luteolytic cascade leading to termination of pregnancy [11].

In addition to the genomic pathway, physiological actions of P4 can be conveyed through activation of non-canonical (non-genomic) pathways by utilizing its membrane bound receptors (mPRs) [12], and P4 receptor membrane component (PGRMC) 1 and −2 [13,14], presenting specific binding sites with high affinity to P4 [15–18]. mPRs belong to the progestin and adipoQ receptor (PAQR) family transduced through the activation of G protein-coupled receptors [12,19]. To date, five distinct subtypes of mPRs have been identified (α, β, γ, δ, ε) [20], with most of the mPR-mediated effects in the reproductive system appearing to arise from activation of the α and β subtypes (encoded by *PAQR7* and *-8*) [21–23]. These receptors are 7 transmembrane proteins binding P4 [19] and activating signaling pathways involving, i.a., MAPKs, leading to the phosphorylation of different transcription factors [24]. Furthermore, PGRMCs, as the transmembrane protein receptors of approximately 24–28 kDa [25,26], can bind to P4 in the presence of SERPINE1 mRNA binding protein (SERBP1) and cause the proliferative and antiapoptotic effects in different cell populations [27–29] through activation of AKT and PKG [30–32]. Recruiting the intracellular signaling elements and second messengers, like cAMP and downstream kinases, e.g., MAPKs and PKG [33,34], nongenomic pathways act faster than genomic pathways in provoking the cellular responses to P4 [35–37]. The expression of both the PGRMC and mPR families has been detected in the reproductive tract of different species [12,28,38–45]. Their functions were associated with the maturation of ovarian follicles [46], sperm motility [23], regulation of uterine function [47], and myocontractile activity [34]. Interestingly, although the importance of a well-orchestrated expression of membrane P4 receptors in the female reproductive tract, and the effects of disruption of these signaling pathways on the physiology of reproductive tissue in pregnant and nonpregnant animals of different species, have been addressed [47–50], there is a lack of knowledge regarding their availability and function in canine uterine and placental compartments.

Consequently, due to their PGR expression, decidual cells have been considered to be the sole placental player in P4 signaling in pregnant bitches. However, we hypothesized that: i) membrane-bound progesterone receptors are present in the canine uterine and placental compartments; ii) their expression is modulated in a spatio-temporal manner throughout gestation; and iii) P4/PGR signaling might be involved in regulating their availability in canine decidual cells, as well as in other cellular compartments of the canine placenta. To fill this knowledge gap and to provide a basis for further studies of P4 signaling pathways in the canine reproductive tract, the current study was designed to investigate the uterine and placental expression of membrane bound P4 receptors (mPRα, -β, and -γ [encoded by *PAQR7, -8* and *-5*], and PGRMC1 and -2) during different stages of pregnancy. The functional approach was achieved by employing our *in vitro* decidualization model with dog uterine stromal (DUS) cells [51,52] and investigating the effects of functional PGR withdrawal on the expression of membrane-bound receptors in decidualized DUS cells.

2. Material and methods

2.1. Tissue sampling

A total of 27 uterine and utero-placental samples from clinically healthy, mature crossbred dogs were used for the current study. All tissue samples used for this study had been used previously, and the procedures for estimation of the pregnancy stages and tissue collection have been described in previous publications [9,52–56]. Animal experimental procedures were performed according to the requirements of animal welfare legislation and approved by the respective authorities of Justus-Liebig University, Giessen, Germany (permit no. II 25.3–19c20-15c GI 18/14 and VIG3-19c-20/15c GI 18,14) and of the University of Ankara, Turkey (permits no. Ankara 2006/06 and 2008-25-124). Samples from animals submitted to routine ovariohysterectomy at the Section of Small Animal Reproduction, Vetsuisse Faculty, Zurich, Switzerland and samples from day 17 of pregnancy collected at the "Hospital Veterinário do Baixo Vouga", Águeda, Portugal, were collected after informed consent of the owners.

In brief, day of ovulation was predicted for each dog based on measuring the circulatory P4 levels using radioimmunoassay [57], and vaginal cytology. The day when the serum concentration of P4 exceeded 5 ng/ml was considered as the day of ovulation. Bitches were mated 2 days after ovulation (day 0 of pregnancy), and uterine and placental samples were collected at different stages of pregnancy. Depending on pregnancy stage, uterine samples or utero-placental complexes (i.e., uterus with adjacent placenta) were allotted to the following groups: non-pregnant (E−, days 8–12 after mating, n = 5), pre-implantation (E+, days 8–12 after mating, n = 5), implantation (Day 17, n = 3), post-implantation (Post-Imp, days 18–25 of pregnancy, n = 5), mid-gestation (Mid-Gest, days 35–40 of pregnancy, n = 5), and prepartum luteolysis (n = 4). In the dog, implantation takes place on day 17 [58,59], thus, for those samples collected prior to implantation (E+), embryo flushing was performed. Animals that did not have embryos recovered by flushing were included in the E−group. For the luteolysis group, samples were collected during active prepartum P4 decline, ascertained by measurements of serum P4 at 6 h intervals beginning on day 58 of pregnancy. Therefore, taking into account that physiologically parturition in dogs occurs 12–42 h following the luteolytic P4 drop [7,60], tissue samples were collected when P4 levels dropped below 3 ng/ml in three consecutive measurements, which was interpreted as the onset of the prepartum luteolysis. Following ovariohysterectomy, tissue samples were trimmed of the connective tissue and washed with cold PBS. To preserve the samples for gene expression profiling, samples were immersed in RNAlater solution (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24 h at +4 °C and stored at −80 °C until further use. For histological studies, samples were fixed with 10% neutral phosphate-buffered formalin for 24 h at +4 °C, and then washed with PBS for 7 days, dehydrated by passage through a graded ethanol series and embedded in the paraffin equivalent Histo-Comp (Vogel, Giessen, Germany).

2.2. Cell culture and in vitro experiments

The previously described methods were utilized in the culture and use of immortalized DUS cells in experimental procedures [52,61,62]. Briefly, cells were cultured in 150 cm^2 cell culture flasks (Corning, New York, NY, USA) using maintenance medium containing DMEM-High Glucose (4.5 g/L; Bio Concept, Allschwil, Switzerland; pH 7.2–7.4), supplemented with 10% heat inactivated fetal bovine serum (FBS; Thermo Fisher Scientific AG, Reinach, Switzerland), 100 U/mL penicillin, 100 ug/ml streptomycin from

PANBiotech (Aidenbach, Germany), and 1% insulin-transferrin-selenium (ITS; Thermo Fisher Scientific AG). After DUS cells reached at least 80% confluency, they were trypsinized, and seeded at 2×10^5 cells per well in 6-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland). The cells were then pre-incubated with maintenance medium for 24 h to adhere and recover, before they were incubated with stimulation medium i.e., maintenance medium containing 0.01% bovine serum albumin (BSA; SUB001, Canvax Biotech, Córdoba, Spain) instead of FBS. Following the previously established protocol [51,52], to induce *in vitro* decidualization, DUS cells were incubated with stimulation medium supplemented with 0.5 mM dbcAMP (D0627, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 72 h. To observe the effects mediated by blockage of PGR on membrane P4 receptors, decidualized DUS cells were incubated with stimulation medium containing different concentrations (0.5, 1.0 and 2.0 μM) of two type II antigestagens, aglepristone (Batch No: 2064665, provided by Virbac, Tierarzneimittel GmbH, 23843 Bad Oldesloe, Germany) or mifepristone (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for an additional 6 h, a time course previously shown to evoke the antigestagen-mediated effects in decidualized DUS cells [62]. The cells treated with stimulation medium without cAMP (non-decidualized cells) or blocking components (decidualized cells) served as controls in experiments utilizing antigestagens. All *in vitro* experiments were performed under standard culture conditions (i.e., 37 °C, 5% CO_2 in air, in a humidified incubator).

2.3. Immunohistochemical (IHC) staining

Immunohistochemical (IHC) staining was used to detect the expression of membrane P4 receptors in uterine and placental compartments according to the previously published indirect peroxidase staining method [63,64]. Briefly, formalin-fixed tissue samples embedded in paraffin were cut into 2–3 μm thick sections, mounted on Super Frost microscope slides (Menzel-Glaeser, Braunschweig, Germany), deparaffinized in xylene and rehydrated through a graded ethanol series to water. Then, sections were heated in 10 mM citrate buffer (pH 6.0) using a microwave oven at 600 W for 15 min for antigen retrieval. Afterwards, endogenous peroxidase activity was quenched by immersing the slides into the 0.3% hydrogen peroxide mixed with methanol for 30 min on a shaker at ambient temperature. To avoid non-specific staining, slides were incubated with TNB blocking buffer (0.1 M Tris-HCL, pH 7.2; 4.5 g NaCl, 0.5 g BSA, 0.5 g Top-Block: 37766, Sigma-Aldrich Chemie GmbH) and 10% goat serum for 30 min at ambient temperature. Then, primary antibodies (Table 1) were added to the slides and incubated at +4 °C overnight, following which the slides were incubated for 30 min at ambient temperature with biotin-labeled secondary antibodies (goat anti-rabbit IgG or horse anti-mouse IgG; both from Vector Laboratories Inc., Burlingame, CA, USA). All antibodies were diluted in IHC buffer (0.8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, and 137 mM NaCl, containing 0.3% Triton X; pH 7.2–7.4). Then the slides were incubated with the avidin-peroxidase Vectastain ABC kit (Vector Laboratories Inc.). The peroxidase activity was detected with a Liquid DAB + substrate kit (Dako Schweiz AG, Baar, Switzerland). Subsequently, slides were immersed in tap water, counter-stained with hematoxylin and mounted with Histokit (Assistant, Osterode, Germany). For isotype (negative) controls, slides were overlaid with IgGs from non-immunized animals from the same species and at the same concentration as for the primary antibodies (rabbit IgG and mouse IgG, all from Vector Laboratories Inc.); representative pictures are shown in Figs. 3 and 4. The complete list and description of primary and secondary antibodies used for this study are given in Table 1.

2.4. Total RNA isolation, reverse transcription (RT) and real time (TaqMan) qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. As for the *in vitro* experiments, at the end of experiments, TRIzol was added to cells which had previously been washed with ice cold PBS. Further steps, including cDNA preparation and performing qPCR, were performed as previously described [63,65]. In short, a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific AG) was used to measure the RNA concentration and the purity of each sample. Possible genomic DNA contamination was removed by RQ1 RNA-free DNase Kit (Promega, Dübendorf, Switzerland) following the manufacturer's instructions, using 1.3 μg RNA of each sample. Then, RT was performed using random hexamers as primers and other RT reagents (Applied Biosystems by Thermo Fisher, Waltham, MA, USA). Semi-quantification of selected genes was performed by real-time (TaqMan) PCR using an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems by Thermo Fisher Scientific). Autoclaved water or non-reverse transcribed RNA (RT-minus control) were used instead of cDNA and considered as negative controls. The detailed description of primers and TaqMan probes used for this study is given in Table 2. While the commercially available TaqMan systems (for *PAQR5*, *PGRMC1*, and *-2*) were ordered from Applied Biosystems, TaqMan systems for *PAQR7* and -8 were not commercially available. Additionally, both canine-specific sequences were available from the GenBank only in their predicted forms. Thus, in order to provide templates for downstream analyses, both partial sequences were amplified and cloned utilizing a pGEM-T vector – mediated subcloning approach and commercially sequenced (Microsynth AG, Balgach, Switzerland). The following primers were used: *PAQR7* forward: 3′-GCG TCC TTC ACC TAC CTC TC-5′ and reverse: 3′-GAA CGC GGT CAG

Table 1
List of antibodies used for immunohistochemistry (IHC) staining and western blot (WB) analysis.

Antibody	Company	Reference Number	Host	Dilution
PGRMC1	Atlas	HPA002877	Rabbit polyclonal	IHC 1:300 WB 1:250
PGRMC2	Sigma	WH0010424M4	Mouse monoclonal	IHC 1:300 WB 1:200
mPRα	Atlas	HPA046936	Rabbit polyclonal	IHC 1:250
mPRβ	Sigma	SAB1302273	Rabbit polyclonal	IHC 1:250
mPRγ	Biorbyt	ORB312687	Rabbit polyclonal	IHC 1:500
ACTB	Santa Cruz Biotechnology	sc-69879	Mouse monoclonal	WB 1:1000
Goat anti rabbit IgG (H + L), biotinylated (secondary IgG)	Vector Laboratories	BA1000	Goat	IHC 1:100
Horse anti-mouse IgG (H + L), biotinylated (secondary IgG)	Vector Laboratories	BA-2000	Horse	IHC 1:100
Goat anti-mouse HRP-labelled secondary IgG	Promega	W402B	Goat	WB 1:15000
Goat anti-Rabbit IgG (H + L) secondary antibody, HRP	Thermo Fisher Scientific	31460	Goat	WB 1:15000

Table 2
List of all TaqMan system used for the semi-quantitative RT-PCR.

Primer Gene Name	Accession Numbers	Primer Sequence		Product Length (bp)
PAQR7	OQ200656	Forward	5′-GCA GGC CAA GTC CGA GTT CT-3′	117
		Reverse	5′-CGG GCT CGA TGG CGT AGT A-3′	
		TaqMan probe	5′-CGT GTA CCA GTT CGG CAG CGC C-3′	
PAQR8	OQ200657	Forward	5′ -GGC TGC TGC TAC GCC AAA T-3′	69
		Reverse	5′ -CAC CAC CTG ACA CAG CTT CCT-3′	
		TaqMan probe	5′ -ACC GCA GGC CTT ACC CGG TCA-3′	
PAQR5	XM_005638542.3	Applied Biosystems, prod nr. Cf02652109_m1		83
PGRMC1	NM_001195148.1	Applied Biosystems, prod nr. Cf02706380_G1		80
PGRMC2	XM_005631808.2	Applied Biosystems, prod nr. Cf02699054_m1		164

GAC ACT G-5' (product size: 657 bp), and PAQR8 forward: 3′-CCT TCT ACT TCG TGG ACT ACG TG-5′ and reverse: 3′-CTG GCC TTG ACT TTG TGT C-5' (product size: 619 bp). Subsequently, in-house designed primers and probes were ordered from Microsynth. The detailed procedure of cloning has been described previously [63,65,66]. The newly sequenced fragments of genes are available from the GenBank: *PAQR7* acc. Number: OQ200656, and *PAQR8* acc. Number OQ200657. The efficiency of assays was evaluated to ensure approximately 100%, as previously described [63,67]. The control group served as the calibrator in the ΔΔCT method, and three reference genes (KDM4A, EIF4H and PTK2) were used for normalization of the CT values [68].

2.5. Protein preparation and western blot analysis

Western blot analysis was performed in accordance with the previously published protocol [69]. In brief, following the termination of each experiment, cells in a 6-well plate were washed with ice cold PBS and were scraped in the presence of NET-2 lysis buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.05% NP-40) containing 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Samples were homogenized via Vibra-Cell 75186 (Sonics & Materials, Inc., Newtown, CT, USA) at 75W, two times for 10s. The protein concentrations were measured by the Bradford assay utilizing a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Munich, Germany) and were normalized with sample buffer (25 mM Tris-Cl, pH 6.8, 1% SDS, 5% mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). Protein samples (20 μg/sample/lane) were subjected to electrophoresis in a 15% polyacrylamide gel at 120 V (Bio-Rad Laboratories) and transferred onto methanol activated polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) for 1 h at 100 V. After blocking any non-specific binding via 5% low-fat dry milk diluted in PBST (PBS/0.25% Tween20) for 1 h at ambient temperature, membranes were probed with primary antibodies overnight at 4 °C (Table 1). The following day, the membranes were washed with PBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at ambient temperature, then washed with PBST five times each for 10 min and soaked in Super Signal West Chemiluminescent Kit substrate (Thermo Fisher Scientific AG, Reinach, Switzerland) according to the manufacturer's protocol. Signals were visualized utilizing the Chemi-Doc XRS + System and Image Lab Software (Bio-Rad Laboratories) and were quantified via ImageJ Software (US National Institutes of Health, Bethesda, Maryland, USA). For this, the signal intensity of each band was normalized to the respective ACTB stained on re-blotted membranes and compared with the other experimental group. The respective results are presented as standardized optical density (SOD). The original western blots are presented in Supplementary File 1. Complete information regarding the antibodies used for the western blot analysis is provided in Table 1.

2.6. Statistical analysis

All cell culture experiments were performed independently at least three times using different consecutive passages (biological replicates [70]). All statistical analyses were performed using GraphPad 3.06 Software (GraphPad Software, San Diego, CA, USA). For each comparison, parametric one-way ANOVA was performed, and if the p value was less than 0.05, this was followed by the Tukey-Kramer multiple comparisons post-hoc test to perform a statistical analysis between all experimental groups. Numerical data are presented as geometric mean (Xg) ± geometric standard deviation (SD) for mRNA relative quantification from tissue, and mean ± standard deviation (SD) for cell culture experiments.

3. Results

3.1. Spatio-temporal distribution patterns of P4 membrane receptors in canine uterine and placental compartments during pregnancy

The expression of transcripts encoding for all investigated mPRs and PGRMCs was detectable in the uterus of non-pregnant, and the uterine and utero-placental compartments of pregnant animals. In comparison with non-pregnant animals (E−), initiation of pregnancy (E+) was associated with decreased expression of *PGRMC1* ($p < 0.001$; Fig. 1A), *PAQR5* (encoding for mPRγ; $p < 0.01$; Fig. 2A) and *PAQR8* (encoding for mPRβ; $p < 0.01$; Fig. 2C), while the presence of free-floating embryos increased the expression of *PGRMC2* ($p < 0.001$; Fig. 1B). Then, towards implantation (Day 17), there was a significant increase in the expression of *PAQR8* compared with E+ ($p < 0.01$; Fig. 2C), with suppression of *PGRMC2* ($p < 0.001$; Fig. 1B). The expression of other receptors was not affected by embryo presence ($p > 0.05$). Following the day of implantation (Day 17), the mRNA levels of *PGRMC1* continued to decrease and were the lowest during prepartum luteolysis ($p < 0.01$; Fig. 1A). Conversely, the expression of *PGRMC2* began to increase, and was significantly elevated in utero-placental compartments starting from Post-Imp until prepartum luteolysis, compared with the day of implantation (Day 17) ($p < 0.01$; statistical details in Fig. 1B). The transcript levels of *PAQR5* were strongly upregulated at mid-gestation compared with all previous gestational stages ($p < 0.05$; statistical details in Fig. 2A) and did not change significantly during prepartum luteolysis ($p > 0.05$). The expression of *PAQR7* (encoding for mPRα) and *-8* was decreased at Post-Imp compared with Day 17 ($p < 0.001$; Fig. 2B and C). Although *PAQR7* mRNA decreased ($p < 0.001$) at Post-Imp and, together with *PAQR8*, remained low, the expression of *PAQR8* was higher in utero-placental compartments during Mid-Gest compared with Post-Imp ($p < 0.05$).

As determined by IHC (Figs. 3 and 4), apparent differences in signal intensity were observed between the early pregnant uteri (E+) and their dioestric counterparts (E−), with membrane

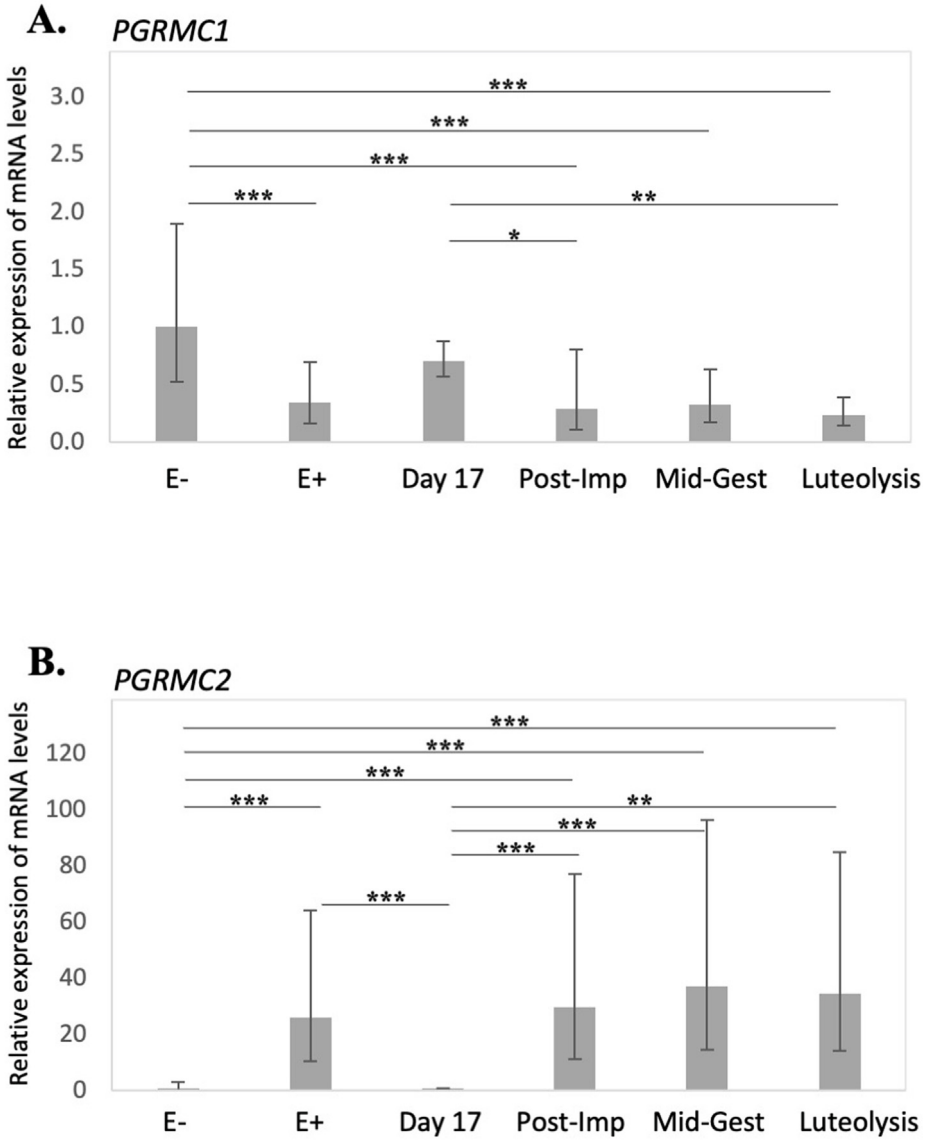

Fig. 1. Relative gene expression of P4 receptor membrane component (*PGRMC*) *1* and *-2* in canine uterine and placental compartments during different stages of pregnancy determined by semi-quantitative real time (TaqMan) PCR. E−/E+ = Embryo −/+ (n = 5), Day 17 = day of implantation (n = 3), Post-Impl = post-implantation (n = 5), Mid-Gest = mid-gestation (n = 5), Luteolysis = prepartum luteolysis (n = 4). One-way ANOVA was applied to test the effects of different pregnancy stages on gene expression, revealing: $p < 0.0001$ for *PGRMC1* (A) and *PGRMC2* (B) throughout pregnancy. When $p < 0.05$, the analysis was followed by a Tukey-Kramer multiple-comparisons test. Numerical data are presented as geometric mean (Xg) ± geometric standard deviation (SD). Bars with different asterisks differ at: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

receptors being predominantly associated with uterine epithelial compartments. The presence of free-floating embryos appeared to have a particularly strong effect on the expression of PGRMC1 and mPRβ (Figs. 3 and 4). The myometrial staining appeared to follow the endometrial staining intensity. Following implantation and placentation, uterine signals of all factors were abundantly present in epithelial compartments of superficial glands (glandular chambers) (Figs. 3 and 4). In the placental labyrinth, the following specific localization patterns were observed: there was clearly detectable specific localization of PGRMC1 in endothelial cells of maternal capillaries (Fig. 3C, panel 1); PGRMC2 stained strongly in decidual cells (Fig. 3G, panel 1); and mPRα and -β seemed to be mostly associated with trophoblast cells, particularly within its syncytiotrophoblast population (Fig. 4C and G, panels 1). Following the progression of pregnancy towards prepartum luteolysis the signal intensity of all factors appeared to be weaker. The mPRγ seemed to be distributed ubiquitously throughout utero-placental compartments with apparently lower signal intensity during prepartum luteolysis (Fig. 4K and L).

3.2. The modulatory effects of PGR signaling on PGRMC1 and -2 in DUS cells

The decidualization of DUS cells was associated with an increased abundance of transcripts for *PGRMC1* and *-2* ($p < 0.05$, details in Fig. 5A, B, 6A, 6B), but did not affect the expression of the other receptors ($p > 0.05$). Antigestagens had relatively weak effects on the expression of *PGRMC1* at the mRNA level in decidualized DUS cells, with only 1.0 μM aglepristone showing a significant effect on its expression ($p < 0.05$; Fig. 5A), while the other dosages of aglepristone or mifepristone treatment had no effect on expression of this factor ($p > 0.05$; Figs. 5A and 6A). Conversely, they had stronger effects on the transcript availability of *PGRMC2*, which were suppressed significantly by all dosages of

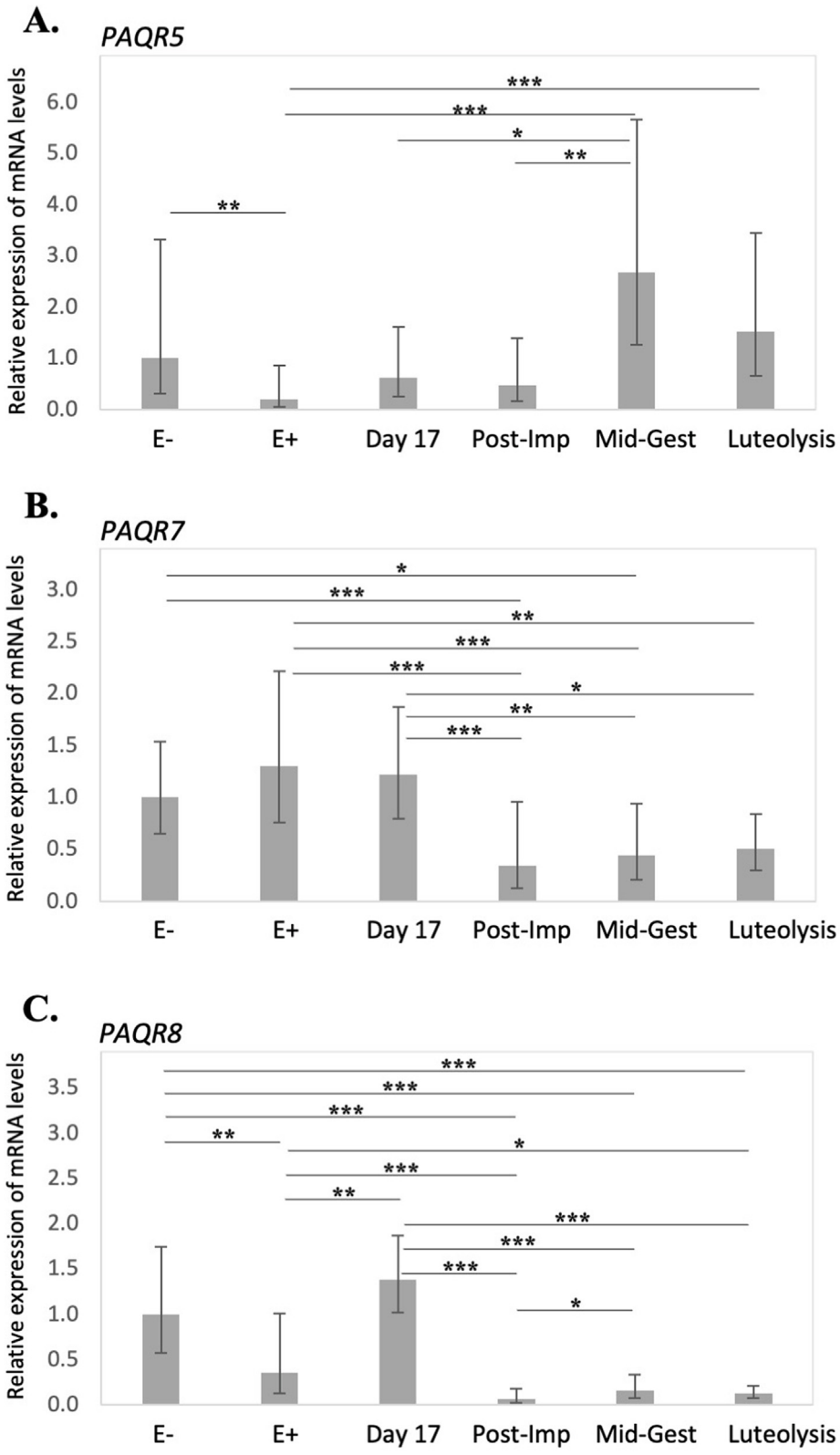

Fig. 2. Relative gene expression of progestin and adipoQ receptor (*PAQR*) *5*, *-7*, *-8* in canine uterine and placental compartments during different stages of pregnancy determined by semi-quantitative real time (TaqMan) PCR. E−/E+ = Embryo −/+ (n = 5), Day 17 = day of implantation (n = 3), Post-Impl = post-implantation (n = 5), Mid-Gest = mid-gestation (n = 5), Luteolysis = prepartum luteolysis (n = 4). One-way ANOVA was applied to test the effects of different pregnancy stages on gene expression revealing: $p < 0.0001$ for *PAQR5* (A), *PAQR7* (B) and for *PAQR8* (C) throughout pregnancy. When $p < 0.05$, the analysis was followed by a Tukey-Kramer multiple-comparisons test. Numerical data are presented as geometric mean (Xg) ± geometric standard deviation (SD). Bars with different asterisks differ at: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

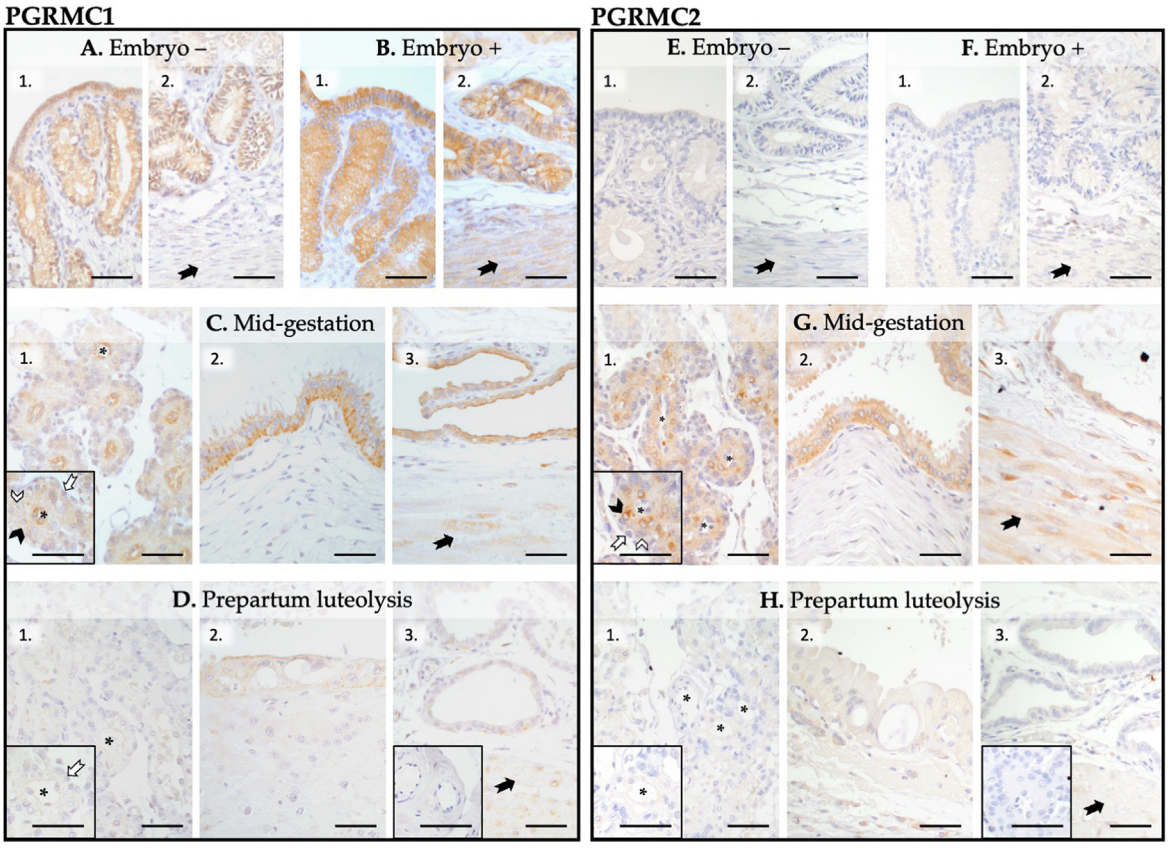

Fig. 3. Immunohistochemical detection of PGRMC1 and -2 in the canine uterine and placental compartments during different stages of pregnancy. **(A–D)**: endometrial PGRMC1 is localized in luminal and uterine glandular epithelium in non-pregnant (panel 1 in A) and early pregnant (panel 1 in B) dogs. Following placentation, superficial glands (glandular chambers) stain positive for PGRMC1 (panel 2 in C). The placental localization is mainly in maternal endothelial cells (panel 1 in C), with apparently stronger signal intensity observed at mid-gestation compared with prepartum luteolysis (panel 1 in D); the signal intensity in other cellular compartments appears weaker. For placental localization, insets with higher magnification are included in panel 1 of C and D. The myometrial staining, shown along with deep uterine glands, appears to follow the intensity of the endometrial signals (panel 2 in A, B and panel 3 in C and D). **(E–H)**: The uterine PGRMC2 signals appear weak in non-pregnant (panel 1 in E) and early pregnant (panel 1 in F) dogs. Following implantation, placental PGRMC2 is predominantly localized in decidual cells (panel 1 in G), followed by syncytiotrophoblast cells; strong signals are localized in superficial uterine glands (panel 2 in G). As for PGRMC1, the myometrial staining shown along with deep uterine glands, appears to follow the intensity of the endometrial signals (panel 2 in E, F and panel 3 in G and H). Termination of pregnancy appears to be associated with weak to undetectable signals for both factors (**D and H**). In C, D and G, H: panel 1 = placental labyrinth, panel 2 = superficial glands (glandular chambers) and uterine stroma, panel 3 = deep uterine glands and myometrium. No staining is observed in the isotype controls (representative pictures shown in inset in panel 3 of D and H). (Asterisk: maternal blood vessel, solid arrow: myometrium, open arrow: cytotrophoblast cells, open arrowhead: syncytiotrophoblast cells, solid arrowhead: decidual cells). Scale bars = 50 μm.

aglepristone ($p < 0.05$, details in Fig. 5B) and by higher dosages of mifepristone ($p < 0.05$; Fig. 6B). Finally, there was no effect of antigestagen treatment upon the expression of transcripts encoding for mPRα (*PAQR7*), -β (*PAQR8*) and -γ (*PAQR5*) ($p > 0.05$, Figs. 5 and 6).

At the protein level, decidualization of DUS cells was associated with increased protein availability of PGRMC1 and -2 ($p < 0.05$; Fig. 7), although this was inhibited by antigestagen treatment ($p < 0.05$, details in Fig. 7).

4. Discussion

Considering the importance of P4 signaling in canine pregnancy, this study was performed to obtain insights into possible uterine and placental activity of nongenomic P4 pathways during canine pregnancy. The gene expression of membrane-bound P4 receptors was detectable in DUS cells, as well as in both uterine and placental samples collected at different time points during pregnancy, although the presence of free-floating embryos had a modulatory effect on some of these receptors. The transcriptional availability of *PGRMC1* and *-2* varied throughout pregnancy, albeit in opposite regulatory directions. The presence of free-floating embryos in the uterus was associated with decreased expression of *PGRMC1*, and with elevated mRNA levels of *PGRMC2*. Interestingly, however, the protein abundance of PGRMC1 appeared to be increased in the endometrial epithelial compartments. The apparent disparity between mRNA and protein levels could be related to the whole uterine cross-section having been used for mRNA analysis, so the localization of uterine *PGRMC1* mRNA expression needs to be investigated. Nevertheless, in comparison with PGRMC2, there seemed to be a lower transcriptional availability of *PGRMC1* during the initiation of pregnancy and following placentation. This finding is supported by similar observations in human and rhesus monkey endometrium, where increased circulatory levels of P4 during the secretory phase suppressed *PGRMC1* expression during the window of implantation [71,72]. The extent to which the expression of membrane-bound P4 receptors is regulated by circulating P4 levels in the dog remains to be investigated. In the human uterus, PGRMC1 function is associated with cell proliferation, and thus is needed for endometrial development during the follicular phase [73], while PGRMC1-P4 interaction exhibits antiapoptotic properties in cultured human and rat granulosa cells [28,74]. Furthermore, balanced expression of this receptor is essential for fertility [48], uterine receptivity and embryo implantation [75], and

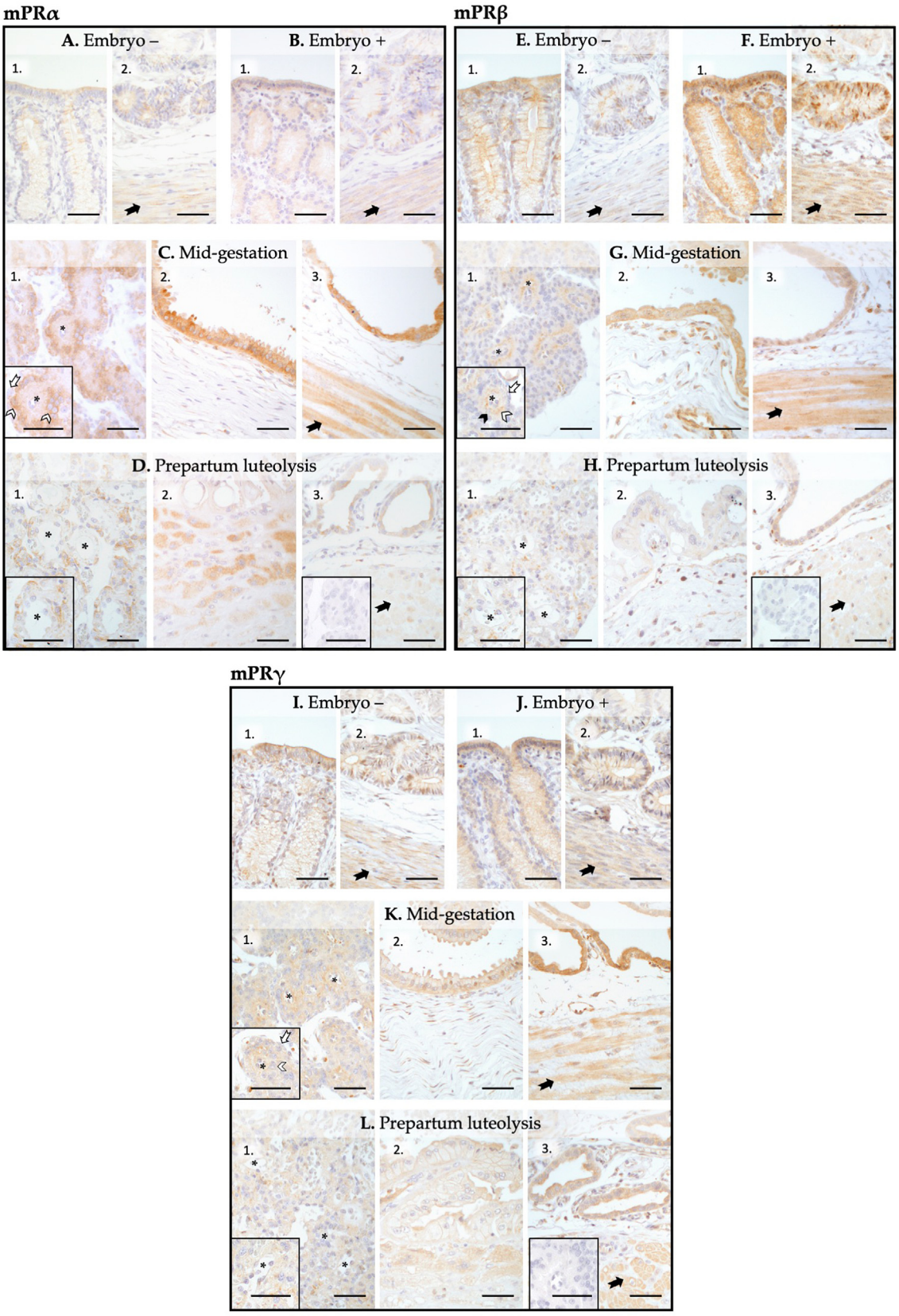

Fig. 4. Immunohistochemical detection of mPRα, mPRβ and mPRγ in the canine uterine and placental compartments during different stages of pregnancy. **(A–D)**: endometrial mPRα is localized in luminal and uterine glandular epithelium in non-pregnant (panel 1 in A) and early pregnant (panel 1 in B) dogs, with varying signal intensity. Following placentation, superficial glands (glandular chambers) stain positive for mPRα (panel 2 in C). The placental localization is abundantly present in syncytiotrophoblast cells at the middle of pregnancy (panel 1 in C). For placental localization insets with higher magnification are included in panel 1 of C and D. The myometrial staining, shown along with deep uterine glands, appears to follow the intensity of the endometrial signals (panel 2 in A, B and panel 3 in C and D). **(E–H)**: The uterine expression of mPRβ is observed in superficial

decidualization [76] in humans and mice.

As for the increased availability of PGRMC2 during placentation, similar observations were made in primates and in mice, in which the expression of *PGRMC2* was elevated in response to the naturally increased P4 level during the secretory phase in macaques [47], or in response to P4 treatment in ovariectomized mice [42]. Conversely, decreased availability of *PGRMC2* was associated with proliferation of granulosa cells and preovulatory follicle development in rats [77,78], while conditional ablation of PGRMC2 in mice after implantation led to pregnancy loss due to post-implantation embryonic death [48,79]. Overall, although the functionality of PGRMC1/-2 is still not fully understood, our observations suggest that both PGRMCs are involved in stabilizing uterine homeostasis [79]. This appears even more plausible when their localization within the canine placental labyrinth is considered (discussed below).

Regarding the PAQR family members (mPRα, -β, and -γ), the initiation of canine pregnancy was associated with decreased availability of *PAQR5* (mPRγ) and *PAQR8* (mPRβ) transcripts, with the latter, however, being highly upregulated by embryo attachment during implantation, and utero-placental availability of *PAQR5* rising at mid-term. The transcriptional activity of *PAQR7* (mPRα), while not affected by either embryo presence or by its attachment to the uterine surface during implantation, decreased following implantation and did not change during placentation. In other species, mPRγ appeared to be implicated in the positive regulation of cell proliferation and migration, showing anti-apoptotic effects in rat Schwann cells (both in primary cells and in an immortalized cell line model), acting in a MAPK-dependent manner [80,81]. When compared with mid-gestation, the natural prepartum luteolysis did not significantly affect the availability of transcripts encoding for membrane-bound progesterone receptors in utero-placental complexes.

While the uterine localization of membrane-bound receptors was predominantly in the epithelial compartments, and to a lesser extent in the stroma and myometrium, their placental localization showed specific, distinguishable patterns. Similar uterine localization of PGRMC1 and -2 was found in the murine uterus, where they were abundantly present in luminal and glandular epithelium following the initiation of pregnancy [42]. In the human uterus, the glandular and epithelial endometrial presence of PGRMC1 and mPRα seemed to be associated with rising circulatory levels of P4 during the establishment of pregnancy [76,82].

In the placenta, PGRMC1 was predominantly localized in endothelial cells while weaker signals were detected in other cellular compartments. This localization pattern appears to be species-specific, as the expression of PGRMC1 in the human placenta was found to be limited to the vascular smooth muscle cells and syncytiotrophoblast cells [42]. Because PGRMC1 is expressed predominantly in the placental maternal vascular endothelial cells in the dog, it could play an important role in the mediating effects of P4 upon microvascular homeostasis. Our recent findings using a transcriptomic approach show that both the natural and aglepristone-mediated withdrawal of PGR function results in the disruption of vascular integrity, in association with an increased expression of proinflammatory intercellular adhesion molecule 1 (ICAM1) [83]. The extent to which these effects are related to membrane-bound receptor-mediated effects remains to be investigated. Conversely, PRGMC2 was mainly associated with decidual cells, and thereby colocalized with PGR, highlighting the possible interaction between their expression within the same cell type, reflected in the functionality of decidual cells. Further, both mPRα and -β were mostly localized in the syncytiotrophoblast. This localization pattern of mPRα was also reported for the human placenta [84]. Their expression in syncytiotrophoblast, surrounding the maternal vessels [84] and associated with the placental localization of endothelin receptor B (ETB; a strong vasodilator) [85], or inhibitors of metalloproteinases (MMPs), e.g. TIMP2 [86–88], suggests that membrane-bound receptors, in particular mPRα and -β, could be further involved in regulating the vascular tonus, vascular integrity and invasive properties of the trophoblast. Indeed, MMPs were identified among the P4-regulated genes represented in the canine term placenta [83]. The signal intensity of virtually all of the membrane-bound receptors appeared weaker in term placenta, i.e. during prepartum luteolysis. This includes the expression of mPRγ, which otherwise seemed to be ubiquitously present. Despite its ubiquitous expression, the mPRγ staining was considered positive in our study based on the negative staining achieved with the respective isotype control and the low or no signal intensity in samples collected at prepartum luteolysis.

Although there have been few studies investigating the luteolysis-associated effects on the expression of membrane-bound P4 receptors in other species, the available knowledge supports our findings, i.e. decreased circulating concentrations of P4 during the late secretory phase reduce PGRMC1 expression in human endometrium [76], and PGRMC1, mPRα and -β are decreased in rat luteal cells during parturition [89].

Thus, it appears plausible that in the canine placenta the expression of membrane-bound P4 receptors is also regulated by endocrine factors. Moreover, it also seems plausible that the membrane-bound P4 receptors could reciprocally modulate their expression, as some placental cell populations stained positive for more than one P4 receptor. Such regulatory effects were observed in human PGR-negative breast cancer cells, where an increased level of PGRMC1 was shown to stimulate the expression of mPRα [90].

Cumulatively, taking into account the uterine and placental spatio-temporal distribution of membrane-bound P4 receptors during canine pregnancy, our study supports the possible broad role of these receptors in the maintenance of placental homeostasis, including cell-to-cell interaction between the fetal and maternal placental compartments, as well as the previously postulated regulation of uterine blood flow [44] and uterine contractile activity during parturition [34].

New, important clues regarding the expression and regulation of decidual cell function can be derived from the findings in our *in vitro* model. In previous studies utilizing this model, we were able to show a reciprocal interaction between P4 and its own PGR receptor. Whereas P4 stimulated PGR expression in decidualizing DUS cells [61], functional blocking of PGR by aglepristone and

and glandular epithelium (panel 1 in E and F). Following placentation, placental mPRβ is predominantly localized in syncytiotrophoblast cells (panel 1 in G); strong endometrial signals are localized in superficial uterine glands (panel 2 in G). For placental localization insets with higher magnification are included in panel 1 of G and H. As for mPRα, the staining of mPRβ in myometrium, shown along with deep uterine glands, appears to follow the intensity of endometrial signals (panel 2 in E, F and panel 3 in G, H). Termination of pregnancy (prepartum luteolysis, (**D, H**)) is associated with weaker signals for mPRα and mPRβ. **(I–L)**: mPRγ appears to be ubiquitously distributed throughout utero-placental compartments. However, prepartum luteolysis appears to be associated with decreased placental availability of this receptor (**L**). Panel 1 in A, B, E, F, I, J = endometrial superficial layers; panel 2 in A, B, E, F, I, J and panel 3 in C, D, G, H, K, L = deep uterine glands and myometrium; panel 1 in C, D, G, H, K and L = placental labyrinth; panel 2 in C, D, G, H, K and L = superficial glands (glandular chambers) and uterine stroma. No staining is observed in the isotype controls (representative pictures shown in inset in panel 3 of D, H and L). (Asterisk: maternal blood vessel, solid arrow: myometrium, open arrow: cytotrophoblast cells, open arrowhead: syncytiotrophoblast cells, solid arrowhead: decidual cells). Scale bars = 50 μm.

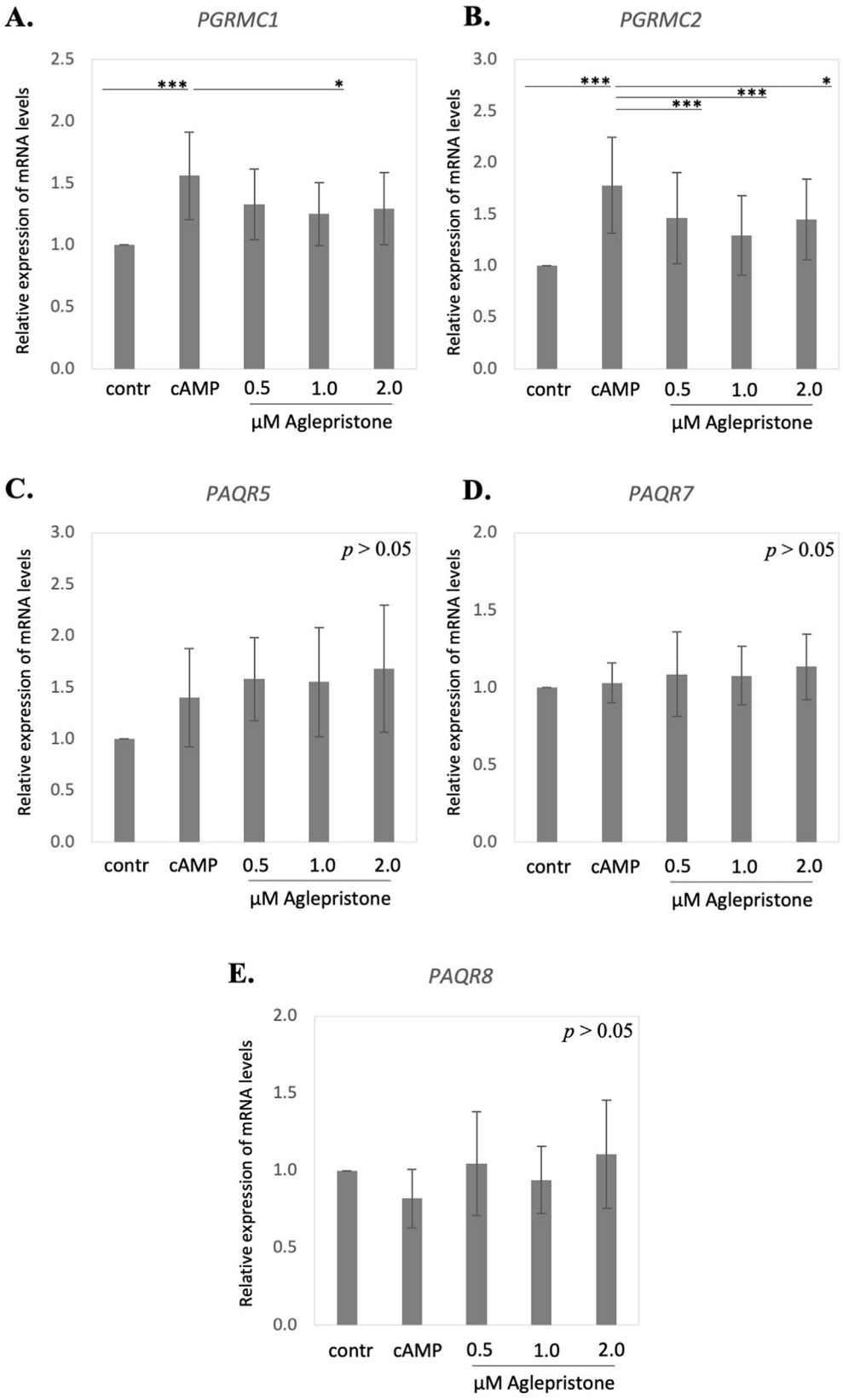

Fig. 5. Aglepristone-mediated effects on the expression of membrane-bound P4 receptors in decidualized DUS cells. Relative gene expression of (A): *PGRMC1*, (B): *PGRMC2*, (C): *PAQR5* (mPR γ), (D): *PAQR7* (mPRα), and (E): *PAQR8* (mPRβ) in DUS cells during *in vitro* decidualization and following the treatment with different dosages of aglepristone was determined by semi-quantitative real-time (TaqMan) PCR. One-way ANOVA was applied, revealing: $p < 0.0001$ for both *PGRMC1* and *-2*, and $p > 0.05$ for the other factors. When $p < 0.05$, the analysis was followed by a Tukey-Kramer multiple-comparisons test. Numerical data are presented as mean ± standard deviation (SD). Bars with different asterisks differ at: (*) $p < 0.05$, (***) $p < 0.001$.

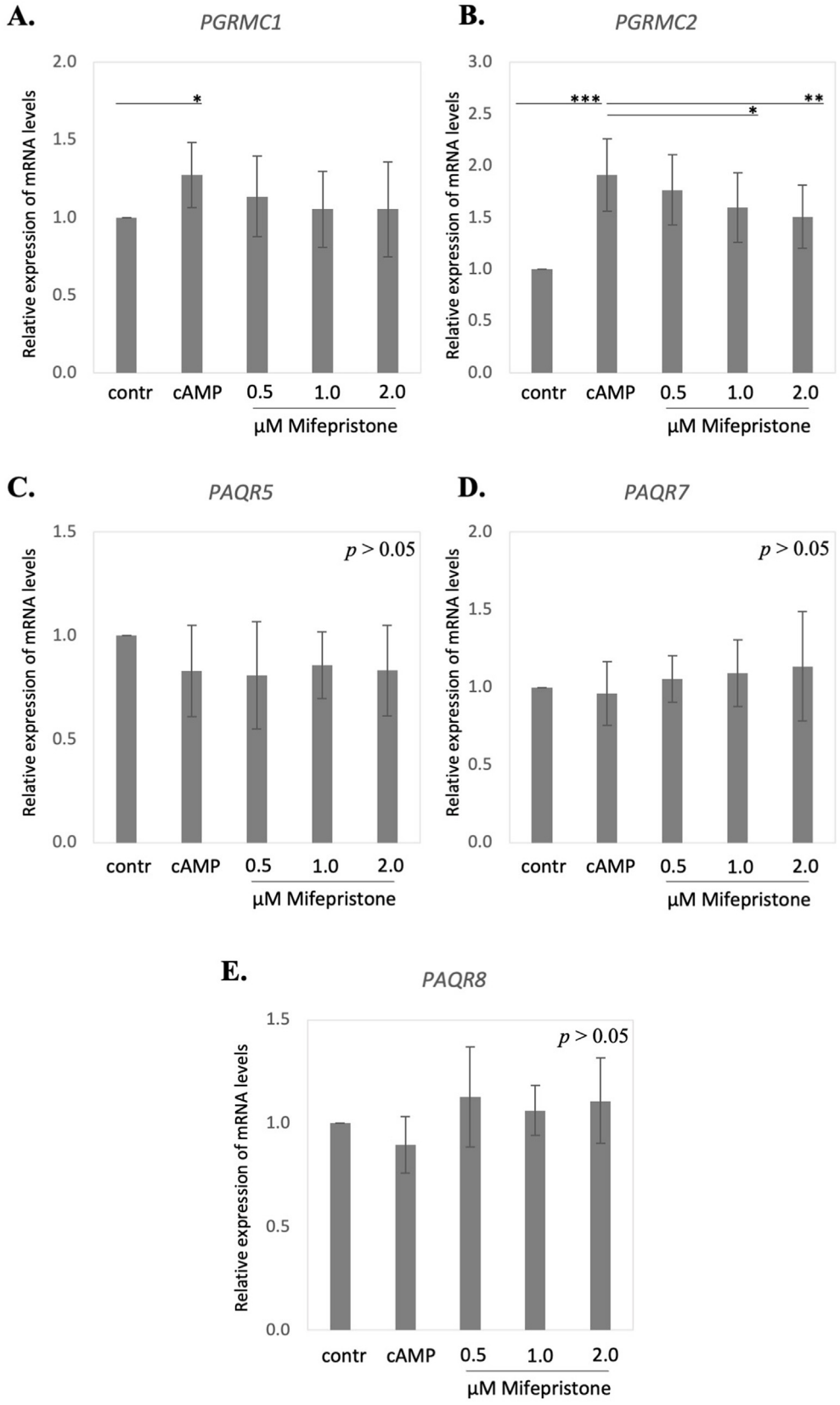

Fig. 6. Mifepristone-mediated effects on the expression of membrane-bound P4 receptors in decidualized DUS cells. Relative gene expression of (A): *PGRMC1*, (B): *PGRMC2*, (C): *PAQR5* (mPR γ), (D): *PAQR7* (mPRα), and (E): *PAQR8* (mPRβ) in DUS cells during *in vitro* decidualization and following the treatment with different dosages of mifepristone was determined by semi-quantitative real-time (TaqMan) PCR. One-way ANOVA was applied, revealing: $p = 0.05$ for PGRMC1, and $p < 0.0001$ for PGRMC2, and $p > 0.05$ for the other factors. When $p < 0.05$, the analysis was followed by a Tukey-Kramer multiple-comparisons test. Numerical data are presented as mean ± standard deviation (SD). Bars with different asterisks differ at: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

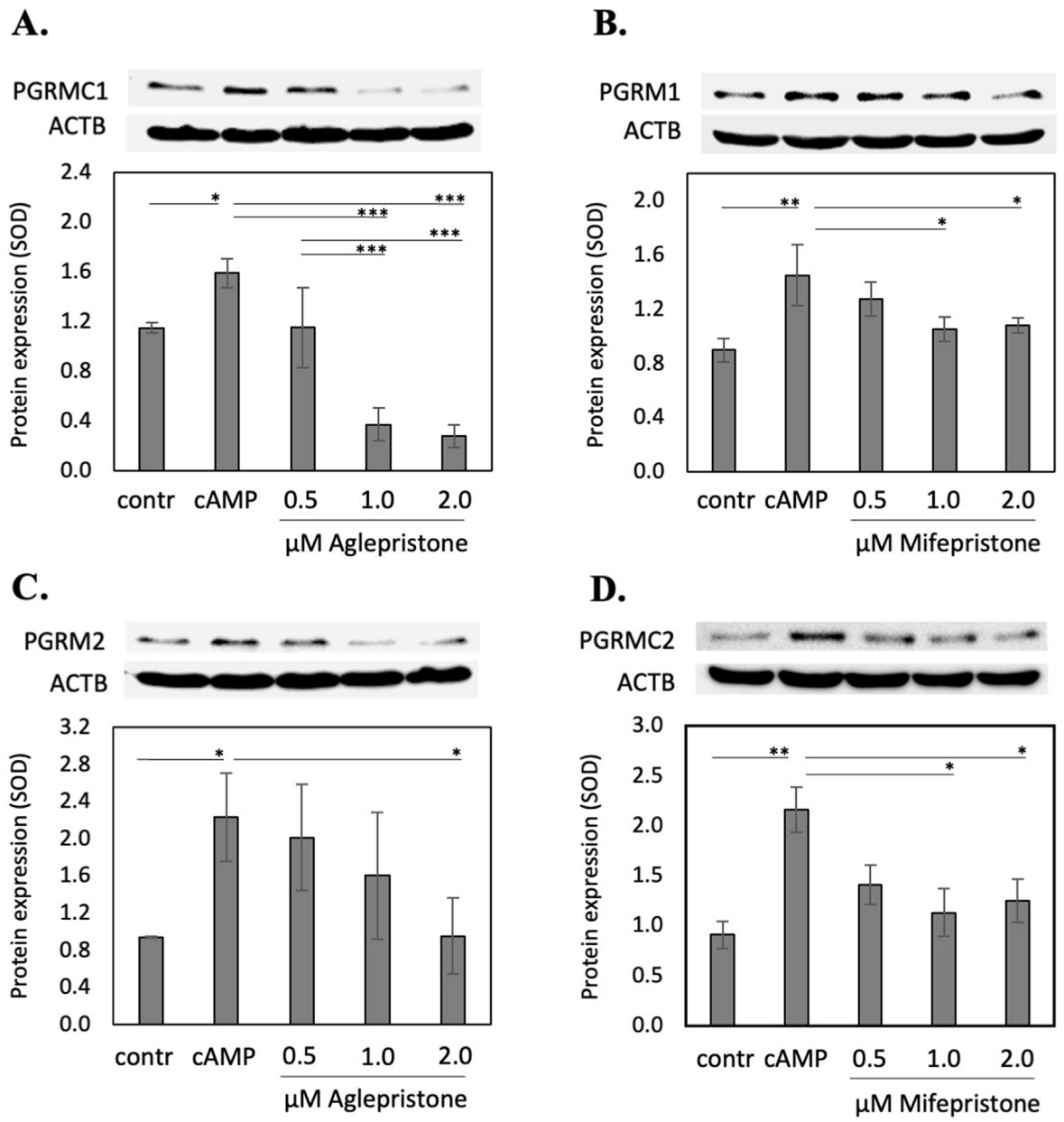

Fig. 7. *In vitro* decidualization- and antigestagen-mediated effects on PGRMC1 and -2 protein expression in decidualizing DUS cells. Standardized optical densities (SOD) of PGRMC1 (25 kDa) and PGRMC2 (25 kDa) were normalized against ACTB (45 kDa). One-way ANOVA was applied, revealing: $p = 0.002$ for PGRMC1 in response to aglepristone, $p = 0.007$ for PGRMC1 in response to mifepristone, $p = 0.01$ for PGRMC2 in response to aglepristone, and $p = 0.008$ for PGRMC2 in response to mifepristone. When $p < 0.05$, the analysis was followed by a Tukey-Kramer multiple-comparisons test. Numerical data are presented as mean ± standard deviation (SD). Bars with different asterisks differ at: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

mifepristone suppressed its protein availability [62]. Here, while we found detectable transcriptional levels of all investigated receptors in DUS cells, only the expression of *PGRMC1* and *-2* increased during *in vitro* decidualization, although this expression was diminished in cells treated with antigestagens, with aglepristone showing negative effects on both receptors. The mRNA expression of mPRs was not affected in decidualized cells, nor was it affected by treatment with antigestagens. After observing the effects exerted by decidualization and antigestagens upon *PGRMC1* and *-2*, we verified and confirmed their respective protein levels, finding clearly decreased expression in response to both aglepristone and mifepristone. Therefore, PGR appears to regulate the availability of important membrane-bound receptors in canine decidual cells, with PGRMC2 being highly abundant in decidual cells *in vivo*. Taking into account its function as a nuclear receptor, membrane-bound receptors PGRMC1 and -2 appear to act as downstream targets of PGR. Their expression patterns clearly indicate the compartmentalization and multidirectional effects of non-genomic P4 signaling, possibly contributing to the proliferative and anti-apoptotic effects of P4 as part of the PGR-mediated signaling [62]. Similarly, the proliferative properties of PGRMC1 were implied in immortalized murine KK1 cells and Leydig tumor cells, with mifepristone showing suppressive/antiproliferative effects [91,92]. With this, a novel finding from the present study is that the application of PGR blockers not only appears to directly affect the canonical genomic pathway of P4 signaling in the canine

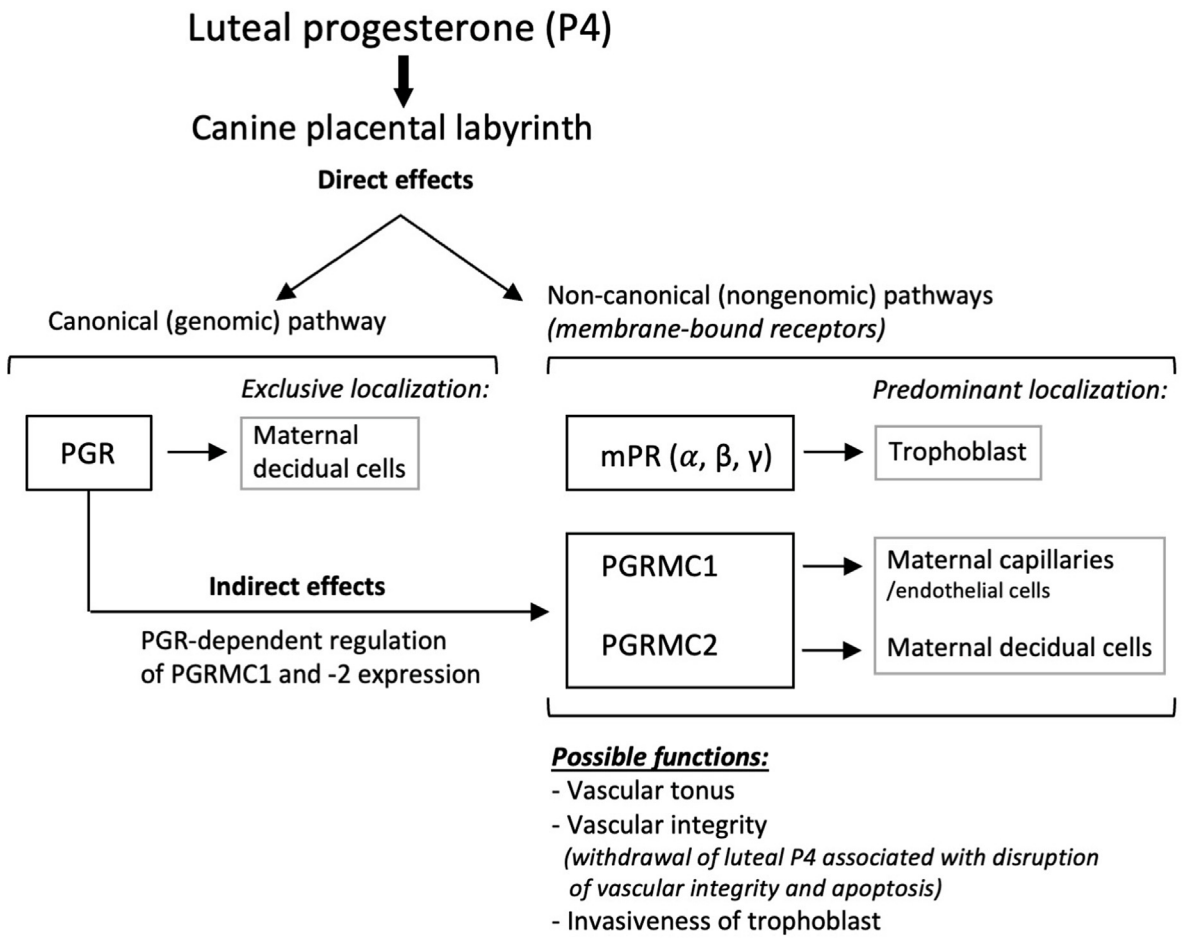

Fig. 8. The summary of the currently proposed model of progesterone (P4)-mediated regulation of placental function in dogs during the maintenance and termination of pregnancy.

placenta, but also indirectly regulates the expression of membrane-bound P4 receptors PGRMC1 and PGRMC2 through PGR-mediated signaling. Future studies are strongly encouraged to investigate the functional importance and biological effects associated with the nongenomic P4 signaling pathways in canine pregnancy.

5. Conclusions

In conclusion and as summarized in Fig. 8, our findings demonstrate the spatio-temporal distribution of membrane-bound P4 receptors (mPRα, -β, -γ, PGRMC1 and -2) in the canine utero-placental compartments, introducing new possible cellular responders mediating the biological functions of P4 in the dog. Adding to previous findings indicating the functional regulatory loop between P4 and its nuclear PGR receptor, by using the *in vitro* model with DUS cells it seems that P4 regulates the expression of its own non-genomic receptors, PGRMC1 and PGRMC2, in a PGR-dependent manner, as evidenced in the antigestagen-treated cells. This regulatory loop is also mirrored in the decidualization-dependent expression of PGRMCs, in particular of PGRMC2, which is abundantly present in decidual cells *in vivo*. Indeed, the cellular distribution of membrane-bound receptors in the placenta showed distinct localization patterns. Besides decidual cells being the main target for PGRMC2, PGRMC1 was mostly localized in endothelial cells, and syncytiotrophoblast appeared to be associated with the presence of mPRα and mPRβ, implying the main cellular targets of these receptors. Taking into account that prepartum luteolysis leading to placental detachment is associated with the disruption of vascular integrity [83], the presence of PGRMC1 in endothelial cells is a particularly interesting finding. It adds a new functional insight into the process of parturition in the dog. It also further highlights the possible role of nongenomic P4 pathways in the underlying biological processes. The possibility of crosstalk between different P4 signaling pathways certainly deserves further elucidation. Moreover, as antigestagens are the drugs of choice for the induction of parturition in dogs, the possible functional interactions with membrane-bound P4 receptors appear to be of clinical importance and should thus be explored in greater detail.

Summary sentence

The non-genomic P4 signaling in the canine placenta is possibly involved in regulating the vascular tonus, vascular integrity, invasive properties of the trophoblast, and decidualization. This paper reveals novel potential P4-mediated mechanisms in regulating the maintenance of canine pregnancy.

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Data availability statement

The gene expression data will be available from the corresponding author, upon reasonable request.

CRediT authorship contribution statement

Ali Kazemian: Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Miguel Tavares Pereira:** Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Selim Aslan:** Resources, Writing – review & editing. **Rita Payan-Carreira:** Resources, Writing – review & editing. **Iris M. Reichler:** Writing – review & editing. **Reha A. Agaoglu:** Resources, Writing – review & editing. **Mariusz P. Kowalewski:** Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.07.005.

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