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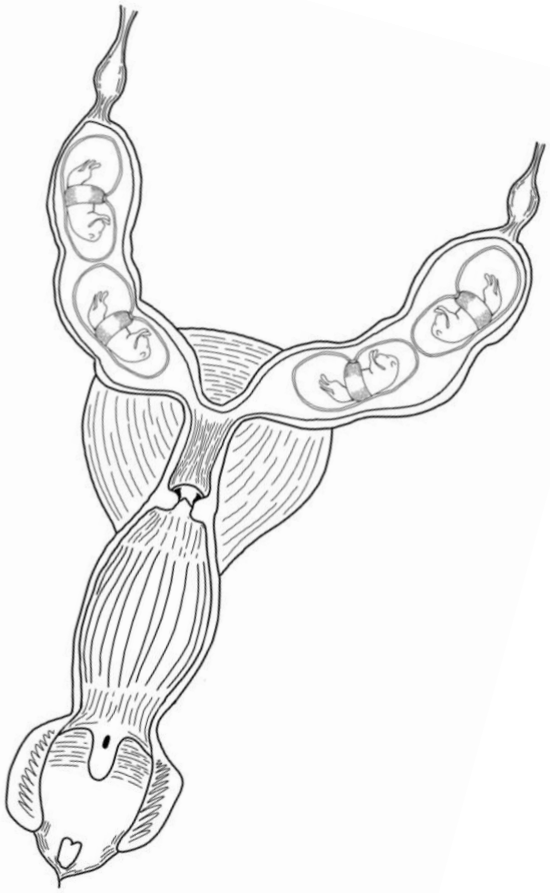

Investigations of the contraction-associated proteins PTGFR, Cx26 and Cx43 and their role in canine primary uterine inertia

Master thesis

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Opgaveformulering	Opgaven omhandler primær uterin inerti (PUI) hos hunde. Det er et case-kontrolstudie, der undersøger protein- og mRNA myometrie ekspression af de kontraktions-associerede proteiner prostaglandin F receptor (PTGFR), connexin 26 (Cx26) og connexin 43 (Cx43) i hunde med PUI sammenlignet med hunde med obstruktiv dystoki. Undersøgelserne sker ved hjælp af metoderne immunhistokemifarvning (IHC) og kvantitativ 'reverse transkriptase' polymerasekædereaktion (RT-qPCR). Formålet med studiet er at bidrage til den samlede forståelse af sygdomspatologien bag PUI hos hunde.

Contents

Preface and Acknowledgements

This master thesis is a result of my work from August 2023 to January 2024 under the supervision of Assoc. Prof. Jan Bojsen-Møller Secher, PhD, DVM at the Section for Veterinary Reproduction and Obstetrics, University of Copenhagen & Prof. Dr. Sandra Goericke-Pesch, Dipl. ECAR, at the Unit for Reproductive Medicine, University of Veterinary Medicine in Hannover. This thesis concludes my master's degree in veterinary medicine at the University of Copenhagen.

First and foremost, I would like to thank my supervisors for their help and guidance throughout this project. I wish to give a special thanks to Sandra for granting me the opportunity to travel to Hannover, Germany, and work alongside her research group. The weeks I spend there, truly benefited my gynaecological and obstetrical knowledge. I also want to thank DVM, PhD student Carolin Jungmann for welcoming me in Hannover and helping me with the data collection process. I am truly grateful for my fellow students who volunteered to proofread and perfect my thesis before submission.

Finally, I wish to thank Dansk Kennel Klub (DKK) and Mads Rousing, DVM for financially supporting my trip to Hannover, Germany.

Resume

Dette studie undersøger de specifikke kontraktions-associerede proteiner (CAPs): prostaglandin receptor F (PTGFR), connexin 26 (Cx26) samt connexin 43 (Cx43) og deres potentielle roller i ætiologien bag primær uterin inerti hos hunde (PUI). En hypotese blev således forslået: At den generelle udtrykkelse af uterine PTGFR, Cx26 og Cx43 er nedsat hos tæver diagnoseret med primær uterin inerti (PUI) sammenlignet med tæver der har obstruktiv dystoki (OD). Yderligere blev der forslået en hypotese om at ekspression af PTGFR er lavere i myometriets longitudinale lag (LM) end i det cirkulære lag (CM). Dette blev gjort for at klargøre om der er overensstemmelse med foreløbige resultater af et in vitro organ studie, hvoraf det blev påvist, at LM laget havde en ringere respons til eksogen prostaglandin sammenlignet med CM laget (Jungmann, Mazzuoli-Weber, et al., 2023). I alt blev 20 hunde inkluderet i studiet (PUI = 12, OD = 8). Alle hundene fik udført et akut kejsersnit grundet underliggende dystoki. Under operationen blev der udtaget en biopsi fra enten et interplacental (IP) og/eller uteroplacental område (UP). Biopsierne blev stabiliseret i paraffinblokke og forberedt til enten immunhistokemi (IHC) farvning og/eller kvantitativ 'reverse transkriptase' polymerasekædereaktion (RT-qPCR). Både RT-qPCR metoden og resultaterne heraf på Cx26 og Cx43 blev udført og analyseret af en tredje part og foræret til forfatteren. Selve evalueringen af IHC-intensiteten af PTGFR, Cx26 og Cx43 i myometriet samt statistisk analyse af resultaterne heraf blev fortaget af forfatteren selv. Baseret på optegnet kuldstørrelser på tæverne, blev der udført statistisk undersøgelse for at undersøge hvorvidt kuldstørrelse havde en betydning for CAP udtrykkelse i myometriet. Angående PTGFR, blev det konkluderet, at der var en signifikant forskel mellem IP lagene ($p = 0{,}001$), hvor CM laget farvede mere intens end LM laget. Der er derfor enighed med foreløbige resultater fra in vitro studiet. Dog kunne hypotesen omkring en lavere PTGFR ekspression i tæver med PUI sammenlignet med OD-gruppen ikke accepteres ($p = 0{,}637$). Angående Cx26 kunne hypotesen heller ikke accepteres, da der blev påvist en højere ekspression i PUI-gruppen sammenlignet med OD-gruppen. Dette var sandt for både protein ($p = 0{,}0022$) og mRNA ($p = 0{,}0018$). Det samme kunne ikke definitivt konkluderes ud fra Cx43 resultaterne, da der kun blev set en signifikant forskel mellem PUI- og OD-gruppen i mRNA ekspression ($p = 0{,}0003$) og ikke protein ($p = 0{,}689$). Der kunne ikke konstateres, at kuldstørrelse havde en påvirkning på CAP udtrykkelse i myometriet hos tæver med PUI. Dette studie svækkede interessen i prostaglandiners rolle i sygdomspatologien bag PUI. Dog blev der vakt særligt interesse i Cx26. Opfølgende studier burde laves på Cx26 og Cx43 for at understrege deres reelle rolle i uterin inerti hos hunde.

Abstract

The author of this thesis conducted a study investigating the potential role of the contraction-associated proteins (CAPs) prostaglandin-F receptor (PTGFR), connexin 26 (Cx26) and connexin 43 (Cx43) and their roles in the aetiology of canine primary uterine inertia (PUI). It was hypothesised that the general myometrial expression of PTGFR, Cx26 and Cx43 would be lower in bitches diagnosed with PUI compared to the control group consisting of dogs diagnosed with obstructive dystocia (OD). Furthermore, it was hypothesised that the PTGFR expression would be lower in the longitudinal layer (LM) of the myometrium compared to the circular layer (CM). Exploring if there is an agreement with preliminary results from previous in vitro organ bath study showing no response in the LM layer to exogenous prostaglandins compared to the CM layer (Jungmann, Mazzuoli-Weber, et al., 2023). In total, 20 dogs were included in the study (PUI = 12, OD = 8) and underwent an acute caesarean section. During surgery, a biopsy from either interplacental site (IP) and/or uteroplacental site (UP) was performed. The biopsies were formalin fixed, paraffin embedded and prepared for either immunohistochemical (IHC) staining or reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Both the RT-qPCR and statistical analysis results hereof were conducted on both Cx26 and Cx43 from a third party and handed to the author. The author of this study evaluated the IHC staining intensity of PTGFR, Cx26 and Cx43 in the myometrial layers of the uterus and performed statistical analysis of the results. Considering the average litter size of each breed compared to the actual litter size of the bitches in the PUI group, it was further statistically analysed if litter size influences myometrial CAP expression in bitches with PUI.

For PTGFR protein expression, it was concluded that there was a significant difference between the IP layers ($p = 0.001$) with the CM layer staining more intensely than the LM layer. These results agree with preliminary findings in the in vitro organ bath study. Though, the hypothesis concerning a lower PTGFR myometrial expression in bitches with PUI compared to the control group could not be accepted ($p = 0.637$). For Cx26 the hypothesis could not be accepted either, seeing as the expression was higher in the PUI group compared to the OD group. This was true for both the protein ($p = 0.0022$) and the mRNA ($p = 0.0018$). The same could not be concluded for Cx43, as only the mRNA expression revealed a significant difference in between groups ($p = 0.0003$), but protein expression did not ($p = 0.689$). Litter sizes were shown not to interfere with myometrial CAP expression, neither in the CM nor LM layer of the PUI group.

This study indicates that PTGFR does not seem to play a role in the aetiology of canine inertia. Yet, it spiked an increasing interest in the role of Cx26. Further studies need to be conducted on Cx26 and Cx43 to emphasize their role in canine primary uterine inertia.

1. Abbreviations

Abbreviations are listed in alphabetic order.

CAPs = Contraction associated proteins

CL = Corpora lutea

CM = Circular myometrial layer

COX2 = Cyclooxygenase 2

Cx26 = Connexin 26

Cx43 = Connexin 43

FFPE = Formalin-Fixed Paraffin Embedded

FSH = Follicle stimulating hormone

GH = Growth hormone

GnRH = Gonadotropin releasing hormone

HPGD = 15-hydroxy prostaglandin dehydrogenase

IHC = Immunohistochemistry

IP = Interplacental

LH = Luteinizing hormone

LM = Longitudinal myometrial layer

mRNA = Messenger ribonucleotide acid

OD = Obstructive dystocia

PG = Prostaglandin

PG_{E2} = Prostaglandin-E2

$PGF_{2\alpha}$ = Prostaglandin-F2 alpha

PGT = Prostaglandin transporter

PTGER = Prostaglandin E receptor

PTGFR = Prostaglandin F receptor

PTGES = Prostaglandin E synthase

PGHS = Endoperoxide-H synthase

PGH2 = Prostaglandin H2

PTGS2 = Prostaglandin-endoperoxide synthase 2

P4 = Progesterone 4

PUI = Primary uterine inertia

PUI-L = Primary uterine inertia large litter

PUI-N = Primary uterine inertia normal litter

PUI-S = Primary uterine inertia small litter

qPCR = Quantitative polymerase chain reaction

RT-PCR = Reverse transcriptase polymerase chain reaction

SD = Standard deviation

SUI = Secondary uterine inertia

UP = Uteroplacental

2. Introduction

Dystocia in bitches is a critical condition that can have life threatening consequences for both the dam and the puppies if not aided. Primary uterine inertia (PUI) is one of the main causes behind dystocia in bitches and is therefore a medical condition that most likely will show in ordinary veterinary practice. PUI is described as a condition where proper uterine contractions with sufficient force cease, and the puppies are therefore not delivered through an otherwise normal and non-obstructed birth canal. To improve the treatment of PUI, a better understanding of the underlying pathophysiology is required. Many theories concerning an aetiology behind PUI has been suggested but the exact pathophysiology behind the condition has not been confirmed.

Low expressions of contraction associated proteins (CAPs) such as prostaglandin receptor F (PTGFR) have earlier been suggested as a possible cause of PUI yet the significance hereof has yet to be fully proven. In a later in vitro organ bath study, it was found that the reaction on exogenous prostaglandin differed between layers in the myometrium of bitches with PUI. Being that the circular layer (CM) had a stronger contractile response than the longitudinal layer (LM), which did not respond at all. The aim of this study is therefor to reinvestigate the potential role of PTGFR in the aetiology of PUI by using a different sample group. There is also a focus on the comparison of myometrial layers (CM versus LM) in the expression of PTGFR to investigate if it is consistent with the previous results from the in vitro organ bath study.

Furthermore, this study investigates the role of the CAPs connexin 26 (Cx26) and connexin 43 (Cx43) in the aetiology of PUI. Previous studies have investigated Cx26 and Cx43 in pregnant bitches, mice, and rats, yet the expression of these gap-junction proteins has yet to be fully investigated in bitches with dystocia. The author of this thesis was handed preliminary RT-qPCR results on both connexins to compare with own IHC results. The potential role of all the CAPs in PUI was investigated through immunohistochemistry (IHC) staining. All the results are lastly gathered based on litter sizes of the bitches, investigating whether litter sizes have an influence on myometrial CAP expression.

The author of this study hypothesizes that bitches with primary uterine inertia (PUI) will have a lower myometrial expression of PTGFR, Cx26 and Cx43 than those who do not suffer from uterine inertia, which in this study is represented by dogs who suffered from obstructive dystocia (OD). Lastly, it is hypothesized that there is a difference in PTGFR expression between the myometrial layers and that the expression is higher in the CM that the LM of PUI patients.

3. Theory

3.1 Eutocia

Eutocia is the definition of normal parturition. The duration of pregnancy in dogs is 63±1 days from ovulation with a range of 56-72 days if calculated from first mating (Forsberg, 2021). Canine parturition can be divided into three stages. Prior to these stages, some physiological changes happen in the myometrium and cervix, ensuring a slow preparation for birth. This includes a rapid softening of the cervix and increased expression of CAPs (see section 3.6) (Taverne & Noakes, 2019).

In the first stage (Opening stage) of parturition, the vaginal and cervical tissue is prepared by relaxation and dilation. Minor regular intermittent uterine contractions take place without abdominal contractions, forcing the foetal membranes ahead of the foetus itself. This stage is often noticed by the owner since the bitch may show signs of increasing restlessness. Typically, this is where the bitch builds her 'nest', pants, shivers and has decreased to non-existing appetite (Forsberg, 2021; Taverne & Noakes, 2019). This stage usually lasts up to 6-12 hours but can prevail for up to 36 hours in primiparous bitches (Forsberg, 2021). 8-24 hours prior to the first stage, a drop of rectal temperature (1° C) can be measured with a range of (0.8-1.2 °C). This drop is believed to be positively correlated with the drop in serum progesterone concentration (< 2 ng/ml) before parturition (Forsberg, 2021; Fusi & Veronesi, 2022; Taverne & Noakes, 2019).

The second stage (Expulsion) of parturition consists of expulsion of the foetus(es). Uterine contractions have become more intense and are accompanied by abdominal straining which is externally visible (Taverne & Noakes, 2019). This stage typically lasts 3-12 hours and each puppy will be delivered with an amniotic membrane. The puppies will normally be birthed within two hours of each other (Forsberg, 2021; Taverne & Noakes, 2019). The expulsion phase of the first puppy of a litter is longer than the following puppies. Furthermore, when giving birth to a large litter, the bitch may stop straining and rest in between deliveries (Forsberg, 2021). The resting phase can take place up to two hours. The second stage may never last more than 24 hours in total and is complete when all foetuses have been delivered (Forsberg, 2021; Taverne & Noakes, 2019).

The third and final stage (Secundinae) involves the expulsion of the placenta and usually follows within 15 minutes of each foetus. In some cases, two or more foetuses can pass without placental expulsion, but all foetal membranes should pass within 4-6 hours after the end of parturition (Forsberg, 2021).

3.2 Hormonal balances in pregnancy and parturition

Several hormones are involved in maintenance of canine pregnancy and parturition.

During the maintenance of pregnancy, the ovarian corpora lutea (CL) are responsible for the production of progesterone (P4) (Taverne & Noakes, 2019). Progesterone maintains pregnancy by 1) inhibiting the hypothalamus in GnRH secretion and thereby endogenous gonadotropin[1] release, 2) closing the cervix and 3) inhibiting uterine smooth muscle contractions. Furthermore, P4 also stimulates growth hormone (GH), which contributes to the growth of the placenta and mammae (Forsberg, 2021). Prolactin is one of most important luteotrophic hormones[2] and supports CL alongside LH from day 25 of gestation (G. England, 2021; Fusi & Veronesi, 2022). From here on, prolactin concentration increases considerably and peaks by the end of pregnancy (Fusi & Veronesi, 2022). Hormones like relaxin has also proven to be luteotropic. Relaxin is specific to canine pregnancy and is released from the placenta from day 25 of pregnancy and acts out by upregulating prolactin (G. England, 2021; Fusi & Veronesi, 2022; Nowak et al., 2018). Furthermore, relaxin acts out its purpose by causing softening of the fibrous connective tissue in the birth canal (G. England, 2021; Fusi & Veronesi, 2022).

During canine parturition, several hormones have been suggested to participate including corticosteroids, oestrogens, prostaglandins, oxytocin, parathyroid and thyroid hormones (Fusi & Veronesi, 2022; Taverne & Noakes, 2019). Contrary to other domestic animals like the sheep and goat (Taverne & Noakes, 2019), corticosteroids have not yet proven to be mandatory for normal parturition in dogs but are considered a sign of increased stress of the bitch during parturition (Fusi & Veronesi, 2022). Similarly, dogs prove to be different from other domestic species when discussing oestrogen concentration. In other species, it is believed that oestrogen is a pro-contractile hormone (Taverne & Noakes, 2019). In the dog, a rise in oestrogen plasma concentrations is not observed during parturition, rather the concentration is either stable or slightly decreased (Fusi & Veronesi, 2022; Hoffmann et al., 1994). With the breakdown of the corpora lutea, the P4 plasma concentration will drop below a critical threshold, allowing the ratio of oestrogen to progesterone concentration to change (Forsberg, 2021). It is therefore implied that it is the oestrogen/progesterone ratio that is of importance in canine parturition and not oestrogen alone (Milani et al., 2021).

When discussing contractile forces during parturition both oxytocin and prostaglandins (PGs) are believed to play an important role (Taverne & Noakes, 2019). Oxytocin is said to play a part in

[1] **Gonadotropins:** Hormones that affects the gonads (reproductive glands), e.g. FSH and LH.
[2] **Luteotrophic hormones**: Acting to preserve the corpora lutea.

cervical ripening as well as a dual role in contractions. It has 1) direct effect on the myometrium and induced contractions through appropriate oxytocin receptors and 2) regulates the release of uterotonic[3] PGs (Gram et al., 2014).

In the pregnant dog, the trophoblasts[4] of the foeto-maternal compartment are responsible for the production of prostaglandin F2-alpha ($PGF_{2\alpha}$). Contrary to the non-pregnant dog, the initiating function of $PGF_{2\alpha}$ hormone is to terminate the luteal phase in pregnant dogs by causing pre-partum luteolysis (Kowalewski, 2014). A rise in PGF2α will commonly be seen 24 hours before parturition (Forsberg, 2021). Both prostaglandin F2-alpha ($PGF_{2\alpha}$) and prostaglandin E2 (PGE_2) are believed to stimulate uterine contractions (Parkinson et al., 2019c). The general availability of the hormones in the uterine tissue and their active part in parturition rely on their synthesis, catabolism, and their mediation through appropriate receptors (see section 3.9.1) (Fusi & Veronesi, 2022; Taverne & Noakes, 2019)

3.3 Dystocia

Dystocia is the failure of the expulsive forces during birth, hindrances in the birth canal or disposition of the foetus (Davidson, 2021; Parkinson et al., 2019b). Dystocia can be subdivided into maternal and foetal causes and is presented in figure 1. The foetal causes can either be related to the foetus' size (foetopelvic disproportion) or its position in the birth canal (e.g., breech presentation). Maternal causes can too be further subdivided into primary uterine inertia (PUI), secondary uterine inertia (SUI) and obstructive dystocia (OD) (Davidson, 2021).

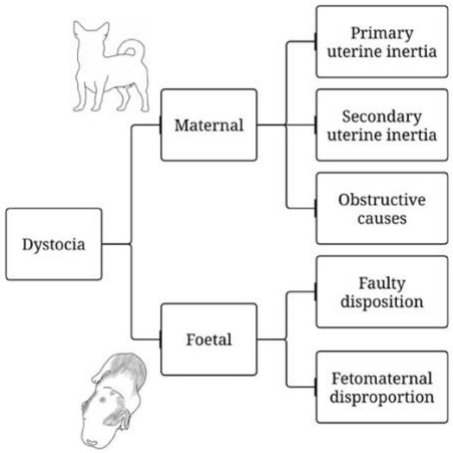


Figure 1: Illustration of dystocia classifications. The illustration is constructed with suggested classifications as mentioned in the publications *(Darvelid & Linde-Forsberg, 1994)* and (Parkinson et al., 2019a).

[3] **Uterotonics:** Agents that increase uterine tone and thereby increase the strength of contractions.
[4] **Trophoblasts:** Specialized placental cells that play an essential role in embryo implantation (Taverne, 2019).

PUI is an idiopathic condition where uterine contractions of the second stage cease and therefore the foetus is not delivered through an otherwise normal and non-obstructed birth canal (Darvelid & Linde-Forsberg, 1994). It is a failure of the myometrium to contract with sufficient force and the second stage of labour is never initiated. With SUI, the expulsion phase is initiated but the progression stops because of uterine fatigue (Egloff et al., 2020; Parkinson et al., 2019b).

While overall prevalence of canine dystocia has been estimated to 5% (Parkinson et al., 2019c), PUI accounts for 49.1-72% cases and is hereby the largest cause for unsuccessful birth in the bitch (Darvelid & Linde-Forsberg, 1994; Dejneka et al., 2020; Parkinson et al., 2019c).

3.3.1 Aetiology of primary uterin inertia

Previous studies have tried to investigate the underlying causes of PUI, but the aetiology is far from understood. Suggested aetiologies in previous publications according to (Parkinson et al., 2019b) are: A) Lack of uterotonic factors such as oxytocin, prostaglandin or their appropriate receptors, B) lack of or decreased expression of contractile proteins, C) lack of uterine stretching (small litter) or overstretching (large litter), D) obesity and the infiltration of fatty tissue in-between myometrial cells and/or E) failure of uterine cell-to-cell communication. Conclusions of scientific publications investigating some of these suggestions are illustrated in figure 2.

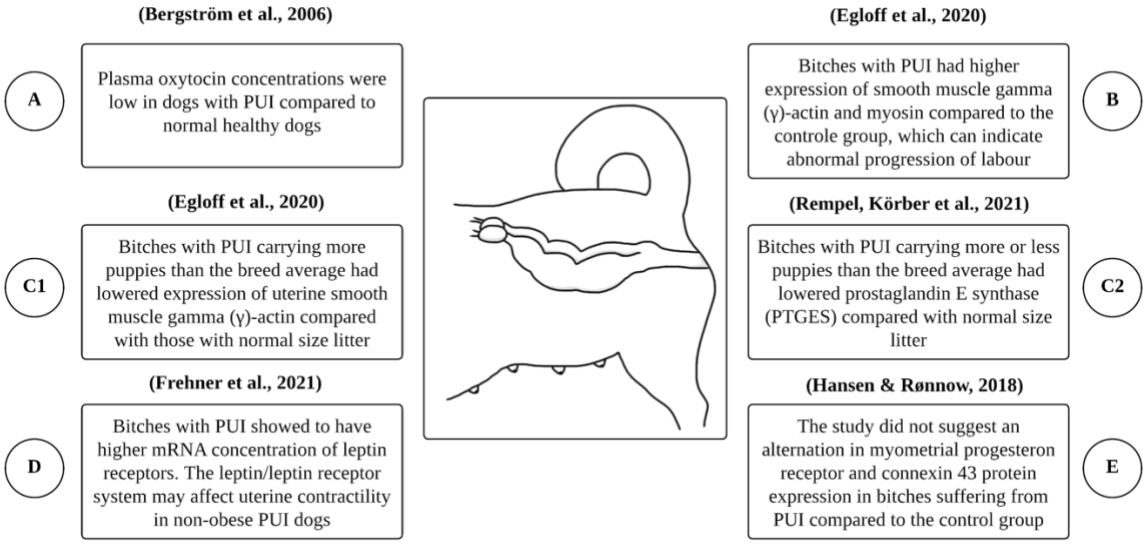


Figure 2: Illustration of conclusions from scientific publications investigating aetiologies behind canine PUI. The markings (A-E) outside each box represents which aetiology suggestion have been implied in the mentioned publication consistent with written in the body text in section 3.3.1 "Aetiology of primary uterine inertia". Above each textbox is a parenthesis with the author(s) and publication year: A) (Bergström et al., 2006), B and C1) (Egloff et al., 2020), C2) (Rempel, Körber, et al., 2021), D) (Frehner et al., 2021) and E) *(Hansen & Rønnow, 2018)*.a

Relevant for this study, it has been investigated if prostaglandins (PGs) play a role in the aetiology of canine inertia, since their presence in eutocia is essential. A study (Rempel, Körber, et al., 2021) hypothesized that prostaglandin E2 (PGE2), 15-hydroxy prostaglandin dehydrogenase (HPGD) and prostaglandin transporter (PGT) had an important role in the aetiology of uterine inertia in dogs. They further expanded their project and investigated the expression of prostaglandin E synthases (PTGES) and their following receptors (PTGER2/4). Lastly, they also screened their groupings for litter size and specifically investigated whether PGE2 expression could be influenced by the number of foetuses. Through RT-PCR, they found that the PTGES, PTGER2, PTGER4, HPGD and PGT mRNA expressions did not differ significantly between the PUI group and OD control group. Most relevantly, they did discover a significantly lower mRNA expression of PTGES in the PUI group with a smaller and larger litter size compared to the PUI with normal litter sizes and OD group. This gives a relevant insight into possible predisposing factors of PUI, seeing a smaller and larger litter size can raise difficulties in prostaglandin synthesis and thereby in parturition itself.

The same research group (Rempel, Lillevang, et al., 2021) investigated the mRNA and protein expression of prostaglandin endoperoxidase synthase 2 (PTGS2), PGF2-α synthase (PGFS) and the corresponding receptor (PTGFR) in interplacental (IP) and uteroplacental sites of the uterus (UP) in bitches with PUI. When compared to the control group, which consisted of dogs with obstructive dystocia (OD), the authors did not find a significant difference in the mRNA expressions between the PUI and OD group, neither in the IP nor the UP layers. The study did identify a significant difference in PTGS2 staining intensity between LM and CM layer in the IP samples with the LM layer staining stronger.

Recent preliminary results from members of the same research group investigated the ex vivo effects of prostaglandins in an organ bath study[5]. The study also showed a difference in contractions strength and frequency between the CM and LM layers when treated with prostaglandin. Here, the CM layer responded strongly compared with the LM layer where no response was seen. The response and difference seen in the layers was true only when treated with LD50 dosages of prostaglandins (Jungmann, Mazzuoli-Weber, et al., 2023).

[5] **Organ bath:** Also known as an isolated tissue bath. A chamber where isolated tissue can be administered with drugs.

3.4 Diagnosis and treatment of primary uterine inertia

Several factors are involved in the complete diagnosis of primary uterine inertia (PUI), such as a thorough history, clinical signs, and a physical examination (Davidson, 2021). Clinical signs of PUI involves a decline in plasma progesterone and a subsequent decline in rectal temperature indicative of initiating labour (Davidson, 2021; Parkinson et al., 2019b), zero puppies birthed yet green discharge from the vulva appear (Davidson, 2021) followed by abdominal and uterine contractions that fail to commence or do not progress (Parkinson et al., 2019b). Diagnostic imaging is recommended to rule out possible obstructive causes and/or to confirm foetal viability (Davidson, 2021). To directly measure uterine activity and therefor make a definite diagnosis of PUI, equipment such as tocodynamometry has shown to be an effective tool (Jayakumar et al., 2019), where spikes indicating abdominal contractions cease to appear. The machine is connected to a uterine sensor and foetal Doppler probe allowing both measurements of intrauterine and intra-amniotic pressure as well as foetal heartrate (Davidson, 2001, 2021). Furthermore, tocodynamometry has shown to visualise effects of medical treatments such as mentioned below (Jayakumar et al., 2019).

According to current knowledge, the preferred medical approach to treating inertia is a combination of exogenous oxytocin and calcium (Davidson, 2021). Treatment with such uterotonic drugs is only recommended when 1) OD is ruled out, 2) the cervix is confirmed dilated, 3) remaining number of foetuses are low and 4) parturition is not too protracted already. Lastly, it is vital that the overall health status of the bitch is good before such treatments are initiated (Davidson, 2021). One study (Jungmann, Pyzik, et al., 2023) analysed different oxytocin concentrations and their effects on the contractility of the myometrium. The study concluded that higher doses of exogenous oxytocin (10 nM and 100 nM) caused the myometrium to spasm. This was true for both layers of the myometrium. The ideal dose was found to be 1 nM oxytocin. In clinical terms, mini doses starting at 0.25 IU/animal subcutaneously or intramuscularly has proven to be effective (Davidson, 2021). Though oxytocin is said to be the most potent uterotonic drug available, it has a failure rate of 54.3%, failure meaning a puppy was not conceived after treatment (Munnich & Küchenmeister, 2009).

The purpose of the calcium gluconate administration is to strengthen the contractions (Davidson, 2021; Parkinson et al., 2019b). A 10% solution is recommended with a dosage of 1.0 ml/5.5 kg (~0.2 ml/kg) subcutaneously and is typically attempted before or alongside oxytocin (Parkinson et al., 2019b). Medical treatment using calcium and/or oxytocin has shown to be useful in only 27.6% of cases (Darvelid & Linde-Forsberg, 1994).

Another ecbolic drug is denaverine hydrochloride. The gathered information on the medicine is limited but it is described as a neurotropic-musculotropic spasmolytic agent. Specifically, it decreases the tone of the muscle in the soft birth canal. One recent study (Jungmann, Pyzik, et al., 2023) found that denaverine failed to increase myometrial contractions with or without oxytocin. They also found that in the parturient bitch, denaverine did not have a priming or spasmolytic effect before oxytocin stimulation. Hence, there are no current studies proving the beneficial interactions of denaverine on solely the canine myometrium. The only beneficial effect on canine parturition proven by denaverine is when combined with oxytocin, where it showed to significantly cause less litters with stillborn and hypoxic puppies compared to when treated with oxytocin alone (Munnich & Küchenmeister, 2009).

If medical approaches have yet to improve the parturition, surgical approach in form of Caesarean section is recommended (Davidson, 2021; Parkinson et al., 2019b).

3.5 Histological construction of the canine uterine wall and placenta

3.5.1 The uterine wall

The uterine wall is composed of three layers: the endometrium, myometrium and perimetrium. The endometrium is the thin most inner layer of the uterus and is rich in vascular and glandular tissue. (Premanandan, 2017; Priedkalns, 2006) The thick inner muscular layer of the uterus is called the myometrium, which consists of two distinctive layers. The innermost layer is called the *stratum circularis*[6] and the outermost is the *stratum longitudinalis*[7] (Priedkalns, 2006). Both layers have very distinctive appearances in a cross-sectional histological sample because of the course of the muscle fibres. Despite the layers given names, when looked in a cross-sectional view, the CM layer visually appears with myocytes running parallel in longitudinal patterns. The visual appearance of the LM layer also shifts, and the myocytes appear rounded in bundles in a circular manner (Priedkalns, 2006). Both muscle layers serve their purpose to contract and relax during parturition (Parkinson et al., 2019b). In between these muscular layers is a vascular layer called the *stratum vascularis* which contains blood vessels, lymph vessels and nerves. It also contains various amounts of myocytes (Priedkalns, 2006). The most peripheral layer of the uterine wall is the perimetrium. The layer is thin and consists of loose connective tissue covered by peritoneal mesothelium (Priedkalns, 2006).

[6] **Stratum circularis:** Earlier referenced to as the circular layer of the myometrium.
[7] **Stratum longitudinalis:** Earlier references to as the longitudinal layer of the myometrium.

3.5.2 The canine placenta

The placenta is a delicate yet extremely important organ which carries out nutrients and oxygen supply to the embryo. It is directly attached to the uterine endometrium and will stay intact during canine parturition to only be disrupted at the expulsion phase (Taverne & Noakes, 2019). Several membranes are present in the placenta: the amnion, yolk sac, chorion, and allantois (Taverne, 2019). Placental types vary amongst species, where members of the canine family all have zonary placentas of endotheliochorial type (Dantzer & Leiser, 2006; Taverne, 2019). The 'zonary' classification refers to the distribution of contact area, or *villi*, between the maternal and foetal compartments, which in the dog is presented as an annular shape of the allantochorion that encircles the embryo (Dantzer & Leiser, 2006). The foetal membranes and placental type are illustrated on figure 3.

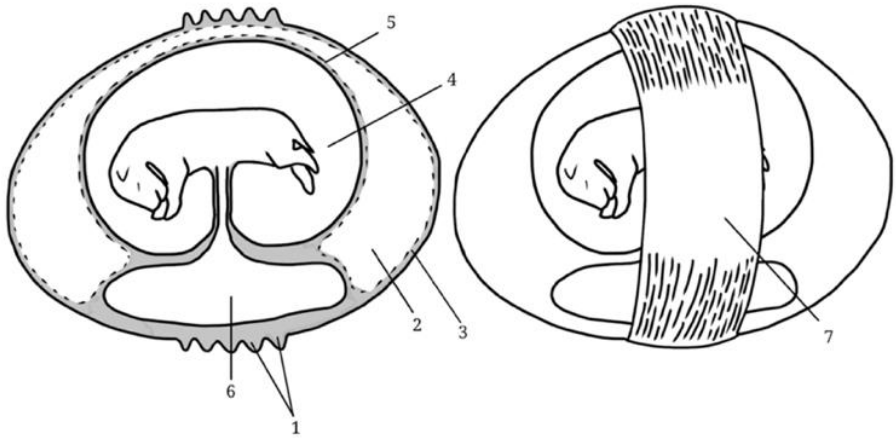


Figure 3: Illustration of foetal membranes of the dog. To the left, a longitudinal cross section of the embryo and foetal membranes are shown. Each digit represents its own membrane: **(1)** Allantochorion involved in the zonar placenta **(2)**, Allantoic cavity, **(3)** Allantochorion outside the zonar placenta, **(4)** Amniotic cavity, **(5)** Allantoamion, **(6)** Yolk sac and **(7)** The zonar placenta with an annular shape around the embryo. The illustration is made taking inspiration from (Taverne, 2019), though modifications has been made to fit the desired presentation.

The placenta is a well vascularised organ. It carries both the foetal and maternal vessels, which grow throughout gestation (Dantzer & Leiser, 2006). Vessels from both parts of the placenta develop a branching network to provide a better supply of nutrients to the growing embryo. The branches of foetal vessels carry closely related trophoblasts with them (Taverne, 2019). These specialised embryonic cells are directly connected to the foetus' blood supply and function by communicating with the mother's specialised endometrial cells called the decidual cells. Decidual cells derive from fibroblasts in the endometrium and have an enlarged, polyhedral appearance (Dantzer & Leiser, 2006). Whereas trophoblasts can vary in appearance depending on their differentiation they often

appear smaller compared to decidual cells (Dantzer & Leiser, 2006). Communication between these two types of cells is especially important during decidualization[8], when the early implantation of embryo takes place (Priedkalns, 2006).

3.6 Contractions-associated proteins (CAPs)

Numerous communications between cells take place during a uterine contraction. One important group of signal molecules in eutocia is the so-called contraction-associated proteins (CAPs). Known CAPs are calcium channels, oxytocin, prostaglandin-F receptor (PTGFR) and connexins (Cx) (Taverne & Noakes, 2019). This paper will specifically focus on the PTGFR, Cx26 and Cx43.

3.6.1 Prostaglandins (PGs) and their role in myometrial contractions

Prostaglandins (PGs) are signal molecules of the eicosanoid[9] family and are synthesized from arachidonic acid via the cyclo-oxygenase pathway (Smith, 1989; Taverne & Noakes, 2019). PGs are produced by nearly all cells within the body and have been known to act locally rather than systemically (Smith, 1989). Both PGs ($PGF_{2\alpha}$ and PGE_2) play a role both in cervical ripening and myometrial contractions by interacting with their appropriate receptors (Taverne & Noakes, 2019). $PGF_{2\alpha}$ has two separate receptors it can act through: The prostaglandin EP4 receptor (EP4) and the prostaglandin F2-alpha receptor (PTGFR). Regarding stimulation of myometrial contractions $PGF_{2\alpha}$ must interact with the PTGFR (Taverne & Noakes, 2019).

3.6.2 The synthesis, regulation and breakdown of prostaglandins

The synthesis of PGs has been shown to occur in most organs and are not restricted to a specific endocrine organ (Park et al., 2006; Smith, 1989). The availability of $PGF_{2\alpha}$ and PGE_2 is dependent on the expression of the enzymes responsible for their synthesis alongside the activity of the enzymes degrading the proteins. Prostaglandins are synthetised through prostaglandin specific synthetases (PGHS) whereas cyclooxygenase 2 (PTGS2, formerly known as COX2), is the key enzyme that allows formation of $PGF_{2\alpha}$ and PGE_2 from the common precursor named prostaglandin H2 (PGH2) (Park et al., 2006; Smith, 1989; Taverne & Noakes, 2019). Additionally, oestrogens play a role in upregulating PTGS2 and thereby $PGF_{2\alpha}$ and PG_{E2} by activating phospholipase A2. The enzyme

[8] **Decidualization:** The morphological changes of the endometrium in the uterus where maternal derived decidual cells form a lining preparing for implantation (Graubner et al., 2017).
[9] **Eicosanoid:** Signaling molecules made by enzymatic or non-enzymatic oxidation of arachnoid acid (Smith, 1989).

phospholipase A2 is the key to converting membrane phospholipids into arachidonic acids, that will later be converted into the PTGS2 (Taverne & Noakes, 2019).

The $PGF_{2\alpha}$ synthases have been recognized in the trophoblast of the feto-maternal contact zone but have shown to be sparse in the myometrium. Furthermore, it has shown to be up regulated during post implantation and declined in the prepartum phase (Gram et al., 2013). Oxytocin, which is a strong uterotonic hormone on itself, plays a role in upregulating $PGF_{2\alpha}$ by stimulating its synthesis and thereby following secretion. Oxytocin and prostaglandins thereby have a synergistic effect on the myometrium (Taverne & Noakes, 2019).

In terms of the downregulation of $PGF_{2\alpha}$, 15-hydroxyprostaglandin dehydrogenase (PGDH) plays a crucial part. The enzyme catabolizes both PGE_2 and $PGF_{2\alpha}$ to their inactive metabolites. (Taverne & Noakes, 2019). PGDH has shown to be upregulated until post-implantation, is low at mid-gestation and suppressed during prepartum in dogs (Gram et al., 2013).

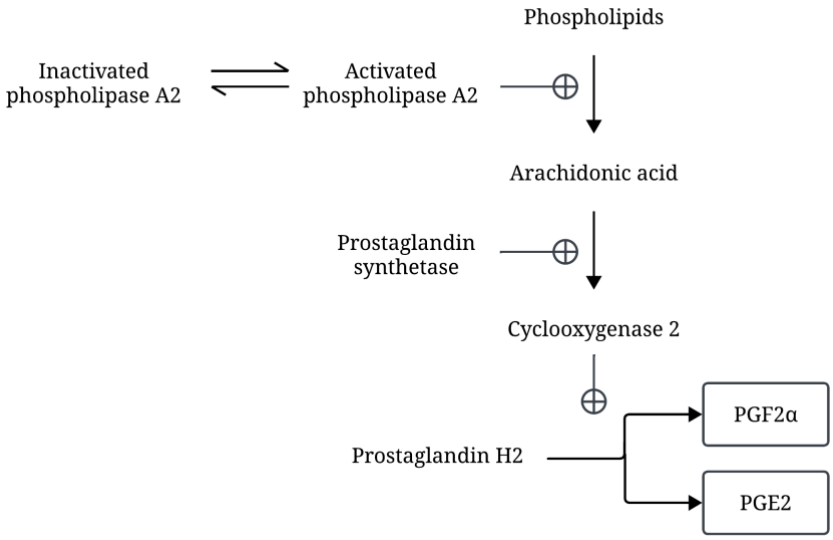


Figure 4: Illustration of the synthesis of PGF2α and PGE2. The simple constructed arrows **(→)** show the general synthesis of intermediate and final products. The lines with encircled crosses (⊕) illustrates what processes the specific enzymes effect. Modified illustration from *(Taverne & Noakes, 2019).*

3.6.3 The structure and function of connexins

Connexins are components of gap junction channel proteins called connexons (Hussain, 2014; Laird, 2006). One connexon is composed of six connexins. Connexins themselves are membrane bound yet consist of different domains that are either transmembrane or extracellular. In total, a single connexin protein consist of six domains; four being transmembrane (M1-M4) and two being extracellular (E1

& E2) (Hussain, 2014; Laird, 2006; Nielsen et al., 2012). Gap junction proteins such as connexons are responsible for intercellular communication and allow passage of molecules up to 1000 dalton, such as nutrients, waste products, metabolites, second messengers or ions (Laird, 2006). While connexins have been found in various type of tissues, four different connexins have been described in uterine tissue: Connexin 26, 40, 43 and 45 (Döring et al., 2006). This paper will focus on the connexin 26 (Cx26) and connexin 43 (Cx43).

In general, connexins have a short half-life of only a few hours. It has been suggested that the short half-life contributes to fast up-and down regulations in response to physiological requirements. Especially when taking into consideration the requirement seen in the myometrium prior to labour, where steroid hormones promote a 5-fold increase in total gap junctions (Laird, 2006).

Cx43 has been shown to be scarce in the myometrium during pregnancy in humans but increase in size and abundance during parturition (Laird, 2006). More recently it has been shown to be essential for uterine contractions during labour. In the extensive ablation of Cx43 in parturient mice, it has been shown that the proper function of SMCs in vitro/vivo was inhibited, and that the length of labour was increased (Döring et al., 2006). Not many studies on dogs have investigated the importance of Cx43 in uterine tissue. One study investigated locations of different selected connexins in the canine myometrium and found Cx43 to be mostly targeted to the maternal endothelium of the placenta (Graubner et al., 2018). Another study investigated the potential role of Cx43 and its role in uterine inertia but did not conclude a significant difference between the PUI and OD group (Hansen & Rønnow, 2018). Several factors are found to respectively up- and downregulate the expression of Cx43. For instance, the physical stretching or distention of the myometrium in rats during labour is required for full expression of myometrial Cx43 (Ou et al., 1997). Furthermore, products such as oestradiol and intracellular calcium are also believed to upregulate the Cx43 expression (Garfield et al., 1980; Nielsen et al., 2012). Progesterone effectively downregulates Cx43. More specifically, when trying to stimulate the Cx43 upregulation through myometrial stretching, administration of progesterone 24 hours before the action proved to block the increase (Döring et al., 2006).

Additionally, **Cx26** has specifically been chosen since it was shown to be highly expressed in the myometrium and placental tissue of rats during late pregnancy (Ou et al., 1997). This rise during late pregnancy was confirmed to be momentary, as Cx26 drops with onset of parturition. Cx26 does not seem to be affected by myometrial stretching analogously to the Cx43, and it has therefore been suggested that Cx26 is not affected by physical foetal growth (Ou et al., 1997). Interestingly, the myometrial Cx26 does appear to be ovarian dependent, seeing the normal rise of Cx26 late in

pregnancy was blocked when rats were ovariectomized. Furthermore, the downregulation of Cx26 could be blocked by the progesterone, proving that contrary to Cx43, Cx26 role is more evident in maintaining uterine quiescence rather than promote contractile forces (Ou et al., 1997). When investigated in canine uterine tissue and the differences in gestation-periods, Cx26 concentrations similarly showed to be most evident through mid-gestation and decreased towards prepartum luteolysis (Graubner et al., 2018). The same study investigated localisation patterns of Cx26 amongst others in uterine prepartum luteolysis though IHC staining. Results showed that Cx26 was present in placental trophoblasts and decidual cells as well as in blood vessels, glandular tissue, and connective tissue in the endometrium. Lastly, it was evident in both smooth muscle cells and blood vessels of the myometrium. To this date, the information on canine uterine connexin regulation is scarce.

4. Materials and methods

4.1 Materials

The experiment in this study was performed in accordance with the animal welfare legislation (*permit no. 2015-15-0201-00513, Dyreforsøgstilsynet, Fødevarestyrelsen; permit no. ZH086/15, Cantonal Veterinary Office Zurich*). This study is based on pregnant dogs presented for medically indicated Caesarean section (C-section) due to dystocia. All the dogs underwent surgery prior to sample collection. The samples were collected from healthy bitches during C-section and with the owners' consents. In this context, healthy means that, apart from dystocia, the bitches did not show any signs of systemic disease. An overview of the data collection process is illustrated in figure 5.

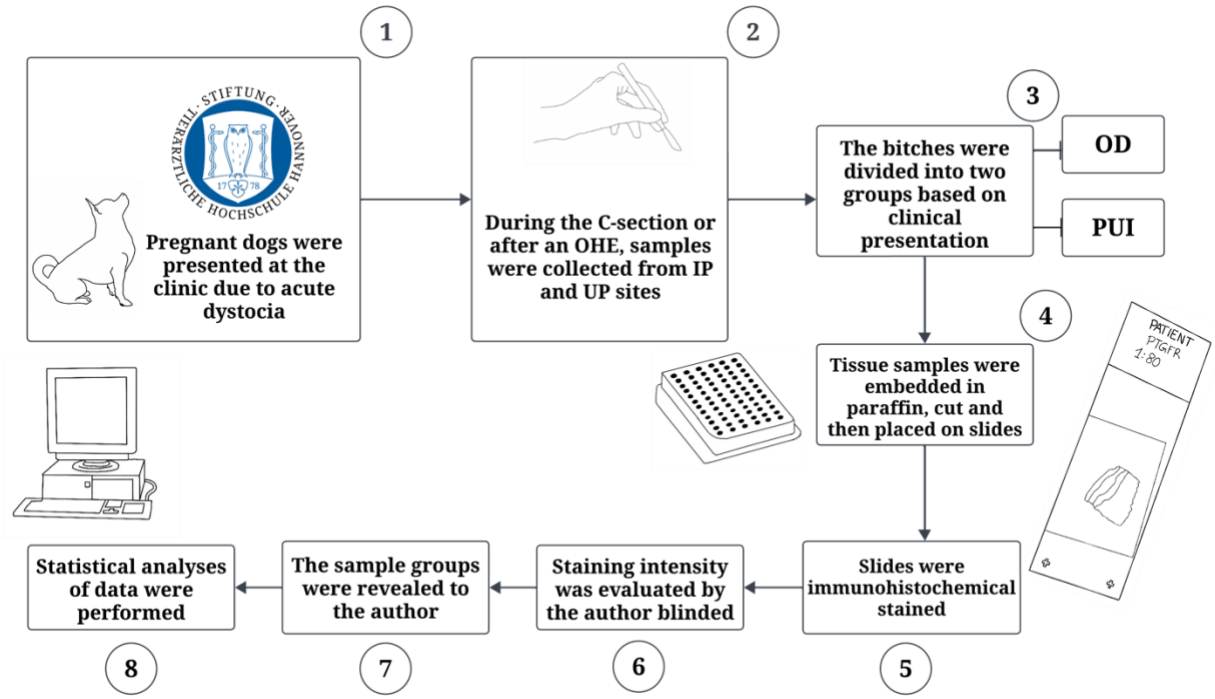


Figure 5: Figure 6: An illustration over the data collection process. The process can be read from the left top corner and followed down by a numerated order (step 1-8). Abbreviations used: **C-section** = Caesarean section, **OHE** = Ovariohysterectomy, **IP** = Interplacental, **UP** = Uteroplacental, **OD** = Obstructive dystocia, **PUI** = Primary uterine inertia.

4.1.1 Samples and groups

During C-section an IP tissue sample was collected from all bitches. If the owner chose to have their bitch spayed with the procedure ovarian hysterectomy (OHE), an UP sample was collected in addition to the IP sample. Bitches included in the study were retrospectively assigned to one of the

two groups: PUI (n = 12) or OD (n = 8). When the bitches were assigned into the PUI or OD group, they had to pass a list of strict inclusion criteria based on their history and clinical presentation. The same criteria are published in (Rempel, Lillevang, et al., 2021) as listed below:

The PUI dogs showed signs of first stage labour with no progression to second stage. Foetal fluids had passed but showed no progression in parturition since. They presented with zero puppies born and weak or total absence of contractions alongside a weak or negative Ferguson reflex[10]. Furthermore, the owners had mentioned the presence of abnormal vaginal discharge. Before making the diagnoses of uterine inertia, the dogs underwent an obstetrical examination including an ultrasound and/or x-ray to rule out possible obstructive causes.

The OD dogs showed normal abdominal and/or uterine contractions with clear signs of second stage (active birth). They would present with strong spontaneous abdominal and/or uterine contractions. Visible straining was observed when vaginal digital manipulation (feathering) was performed. The bitches could have given birth to zero, one or more puppies before showing signs of dystocia. Dystocia was suspected when >4 hours had passed in-between the puppies. An obstetrical examination and/or diagnostic imaging was used to rule in an obstructive cause.

Besides the clinical presentation and classification of dystocia (PUI/OD), records of age, breeds, parity, body weight, litter sizes and P4 values of all dogs were included. The litter sizes of the PUI group were further divided into three categories: 1) Normal litter size, 2) Small litter size and 3) Large litter size. The normal litter size was defined by the average litter size of the respective breed (N) ±1 (Borge et al., 2010). To summarize, 12 dogs were categorized in the PUI group whilst 8 were categorized in the OD group making in total 20 dogs which underwent C-section with collection of IP biopsies (IP n = 20). Out of these dogs, 4 from each group, meaning 8 in total, underwent OHE and thus collection of UP samples was possible (UP n = 8). All the tissue samples were formalin fixated, embedded in paraffin, and used to cut 3 µm sections that were mounted on microscope slides. These slides were used for immunohistochemical staining. For PGTFR and Cx43, all IP and UP slides were included. Only for Cx26, one IP slide was excluded because of default staining, meaning that no staining intensity was noticed in any of the layers. The classification of the dogs alongside litter sizes and included samples can be seen in table 1.

[10] **Ferguson reflex:** A neuroendocrine reflex initiated by physical pressure at the cervix resulting in uterine contractions.

Table 1: Classification of samples and groups. The table shows the breeds alongside included biopsy samples (IP/UP) in the PUI and OD group. If an "X" has been marked in the IP and/or UP box, it illustrates that the biopsy was included from the patient. If an hyphen, "-", has been marked, it illustrates the lack of a biopsy. If an asterisk symbol, "*", has been added to the hyphen it illustrates that a biopsy was made but later excluded in the IHC evaluation step.

Group	Breed	Litter size group (S/M/L)	PTGFR		Cx26		Cx43	
			IP	UP	IP	UP	IP	UP
PUI	Dachshund	Small	X	-	X	-	X	-
(*n* = 12)	Boxer	Small	X	-	X	-	X	-
	French Bulldog	Small	X	-	X	-	X	-
	French Bulldog	Small	X	-	X	-	X	-
	Maremmano	Small	X	X	X	X	X	X
	Maltese	Normal	X	-	X	-	X	-
	Broholmer	Normal	X	-	X	-	X	-
	German Sherpherd	Normal	X	-	X	-	X	-
	Labrador	Normal	X	X	X	X	X	X
	Maremmano	Large	X	-	X	-	X	-
	Maltese	Large	X	X	X	X	X	X
	Beagle	Large	X	X	-*	X	X	X
OD	Cairn Terrier		X	-	X	-	X	-
(*n* = 8)	Staffordshire Bull Terrier		X	-	X	-	X	-
	Mix		X	-	X	-	X	-
	Chihuahua		X	-	X	-	X	-
	West-Highland White-Terrier		X	X	X	X	X	X
	Yorkshire Terrier		X	X	X	X	X	X
	Zwergschnauzer		X	X	X	X	X	X
	Chihuahua		X	X	X	X	X	X

4.2 Methods in detection of contraction associated proteins

Prior to descriptions of methods, it is valid to mention that the author of this study only participated in the IHC staining process of PTGFR and not Cx26 nor Cx43. The author evaluated all the completed IHC slides of PTGFR, Cx26 and Cx43 and participated in the statistical analysis of the results. The RT-qPCR process and statistical analysis hereof were completed by a third party and handed to the author for incorporation into the master thesis.

4.2.1 Immunohistochemical detection of PTGFR

The tissue samples used for the IHC staining were formalin fixed and then paraffin embedded before being cut in 3 µM sections and mounted on microscope slides (*Thermo Scientific/Menzel-Gläser, Braunschweig, Germany).*

Deparaffinization and rehydration: For the preparation of the IHC slides, each slide was deparaffinized with Xylene for 10 minutes twice, followed by rehydration bath in decreasing alcohol concentrations (100%, 96% and 75%). The slides soaked for 2 minutes in each concentration. They were then washed for 5 minutes using regular cold tap water.

Antigen-unmasking: To prepare for the antigen unmasking, the slides were transferred to a citrate buffer *(9 ml solution I + 41 ml solution II + 500 mL double destilled water)* for 5 minutes at room temperature. Next, the slides were transferred to another buffer bath with the same content, only it had been prewarmed in the microwave. The buffer solution with the slides was placed in the microwave for 5 minutes at 560 watt 3 times in total. Afterwards the citrate buffer with the slides were left to cool down at room temperature for 20 minutes. Slides were then washed for 5 minutes under cold tap water.

Blocking of the endogenous peroxidase reaction: The slides were placed in a closed container for 30 minutes with a mix of 7,5 ml 30% hydrogen peroxide (H_2O_2) and 241 ml Methanol.

Blocking of the non-specific antibody: To block any non-specific antibody reactions, the slides were washed with ICC buffer (*6 ml TritonX, 200 ml 10xICC, 1800 ml Aqua bidest*).

Peptide blocking: The slides were placed in a Shandon cover plate and the appurtenant racks. They were then incubated with 100µl serum for 30 minutes. The serum used contained 10% goat serum combined with 5% bovine serum albumin resolved in phosphate buffered saline (BSA). Afterwards, the slides were washed 3 times with ICC buffer, letting the buffer run completely over the slide in-between washes.

Specific antibody-binding: The specific IgG PTGFR-antibody "Anti-Prostaglandin F2 alpha Receptor/PTGFR antibody" from a rabbit host (*Abcam, Cambridge, England*) were pipetted onto the slides using exactly 90µl (diluted in ICC-buffer 1:80) per slide. The slides were incubated overnight in a fridge (4°C) for 20 hours.

When the hours had passed, they were removed from the fridge and left on the counter at room temperature for 30 minutes. They were then washed in their chambers with ICC-buffer 3 times. Slides were then incubated with the biotinylated goat anti-rabbit IgG secondary antibody (*BA-1000, Vector Labaoratories Burlingame, CA, USA*) in 10% goat serum in 5% BSA, using 90 µl per slide (diluted

in ICC buffer 1:200). Following 30 minutes incubation of the secondary antibody, washing with ICC-buffer was repeated 3 times to remove the antibody. An ABC solution (*2.5 ml ICC buffer + 1 drop of solution A + 1 drop solution B*) was added using 100µl per slide and incubated for 30 minutes and washed with ICC-buffer 3 times again. A Pap-pen was carefully used a circular section around the tissue sample to prevent the upcoming staining from running off the slide.

Staining: Vector NovaRED© (*Vector Laboratories, California, United States*) was added (90µl per slide) and covered with tinfoil to protect it from natural light. The staining was checked under the microscope and the NovaRED was removed when sufficient staining was achieved (9 minutes). The slides were then washed with cold tap water for 10 minutes to wash excess stain off.

Counterstaining: Lastly the slides were counterstained with Hämatoxilin and Eosin (Dipped 3 times and left for 30 minutes) and then rewashed with cold tap water for 10 minutes. The slides were then dehydrated and therefor left in 95% alcohol for 2 minutes, then 100% alcohol twice for 2 minutes and lastly Xylene twice for 3 minutes. The slides were then covered with a cover glass secured with glue (*Histokit, Valladolid, Spain*). All the samples were left out to dry under air suction for 3 days.

Negative control and isotype control: For the negative control the ICC buffer solution was used instead of the specific IgG PTGFR antibody. The slide for the isotype control was incubated with rabbit IgG, control antibody (*I-200, Vector Labaoratories Burlingame, CA, USA)* and was pipetted on instead of the primary antibody.

4.2.2 Immunohistochemical detection of Cx26 and Cx43

The author of this thesis did not assist the IHC staining process of Cx26 and Cx43, as it was conducted by a third party. The description of the used IHC staining protocols can be found in previous publications. For Cx26, the protocol is published in (Graubner et al., 2018), whilst the Cx43 protocol can be found in (Reifarth et al., 2023). An overview of the used antibodies and isotype controls for the generated IHC slides are depicted in table 2.

Table 2: Overview of used antibodies in IHC staining. The table includes the name of the specific antibody as listed by the clone, host species, protein concentration, applied dilution and antibody isotype. The dilution is noted in µg/µl and S:D = Solute to diluent. (* Abcam, Cambridge, England; ** Thermo Fisher Scientific Inc., Waltham, MA, USA.; *** Cell Signalling Technology., Danvers, MA, USA; **** Vector Laboratories Burlingame, CA, USA).

Antibody	Clone/description	Source	Protein Concentration µg/µl	Dilution µg/µl (S:D)	Isotype control
PTGFR	Anti-Prostaglandin F2 alpha Receptor/PTGFR Polyclonal antibody, Catalog #ab203342*	Rabbit	0.013	0.013 (1:80)	Rabbit IgG (I-1000-5 Control Antibody)****
Cx26	Connexin 26 Polyclonal Antibody, Catalog #71-0500**	Rabbit	0.0013	0.005 (1:200)	Rabbit IgG (I-1000-5 Control Antibody)****
Cx43	Connexin 43 Polyclonal Antibody, Catalog #351***	Rabbit	0.002	0.02 (1:50)	Rabbit IgG (I-1000-5 Control Antibody)****

4.2.3 Evaluation of immunohistochemical samples

For each sample an overview was made of which cells had stained; Myometrial cells, endothelial cells and cuboidal glandular cells in both IP and UP samples and further trophoblast and decidual cells in the UP samples. If the layers of the myometrium had been deemed 'correctly stained' a more thorough evaluation of the staining intensity was made. For the evaluation of IHC staining intensity, a semi-quantitative method was applied. The staining intensity was subjectively evaluated by a qualitative manner yet processed to fit a numerical ordinal[11] scale.

It was decided to use a scale from [1-3] to describe the staining intensity. A score of [1] meant a very weak perinuclear (PN) and/or cytoplasmatic (CP) stain or no stain at all, a score of [2] meant a moderate PN and/or CP stain and lastly a score of [3] meant a strong PN and or CP stain. The grading system used for the PTGFR samples are shown in table 3, and the Cx26 and Cx43 samples are shown in table 4.

[11] **Ordinal:** Data that originates in qualitative measurements yet is grouped into numerated categories.

Table 3: Examples of intensity scores of PTGFR samples. The table illustrates the staining scores [1-3] in the PTGFR slides via photo examples. Both samples from the CM layer and LM layer are included to illustrate how the layers can appear different visually yet still have the same staining score.

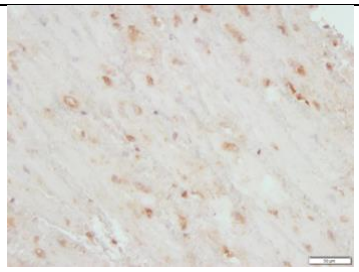
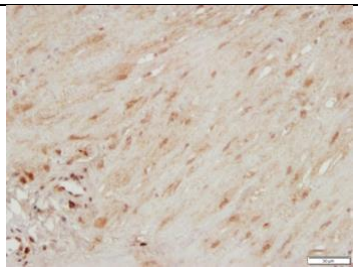
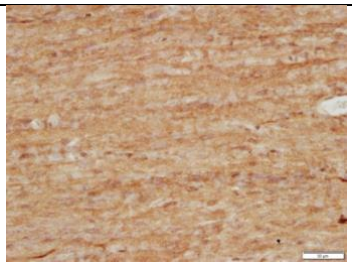
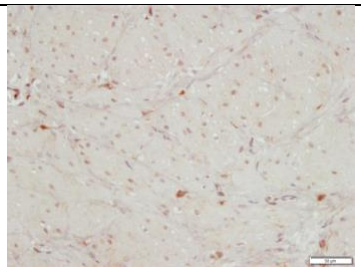
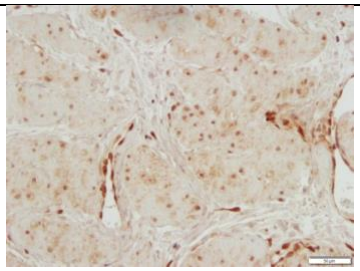
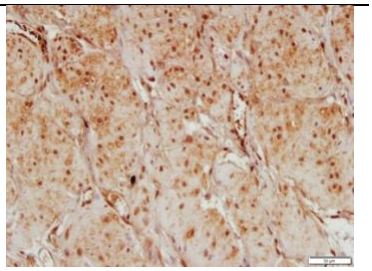
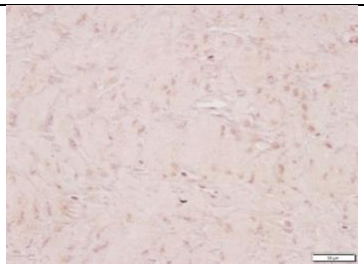
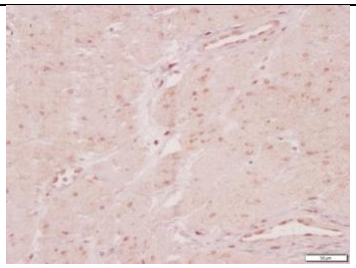
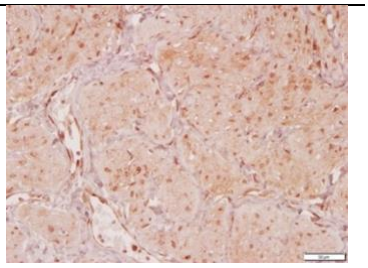
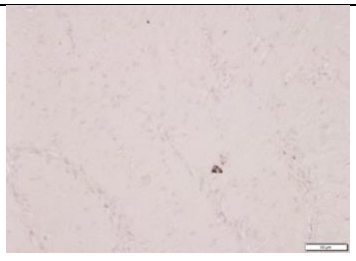
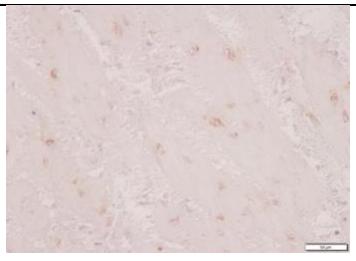
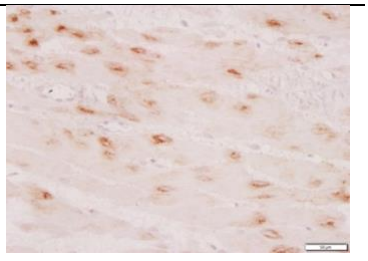
PTGFR samples			
Score	1	2	3
Intensity	None/weak CP/PN stain	Moderate CP/PN stain	Strong CP/PN stain
CM			
LM			

Table 4: Examples of intensity scores of connexin samples. The table illustrates the staining scores [1-3] in the Cx26 and Cx43 slides via photo examples. The photos included are a mix of the CM and LM.

Connexin samples			
Score	1	2	3
Intensity	None/weak PN stain	Moderate PN stain	Strong PN stain
Cx26			
Cx43			

The grading of the IHC slides was solely made in the CM and LM layers of the myometrium. Each set of samples (PTGFR/Cx26/Cx43) contained at least one negative control and isotype control. The author evaluated the slides blinded, meaning there was no knowledge of the reasoning behind the surgical intervention (PUI/OD). Only the names of the submitted dogs were known and if the biopsy had been taken from IP or UP site.

In each sample, the staining intensity was evaluated centrally in both the CM and LM layers, meaning approximately fifty percent in length and width of the layers when deemed possible. This was done to avoid the known 'edge phenomenon'. The phenomenon describes how specific staining methods like IHC usually allow the periphery of the tissue samples to have a more intense staining than the centre. The author looked at each slide initially to be more familiar with the layers of the uterus. Each sample was thereafter evaluated twice. At least two days would pass in-between round of evaluations and by the time the second evaluation came, the author would blind themself to the gradings of round one. After both rounds of evaluations, a Kohens Cappa was calculated to evaluate inter-exam agreement.

The slides were evaluated and graded using a standard light microscope (*Olympus BX45, Olympus Europa SE & Co. KG, Hamburg, Germany*). The microscope was connected to a desktop computer which displayed the ocular view on the monitor with assistance from the CellsSens Dimensions software (*Olympus Europa SE & Co. KG, Hamburg, Germany).* Two magnifications were used on the microscope: 4x for an overview of the layers and 20x for grading and pictures. The program offered a fixed artificial lighting setting which was used and set to 1.3 milliseconds. On the microscope, the light setting was set precisely on 3.0. These settings were used for all the given samples (PTGFR/Cx26/Cx43).

4.2.4 qPCR detection of Cx26 and Cx43

A third party performed quantitative reverse transcriptase Polymerase Chain Reaction (RT-qPCR) on samples from the IP and UP compartments to investigate the mRNA expression of connexin 26 (Cx26) and connexin 43 (Cx43). The included data, amongst other, were collected for a current project investigating intercellular communication and its role in PUI. The RNA isolation and RT-qPCR were performed according to previous publications by the same research group (Rempel, Körber, et al., 2021). Data analysis was made by the third party as well and the results were handed to this author for evaluation and comparison between the qPCR and IHC results.

4.2.5 Statistical evaluation of data PTGFR, Cx26 and Cx43

The raw data from the IHC evaluations were noted and processed in Microsoft Excel 2023 (*version 16.76, Microsoft Corporation, Redmond, WA, USA*). From here on the data were statistically analysed in the program GraphPad Prism (*version 8.3.0 2019, GraphPad Software, Boston, USA*).

To truly determine the difference between the layers of the PUI and OD group, the following groups were constructed and statistically compared: 1) PUI IP CM and LM, 2) PUI UP CM and LM, 3) OD IP CM and LM, 4) OD UP CM and LM, 5) All IP CM and LM and 6) All UP CM and LM. The latter not taking PUI or OD grouping into considerations. Another addition was added called "MyoS", short for myometrial staining score. This group combined the CM and LM together in both the PUI and OD group, still separating the IP and UP samples.

Furthermore, it was chosen to determine if litter size in the PUI group asserted any difference in protein expression between the layers. Therefore, the following groups were made and statistically compared: 1) Litter size PUI S/M/L CM and 2) Litter size PUI S/N/L LM. All the groups that were statistically compared are shown in table 5.

The analyses chosen for the raw data depended on whether the data was normally distributed or not. The Shapiro-Wilk test was used to determine if the data was normally distributed. Normality was confirmed if $p>0,05$. If the data was normally distributed, a parametric test would have been used. Analysing the frequency distribution of the mean IHC gradings, none of analysed IP sample results in any of the investigated proteins presented with a normal distribution. Only uteroplacental (UP) of the obstructive dystocia (OD) group presented with a normal distribution. The results of the normality tests are gathered in table 17 in the appendix. Seeing most of the data was not normally distributed, a non-parametric test was necessary. Non-parametric tests used were Mann-Whitney test, the Wilcoxon matched-pairs test and the Kruskal Wallis test. The Mann-Whitney test was applied for unpaired samples (e.g. PUI versus OD) while the Wilcoxon matched-pairs test was applied for paired samples (e.g. All IP CM versus All IP LM). The Kruskal Wallis test was applied when more than two samples were compared (e.g. Litter sizes). The data was analysed in cooperation with Prof. Dr. Sandra Goericke-Pesch and DVM Carolin Jungmann.

Table 5: An overview of all the statistically compared groups. The double headed arrow "⇔" in between the groups depicts that a comparison was made.

Groups statistically compared				
PUI IP CM		⇔		PUI IP LM
PUI UP CM		⇔		PUI UP LM
OD IP CM		⇔		OD IP CM
OD UP CM		⇔		OD UP LM
All IP CM		⇔		All IP LM
All UP CM		⇔		All UP CM
MyoS PUI IP		⇔		MyoS OD IP
MyoS PUI UP		⇔		MyOS OD UP
PUI CM Small	⇔	PUI CM Medium	⇔	PUI CM Large
PUI LM Small	⇔	PUI LM Medium	⇔	PUI LM Large

5. Results

5.1 Results of PTGFR

5.1.1 Expression of uterine and placental PTGFR

All the PTGFR sample slides had a positive staining. The signal was noticed in the smooth muscle cells of the myometrium. It was specifically noticed that both the LM and CM cells had staining in every sample. In the myocytes, the staining appeared both perinuclear and cytoplasmatic but was more apparent in the cytoplasm. Staining in the endometrial endothelial cells and glandular cuboidal cells was seen in every sample as well. When screening the placental tissue, a marked staining was noticed around the vessels, with the vascular endothelial cells being brightly stained. The decidual and trophoblast cells that were closely related to vascular tissue, seemed to show positive staining but no obvious intense staining was seen when the cells were isolated from vascular epithelial. An illustration of the placental tissue is visualised in figure 7.

It was generally obvious that the outer rim of the tissue samples had a more intense signal when compared to the centre. Furthermore, a faded staining was noticed in all the UP slides, to the point where only 70-80% of the slides were stained lengthwise. The negative control and isotype control showed no staining signal in any of the tissue layers.

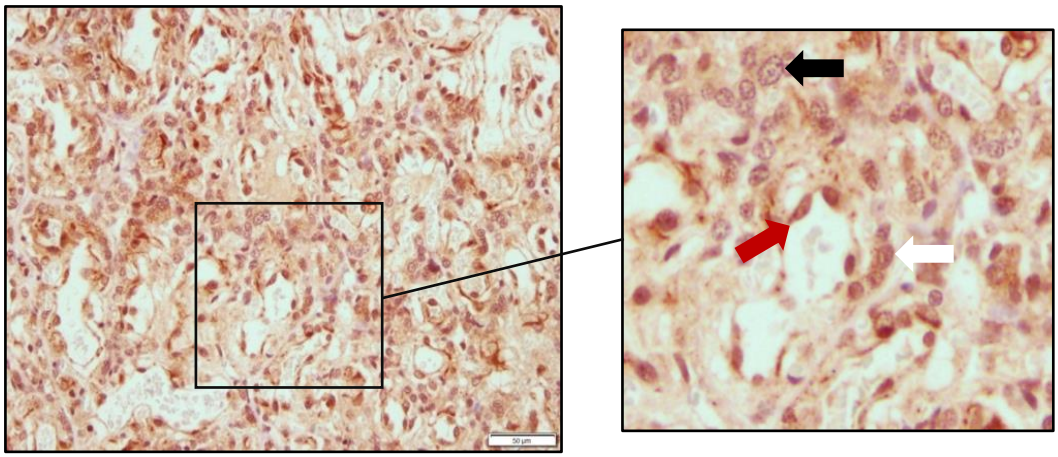


Figure 7: Microscopic illustration of placental tissue from a PTGFR sample. The illustration shows a random spot in placental tissue of one of the PTGFR IHC tissue samples. To the right, a selected area has been further magnified to visualize staining intensity in the cells. The **RED** arrow points to an endothelial cell, whilst the **WHITE** arrow points to closely related trophoblasts. Lastly, the **BLACK** arrow points to a decidual cell.

5.1.2 Interplacental and uteroplacental PTGFR expression

When evaluating and comparing the IP and UP, statistical analysis was made comparing CM and LM layers of both groups. This comparison was made to evaluate if there was a true difference between the two layers. All the IP results from the PUI and OD group were gathered into one, only letting the layers (CM versus LM) differentiate them. Evaluation of the staining intensity showed a significant difference between the CM and LM in the interplacental samples ($p = 0.001$) with the CM (2.55 ± 0.69 [2.25;2.85]) staining stronger than the LM ($1.80 \pm$ [1.43;2.17]) as shown in figure 8 and table 6. In this study, the focus is primarily on the IP group when analysing all protein expressions.

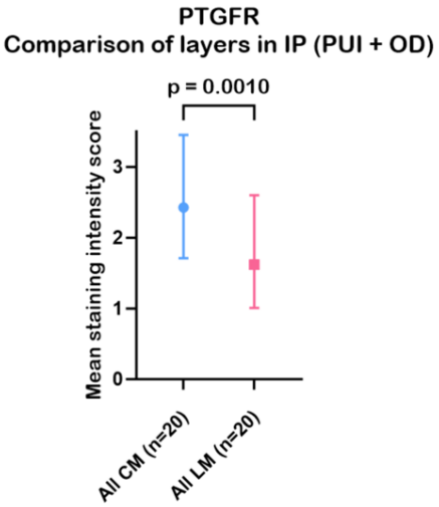


Figure 8: Comparison of myometrial PTGFR expression between layers in combined IP groups. The model contains all circular myometrial (CM) and longitudinal myometrial layers (LM) in interplacental (IP) samples from both primary uterine inertia (PUI) and obstructive dystocia (OD). Data are presented as mean ± SD.

Table 6: Interplacental comparison between layers: The table shows the parameters that are analysed/compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the significant p-value.

Parameters	**Sample size**	**Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
All IP CM	20	2.55 ± 0.69		[2.25;2.85]	
vs			Wilcoxon		p = 0.001
All IP LM	20	1.80 ± 0.83	paired t-test	[1.43;2.17]	

5.1.3 Comparison of study groups

When comparing the PUI versus OD group, there was no significant difference between the CM layers (p = 0.328) nor the LM layers (p = 0.378) in the IP group. The same comparison was made in the UP samples, where a significant difference was seen in the CM layers between the PUI and OD group (p = 0.028) but not in the LM layer (p = 0.228) as shown in figure 9. The relevance of the significant difference in the UP group will be referenced to later in the discussion section. Furthermore, to evaluate whether there was a true difference between PUI and OD groups, a gathered group named "MyoS" contained both the LM + CM of IP samples. Despite the gathering of evaluations, no significant difference was seen between the groups (p = 0.637). The results from comparisons of study groups are shown in table 7.

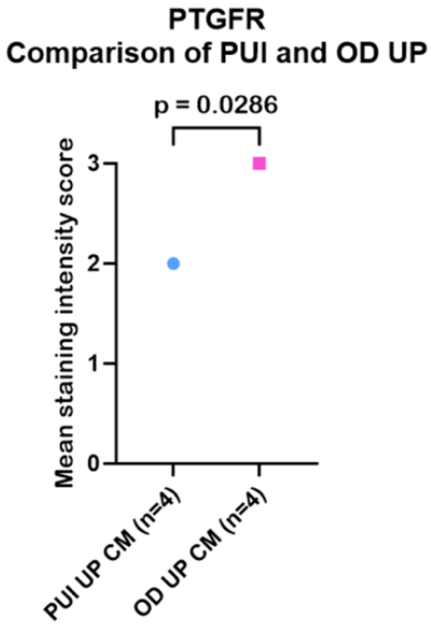


Figure 9: Comparison of myometrial PTGFR expression in UP samples between PUI and OD. The model contains data from circular myometrial layers from uteroplacental samples in both the PUI and OD group. Data are presented as mean ± SD. Notice that there is not a SD depicted, seeing the SD = 0 in both data sets, yet a significant difference (p < 0.05) could still be noted.

Table 7: Comparison of PTGFR expression between PUI and OD: The table shows the parameters that were compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the p-value.

Parameters	**Sample size**	**Distribution Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
PUI UP CM	4	2.00 ± 0.00		[0.00;0.00]	
vs			Mann-		p = 0.028
OD UP CM	4	3.00 ± 0.00	Whitney Test	[0.00;0.00]	

PUI UP LM	4	1.50 ± 0.57		[0.93;2.06]	
vs			Mann-		p = 0.228
OD UP LM	4	2.5 ± 1	Whitney Test	[1.52;3.47]	
PUI IP CM	12	2.67 ± 0.65		[2.30;4.96]	
vs			Mann-		p = 0.328
OD IP CM	8	2.38 ± 0.74	Whitney test	[1.86;2.98]	
PUI IP LM	12	1.92 ± 0.79		[1.47;3.38]	
vs			Mann-		p = 0.378
OD IP LM	8	1.63 ± 0.92	Whitney test	[0.99;2.26]	
PUI IP MyoS	24	2.29 ± 0.81		[1.97;2.61]	
vs			Mann-		p = 0.637
OD IP MyoS	16	2.0 ± 0.89	Whitney test	[1.56;2.44]	

5.1.4 Comparison of litter sizes

When comparing the IP CM and LM layer in the different litter sizes (S/M/L) of the PUI group, there was no significant differences noticed. This was both true for the CM layers ($p = 0.545$) and the LM layers ($p = 0.199$). The results from statistical analyses on litter sizes are gathered in table 14 in the appendix.

5.1.5 mRNA expression of PTGFR

When analyzing full thickness uterine samples by qPCR, no significant difference was detected between OD and PUI ($p > 0.05$). These results are also shown in the publication by (Rempel, Lillevang, et al., 2021). The aim of this study was initially to analyze PTGFR mRNA expression in the myometrial layers only by RT-qPCR analysis. Though we were able to extract mRNA and to write cDNA from formalin-fixed paraffin-embedded (FFPE) samples, we failed to get a proper standard curve using the primers that had worked previously. For this reason, we performed a conventional RT-PCR from fresh-frozen myometrial tissue confirming the presence of the PTGFR gene.

5.2 Results of Cx26

5.2.1 Expression of uterine and placental Cx26

In all the Cx26 samples, IHC signal was noticed in the smooth muscle cells of the myometrium. Both the LM and CM layer had positive signal but varied in amount. Specifically, the staining was seen located solely to the perinuclear area of the myocytes. When evaluating the IHC staining in the endometrium, the Cx26 staining intensity was intense in both vascular endothelial and glandular cuboidal cells. Moreso, the glandular cuboidal cells seemed to stain brighter than vascular endothelial cells. When evaluating the placental compartment of the UP samples, there was no signalling in the trophoblasts nor decidual cells. The vascular endothelium in the placental compartment did not stain either. The negative control and isotype control showed no staining signal in any of the tissue layers. Staining examples of Cx26 are shown in figure 10.

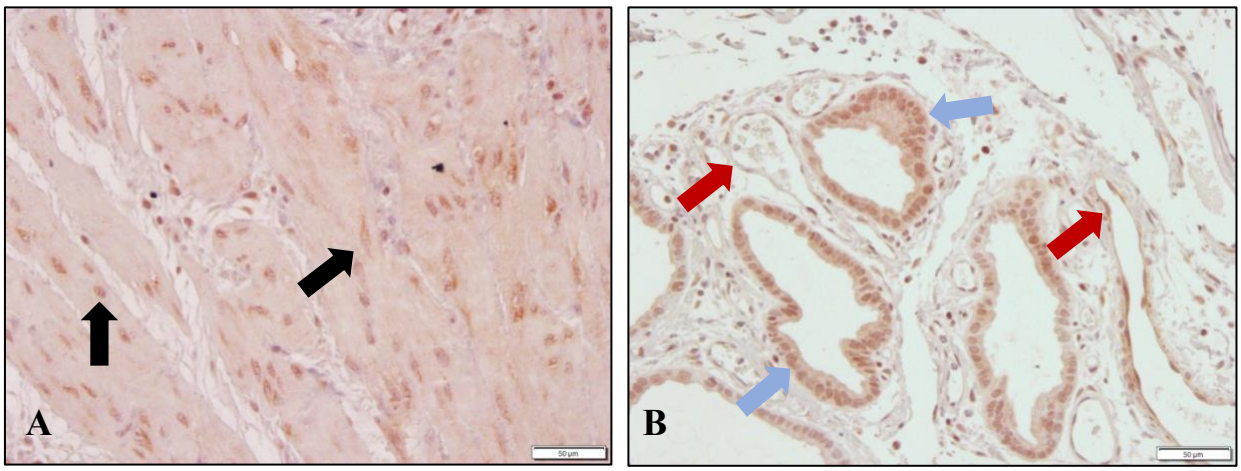


Figure 10: Microscopic illustration of Cx26 expression in myometrial and endometrial. Picture **(A)** is a microscopic image of the myometrium of a Cx26 IHC sample. The **BLACK** arrows present the perinuclear staining of the myocytes. Picture **B)** is an image of the endometrium. The **RED** arrows represent the vascular endothelium, while the **BLUE** arrows represent the glandular epithelium. The illustration is made to visualize the location of Cx26 staining and intensity in the uterus.

5.2.2 Interplacental and uteroplacental Cx26 expression

When evaluating and comparing the IP and uteroplacental UP samples of Cx26, statistical analysis was made on comparing CM and LM layers of both groups (PUI+OD). This comparison was made to evaluate if there was a true difference in Cx26 expression between the two layers. The results did not show a significant difference between the IP CM and LM ($p = 0.0566$), yet it was slightly close to significant. The comparison is shown in figure 11 and table 9.

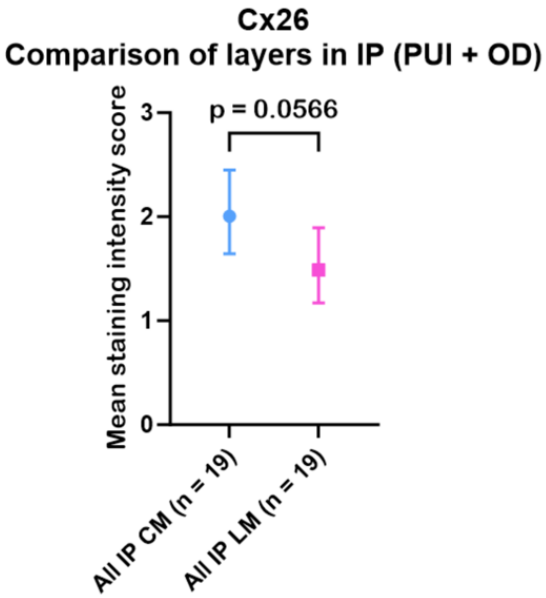


Figure 11: Comparison of myometrial Cx26 expression between layers in combined IP groups. The model contains all circular myometrial (CM) and longitudinal myometrial layers (LM) in interplacental (IP) samples from both primary uterine inertia (PUI) and obstructive dystocia (OD) group. Data are presented as mean ± SD.

Table 8: Interplacental comparison between layers: The table shows the parameters that analysed/compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the significant p-value.

Parameters	**Sample size**	**Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
All IP CM	19	2.15 ± 0.76		[1.84;2.47]	
vs			Wilcoxon		p = 0.0566
All IP LM	19	1.68 ± 0.88	paired t-test	[1.32;2.04]	

5.2.3 Comparison of study groups

When comparing the PUI versus OD group, there was no significant difference between the CM layers in the IP group (p = 0.066), yet there was a significant difference in the LM layers (p = 0.029). Furthermore, the comparison of "MyoS" between PUI and OD was made. Here a significant difference was observed between the groups (p = 0.0022) with the PUI group staining more intensily (2.27 ± 0.76 [1.95;2.59]) than the OD group (1.43 ± 0.72 [1.08;1.79]). Both comparisons are shown in figure 12.1, 12.2 and table 10.

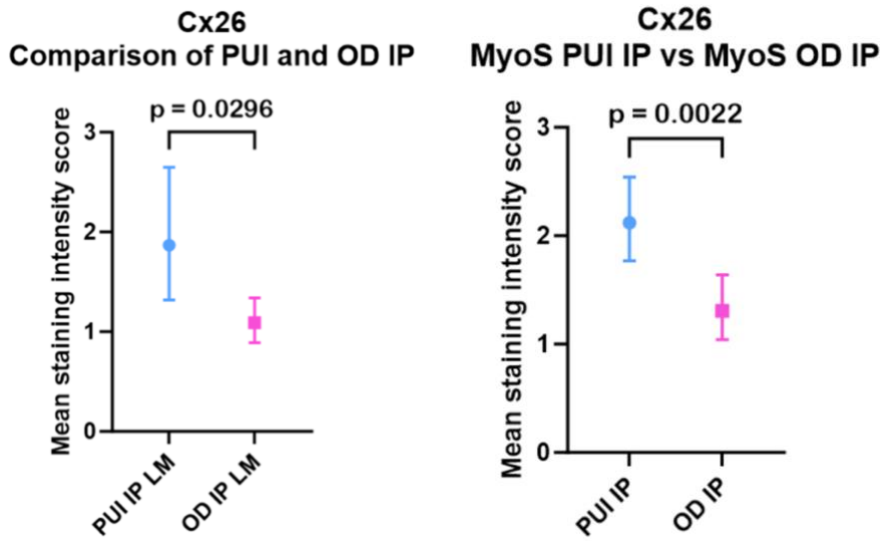


Figure 12.1 and 12.2 Comparison of myometrial Cx26 expression in IP samples between PUI and OD. Figure 12.1 shows data from the longitudinal myometrial (LM) layer in interplacental sites (IP) from both the PUI and OD group and compares them statistically. Figure 12.2 shows data from the combined "MyoS" (CM+LM) in Interplacental sites from the PUI and OD group and compared them. Data are presented as mean ± SD. Here both p-values are depicted and measures p = <0.05, meaning a significant difference is seen.

Table 9: Comparison of Cx26 expression between PUI and OD: The table shows the parameters that was compared: their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the p-value.

Parameters	**Sample size**	**Distribution Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
PUI IP CM	11	2.45 ± 0.52		[2.14;2.76]	
vs			Mann-		p = 0.0668
OD IP CM	8	1.75 ± 0.88	Whitney test	[1.13;2.36]	
PUI IP LM	11	2.09 ± 0.94		[1.53;2.64]	
vs			Mann-		p = 0.0296
OD IP LM	8	1.12 ± 0.35	Whitney test	[0.88;1.36]	
PUI IP MyoS	22	2.27 ± 0.76		[1.95;2.59]	
vs			Mann-		p = 0.0022
OD IP MyoS	16	1.43 ± 0.72	Whitney test	[1.08;1.79]	

5.2.3 Comparison of litter sizes

When comparing the protein expression of Cx26 in IP CM and LM layers of the different litter sizes (S/M/L) of the PUI group, there was no significant difference noticed. This was both true for the CM layers ($p = 0.567$) and the LM layers ($p = 0.307$). The gathered results are shown in table 15 in the appendix.

5.2.4 mRNA expression of Cx26

The results from the third-party RT-qPCR analyses were received and interpreted. In the IP tissue samples there was a significant difference in Cx26 expression between PUI and OD groups ($p = 0.0018$), with the mRNA expression being greater in the PUI group than in the OD group. In the UP samples, PUI and OD did not differ significantly.

5.3 Results of Cx43

5.3.1 Expression of uterine and placental Cx43

In all the Cx43 samples, IHC signal was noticed in the smooth muscle cells of the myometrium. Both the CM and LM layer had positive signal but varied in amount. Like the Cx26 samples, the staining was also seen located solely to the perinuclear area of the myocytes. When evaluating the IHC staining in the endometrium, the Cx43 staining intensity was absent in both vascular endothelial and glandular cuboidal cells. When evaluating the placental compartment of the IP samples, there was no signalling in the trophoblast nor decidual cells. There was slight evidence of weak/moderate signalling in the vascular endothelium of the placenta in some of the Cx43 samples. The negative control and isotype control showed no staining signal in any of the tissue layers.

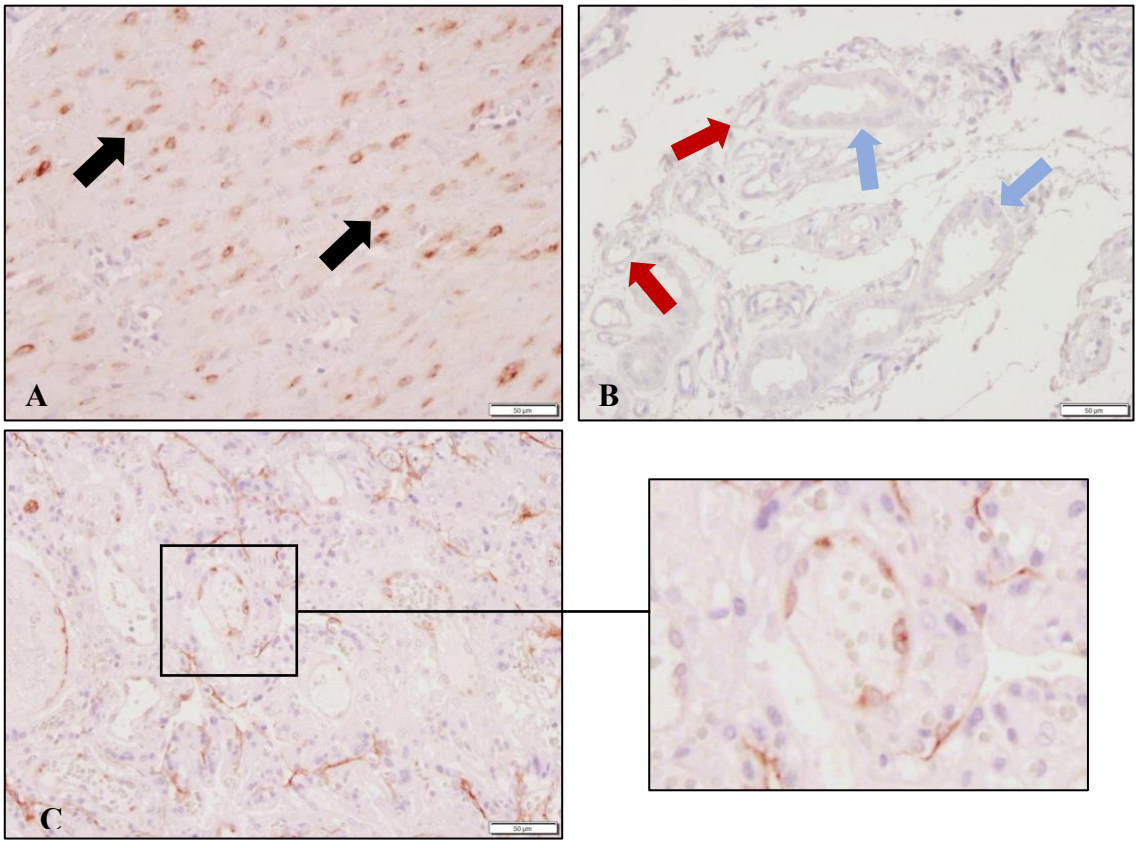


Figure 13: Microscopic illustration of Cx43 expression in uterine and placental tissue. Picture **(A)** is a microscopic image of the myometrium of a Cx43 IHC sample. The **BLACK** arrows present the perinuclear staining of the myocytes. Picture **B)** is an image of the endometrium from a Cx26 sample. The **RED** arrows represent the vascular endothelium, while the **BLUE** arrows represent the glandular epithelium. Picture **(C)** is an image of vascular endothelium from the placental compartment and a magnification to better visualize the staining. These illustrations are made to visualize the location of Cx43 staining in the uterus and placenta.

5.3.2 Interplacental and uteroplacental Cx43 expression

When analysing the IHC intensity of Cx43 there was a significant difference between the ALL IP CM and ALL IP LM groups (p = 0.0137) with the CM staining (2.2 ± 0.89 [1.84;2.55]) being more intense than the LM staining (1.6 ± 0.82 [1.27;1.92]). The results are shown in figure 14 and table 13.

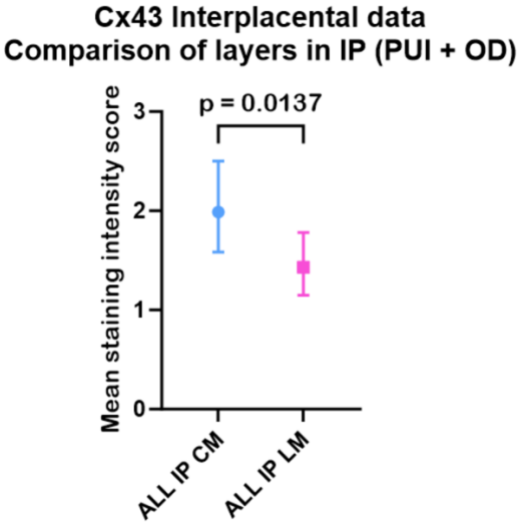


Figure 14: Comparison of myometrial Cx43 expression between layers in combined IP groups. The model contains all circular myometrial (CM) and longitudinal myometrial layers (LM) in interplacental (IP) samples from both primary uterine inertia (PUI) and obstructive dystocia (OD) group. Data are presented as mean ± SD.

Table 10: Interplacental comparison between layers: *The table shows the parameters that was compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the significant p-value.*

Parameters	**Sample size**	**Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
All IP CM	20	2.2 ± 0.89		[1.84;2.55]	
vs			Wilcoxon		p = 0.0137
All IP LM	20	1.6 ± 0.82	paired t-test	[1.27;1.92]	

5.3.3 Comparison of study groups

When comparing the protein expression in the PUI and OD group, no significant differences was seen. This was both true for the CM layer (p = 0.994) and the LM layer (p = 0.395). When gathering the CM and LM layers in the "MyoS" group, there was still no significant difference between the PUI and OD group (p = 0.689). The results are also shown in table 14.

Table 11: Comparison of Cx43 expression between PUI and OD: The table shows the parameters that analysed/compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the p-value.

Parameters	**Sample size**	**Distribution Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
PUI IP CM	12	2.16 ± 0.94		[1.64;2.69]	
vs			Mann-Whitney test		p = 0.994
OD IP CM	8	2.25 ± 0.88		[1.63;2.86]	
PUI IP LM	12	1.75 ± 0.86		[1.26;2.23]	
vs			Mann-Whitney test		p = 0.395
OD IP LM	8	1.37 ± 0.74		[0.85;1.89]	
PUI IP MyoS	24	1.95 ± 0.90		[1.59;2.32]	
vs			Mann-Whitney test		p = 0.689
OD IP MyoS	16	1.81 ± 0.91		[1.36;2.25]	

5.3.4 Comparison of litter sizes

When comparing Cx43 staining intensity between litter sizes (S/M/L), no significant difference was seen. This was both true for the CM layers (p = 0.740) and LM layers (p = 0.179). The gathered results are shown in table 16 in the appendix.

5.3.5 mRNA expression of Cx43

The results from the third-party RT-qPCR analyses were received and interpreted. In the IP tissue samples there was a significant difference in Cx43 expression between PUI and OD groups (p = 0.0003), with the expression being greater in the PUI group. Like Cx26, in the UP, PUI and OD did not differ significantly.

5.4 Agreement in the evaluation of the IHC slides

To determine the author's reliability when categorising the IHC samples from [1-3], a Cohen's Kappa was calculated. The Cohen's Kappa was calculated for the PTGFR total CM and LM layers, and the results were (0.93) and (0.83), respectively. The same calculations were made for Cx26 and Cx43 and were (CM: 0.75 / LM: 0.83) and (CM: 0.94 / LM: 0.90), respectively. With all values being equal to or greater than 0.75 and close to 1.00 it correlates to an 'almost perfect' agreement.

6. Discussion

6.1 Discussion of methods and materials

In total, 20 dogs were included in the study (PUI n = 12, OD n = 8). As convenient as it could be to have a larger sample group for statistical analysis, the author also acknowledges the difficulty in making the definite diagnosis of primary uterine inertia. While the dogs included in this study were grouped based on clinical signs and diagnostic imaging, no tocodynamometry to document uterine contractility was performed. To avoid any bias, strict selection criteria were used why significantly more samples were collected than finally included. A large range of breeds was observed in the sample group, meaning there was a high amount of heterogenicity. This increases the potential of inconsistency, thereby possibly decreasing the validity of the study and the possibility to identify significant differences. On the other hand, it can be argued that heterogenicity is representative for the actual breeding dog population.

It can also be questioned whether the use of bitches diagnosed with obstructive dystocia is ideal as controls, seeing they do not meet the requirements of a normal control group. The optimum control group would have included healthy pregnant dogs. It could be argued that the OD group is an acceptable control group, seeing there should be no reason to believe obstructive dystocia is positively correlated to PUI, yet one condition does not exclude the other. An ideal control group would be that of a dog with eutocia, yet performing a C-section on healthy dog serves as an ethical dilemma. Hence, why the OD group was opted for as an alternative, seeing the group already needed surgical intervention in form of C-section. It could be discussed whether a new control group should be added in forms of recruiting bitches who are scheduled for a planned caesarean section and therefor the closest option for a 'healthy' control group. Yet, in can also be argued that dogs scheduled for a planned caesarean section can't be classified as 'healthy', seeing that they in the past had a difficult parturition or eutocia did not seem like a possible outcome, hence why the C-section was planned.

When evaluating the IHC intensity, a standard score of [1-3] was chosen. Despite the method being semi-quantitative, it can be argued that using a broader scoring system (e.g., 0.25 decimals) would make a more precise scoring. If doing so, the data might be closer to normal distribution. Only, an ordinal scoring system of [1-3] was chosen to make the scoring process simplified and to ensure a consistency when rating the slides. The consistency of scoring was proven by calculating the Cohen's Kappa and proved to be 'an almost perfect agreement'. Yet the true subjectivity of the scoring must still be taking into consideration when evaluating the validity of the study. One attribute that

strengthens the IHC intensity results of Cx26 and Cx43 is the concurrent third-party RT-qPCR results. Especially since there is an agreement between the Cx26 results from IHC and RT-qPCR, showing a significant difference between the PUI and OD group. Unfortunately, the same attribute cannot be said about the PTGFR results, seeing that full thickness qPCR analysis did not reveal significant differences and that RNA obtained from FFPE samples did not allow for qPCR. The error of the qPCR on PTGFR is possibly due to the samples having insufficient quality.

For statistical analyses, non-parametric methods were chosen (Wilcoxon, Mann-Whitney, Kruskal Wallis). Non-parametric methods were chosen based on the data not being normally distributed. This type of analyses seemed suitable for the ordinal data included. Despite the scoring method being chosen because of its simplicity, there is a conversion of qualitative evaluations to categorical quantitative values which can cause uncertainty when choosing a statistical analysis. It can be argued whether a logistic regression was more suitable for this set of data, seeing an outcome could be calculating an odds ratio, which is the usual result of a case-control study. Yet, this study was based on a hypothesis regarding PUI, seeing an alternative to independent t-test was used, hence the use of a non-parametric test. More so, the results of this study contribute to other publications regarding PTGFR and cell-to-cell communications where similar statistical methods are used.

6.2 Discussion of results

Concomitant with the C-section, ovariohysterectomy was performed in 8 bitches, 4 in each group (PUI/OD), either because of medical indication or for owner's choice, making the uteroplacental (UP) sample size very small. As to why the statistical comparisons of UP results generally weren't included in the result section. Only in section 5.1.3 "*Comparison of study groups*" were UP results concluded because a significant difference in PTGFR expression was observed in the CM layer between the PUI and OD group ($p = 0.028$). Even though a high strength of significance is shown, it can be argued if a statistical difference is truly present because of the small sample group. The small sample size can undermine the validity of the study and these results could be of type I error[12]. Contrary, seeing that the probability is low and far from 0.5 in a very small group, it can be representative of the population and prove a strong difference. It would be of interest to have a larger UP sample group to see if the significance difference withholds.

[12] **Type I error:** Also known as "false positive", where one rejects a null hypothesis that is actually true.

Still considering the myometrial biopsy sites (IP/UP) of the PTGFR samples, the UP slides showed to be faulty stained, seeing only 70-80% percent of each slide was stained. Even though this was considered when evaluating the IHC staining intensity, a source of error could be that the 'edge phenomenon' reached closer to the evaluation centre than it would in a correctly stained slide, meaning that the author could have misevaluated the slide. The fault in the uteroplacental PTGFR slides could be due to the placement in the Shandon racks, seeing it was the edge close to label area pointing upwards that appeared faulty stained. A possible explanation could be the size of the tissue of UP samples, seeing that inclusion of the placental department made the tissue sample larger compared to the IP samples.

More so, there was a consistency for all investigated parameters, PTGFR, Cx26 and Cx43, regarding a stronger staining intensity in the CM layer compared to the LM layer. This could also be a source of error in the evaluation process. A possible cause could be the cutting direction of muscle fibres: When making a longitudinal cut in the LM layer, the muscle cells will appear round and each cell will present with a lower surface area. This can also allow the staining to appear less frequent or vaguer compared to the circular layer where in the same cut these myocytes appear longitudinally cut with a larger surface. When calculating the Cohens Kappa in section 5.4 "*Agreement in the evaluation of the IHC slides*", there was a lower agreement rate in the LM layer of PTGFR and Cx43 compared to the CM. This could support the hypothesis that the LM layer is more difficult to evaluate than the CM layer.

The RT-qPCR conducted for Cx26 and Cx43 showed a significant difference between the PUI and OD group. Yet only the Cx26 showed a significant difference between the groups in the IHC staining. While these gathered results strengthen the hypothesize of a true difference in the Cx26 expression between the groups, there is still lack of information on the Cx43. Reasons for indifference in the results of Cx43 could be of three origins: 1) Faulty in the evaluation of IHC slides, 2) fault in the RT-qPCR process or 3) simply the difference in methods, seeing RT-qPCR is an objective quantified method, while IHC is a semi-quantified subjective measurement.

In section 3.6.3 "*The structure and function of connexins*" it was stated by a former study, that Cx26 in rats was shown to be more related to uterine quiescent during pregnancy rather than contractile forces during parturition. The same was evident in canine uterine tissue, seeing that there was a decrease in protein expression close to prepartum luteolysis. Contrary to the hypothesis of this study, the results show that Cx26 expression is significant different between the PUI and OD group, with the expression in the PUI control group being higher than the OD group. The theory of the PUI

bitches having a ‘lack of cell-to-cell communication’ and thus the reason for not producing adequate contractions now lacks evidence when the contrary is shown. With Cx26 expression being greater in the PUI group, it raises the question of whether the fault lies in the initiating stages of uterine contractions. Even more so it questions whether the bitches initiate labour correctly. In the study concerning Cx26 in canine uterine tissue (Graubner et al., 2018), uterine biopsies were collected through OHE on different days of gestation: From early gestation (day 10-12) to mid-gestation (day 35-40) and prepartum luteolysis. Such a thorough investigation in CAPs would be of interest in dogs diagnosed with PUI to envision the expression of Cx26 throughout gestation and its course when parturition is initiated. Though such a study carries great dilemmas, seeing it is 1) unethical to perform C-section on otherwise healthy pregnant dogs, 2) difficult to gather owners who want their bitches spayed in late gestation and/or 3) difficult if not impossible to predict if the bitches will suffer from PUI before dystocia arises.

7. Conclusion

The results of this thesis contribute to the general understanding of canine primary uterine inertia. For PTGFR, it could be concluded that there was a significant difference between the interplacental layers with the circular layer staining more intensely than the longitudinal layer. It therefor corresponds well with the recent in in vitro organ bath results showing a contractile response to PGF2α only in the CM layer. Though, the hypothesis concerning a lower PTGFR myometrial expression in bitches with PUI compared to the OD group could not be accepted. This study alongside previous results questions whether prostaglandins have a true role in the pathophysiology of canine primary uterine inertia.

In this study, litter size did not interfere with myometrial CAP expression, neither in the CM nor LM layer of the PUI group. The hypothesis concerning a lower Cx26 myometrial expression in bitches with PUI compared to the control group could not be accepted, seeing the Cx26 mRNA and protein expression showed to be greater in bitches with PUI. These results contribute to an understanding of PUI, questioning whether the bitches with the condition can properly regulate contraction associated proteins such as connexins associated with initiating stages of labour. The same hypothesis regarding Cx43 could neither be discarded nor fully accepted, seeing there was not an agreement between IHC and RT-qPCR results. The RT-qPCR results showed a significant difference between the PUI and OD group, with the PUI group having a greater mRNA expression. The same could not be concluded from the IHC staining. Both Cx26 and Cx43 therefore need to be further investigated to determine their true relevance in the aetiology canine primary uterine inertia.

8. Perspectives

For future reference, the results of Cx26 and Cx43 could be strengthened by redoing the experiment on a larger sample group to truly see if the intercell communication and lack of modification in gap junctions contribute to the aetiology of canine PUI. Furthermore, increasing the sample size of UP samples would be beneficial and allow for a proper comparison of biopsy sites. If the study was repeated, it would further strengthen the results if a more homogenous population was gathered, meaning a smaller range of breeds would be preferable.

Literature

Bergström, A., Fransson, B., Lagerstedt, A.-S., & Olsson, K. (2006). Primary uterine inertia in 27 bitches: aetiology and treatment. *Journal of Small Animal Practice*, *47*(8), 456–460. https://doi.org/10.1111/J.1748-5827.2006.00071.X

Borge, K. S., Tønnessen, R., Nødtvedt, A., & Indrebø, A. (2010). Litter size at birth in purebred dogs--a retrospective study of 224 breeds. *Theriogenology*, *75*(5), 911–919. https://doi.org/10.1016/J.THERIOGENOLOGY.2010.10.034

Dantzer, V., & Leiser, R. (2006). Placentation. In J. A. Eurell & B. L. Frappier (Eds.), *Dellmann's Textbook of Veterinary Histology* (6th ed., pp. 279–297). Blackwell Publishing.

Darvelid, A. W., & Linde-Forsberg, C. (1994). Dystocia in the bitch: A retrospective study of 182 cases. *Journal of Small Animal Practice*, *35*(8), 402–407. https://doi.org/10.1111/J.1748-5827.1994.TB03863.X

Davidson, A. (2001). Uterine and fetal Monitoring in the Bitch. *Clinical Theriogenology*, *31*(2), 305–313.

Davidson, A. (2021). Problems during and after parturition. In G. England & A. von Heimendahl (Eds.), *BSAVA Manual of Canine and Feline Reproduction and Neontology* (2nd ed., pp. 121–134). British Small Animal Veterinary Assosiciation.

Dejneka, G. J., Ochota, M., Bielas, W., & Niżański, W. (2020). Dystocia after Unwanted Mating as One of the Risk Factors in Non-Spayed Bitches-A Retrospective Study. *Animals*, *10*(9), 1–9. https://doi.org/10.3390/ani10091697

Döring, B., Shynlova, O., Tsui, P., Eckardt, D., Janssen-Bienhold, U., Hofmann, F., Feil, S., Feil, R., Lye, S. J., & Willecke, K. (2006). Ablation of connexin43 in uterine smooth muscle cells of the mouse causes delayed parturition. *Journal of Cell Science*, *119*(9), 1715–1722.

Egloff, S., Reichler, I. M., Kowalewski, M. P., Keller, S., Goericke-Pesch, S., & Balogh, O. (2020). Uterine expression of smooth muscle alpha-and gamma-actin and smooth muscle myosin in bitches diagnosed with uterine inertia and obstructive dystocia. *Theriogenology*, *156*, 162–170. https://doi.org/10.1016/j.theriogenology.2020.06.033

England, G. (2021). Physiology and endocrinology of the female. In G. C. W. England & A. von Heimendahl (Eds.), *BSAVA Manual of Canine and Feline Reproduction and Neonatology* (2nd ed., pp. 1–12). British Small Animal Veterinary Association.

Forsberg, C. L. (2021). Pregnancy diagnosis, normal pregnancy and parturition in the bitch. In G. C. W. England & A. von Heimendahl (Eds.), *BSAVA Manual of Canine and Feline Reproduction and Neonatology* (2nd ed.). British Small Animal Veterinary Association.

Frehner, B. L., Reichler, I. M., Kowalewski, M. P., Gram, A., Keller, S., Goericke-Pesch, S., & Balogh, O. (2021). Implications of the RhoA/Rho associated kinase pathway and leptin in primary uterine inertia in the dog. *Journal of Reproduction and Development*, *67*(3), 207–215. https://doi.org/10.1262/JRD.2020-141

Fusi, J., & Veronesi, M. C. (2022). Canine parturition: what is known about the hormonal setting? *Domestic Animal Endocrinology*, *78*, 106687–106687. https://doi.org/10.1016/j.domaniend.2021.106687

Garfield, R. E., Kannan, M. S., & Daniel, E. E. (1980). Gap junction formation in myometrium: control by estrogens, progesterone, and prostaglandins. *American Journal of Cellular Physiology: Cell Physiology*, *7*(2), C81–C89.

Gram, A., Boos, A., & Kowalewski, M. (2014). Uterine and Placental Expression of Canine Oxytocin Receptor During Pregnancy and Normal and Induced Parturition. *Reproduction in Domestic Animals*, *49*(2), 41–49. https://doi.org/10.1111/rda.12295

Gram, A., Bü, U., Boos, A., Hoffmann, B., & Kowalewski, M. P. (2013). Biosynthesis and Degradation of Canine Placental Prostaglandins: Prepartum Changes in Expression and Function of Prostaglandin F2alpha-Synthase (PGFS, AKR1C3) and 15-Hydroxyprostaglandin Dehydrogenase (HPGD). *Biology of Reproduction*, *89*(1), 1–12. https://doi.org/10.1095/biolreprod.113.109918

Graubner, F. R., Alois, B., Aslan, S., Kücükaslan, I., & Kowalewski, M. P. (2018). Uterine and placental distribution of selected extracellular matrix (ECM) components in the dog. *Reproduction (Cabridge, England)*, *155*(5), 403–421.

Graubner, F. R., Reichler, I. M., Rahman, N. A., Payan-Carreira, R., Boos, A., & Kowalewski, M. P. (2017). Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines. *Reproduction in Domestic Animals*, *52*(2), 137–147.

Hansen, A. D., & Rønnow, A. F. (2018). *Investigations on the Myometrial and Placental Expression of Connexin 43 and Progesterone Receptor in Canine Dystocia*. University of Copenhagen.

Hoffmann, B., Höveler, R., Nohr, B., & Hasan, S. H. (1994). Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. *Experimental and Clinical Endocrinology*, *102*(3), 185–189.

Hussain, M. U. (2014). Molecular Components and Nomenclature of Gap Junctions. In *Connexins: The Gap Junction Proteins* (1st ed., Vol. 1, pp. 5–6). Springer New Delhi.

Jayakumar, C., Raheema, S., Sophia, X., Vidhya, S., & Kurien, M. (2019). Uterine contraction monitoring in dogs using tocodynamometer. *Indian Journal of Canine Practice*, *11*(2), 135–139. https://doi.org/10.29005/IJCP.2019.11.2.135-139

Jungmann, C., Mazzuoli-Weber, G., & Goericke-Pesch, S. (2023). The ex vivo response of canine pregnant myometrium to exogenous PGF2α. *Reproduction in Domestic Animals* , *58*(2), 202–202.

Jungmann, C., Pyzik, S.-C., Packeiser, E.-M., Körber, H., Hoppe, S., Mazzuoli-Weber, G., & Goericke-Pesch, S. (2023). The In Vitro Contractile Response of Canine Pregnant Myometrium to Oxytocin and Denaverine Hydrochloride. *Biology (Basel, Switzerland)*, *12*(6), 860.

Kowalewski, M. P. (2014). Luteal regression vs. prepartum luteolysis: Regulatory mechanisms governing canine corpus luteum function. *Reproductive Biology*, *14*(2), 89–102.

Laird, D. W. (2006). Life cycle of connexins in health and disease. *Biochemical Journal*, *394*(3), 527–543.

Milani, C., Rota, A., Olsson, U., Paganotto, A., & Holst, B. S. (2021). Serum concentration of mineralocorticoids, glucocorticoids, and sex steroids in peripartum bitches. *Domestic Animal Endocrinology*, *74*(1), 106558–106558. https://doi.org/10.1016/j.domaniend.2020.106558

Munnich, A., & Küchenmeister, U. (2009). Dystocia in Numbers – Evidence-Based Parameters for Intervention in the Dog: Causes for Dystocia and Treatment Recommendations. *Reproduction in Domestic Animals*, *44*(2), 141–147. https://doi.org/10.1111/J.1439-0531.2009.01405.X

Nielsen, M. S., Axelsen, L. N., Sorgen, P. L., Verma, V., Delmar, M., & Holstein-Rathlou, N. (2012). Gap Junctions. *Comprehensive Physiology*, *2(3)*(3), 1981–2035.

Nowak, M., Boos, A., & Kowalewski, M. P. (2018). Luteal and hypophyseal expression of the canine relaxin (RLN) system during pregnancy: Implications for luteotropic function. *PLOS One*, *13*(1), e0191274–e0191274. https://doi.org/10.1371/journal.pone.0191374

Ou, C.-W., Orsino, A., & Lye, S. J. (1997). Expression of Connexin-43 and Connexin-26 in the Rat Myometrium during Pregnancy and Labor Is Differentially Regulated by Mechanical and Hormonal Signals. *Endocrinology (Philadelphia)*, *148*(12), 5398–5407.

Park, J. Y., Phillinger, M. H., & Abramson, S. B. (2006). Prostaglandin E2 synthesis and secretion: The role of PGE2 synthases. *Clinical Immunology (Orlando, Fla)*, *119*(3), 229–240.

Parkinson, T. J., Vermunt, J. J., & Noakes, D. E. (2019a). Approach to an Obstetrical Case. In D. E. Noakes, T. J. Parkinson, & G. C. W. England (Eds.), *Veterinary Reproduction and Obstetrics* (10th ed., pp. 203–2013). Elsevier.

Parkinson, T. J., Vermunt, J. J., & Noakes, D. E. (2019b). Maternal Dystocia: Causes and Treatment. In D. E. Noakes, T. J. Parkinson, & G. C. W. England (Eds.), *Veterinary Reproduction and Obstetrics* (10th ed., pp. 236–249). Elsevier.

Parkinson, T. J., Vermunt, J. J., & Noakes, D. E. (2019c). Prevalence, Causes and Consequences of Dystocia. In D. E. Noakes, T. J. Parkinson, & G. C. W. England (Eds.), *Veterinary Reproduction and Obstetrics* (10th ed., pp. 214–233). Elsevier.

Premanandan, C. (2017). Female Reproductive System. In R. Jennings & C. Premanandan (Eds.), *Veterinary Histology* (1st ed., pp. 207–224). Ohio State University Libraries.

Priedkalns, J. (2006). Female Reproductive System. In J. A. Eurell & B. L. Frappier (Eds.), *Dellmann's Textbook of Veterinary Histology* (6th ed., pp. 256–278). Wiley Blackwell.

Reifarth, L., Körber, H., Packeiser, E.-M., & Goericke-Pesch, S. (2023). Detection of spermatogonial stem cells in testicular tissue of dogs with chronic asymptomatic orchitis. *Frontiers in Veterinary Science*, *10*, 1205064–1205064. https://doi.org/10.3389/fvets.2023.1205064

Rempel, L. M., Körber, H., Reichler, I. M., Balogh, O., & Goericke-Pesch, S. (2021). Investigations on the potential role of prostaglandin E2 in canine uterine inertia. *Theriogenology*, *175*, 134–147.

Rempel, L. M., Lillevang, K., Straten, A.-K., Friðriksdóttir, S., Körber, H., Wehrend, A., Kowalewski, M., Reichler, I., Balogh, O., & Goericke-Pesch, S. (2021). Do uterine PTGS2, PGFS, and PTGFR expression play a role in canine uterine inertia? *Cell and Tissue Research*, *385*, 251–264. https://doi.org/10.1007/s00441-021-03427-6

Smith, W. L. (1989). The eicosanoids and their biochemical mechanisms of action. *Biochemical Journal*, *259*(2), 315–324.

Taverne, M. (2019). Fertilisation and Development of the Conceptus. In D. E. Noakes, T. J. Parkison, & G. C. W. England (Eds.), *Veterinary Reproduction and Obstetrics* (10th ed.). Elsevier.

Taverne, M., & Noakes, D. E. (2019). Parturition and the Care of Parturient Animals and the Newborn. In D. E. Noakes, T. J. Parkinson, & G. C. W. England (Eds.), *Veterinary Reproduction and Obstetrics* (10th ed.). Elsevier.

Appendix

Table 12: Comparison of PTGFR expression between Interplacental sites in in different litter sizes. The table shows the parameters that analysed/compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the p-value.

Parameters	Sample size	Distribution Mean ± SD	Test performed	Distribution [CI lower;CI upper]	p-value
PUI IP CM S	5	2.60 ± 0.89		[1.82;3.38]	
vs			Kruskal-		
PUI IP CM M	4	2.50 ± 0.57	Wallis test	[1.93;2.50]	p = 0.545
vs					
PUI IP CM L	3	3.00 ± 0.00		[0;0]	
PUI IP LM S	5	1.6 ± 0.98		[0.81;1.6]	
vs			Kruskal-		
PUI IP LM M	4	1.75 ± 0.50	Wallis test	[1.26;2.24]	p = 0.199
vs					
PUI IP LM L	3	2.66 ± 0.57		[2.01;3.32]	

Table 15: Comparison of Cx26 expression between Interplacental sites in in different litter sizes. The table shows the parameters that was analysed and compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the p-value.

Parameters	Sample size	Distribution Mean ± SD	Test performed	Distribution [CI lower;CI upper]	p-value
PUI IP CM S	5	2.60 ± 0.54		[2.12;3.08]	
vs			Kruskal-		
PUI IP CM M	4	2.50 ± 0.57	Wallis test	[1.93;3.06]	p = 0.567
vs					
PUI IP CM L	2	2.00 ± 0.00		[0;0]	
PUI IP LM S	5	2.6 ± 0.54		[2.11;3.08]	
vs			Kruskal-		
PUI IP LM M	4	1.50 ± 1.00	Wallis test	[0.52;2.24]	p = 0.307
vs					
PUI IP LM L	2	2.00 ± 1.41		[0.04;3.95]	

Table 16: Comparison of Cx43 expression between Interplacental sites in in different litter sizes. The table shows the parameters that analysed/compared, their sample sizes, the mean +/- the standard deviation (SD), what analytic test was performed in Prism, the distribution, and the p-value.

Parameters	**Sample size**	**Distribution Mean ± SD**	**Test performed**	**Distribution [CI lower;CI upper]**	**p-value**
PUI IP CM S	5	2.00 ± 1.00		[1.12;2.87]	
vs			Kruskal-		
PUI IP CM M	4	2.50 ± 1.00	Wallis test	[1.52;3.47]	p = 0.740
vs					
PUI IP CM L	3	2.00 ± 1.00		[0.86;3.13]	
PUI IP LM S	5	1.8 ± 0.83		[1.06;2.53]	
vs			Kruskal-		
PUI IP LM M	4	2.25 ± 1.00	Wallis test	[1.31;3.18]	p = 0.179
vs					
PUI IP LM L	3	1.00 ± 0.00		[0;0]	

Table 17: Results of normality test. The table lists the values of probability of normality tests run on different groups on the researched contraction associated proteins (CAPs). The choice of method was Shapiro-Wilk test and listed at the top of the table. The CAPs (PTGFR, Cx26 and Cx43) are listed horizontally in each column while the value of probability (p-value) is listed vertically in each row. When the $p<0.05$ the data did not pass the normality test. When the $p \geq 0.05$ the data passes the normality test. When "Invalid" is listed, the reason is zero variation (0 distribution). If an asterisk symbol, "*", has been added, it symbolizes $p \geq 0.05$ and thereby a passed normality test.

Choice of method		Shapiro-Wilk test		
	Researched CAPs	PTGFR	Cx26	Cx43
	Groups			
Value of probability	PUI IP CM	<0.0001	0.001	0.0020
	PUI IP LM	0.0180	0.0023	0.0038
	PUI UP CM	Invalid	0.0012	0.0012
	PUI UP LM	0.0239	0.0012	0.0239
	OD IP CM	0.0270	0.0185	0.0185
	OD IP LM	0.0019	<0.0001	0.0002
	OD UP CM	Invalid	0.6830*	0.0239
	OD UP LM	0.0012	0.6830*	0.0239