# Effect of pegbovigrastim on metritis in primiparous and multiparous Holstein-Friesian cows on a commercial farm in Uruguay

**Master Thesis Veterinary Medicine University Utrecht**

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# 1. Relevance

Over decades, with the help of intense genetic selection for higher milk yields as well as improved nutrition and management, milk yields have increased so that 9000 kg of milk are not uncommon (Ingvartsen et al., 2003; Barkema et al., 2015). With this increase comes the concern of health and fertility problems, which increases the costs of veterinary treatments and replacement of animals (Ingvartsen et al., 2003). The greatest impact on health and production during parturition, and fertility after parturition is associated with microbial contamination of the uterine lumen after parturition (Sheldon et al., 2008). Antimicrobial treatment of the uterus is then often used to resolve the infection of the reproductive tracts. However, since antimicrobial resistance has become an important public health hazard, veterinarians and farmers are required to be less reliant on the use of antimicrobials (Barkema et al., 2015). Therefore, it is important to find tools to reduce uterine disease and in this way reduce the use of antimicrobials.

## 1.1 Transition period

The period of highest risk for production disease is the period immediately after parturition, which is included in the transition period (-3 weeks to + 3 weeks regarding to calving (Drackley, 1999)). This period is characterised by marked changes in the endocrine status of the animal and a reduction in feed intake when nutrient demand for the developing conceptus and the impending lactogenesis are increasing (Kimura et al., 2014; Mulligan et al., 2008). Thereby a disbalance of energy and most often a negative energy balance is created. Besides, dairy cows also experience immunosuppression in the peri-parturient period and often have to cope with sudden dietary changes that cause digestive disturbances. They are also likely to experience environmental stressors arising from the normal group changes that are associated with dairy farm management of dry and lactating cows. These effects combined, it is not surprising that the period of highest risk for production disease is the period immediately after parturition.

Most production diseases are aetiologically inter-related. Uterine infection after parturition is a manifestation of a reduced immunity in the peri-parturient period (Mulligan et al., 2008).

## 1.2 Metritis

Most (80-100%) animals have bacteria in their uterine lumen within the first two weeks after calving and up to 40% of animals still have a bacterial infection one week later. This does not always imply severe clinical disease, but it could induce clinical or subclinical metritis and subsequently endometritis (Sheldon et al., 2008).

Typically, 25-40% of the animals have clinical metritis in the first two weeks after calving, and disease persists in up to 20% of animals as clinical endometritis. This persistence of disease depends on the balance between factors such as the animal immunity, the number and pathogenicity of the microbes, and the uterine environment. Because an immune and inflammatory response disrupt endocrine function in the female reproductive tract of cattle (Sheldon et al., 2008), metritis contributes to increased days to first breeding, decreased conception and pregnancy rate and increased culling (Hammon et al., 2006), thus causing important economic losses. Therefore, it is especially important to reduce the risk of metritis that dairy cows experience during the peri-parturient period.

Rodriguez et al. (2017) showed that the occurrence of retained placenta, and metritis was 3.4, and 4.3 times more likely, respectively, in cows that had subclinical hypocalcemia than in cows with normocalcemia. Furthermore, the risk of incurring retained placenta or metritis increases in multiparous cows as serum calcium concentrations decreased compared with that in primiparous cows. Besides, the negative energy balance –that is reflected by non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) concentrations – is related to the

presence of disease. Indeed, BHB concentrations of more than 1.2 mmol/L increase by 3 times the risk of metritis (Duffield et al., 2009). However, most studies have been performed in confined conditions, and there is scares information in milk production systems based in pasture.

The postpartum environment of the uterine lumen supports the growth of a variety of aerobic and anaerobic bacteria, many of which are removed by a range of uterine defence mechanisms (Sheldon et al., 2008). One of the key mechanisms to remove invading bacteria is phagocytosis and killing these bacteria by polymorphonuclear cells.

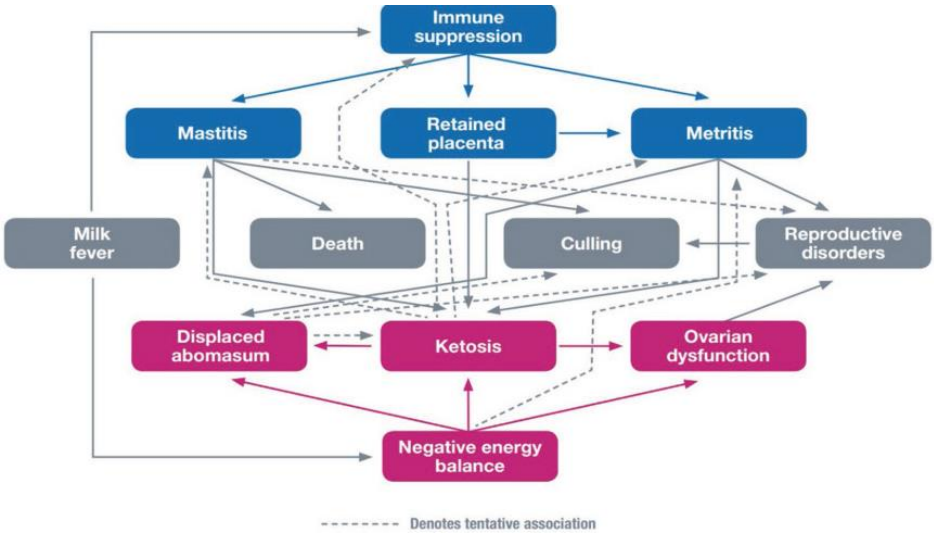



**Figure 1. The effect and the consequences of the negative energy balance and immune suppression (Ruiz et al., 2017).**

## 1.3 Polymorphonuclear cells or neutrophils

Neutrophils play an important role in the uterine defence mechanisms, as they provide the first-line of cellular defence against bacterial colonization within the uterus (Hammon et al., 2006). Previous studies indicate that bovine polymorphonuclear neutrophil (PMN) functions begin to decline 3 to 5 weeks prior to parturition and slowly return to prepartum levels at 2 to 4 weeks postpartum (Hammon et al., 2006). Peripheral blood neutrophil (PMN) function of peri-parturient dairy cows is impaired relative to non-peri-parturient cattle (Hammon et al., 2006), which makes these cattle more susceptible to uterine infection after parturition. However, the mechanisms responsible for PMN function impairment in peri-parturient dairy cows are poorly understood. The metabolic challenges associated with late gestation and the onset of lactation could be responsible in part for PMN function impairment during this time (Hammon et al., 2006; Kimura et al., 2014).
The peri-parturient immunosuppression that cows experience in the first weeks after calving is associated with a high incidence of diseases, including mastitis, metritis and endometritis (Galvao et al., 2010; Kimura et al., 2014).

## 1.4 Uterine involution

Uterine health plays a central role in determining the reproductive efficiency of dairy herds. Clinical metritis can reduce fertility in lactating dairy cows (Baez et al., 2016). Metritis might delay uterine involution, the interval of first ovulation postpartum, reduce conception rate to

first insemination, increase the risk of culling for infertility (Sheldon, 2004) and decreases herd profitability (Roche, 2006).
A study by Young (2011) showed that cows with a reproductive tract score 3 had reduced conception rates compared to cows with a reproductive tract score 1 (Baez et al., 2016). The involution of the uterus is generally complete by day 12 postpartum (Leslie, 1983). By day 10 the uterus could be completely manually evaluated by rectal palpation. From days 10 to 14 postpartum uterine tone increases and uterine size decreases which coincide with the onset of the first estrus (Morrow et al., 1968; Leslie, 1983). In healthy dairy cows uterine involution completes by 28 days postpartum assessed by rectal palpation using a 6-point reproductive tract scoring system (Krueger et al., 2009; Baez et al., 2016). Involution of the cervix is slower than that of the uterus. Normally, the cervical involution completes by day 30 postpartum (Leslie, 1983).
Normal uterine involution progresses more slowly in cows with periparturient diseases, such as milk fever, ketosis, RP (Marion and Gier, 1968; Leslie, 1983) and metritis. The uterus does not reach nongravid size until approximately day 30 in cows with such diseases (Morrow et al., 1968; Leslie, 1983).

### 1.5 Calcium, NEFA and BHBA

Besides uterine health, there are some factors that affect progression of involution, like retained placenta, dystocia and the occurrence of metabolic disorders such as milk fever (Roche, 2006). Milk fever and ketosis affect uterine muscle contractions (Roche, 2006). Gröhn and Rajala-Schultz (2000) found retained placenta, metritis and cysts key risk factors for delayed conception and Maizon et al. (2004) found that days from first breeding to conception increased in cows with dystocia, stillbirth, retained placenta, metritis or ovulation dysfunction and López-Gatius et al. (2005) showed that delayed conception can be related to sub-clinical disease, stressful environmental, social conditions and nutritional stress (Roche, 2006).
Hypocalcemia and a negative energy balance suppress immune function (Hammon et al., 2006; Kimura et al., 2006; Galvao et al., 2010). Hypocalcemic cows have a reduced proportion of neutrophils with phagocytic activity (Ducusin et al., 2003). A study by Martinez et al., (2012) showed that neutrophils of cows with subclinical hypocalcemia were less capable of phagocytizing and killing pathogenic bacteria in vitro. Inadequate concentrations of Ca in the blood are likely to influence the availability of Ca for cellular function (Martinez et al., 2012).
Cows with puerperal metritis have higher concentrations of BHB and NEFA (Hammon et al., 2006, Galvao et al., 2010). Plasma NEFA (Hammon et al., 2006) and BHB (Suriyasathaporn et al., 2000) are associated with PMN function. Cows that developed metritis and later subclinical endometritis had a reduction in PMN function, including reduced killing capacity during the period of negative energy balance and neutrophils with less intracellular glycogen (Galvao et al., 2010).

### 1.6 Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) is a polypeptide, haematopoietic growth factor which stimulates the bone marrow to produce stem cells, stimulates the proliferation

and differentiation of neutrophil precursor cells, as well as some of the functional properties of mature neutrophil granulocytes (Demetri et al., 1991; Kimura et al., 2014).
Pegylation is the modification of native proteins, like G-CSF, by covalent binding to polymers, such as polyethylene glycol. It has shown to extend the duration of activity of these proteins by increasing their hemodynamic volume, reducing first-pass renal clearance, and reducing their proteolytic degradation (Molineux, 2003; Ruiz et al., 2017).
By the use of recombinant technology, pegylation of the natural occurring cytokine, bovine G-CSF has allowed the use of bovine G-CSF at a commercial level (Ruiz et al., 2017). A commercially available long-acting analog of bovine G-CSF is pegbovigrastim injection, **Imrestor** (Elanco Animal Health, Greenfield, IN).

Imrestor is a sterile, injectable formulation of pegbovigrastim (polyethylene glycol-conjugated bovine granulocyte colony-stimulating factor, Elanco Animal Health) in single 2.7 mL dose syringes. Each syringe of Imrestor contains pegbovigrastim (15 mg), L-arginine hydrochloride (94 mg), L-arginine (40 mg), and citric acid monohydrate (17 mg).

Mature and immature PMN increased in cows injected with PEG rbG-CSF. The number of mature PMN cells remained high even after two weeks of injection (Kimura et al., 2014). A study of Canning et al. (2017) also showed a significant increase in circulating neutrophil numbers relative to saline-treated controls within 24 hours of administering the first dose in animals treated with pegbovigrastim (Fig 2). Circulating neutrophil numbers remained significantly elevated relative to the saline-treated controls 7 days after the second dose of pegbovigrastim, administered within 24 hours of calving.

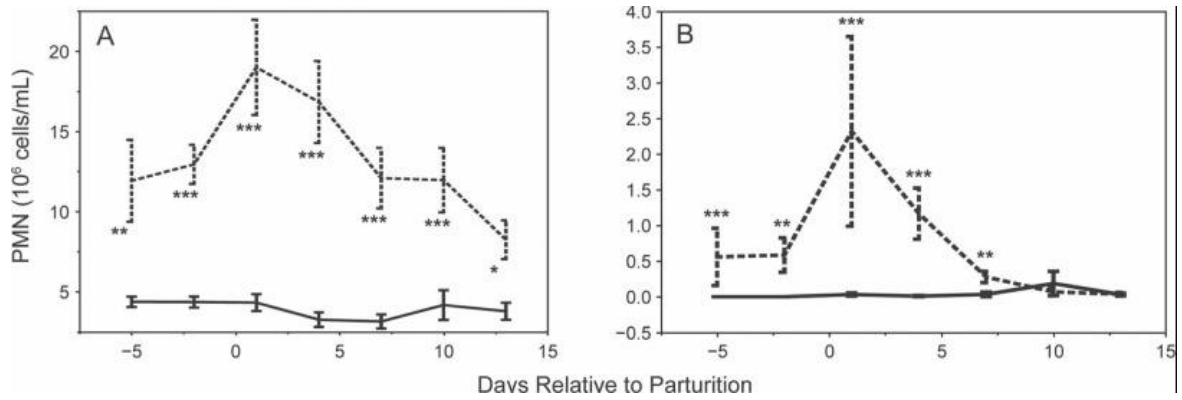



**Figure 2. The effect of recombinant bovine granulocyte colony stimulating factor covalently bound to polyethylene glycol (PEG rbG-CSF) on (A) total polymorphonuclear neutrophil (PMN) number and (B) band form neutrophil number. A solid line represents control (CONT) cows, and dashed line represents PEG rbG-CSF-injected (GCSF) cows. An asterisk (*) represents P<0.05; two asterisks (**) represents P<0.01; three asterisks (***) represents P<0.005 in comparison between groups at the same sampling point (Kimura et al., 2014).**

## 1.7 Hypotheses:

The primary objective of this project is to evaluate whether peri-parturient treatment of dairy cows with PEG rbG-CSF will result in a decrease of the frequency, duration and/or severity of metritis in primiparous and multiparous cows. Besides, the uterus involution will be registered and variables indicative of the metabolic status (NEFA, BHB and calcium) during the peripartum will be determined.

The null hypothesis is that there is no significant difference in the occurrence of metritis between the treated group primi- and multiparous cows with PEG rbG-CSF and the control group primi- and multiparous cows.
The alternative hypothesis is that there is a significant difference in the occurrence of metritis between the group primi- and multiparous cows treated with PEG rbG-CSF and the control group primi- and multiparous cows.

# 2. Material and methods

## 2.1 Experimental design/Animals (selection, treatment, sampling etc.)

Holstein cows (n=55), approximately 65% multiparous and 35% primiparous, were selected in a farm in Uruguay of mixed productive system. These are farms with at least one grazing session throughout the year. Cows are kept in pastures at least one of the periods between the two milking and at least 40% of the DMI coming directly from the grazing sessions.
Cows with expected calving in May and June were selected. A clinical check was carried out between -10 to -7 days of the expected calving date, animals that were experiencing a health disorder or had been treated within 2 weeks before calving were excluded of the study. Selected animals were identified by a tie wrap in the earmark, and half of the animals were randomly (electronic ID national numbers odds/even numbers) treated with PEG rbG-CSF and the other half remained as untreated control (L1 CSF (n=10), L1 control (n=10), L2 CSF (n=18) and L2 control (n=18)).
Treatment was applied according to the label of the product *Imrestor*: two doses of 15 mg of PEG rbG-CSF in a 2.7 mL pre-filled syringe was administered subcutaneously. The first dose was administered -7 days relative to the expected calving date (based on previous reproductive data) while the close up pen was observed two times a week. Cows that were exhibiting clinical signs of calving like swelling of vulva and filling of udder were carefully checked and if appropriate moved to the calving area. The second dose was administered within 24h after calving. Only cows that received both doses were included in the study.

## 2.2 PEG rbG-CSF

Two doses of 15 mg of PEG rbG-CSF in a 2,7 mL pre-filled syringe were administered subcutaneously. The first dose was administered -7 days before expected calving date, calculated based on previous reproductive data and physical changes like swelling of vulva and filling of udder. The second dose was administrated within 24h after calving.

## 2.3 Disease diagnose

A trained veterinary technician in the farm diagnosed and recorded every clinical event, including: clinical mastitis (Pinzón-Sanchez and Ruegg, 2011), metabolic disorders (milk fever, ketosis and displacement of the abomasum), lameness, retention of placenta and metritis (Sheldon et al., 2006). Definitions of these clinical events were carefully described based on literature and all farm personnel was trained in the recognition of these disorders. All diagnoses on the farm were ultimately confirmed by these trained veterinary technicians. All antimicrobials drug products and other treatment products used were recorded.

### *2.3.1 Metritis*

All cows between 3 and 21 days postpartum were scored for metritis by the same veterinary student, twice a week. The definition of clinical metritis of Ruiz et al. (2017) was used. Clinical metritis is recognized by an abnormal (smelly and watery) uterine discharge within 21 d of calving (Table 1). On palpation per rectum, the uterus appears flaccid, not contracting normally, and fluid filled. Metritis is subdivided into 3 levels of severity (Benzaquen et al., 2007) based on clinical signs, mild, moderate and severe or puerperal metritis. Mild clinical metritis is a metritis without clinical signs apart from the uterine changes and foul smelling discharges, muco-purulent but not watery, from the vulva. Moderate clinical metritis is a metritis with uterine changes, foul smelling watery discharges from the vulva, without the presence of clinical signs of systemic illness. And severe or puerperal metritis is a metritis with uterine changes, discharges from the vulva and the presence of clinical signs that may include fever, depression and lack of appetite (Table 1).
Duration of metritis is defined as the duration between the first day the veterinary student

notice an odor and/or discharge and the last day the veterinary student notice an odor and/or discharge.

*2.3.2 Uterus involution*

Transrectal palpation of the reproductive tract of all the cows enrolled was performed on two occasions during postpartum: days 13 to 16 postpartum, and again on days 28 to 31 postpartum by a trained veterinary student. The uterus involution was scored by the cervical diameter and the size of the uterus (Table 2).

*2.3.3 Blood sampling*

Blood samples of all the cows enrolled were collected on two occasions: between day 5 and 8 postpartum, and when the first transrectal palpation was performed (days 13 to 16). Blood samples were collected from the coccygeal vessel into 10-mL sterile heparinized tubes and centrifuged at 3000 X *g* for 20 min. Plasma was stored frozen (-20°C) until further analysis for BHB, NEFA and calcium concentrations at the animal endocrine and metabolism laboratory, Veterinary faculty, Montevideo, Uruguay. Metabolites were measured by colorimetric assays on Vitalab Selectra II autoanalyzer (Vital Scientific, Dieren, The Netherlands) using commercial kits: NEFA, Wako NEFA-HR(2), Wako Pure Chemical Industries Ltd., Osaka Japan; BHB, Randox Laboratories Limited, 55 Diamond Road, Crumlin, Country Antrim, BT29 4QY, United Kingdom; Calcium, Wiener Laboratories S.A.I.C. Riobamba, Rosario, Argentina.

**Table 1. Metritis classification.**

| Category | CIS Score[a] | Uterine Discharge Evaluation |
|---|---|---|
| No Metritis Detected | 0 | Examined<br>No Odor AND No Watery discharge<br>Discharge maybe mucus or mucus with flecks of pus. |
| Mild Clinical Metritis | 1 | Examined<br>Odor Present AND No-Watery Discharge [on Palpation if Not Visible]<br>Discharge maybe mucopurulent or purulent |
| Moderate Clinical Metritis | 2 | Examined<br>Odor Present AND Watery Discharge [on Palpation if Not Visible]<br>Watery discharge is often putrid: red/brown, watery and foul smell.<br>No signs of systemic illness[b] |
| Puerperal Metritis | 3 | Examined<br>Odor Present AND Watery Discharge [on Palpation if Not Visible]<br>Watery discharge is often putrid: red/brown, watery and foul smell.<br>Systemic Illness[b] |

[a]Adapted from Dohmen et al. (1995), Overton et al. (2003), Urton et al. (2005), Sheldon (2006), Benzaquen et al. (2007), and Huzzey et al (2007).

[b]Signs of systemic illness include, but are not limited to, body temperature $\geq$ 39.5°C, decreased milk yield, dullness or other signs of toxemia, decreased dry matter intake, elevated heart rate, and dehydration (Sheldon et al., 2008; Haimerl and Heuwieser, 2014)

**Tabel 2. Transrectal palpation classification.**

| Cervical diameter | | Size of uterus | |
|---|---|---|---|
| < 3 fingers | 1 | Entirely within the pelvis, size of one hand | 1 |
| 3-4 fingers | 2 | Over the pelvic brim, but completely palpable after retraction | 2 |
| >4 fingers | 3 | Over the pelvic brim, not completely palpable | 3 |

*Adapted from LeBlanc et al., 2002*

## 2.4 Statistical analyses

Statistical analyses were performed using SPSS statistics 25. The incidence of clinical metritis was compared between the treatment and control group in both first, and second and higher lactation animals. To test whether the occurrence of metritis dependent on the treatment and the age of the cows, we performed a Fisher Exact test. We also performed a Fisher Exact test

to analyse whether the severity of metritis depended on the treatment and the age of the cows. Prior to analyses, we tested whether duration of metritis, the first day that cows get metritis, both cervix score and uterus score and blood metabolites (calcium concentration, NEFA concentration and BHB concentration) were normally distributed using a Shapiro-Wilk test. When these variables were not normally distributed, we performed Kruskal-Wallis test to analyse whether the duration of metritis and the first day cows got metritis differed among treated primiparous, not treated primiparous, treated multiparous and not treated multiparous cows. We performed a Dunn test to determine which of these four groups differed from each other. We also performed Kruskal-Wallis tests to analyse whether to uterus and cervix score, both 15 days postpartum and 30 days postpartum, differed among these four groups. Moreover, we tested whether uterus and cervix scored 15 days postpartum differed from these scores 30 days postpartum, using a Kruskal-Wallis test. Finally, we used a Kruskal-Wallis test to analyse whether blood metabolites (calcium, NEFA and BHB concentration) differed among treated primiparous, control primiparous, treated multiparous and control multiparous cows and we performed a Dunn test to determine whether which of these four groups differed from each other.

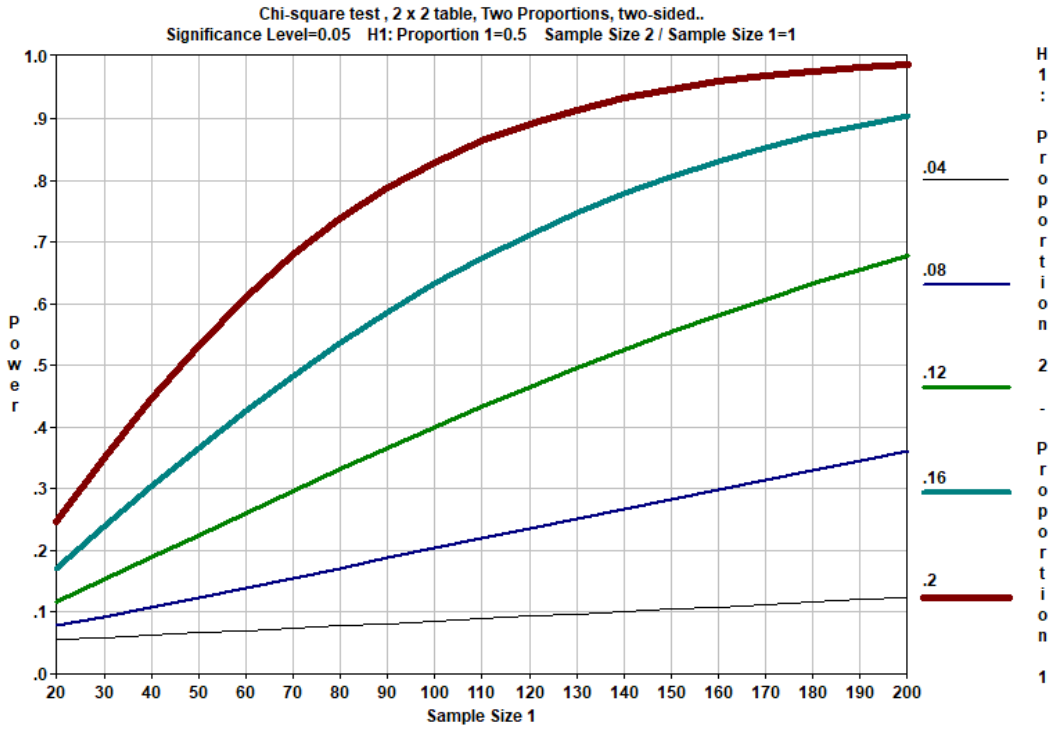
## 2.5 Power calculation

If we use the data of Ruiz (Ruiz et al., 2017) to calculate the sample size; incidence of metritis of 8.36% vs 9.79% control and treated respectively, the sample size are

| | Sample size | | |
|---|---|---|---|
| Power (%) | Control | Treated | Total |
| 60 | 4.092 | 4.092 | 8.184 |
| 65 | 4.578 | 4.578 | 9.156 |
| 70 | 5.12 | 5.12 | 10.24 |
| 75 | 5.74 | 5.74 | 11.48 |
| 80 | 6.473 | 6.473 | 12.946 |

If we use the data of Freicks paper (Freick et al., 2018) to calculate the sample size; incidence of metritis of 43.9% vs 22.7% control and treated respectively, the samples sizes are:

| | Sample size | | |
|---|---|---|---|
| Power (%) | Control | Treated | Total |
| 60 | 58 | 58 | 116 |
| 65 | 63 | 63 | 126 |
| 70 | 70 | 70 | 140 |
| 75 | 77 | 77 | 154 |
| 80 | 86 | 86 | 172 |




As a consequence, the design of our study is only suited to identify a very large difference in incidence of metritis between treated and control cows. The difference in incidence of metritis should be approximately 25% or larger to obtain a sufficient power to observed such a difference in our current study.

# 3. Results

In total there were 54 cows used in this study, 23 cows were not treated with *Imrestor* (42,6%) and 31 were treated with *Imrestor* (57,4%). Of the 54 cows, 16 cows were primiparous (29,6%) and 38 were multiparous (70,4%). In the primiparous group 7 were treated (43,8%), against 24 (63,2%) in the multiparous group (Table 3). One cow was culled during the study.

**Table 3. Number of cows per treatment.**

| | | N | % |
|---|---|---|---|
| Control | Primiparous | 9 | 39,1 |
| | Multiparous | 14 | 60,9 |
| | Total | 23 | 100 |
| Treated | Primiparous | 7 | 22,6 |
| | Multiparous | 24 | 77,4 |
| | Total | 31 | 100 |

## 3.1 Disease diagnose/metritis

### *3.1.1 Occurrence of metritis*

Among the primiparous cows, 71% that were treated had metritis while 67% of the primiparous cows that were not treated had metritis. Among the multiparous cows, 58% that were treated had metritis while 50% of the multiparous cows that were not treated had metritis (Table 4). The proportion of cows that had metritis did not differ significantly among these four groups (P=0.84). Also, the proportion of cows that had different severity of metritis did not differ significantly among these group (P=0.56; Table 5).

**Table 4. Occurrence of metritis per group. Primiparous treated; N=7, primiparous control; N=9, multiparous treated; N=24, multiparous control; N=14.**

| | Primiparous | | Multiparous | |
|---|---|---|---|---|
| | Treatment | Control | Treatment | Control |
| Metritis | 5 | 6 | 14 | 7 |
| No metritis | 2 | 3 | 10 | 7 |

**Table 5. Severity of metritis per group. Primiparous treated; N=7, primiparous control; N=9, multiparous treated; N=24, multiparous control; N=14. Score 1=mild clinical metritis, score 2=moderate clinical metritis, score 3=puerperal metritis.**

| | Primiparous | | Multiparous | |
|---|---|---|---|---|
| | Treatment | Control | Treatment | Control |
| No Metritis | 2 | 3 | 10 | 7 |
| Metritis score 1 | 1 | 3 | 4 | 1 |
| Metritis score 2 | 4 | 2 | 10 | 6 |
| Metritis score 3 | 0 | 1 | 0 | 0 |

*3.1.2 Duration of metritis*

Primiparous cows that were treated had on average metritis for 8 days (n=5, se=2.55), while primiparous cows that were not treated had on average metritis for 2.5 days (n=6, se=1.15). Multiparous cows that were treated had on average metritis for 2.86 days (n=14, se=0.76), while multiparous cows that were not treated had on average metritis for 4.43 days (n=7, se=2.02). The duration of metritis did not differ significantly among these four groups (H = 4.63, df = 3, p-value = 0.20; Fig. 3).

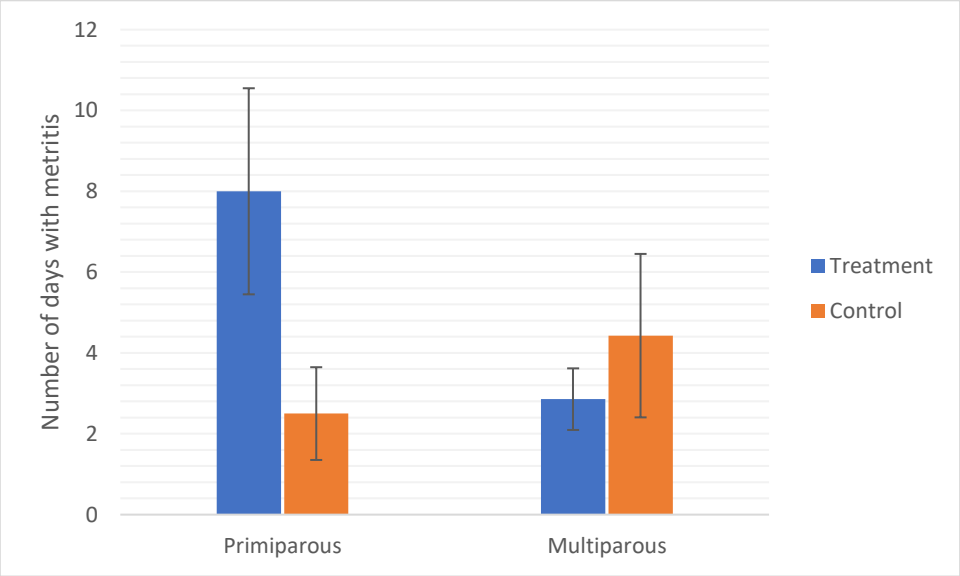



**Fig. 3. Barplot. Duration of metritis. Treated primiparous group had an average of 8 days (n=5, se=2.55), control primiparous group had an average of 2.5 days (n=6, se=1.15), treated multiparous group had an average of 2.86 days n=14, (se=0.76), control multiparous group had an average of 4.43 days (n=7, se=2.02). No significant difference among the four groups (H=4.63, df=3, P=0.20).**

*3.1.3 First day metritis*

The day on which cows got metritis differed significantly among the four different groups (H =8.26, df =3, P=0.04; Fig. 4). Primiparous cows which were treated tended to get on average metritis earlier, on day 3.8 postpartum (n=5, se=0.58), compared to primiparous cows that were not treated. Primiparous cows that were not treated got on average metritis on day 8.17 postpartum (n=6, se=2.43). However, this difference was not statistically significant (P=0.18). Multiparous cows that were treated and not treated got on average metritis on day 8.5 p.p. (n=14, se=0.84) and day 8.14 p.p. (n=7, se=1.16) respectively. The first day on which treated primiparous cows got metritis did not differ significantly from the first day control primiparous cows got metritis (P=0.65). Similarly, the first day on which treated multiparous cows got metritis did not differ from the first day on which control multiparous cows got metritis (P=0.80). Treated primiparous cows got metritis earlier than both treated (P=0.005) and control multiparous cows (P=0.02). The day on which control primiparous cows got metritis did not differ significantly from the day that both treated (P=0.45) and control multiparous cows got metritis (P=0.65).

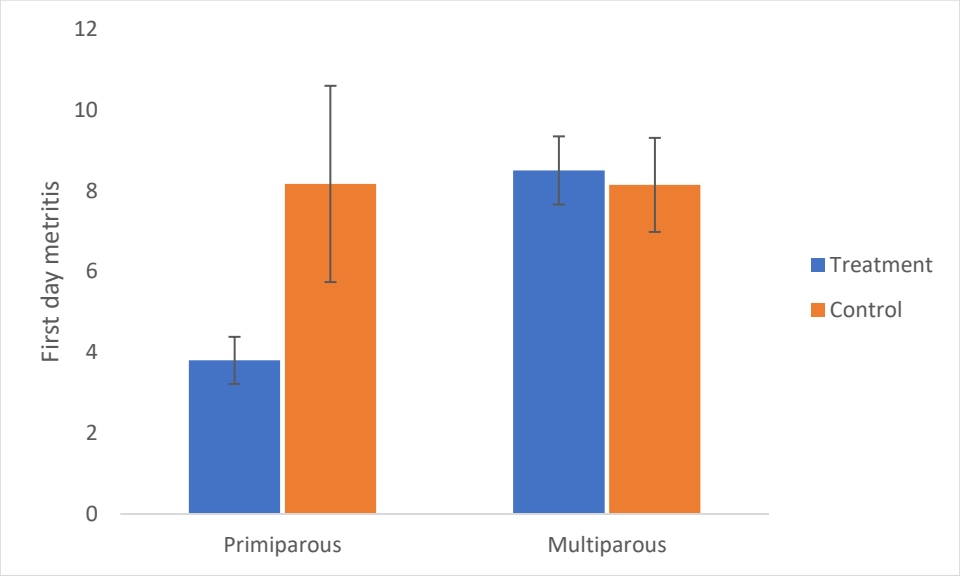



**Fig. 4. Barplot. First day of metritis. Treated primiparous group had an average of 3.8 days p.p. (n=5, se=0.58), control primiparous group had an average of 8.17 days p.p. (n=6, se=2.43), treated multiparous group had an average of 8.5 days p.p. (n=14, se=0.84), control multiparous group had an average of 8.14 days p.p. (n=7, se=1.16). P=**

**Tabel 6. Incidence of metritis per group, duration of metritis per group, severity of metritis per group (0= no metritis, 1=mild clinical metritis, 2=moderate clinical metritis, 3=puerperal metritis) and the average first day p.p. of metritis.**

| | Primiparous | | Multiparous | |
|---|---|---|---|---|
| | Treatment | Control | Treatment | Control |
| N of animals | 5 | 6 | 14 | 7 |
| Occurence of metritis | 71% | 67% | 58% | 50% |
| First day of metritis | 3.8 | 8.2 | 8.5 | 8.1 |
| Duration of metritis | 8 d | 2.5 d | 2.86 d | 4.43 d |
| Severity score | | | | |
| - No metritis | 29% | 33% | 42% | 50% |
| - Score 1 | 14% | 33% | 17% | 7% |
| - Score 2 | 57% | 22% | 42% | 43% |
| - Score 3 | | 11% | | |
| First day (p.p.) | 3.8 | 8.17 | 8.5 | 8.14 |

These data appear to indicate that treated primiparous animals showed a trend towards more, earlier, longer and more severe metritis compared to primiparous not treated cows.

## 3.2 Uterus involution

### *3.2.1 Uterus score*

At the observations performed on day 15 p.p., primiparous cows that were treated had an average uterus score of 1.8 (n=5, se=0.37), while non treated primiparous cows had an average uterus score of 1.88 (n=8, se=0.30). Multiparous cows that were treated had an average uterus score of 1.95 (n=19, se=0.18), while non treated multiparous cows had an average uterus score of 2 (n=12, se=0.17). This uterus score did not differ significantly among these four groups (H=0.37, df =3, P=0.95; Fig. 3).

At the observations performed on day 30 p.p., primiparous cows that were treated had an average uterus score of 1.2 (n=5, se=0.2), while non treated primiparous cows had an average uterus score of 1.17 (n=6, se=0.17). Multiparous cows that were treated had an average uterus score of 1.43 (n=7, se=0.20), while non treated multiparous cows had an average uterus score of 1.75 (n=8, se=0.25). Uterus score 30 days p.p. did not differ significantly among these four groups **(**H =4.18, df =3, P=0.24; Fig. 5). Among primi- and multiparous cows, uterus score 30 days p.p. was lower than uterus score 15 days p.p. (H =8.26, df =1, P=0.004).

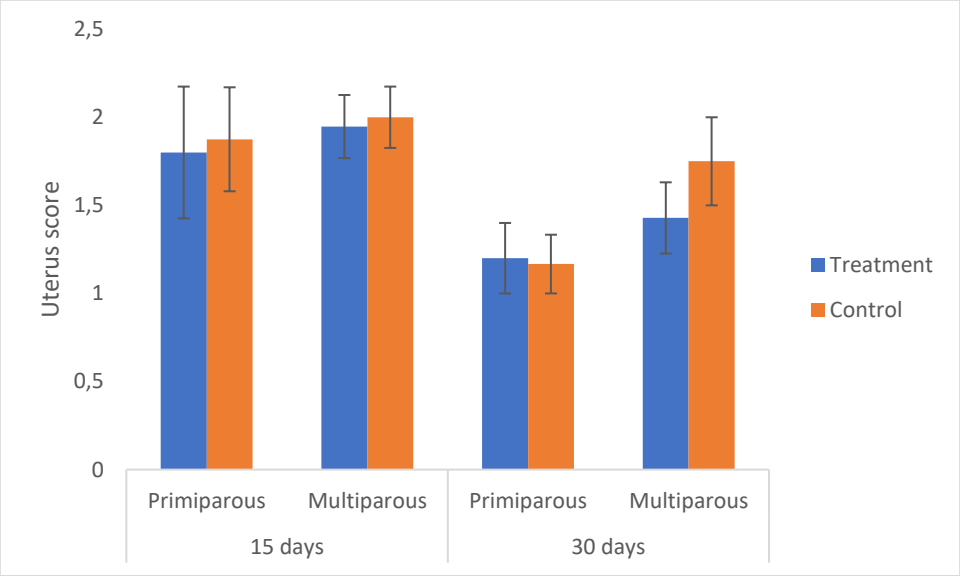



**Fig. 5. Barplot. Uterus score per group. Primiparous treated group had an average score of 1.8 (n=5, se=0.37) at 15 days p.p. and an average score of 1.2 (n=5, se=0.2), primiparous control group had an average score of 1.88 (n=8, se=0.30) at 15 days p.p. and an average score of 1.17 (n=6, se=0.17), multiparous treated group had an average score of 1.95 (n=19, se=0.18) at 15 days p.p. and an average score of 1.43 (n=7, se=0.20) at 30 days p.p. and multiparous control group had an average score of 2 (n=12, se=0.17) at 15 days p.p. and an average of 1.75 (n=8, se=0.25) at 30 days p.p.**

*3.2.2 Cervix score*

At the observations performed on day 15 p.p., primiparous cows that were treated had an average cervix score of 1.8 (n=5, se=0.37), while non treated primiparous cows had an average cervix score of 1.75 (n=8, se=0.31). Multiparous cows that were treated had an average cervix score of 1.68 (n=19, se=0.20), while non treated multiparous cows had an average cervix score of 1.91 (n=12, se=0.19). This cervix score did not differ among these four groups (H =1.04, df =3, P=0.79; Fig. 6)

At the observations performed on day 30 p.p., both primiparous cows that were treated and not treated had an average cervix score of 1 (n=5, se=0 treated and n=6, se=0 not treated). Also both multiparous cows that were treated and not treated had an average cervix score of 1 (n=7, se=0 treated and n=8, se=0 not treated). Because there was no variation within groups, we could not analyse whether the cervix score after 30 days of the different groups was significantly different. Among primi- and multiparous cows, cervix score 30 days p.p. was lower than cervix score 15 days p.p. (H =20.39, df =1, P<0.001).

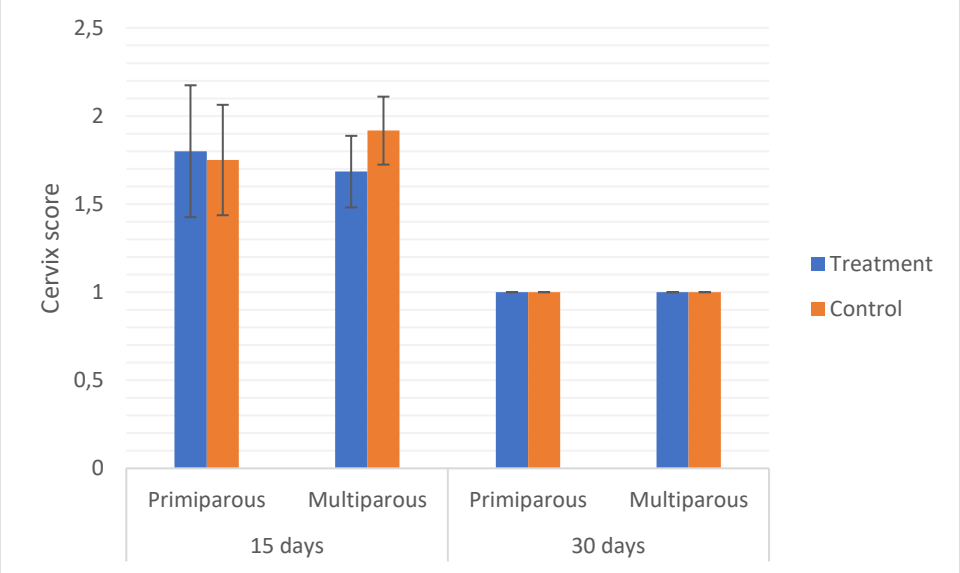



**Fig. 6. Barplot. Cervix score per group. Primiparous treated group had an average score of 1.8 (n=5, se=0.37) at 15 days p.p. and an average score of 1 (n=5, se=0), primiparous control group had an average score of 1.75 (n=8, se=0.31) at 15 days p.p. and an average score of 1 (n=6, se=0), multiparous treated group had an average score of 1.68 (n=19, se=0.20) at 15 days p.p. and an average score of 1 (n=7, se=0) at 30 days p.p. and multiparous control group had an average score of 1.91 (n=12, se=0.19) at 15 days p.p. and an average of 1 (n=8, se=0) at 30 days p.p.**

## 3.3 Blood metabolites

### *3.3.1 NEFA*

NEFA concentration was significantly different among these four groups (H =16.18, df =3, P=0.001; Fig. 7).

Primiparous cows that were treated had an average NEFA concentration of 1.30 mM (n=3, se=0.26), while non treated primiparous cows had an average NEFA concentration of 1.50 mM (n=8, se=0.20).

Multiparous cows that were treated had an average NEFA concentration of 0.72 mM (n=23, se=0.09), while non treated multiparous cows had an average NEFA concentration of 0.74 mM (n=13, se=0.12).

NEFA concentration of treated and control primiparous cows did not differ significantly (P=0.68).

Similarly, NEFA concentration of treated multiparous and control multiparous cows did not differ (P=0.82).

Non treated primiparous cows had a significant higher NEFA concentration than both non treated (P=0.002) and treated multiparous cows (P<0.001).

Treated primiparous cows tended to have a higher NEFA concentration than both non treated (P=0.08) and treated multiparous cows (P=0.05).

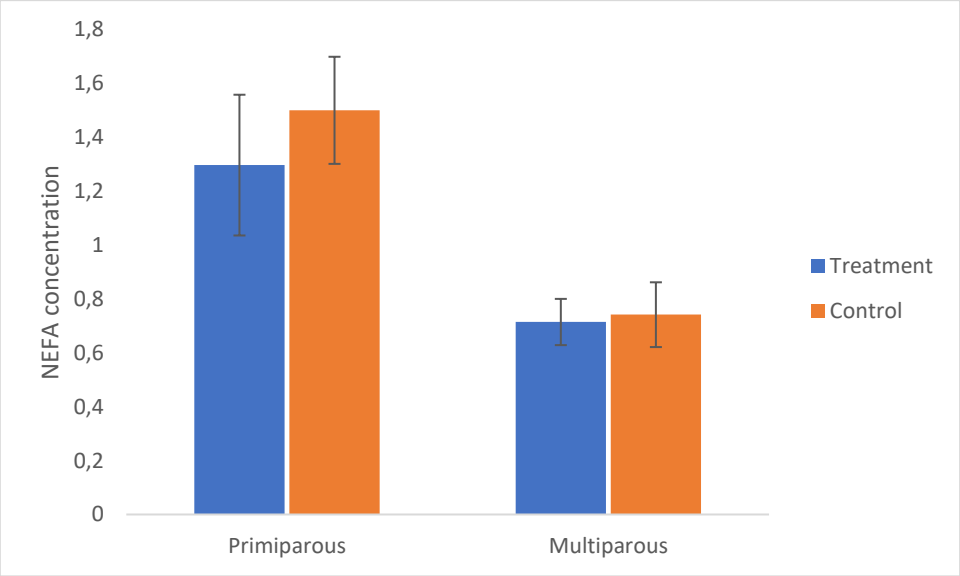



**Fig. 7. Barplot. Concentration NEFA per group at 15 days p.p. Treated primiparous group had an average concentration of 1.30 mM (n=3, se=0.26), control primiparous group had an average concentration of 1.50 mM (n=8, se=0.20), treated multiparous group had an average concentration of 0.72 mM (n=23, se=0.09) and control multiparous group had an average of 0.74 mM (n=13, se=0.12).**

*3.3.2 Calcium*

Calcium concentration was significantly different among these four groups (H =11.61, df =3, P=0.009; Fig. 8).

Primiparous cows that were treated had an average calcium concentration of 2.22 mM (n=3, se=0.08), while non treated primiparous cows had an average calcium concentration of 2.17 mM (n=3, se=0.04).

Multiparous cows that were treated had an average calcium concentration of 1.94 mM (n=13, se=0.06), while non treated multiparous cows had an average calcium concentration of 1.69 mM (n=6, se=0.06).

There was no significant differences in the calcium concentration of treated and control primiparous cows (P=0.80).

Treated multiparous cows had higher calcium concentration than control, multiparous cows (P=0.05).

Moreover, control primiparous cows had a higher calcium concentration than control multiparous cows (P=0.009), but their calcium concentration did not differ significantly from the concentration of treated multiparous cows (P=0.16).

Treated primiparous cows had a higher calcium concentration than control multiparous cows (P=0.004) and tended to have a higher calcium concentration than treated multiparous cows (P=0.09).

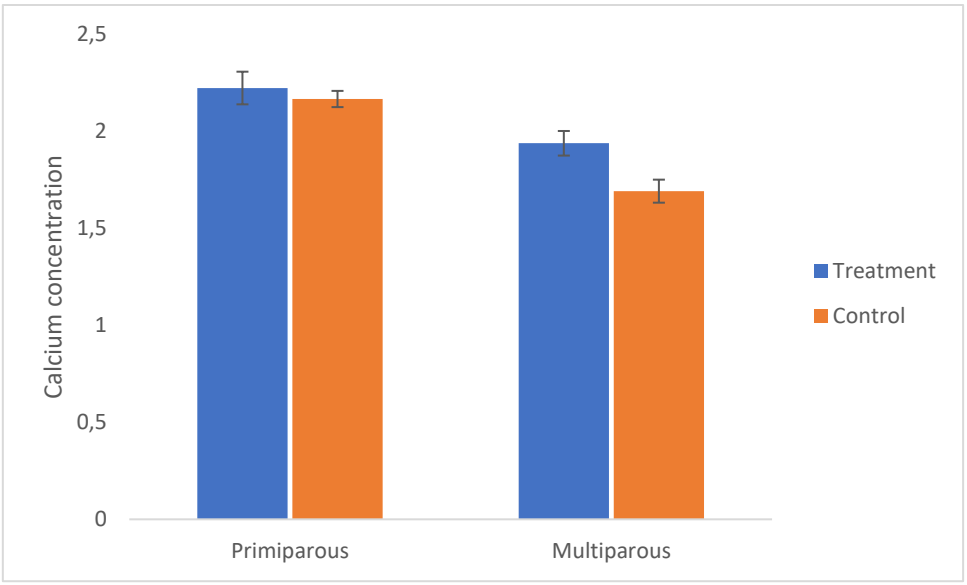



**Fig. 8. Barplot. Calcium concentration per group at days 15 p.p. Treated primiparous group had an average concentration of 2.22 mM (n=3, se=0.08), control primiparous group had an average concentration of 2.17 mM (n=3, se=0.04), treated multiparous group had an average concentration of 1.94 mM (n=13, se=0.06) and control multiparous group had an average concentration of 1.69 mM (N=6, SE=0.06).**

*3.3.3 BHB*

Primiparous cows that were treated had an average BHB concentration of 1.21 mM (n=3, se=0.55), while non treated primiparous cows had an average BHB concentration of 0.84 mM (n=8, se=0.13). Multiparous cows that were treated had an average BHB concentration of 0.97 mM (n=23, se=0.08), while non treated multiparous cows had an average BHB concentration of 0.99 mM (n=13, se=0.12). BHB concentration did not differ significantly among these four groups (H=1.19, df =3, P=0.76; Fig. 9).

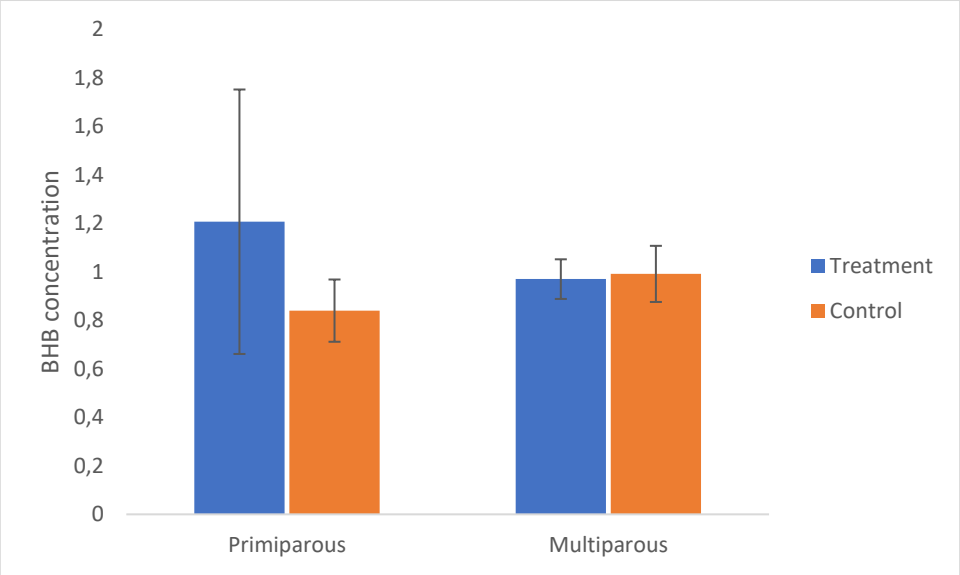



**Fig 9. Barplot. BHB concentration per group at 15 days p.p. Treated primiparous group had an average concentration of 1.21 mM (n=3, se=0.55), control primiparous group had an average concentration of 0.84 mM (n=8, se=0.13), treated multiparous group had an average concentration of 0.97 mM (n=23, se=0.08) and control multiparous group had an average concentration of 0.99 mM (n=13, se=0.12).**

3.4 Power calculation

Considering the data, 13 out of 23 control animals had metritis (56.5%) while 19 out of 31 treated cows had metritis (61%). So, the relative difference was an increase of 4.5% in the treated group. The power analysis [P= 0.05; ratio between groups (control/treated) = 0.74] shows a power of 2.8%.

| Sample Size (n) | Power (%) |
|---|---|
| 40 | 2.8 |
| 42 | 2.6 |
| 44 | 2.5 |
| 46 | 2.5 |
| 48 | 2.5 |
| 50 | 2.6 |
| 52 | 2.7 |
| 54 | 2.8 |
| 56 | 2.8 |
| 58 | 2.9 |
| 60 | 3 |

To reach a power of 80% with this data set, a sample size of approximately 1922 cows per treatment group is needed.

| | Sample size | | |
|---|---|---|---|
| Power (%) | Control | Treated | Total |
| 60 | 1.217 | 1.217 | 2.434 |
| 65 | 1.361 | 1.361 | 2.722 |
| 70 | 1.521 | 1.521 | 3.042 |
| 75 | 1.705 | 1.705 | 3.41 |
| 80 | 1.922 | 1.922 | 3.844 |

As a consequence of the low power, the observation of no significant difference is not strongly supported by the data. Based on the available number of observations is may be concluded with a reasonable power that the difference in metritis incidence is not more than 25%.

# 4. Discussion

This study is one of the first studies that investigated whether injecting cows with *Imrestor* could lower the occurrence of metritis (Ruiz et al., 2017; Freick et al., 2018), and the first one to test it under grazing conditions. Given the low power in our study, it was not surprising that we did not find a significant difference in metritis between the control and treated cows, neither among primi- nor multiparous cows. In contrast to our study, previous much larger studies that did find treated cows had more metritis than the control group did not distinguish between primi- and multiparous cows (Ruiz et al., 2017) or only used primiparous animals (Freick et al., 2018). Freick et al. (2018) did not divide metritis into different scores and they used rectal temperature as an important parameter for the diagnosis of metritis. Instead, the criteria used here to diagnose mild and/or moderate metritis, using smell and visible discharge, is relatively subjective. For example, there was no criteria for the amount of visible discharge or colour. As indicated, it is likely that we did not find significant differences between the different groups due to the relatively low sample size. The power of this study was much lower than the power of the other two studies (Ruiz et al., 2017; Freick et al., 2018).

We did find that multiparous cows that were treated with *Imrestor* tended to have a shorter duration of metritis than untreated multiparous cows, although this was not statistically significant. Kimura et al. (2014) showed that treated cows have more circulating neutrophils than the control group and it is likely that these circulating neutrophils can clean the uterus faster. Surprisingly, the duration of metritis appeared to be longer in treated primiparous cows compared to the untreated primiparous cows. One possible explanation would be that metritis may be easier and better detected among treated primiparous cows than untreated. Because treated primiparous cows have more circulating neutrophils than the control group, the immune response is more intense (Hassfurther et al., 2015). A hypothesis for the difference between the primi- and multiparous groups could be that the increased circulating neutrophils in multiparous cows are mostly memory cells, contrary to primiparous, and thus need less time to clean the uterus.

Treated primiparous cows had metritis earlier than control primiparous cows and both treated and control multiparous cows. Hassfurther et al. (2015) hypothesised that neutrophils might have an increased ability to eliminate infections before an inflammatory response is clinically apparent, but once the immune response is overwhelmed and an acute inflammatory response is triggered, the severity and amount of time required for resolution remain unchanged. It is possible that the immune response of primiparous cows gets overwhelmed faster than multiparous cows, and because *Imrestor* increases circulating neutrophils (Van Schyndel et al., 2015), metritis is detected earlier.

In accordance with the results that the occurrence of metritis did not differ among treated and non treated cows, we did not find significant difference among treated and non treated cows and indirect indicators of metritis, namely uterus size and cervix score. Uterus size and cervix score were measured both at 15 and 30 days. Cows that have metritis are expected to have a higher uterus – and cervix score. Although we did not find significant difference in uterus and cervix score among both treated and non treated primiparous and multiparous cows, we did find trends that may indicate that treated primiparous and multiparous cows ultimately experience less severe metritis. At 15 days, both treated primi- and multiparous cows had a lower uterus score than non treated cows, although these differences were not significant. Also at 30 days p.p., treated multiparous cows had a lower uterus score than non treated multiparous cows. In accordance to our results, Morrow et al. (1968) found no significant

difference in cervix – and uterus size at 30 days p.p. However, in the same study they did found a significant difference in uterus and cervix size at 15 days p.p. It is likely that we did not find significant difference due to the low sample size. The precision of classifying the uterus and cervix size would also likely improve with increased experience.

In our data, we also did not observe differences in calcium concentration, BHB concentration and NEFA concentrations. We did found significant differences between the primi- and multiparous groups, but in these groups we did not found significant differences between treatment and control. Although there were no significant differences found between the treated and control primiparous and treated and control multiparous cows, both treated primi- and multiparous cows tended to have higher concentrations of calcium and lower NEFA concentrations compared with the non treated groups. Among multiparous cows this difference in calcium concentration was actually significant. Also, BHB concentration tended to be lower among treated multiparous cows compared to non treated multiparous cows. In contrast, among primiparous cows, BHB concentration tended to be higher among treated cows compared to the control group.

Interestingly, both calcium and NEFA concentrations were higher among primiparous cows compared to multiparous cows, independent of whether they were treated with *Imrestor* or not. Indeed, based on previous observations, it is expected that both the primiparous groups had a significant higher calcium concentration than multiparous control group (Horst et al., 1997).
Martinez et al. (2012) showed that neutrophils of cows with subclinical hypocalcaemia were less capable of phagocytizing and killing pathogenic bacteria, in this study the multiparous control group had the lowest calcium concentration. This group and the treated multiparous group had an average calcium concentration below 2,1 mmol/L and thus hypocalcaemia (Martinez et al., 2012). Unexpectedly, they are not the group with the longest duration, respectively 4,43 days and 2,86 days. But they do have the lowest percentage of occurrence of metritis, which could mean that because the neutrophils work less, metritis is often missed. Based on the results of Galvao et al. (2010), that showed that NEFA concentration plays a role in PMN function, we would expect that the primiparous group, which had a higher NEFA concentration, had a higher occurrence of metritis or a longer duration of metritis, compared to multiparous cows. The study of Galvao et al. (2010) indeed found a higher occurrence of metritis in the primiparous group, but this group also had the shortest duration of metritis. It could be explained that because the reduced PMN function it actually takes longer to clean the uterus, but the immune response is not so fierce and thus more often missed.

We strongly encourage further investigation on the effectiveness of *Imrestor* for preventing metritis. This study was conducted on only one dairy farm. Consequently, herd effects, like housing, feeding conditions and accuracy of time of calving, can have a significant impact on the immune response and thus could influence the effect of *Imrestor* (Freick et al., 2018). Furthermore, future studies should include placebo administration in cows of the control group. Unfortunately in this study we were unable to account for this effect and therefore effects of the injection, like stress, and/or additional ingredients of the pharmaceutical on study outcomes cannot be excluded completely. It would also be interested to know the conception rate between the four different groups to be able to evaluated the possible economical advantage of *Imrestor*.

The power of this research is low and this is a restriction to the observed results. We did not find a difference in incidence of metritis, but according to the power calculation we did

afterwards, there is only a 2.8% chance of finding a difference of the observed size in incidence of metritis in this study. Freick et al. 2018 had 157 animals and so according to our power calculation a power of 75-80% to observe a difference of approximately 20%. Ruiz et al., 2017 had 10.166 animals and according to our power calculation this study had a power of 65-70% to observed a difference of approximately 1.5%. So while we were not able to identify a difference in incidence, other studies did. Those studies had a much higher power, the results of these studies are more reliable than this study. It would be reasonable to rewrite the hypothesis and focus our hypotheses on severity and duration instead of the occurrence of metritis. This study was designed to show only very large effects of pegbovigrastim on the incidence, severity and duration of metritis. Eventually we did observe a numerical difference in duration of metritis among primiparous animals.

# 5. Conclusion

In this study we did not find a statistically effect of *Imrestor* on the occurrence, severity or duration of metritis in either primi- or multiparous cows. In accordance, we did not find significant difference among cows that were treated with *Imrestor* and cows that were not treated with regard to the indirect indicators of metritis that we measured, namely uterus size, cervix score, calcium concentration, BHB – and NEFA concentration. However, uterus size, cervix score, BHB – and NEFA concentration tended to be lower and calcium concentration tended to be higher among treated cows, especially multiparous cows. We also observed that in treated primiparous cows, metritis occurred numerically earlier in lactation.

We might have not find a significant effect of *Imrestor* on the occurrence of metritis because some of the criteria we used to diagnose metritis, using smell and visible discharge, have a relatively low incidence.

Further studies should investigate the effect of *Imrestor* on metritis in more detail, including mulyiple dairy farms and comparing conception rate between treated and non treated animals before strong generalization can be made and to get more insight in the possible economical advantage of *Imrestor*.

## 6. Acknowledges

First of all I would like to thank Prof. Dr. Ynte Schukken for providing me the opportunity to fulfil my research project abroad and be a part of a bigger PhD project. I also want to thank him for all his general guidance, feedback and help with statistics.

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## Attachments

| ID | Calving date |
|---|---|
| | |

| Days post partum | |
|---|---|
| Odor | |
| Discharge | |
| Systemic illness | |
| Score | |

| Days post partum | |
|---|---|
| Odor | |
| Discharge | |
| Systemic illness | |
| Score | |

| Days post partum | |
|---|---|
| Odor | |
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| Odor | |
| Discharge | |
| Systemic illness | |
| Score | |

| **Rectal palpation** | Days post partum | |
|---|---|---|
| | 14 | 28 |
| Score | | |