The effect of pegbovigrastim on health, fertility, culling and economic performance during a full lactation in grazing dairy cows

Joaquín Barca

Propositions

1. In dairy cows, prepartum metabolic challenge is associated with negative outcomes spread out over the full lactation, ranging from reduced postpartum immune cell counts to impaired overall economic performance.
(this thesis)

2. Immune restoration due to bovine granulocyte colony stimulating factor is particularly beneficial for dairy cows undergoing prepartum metabolic challenge.
(this thesis)

3. Animal health is strongly associated with human welfare.

4. Success of a scientist is partially explained by her/his generosity and the capacity to recognize how much she/he knows.

5. Innate abilities are important to develop any discipline, but hard work and commitment are essential.

6. Music stimulates cognitive development and provides quality of life.

Propositions belonging to the thesis, entitled

The effect of pegbovigrastim on health, fertility, culling and economic performance during a full lactation in grazing dairy cows

Joaquín Barca
Wageningen, 30 August 2022

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Thesis

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Abstract

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Early lactation clinical diseases, which affect up to 50% of modern dairy cows, are associated with impaired productive and reproductive performance and reduced longevity; thus, they have an important impact on the economic performance of dairy farming. Around parturition, both immune dysfunction and metabolic challenge, a major factor associated with immune dysfunction, have been linked to early lactation clinical disease. Immune stimulation therapies may be an innovative development that could mitigate this problem. Recently, a long-lasting analogue of bovine granulocyte colony stimulating factor (pegbovigrastim; PEG) has been developed, as a tool to improve immune dysfunction around parturition. This PhD thesis was based on a large randomized clinical trial, in which I evaluated the use of PEG under commercial conditions in grazing dairy farms. In addition, I evaluated whether the metabolic status of the transition dairy cow, as measured by prepartum body condition score and prepartum nonesterified fatty acids (NEFA) concentration, was associated with the effect of PEG treatment. First, I focused on the effect of PEG treatment on postpartum [5 to 8 days in milk (DIM)] circulating white blood cell counts. Pegbovigrastim treatment reversed the negative association of prepartum NEFA concentration with circulating white blood cell counts. Then, I showed that PEG treatment reduced the occurrence of a first case of clinical mastitis (CM) during the first 30 DIM, particularly in cows with an over body condition and in cows with elevated prepartum NEFA concentration. Moreover, PEG treatment reduced the hazard of a first case and the rate of total cases of CM during the full lactation, and in cows that experienced metritis, PEG treatment reduced the incidence of subsequent endometritis. In addition, I investigated the effects of PEG on fertility, culling and on the economic performance of cows. I showed that the effect of PEG treatment on fertility and culling interacts with prepartum NEFA concentration. In cows with low prepartum NEFA concentration, no treatment effect was detected. In cows with elevated prepartum NEFA concentration, PEG treatment increased the rate of first insemination and counteracted the negative association of a first case of CM during the first 30 DIM and uterine disease (i.e., retained placenta, metritis or both) with the rate of pregnancy. At the same time, in cows with elevated prepartum NEFA concentration, PEG treatment decreased the hazard of culling. Ultimately, we found that PEG treatment resulted in an overall economic

benefit, mostly explained by a reduced cost of culling in PEG treated cows. Altogether, I showed that the beneficial effect of PEG treatment depends on the metabolic status of periparturient dairy cows, and that PEG treatment was particularly beneficial for cows undergoing prepartum metabolic challenge.

Contents

CHAPTER 1

General introduction

1.1. Background

The last decades have seen major improvements in the incidence of clinical disease during the transition from late gestation to early lactation (Ingvartsen et al., 2003, LeBlanc, 2010; Overton et al., 2017). However, this critical period in the productive life of the dairy cow remains one of the biggest challenges for the dairy sector in many regions of the world (Kay et al., 2015; Overton et al., 2017; Gross and Bruckmaier, 2019). Early lactation clinical diseases affect up to 50% of modern dairy cows (Ingvartsen et al., 2003; Leblanc, 2010; Galvao, 2013), and these early lactation diseases are associated with impaired productive and reproductive performance, reduced longevity, and have an important impact on the economic result of dairy farming (Hogeveen et al., 2017).

A dysfunctional immune response has been linked to these early lactation clinical diseases (Waller, 2000; LeBlanc, 2014; Pomeroy, et al., 2017). Around parturition, dairy cows experience a decreased immune response (Kehrli et al., 1989; Sordillo et al., 1995; Kimura et al., 1999). Among other causes, the metabolic burden to cope with milk production and the resulting negative energy balance (NEB) have been identified as major factors associated with immune dysfunctionality (Galvao et al., 2010; Ingvartsen and Moyes, 2015). Metabolites related to NEB, such as nonesterified fatty acids (NEFA) and β-hydroxybutyrate, have been identified as immunosuppressants (Ingvartsen and Moyes, 2015). Elevated NEFA concentrations were associated with decreased white blood cell (WBC) counts (Hachenberg et al., 2007). Epidemiological studies have shown that elevated NEFA concentrations are associated with increased risk of diseases such as clinical mastitis (CM), retained placenta (RP) and metritis (LeBlanc et al., 2004; Melendez et al., 2009; Galvão et al., 2010).
Immune stimulation therapies such as the use of bovine granulocyte colony-stimulating factor (G-CSF) is an innovative development that could reduce the occurrence of early lactation clinical disease.

In the next paragraphs, I will provide an in-depth description of the transition period, focused on the epidemiology of clinical diseases and the metabolism of the dairy cow. Moreover, I will review the information available on the use of G-CSF in dairy cows and I will discuss knowledge gaps that form the basis of the aims of this thesis.

1.2. The transition period: from late gestation to early lactation

1.2.1. Epidemiology of clinical diseases

The transition period is associated with a high incidence of clinical metabolic and infectious diseases (Ingvartsen et al., 2003; Leblanc, 2010; Galvao, 2013). We decided to focus on the infectious diseases CM and uterine disease, as they appear to be closely related to immune system responsiveness (Schukken et al., 2011; Sheldon et al., 2020; Leblanc, 2020).

1.2.1.1. Clinical mastitis

Clinical mastitis is one of the most widespread, painful and economically important diseases that dairy cows have experienced for many decades (Murphy, 1956; Barkema et al., 1998; De Vliegher et al., 2012). Despite many improvements in management, CM still affects a large number of modern dairy cows (Gao et al., 2017; Cruz et al., 2021). Reported incidences differ widely, with mean values around 40 cases per 100 cows per year (Santman-Berends et al., 2015; Gao et al., 2017; Jamali et al., 2018), although some farms can record more than 95 cases per 100 cows per year (Olde Riekerink et al., 2008; Gao et al., 2017). Early lactation is typically the period with the highest risk for CM (Barkema et al., 1998; Compton et al., 2007). Multiparous cows present a higher incidence of CM during the full lactation, although during the first days of lactation, it is higher in primiparous cows (Steeneveld et al., 2008; De Vliegher et al., 2012). Figure 1.1, taken from Steeneveld et al. (2008) with permission, illustrates the risk for CM in primiparous and multiparous cows during lactation. Clinical mastitis, defined as altered milk secretion with or without local signs of inflammation and/or systemic illness (Ruegg, 2017), is the inflammatory response against intramammary bacterial infections (IMI) (Bannerman, 2009). It has been indicated that over 60% of the CM cases that occur during the first 15 DIM may be caused by IMI that are present in late gestation (Green et al., 2002). During transition, and especially during colostrogenesis, dairy cows become more susceptible to IMI (Smith et al., 1985), because, among other factors (e.g. low lactoferrin concentration within the udder, concomitant disease, etc.), phagocytes are reduced in number and in functionality (Burvenich et al., 2007). Furthermore, it has been shown that immune responses against IMI are biased towards an anti-inflammatory profile during late gestation (Quesnell et al., 2012; Gurjar et al., 2013, Pomeroy et al., 2015), potentially increasing the susceptibility to IMI.

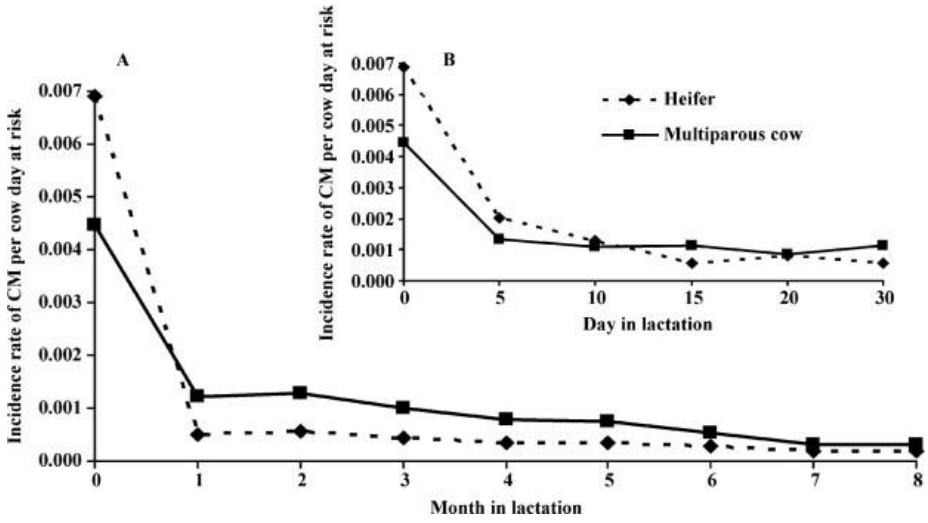

Figure 1.1. Incidence rate of clinical mastitis (CM) per cow-day at risk for a heifer and a multiparous cow (A). Incidence rate for CM for the same cows in the first month of lactation (B). Taken from Steeneveld et at. (2008), with permission.

Intramammary infection pathogens may be classified as contagious pathogens, for which the principal propagation pathway is from cow to cow, usually during milking, or as environmental, for which the principal propagation pathway is from the environment to cow. The main contagious pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Corynebacterium bovis*, while the main environmental pathogens are *Escherichia coli*, *Streptococcus uberis* and *Streptococcus dysgalactiae* (Zadoks and Schukken, 2006). However, it has been shown that specific strains may behave as both contagious and environmental pathogens (Zadoks and Schukken, 2006; Budd et al., 2015; Davies et al., 2016).

The development of mastitis control programs has achieved a fairly effective control of contagious pathogens. Some, such as *Streptoccocus agalactiae,* can be considered eradicable if the control program is implemented properly at the farm. As reviewed by Kingwill (1980), this control program is based on the prevention of new IMI and the elimination of existing IMI. However, as Neave et al. (1966) stated early on, this control program has a major constraint: thousands of farm workers with different skills have to perform specific working routines with a high degree of compliance. In recent times,

social sciences have identified that there are limitations on how to persuade veterinarians, farmers or employees to comply with udder health programs (Jansen et al., 2016).

In modern dairy farms, IMI caused by environmental pathogens are the most prevalent cause of CM in many regions around the world, and under different production systems (Compton et al., 2007; Oliveira and Ruegg, 2014; Verbeke et al., 2014; Oliveira et al., 2015). Controlling the incidence of IMI due to environmental pathogens, which have a much higher rate of CM compared to contagious pathogens (Smith et al., 1985), is complex. Eradication is not possible, as the source of infection is the cow's environment (soil and feces). Thus, it has been suggested that the only preventive strategy is to minimize exposure of the teat to pathogens and maximize immunity of the animal to an IMI (Smith et al., 1985). One of the main effectors of mammary immune defense against mastitis causing pathogens are blood derived neutrophils (Burton and Erskine, 2003). Milk in healthy udders is populated by immune cells, constituted mainly by macrophages, neutrophils and lymphocytes (Sordillo, 2005). As reviewed by Schukken et al. (2011), the ability to recruit neutrophils into the mammary gland during the bacterial growth phase is considered pivotal; the faster the host can recruit neutrophils, the earlier bacterial growth will be decelerated. Cows with very low somatic cell counts (SCC; i.e. < 20,000 cells/mL) in milk showed to be at higher risk for more severe IMI (Wellnitz et al., 2010). At quarter level, very low cell counts tend to affect the response to an IMI challenge and these quarters show higher incidences of CM (Schukken et al., 2011).

Clinical mastitis has important direct economic effects such as production loss, discarded milk during the withdrawal period after medical treatment and the actual cost of treatments (Hogeveen et al., 2011; Hogeveen et al., 2019). Moreover, CM has been associated with impaired fertility. A recent meta-analysis (Dolecheck et al., 2019) showed that time to first insemination and time to pregnancy in an animal with CM is increased by 13 and 32 days, respectively. Moreover, both CM and poor fertility are major reasons for culling (Kossaibati and Esslemont, 1997; Bar et al., 2008; Hertl et al., 2018). Impaired fertility and increased culling are therefore indirect economic effects of CM. Culling in particular makes an important contribution to the costs of CM (Heikkila et al., 2012; Rollin et al., 2015).

The cost of culling is determined by the rearing cost of a replacement heifer, which is one of the main dairy production costs (Vredenberg et al., 2021), and lower milk returns due

to shorter productive lifetime (De Vries and Marcondes, 2020; Schuster et al., 2020). As indicated by Vredenberg et al. (2021), an increased hazard of culling implies higher total rearing costs, because more replacements are needed, which, at the same time, will be spread out over a shorter productive life. Moreover, premature culling will result in more cows in lower producing parity groups, and thus a lower average milk production of the herd (Vredenberg et al., 2021). In addition, culling is negatively associated with environmental sustainability and animal welfare, which are major social concerns (Barkema et al., 2015; Overton and Dhuyvetter, 2020)

1.2.1.2. Uterine diseases

Parturition represents a dramatic event that causes physical damage and bacterial contamination to the reproductive tract (Sheldon et al., 2020). Uterine disease has a high incidence in modern dairy cows, as it can affect up to 50% of animals (Sheldon et al., 2008; Galvao, 2013). Retained placenta (RP), defined as fetal membranes (placenta) still visible hanging from the cows´ vulva 24 h after calving (Kelton et al., 1998), affects 5 to 10% of calved dairy cows, although in some studies, much higher incidences were reported (Kimura et al., 2002; Sheldon et al., 2008). Metritis, characterized by fetid reddish-brown uterine discharge within the first 21 d postpartum (Sheldon et al., 2006), typically affects 25-40% of the animals (Sheldon et al., 2008; Galvao, 2013). Clinical endometritis, defined by Sheldon et al. (2006) as the presence of purulent uterine discharge detectable in the vagina 21 d or more postpartum, or mucopurulent discharge detectable in the vagina after 26 d postpartum, affects 20% of dairy cows, although the prevalence may be higher than 30% in some herds (Galvao, 2013).

Uterine diseases are multifactorial in nature. The predominant risk factors for postpartum uterine disease are trauma to the genital tract followed by colonization with pathogenic bacteria (Sheldon et al., 2020). Trauma to the genital tract is more likely in the primiparous cows, after induction of parturition, or following dystocia, stillbirths, twins, male calves, or RP (Sheldon et al., 2020). Although most cows are infected after calving, few pathogenic bacteria species, which may be present simultaneously and develop synergy, are consistently isolated from the affected uterus. These bacteria species include *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium species*, and *Prevotella melaninogenica* (Galvao, 2013; Sheldon et al., 2020). Epidemiological studies have

associated prepartum NEB (i.e. elevated NEFA concentration) with increased risk for metritis (Ospina et al., 2010; Duboc et al., 2011; Giuliodori et al., 2013).

The immune system plays a pivotal role in restoring uterine physiology after calving, and in mounting a response against intra-uterine pathogens (LeBlanc, 2014). Recently, Sheldon et al. (2020) proposed that prevention of uterine disease depends on three cornerstones. First, limiting the uterus exposure to pathogens by means of physical barriers (vulva, vagina, cervix, and cervical mucus) that avoid bacteria ascending the genital tract into the uterus. However, trauma to the genital tract harms these structures and will allow bacteria to invade the uterus. Secondly, limiting the damage caused by pathogenic bacteria. Mechanisms against damage caused by uterine pathogens include functional barriers to bacterial infection, neutralization of bacterial toxins, repair of tissue damage, and adaptive metabolic responses. Thirdly, limiting the number of pathogenic bacteria. A rapid and robust immune response efficiently controls pathogens, whereas a delayed or blunted immune response leads to disease. Thus, Sheldon et al. (2020) argue that the innate immune system is particularly important in generating an immediate, nonspecific response to pathogens that does not depend on prior exposure to pathogens.

This innate immune response appears to be suitable for uterine infections, which are multi-bacterial and fluctuate during the postpartum period (Sheldon et al., 2020). As suggested by LeBlanc (2014), the effectiveness of the immune response will determine the incidence of reproductive tract diseases. Cows that are capable of recruiting large numbers of neutrophils rapidly to the uterus in the immediate postpartum period are less likely to suffer long-term bacterial infections and more likely to have a healthy postpartum uterine restoration (Gilbert and Santos, 2016). Neutrophil migration and killing capacity (measured as chemotaxis and myeloperoxidase activity) are a determining factor for the development of RP (Kimura et al., 2002), metritis and endometritis (Hammon et al., 2006). Thus, besides controlling risk factors for trauma to the genital tract to prevent postpartum uterine disease, supporting immune function and thereby promoting a physiological uterine restoration appears to be essential (LeBlanc, 2014; Gilbert and Santos, 2016; Sheldon et al., 2020).

Uterine disease has important direct economic effects such as production loss, discarded milk during the withdrawal period after medical treatment and the actual cost of treatment (Pérez-Báez et al., 2021). Moreover, uterine diseases have been linked to impaired

fertility, where RP, metritis and endometritis delayed time to first insemination and time to pregnancy (Fourichon et al., 2000; LeBlanc et al., 2002; Toni et al., 2015). Uterine disease and poor fertility are major reasons of culling (Kossaibati and Esslemont, 1997; Pérez-Báez et al., 2021). Thus, poor fertility and culling are indirect economic effects of uterine diseases. Several studies showed that culling makes an important contribution to the costs of uterine diseases (Galligan, 2006; Overton and Fetrow, 2008; Pérez-Báez et al., 2021).

1.2.2. Energy balance and immune response

The transition from late gestation to lactation in modern dairy cows is characterized by a sudden increase in energy requirement to cope with milk production, while feed intake lags behind (Grummer, 1995). Consequently, metabolic stress arises and homeorhetic changes lead to NEB. Body fat mobilization closes the gap between energy inputs and outputs (Grummer, 1995; Gross and Bruckmaier, 2019). Metabolites related to NEB, such as NEFA and β-hydroxybutyrate, have been linked to the development of metabolic and infectious diseases (Ingvartsen, 2006). Epidemiological studies have shown that elevated NEFA concentrations are associated with increased risk of diseases such as CM, RP, and metritis (LeBlanc et al., 2004; Melendez et al., 2009; Galvão et al., 2010). Several studies have shown a decline in neutrophil and lymphocyte counts during transition, often in combination with impaired functionality such as a reduced chemotaxis, phagocytosis, oxidative burst, and reduced proliferative capacity (Kehrli et al., 1989; Kimura et al., 1999; Gilbert et al., 1993; Ingvartsen and Moyes, 2015). Although there are reports on the negative effect of NEB on neutrophil and lymphocyte function (Kimura et al., 1999; Lacetera et al., 2005; Hammon et al., 2006), there are few reports on the effect of NEB on the counts of these immune cells. Elevated NEFA concentrations were associated with decreased white blood cell (WBC) counts in one study (Hachenberg et al., 2007). It has been reported that NEFA concentrations decreased the viability and increased necrosis of neutrophils (Scalia et al., 2006). LeBlanc (2020) argues that there is sufficient evidence to conclude that exposure to elevated concentrations of NEFA or β-hydroxybutyrate affects neutrophil responsiveness.

The rate and extent of fat mobilization has also been linked to an increased risk of periparturient metabolic and clinical disorders during the transition period (Roche et al., 2009; Ingvartsen and Moyes, 2015). Roche et al. (2009) reported that over-conditioned cows (BCS > 3.5), that mobilize more fat reserves than cows with a moderate BCS (3–3.5), had more mastitis and suggested that high BCS is related to an impaired energy and lipid metabolism that may affect the immune response in these cows. Although the relationship between thin cows and the risk of periparturient metabolic disorders is less consistent (Roche et al., 2009), cows with a low BCS (BCS < 3) had more mastitis relative to cows with a moderate BCS, and low BCS has also been reported as a risk factor for uterine disease (Roche et al., 2009; Loker et al., 2012). A schematic representation of the energy lactation cycle, BCS, energy balance (as NEFA concentration) and neutrophil counts during the periparturient period is provided in Figure 1.2.

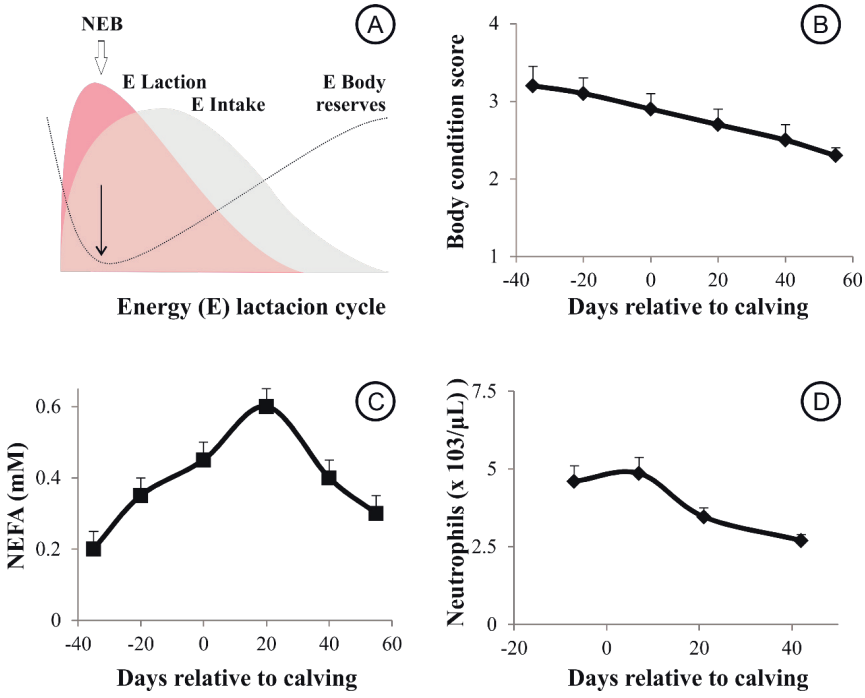

Figure 1.2. Schematic representation of the energy lactation cycle (A), BCS (B), energy balance (as NEFA concentration; C) and neutrophil counts (D) during the periparturient period (Adapted from Gilbert et al., 1993; Meikle et al., 2004).

Parity is an important factor determining the metabolic status during transition. Compared with multiparous cows, primiparous cows had a less pronounced NEB (in terms of NEFA

concentration) in confined animals (Wathes et al., 2007). However, in pasture-based herds it has been reported that primiparous cows have a more pronounced NEB compared to multiparous animals; primiparous cows showed a larger decrease in BCS and had higher NEFA concentrations (Meikle et al., 2004; Adrien et al., 2012).

1.3. Use of Granulocyte Colony-Stimulating Factor in Dairy Cows

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that regulates the proliferation, differentiation, and survival of haematopoietic stem cell precursors and mature peripheral effector cells (Almenar Cubells et al., 2013). In dairy cows, early studies evaluating the use of G-CSF were conducted 30 years ago (Nickerson et al., 1989; Kehrli et al., 1991). Nickerson et al. (1989) assayed the effects of daily injections of human G-CSF (hG-CSF) on *Staphylococcus aureus* mastitis in twelve lactating dairy cows (from 30 to 60 DIM), reporting an elevated marginal pool of circulating neutrophils and a reduction of 47% in quarter level IMI in cows that received hG-CSF compared with controls. Kehrli et al. (1991) reported that daily doses of recombinant bovine G-CSF (rbG-CSF) increased the number of circulating polymorphonuclear cells and improved their capacity to ingest bacteria and cytotoxicity, but impaired their ability to move randomly or toward a chemotactic target. Cai et al. (1994) did not find *in vitro* effects of hG-CSF on migration, chemotaxis, ingestion or killing capacity of neutrophils from clinically normal or postparturient cows that recorded RP, metritis, or mastitis. Mitchell et al. (2003) reported that *in vitro* or *in vivo* exposure to G-CSF enhanced the subsequent oxidative burst and phagocytic responses of bovine neutrophils. Under practical conditions, to inject cows with G-CSF on a daily basis is difficult, if not impossible. Pegylation (covalent binding to polyethylene glycol) of G-CSF results in a molecule with longer duration of bioactivity (Molineux, 2003). More recently, a pegylated form of recombinant bovine G-CSF [pegbovigrastim (PEG), marketed as Imrestor® by Elanco Animal Health] was developed. It has been shown that, when administered at approximately 6 d before calving and again within 24 h after calving, treated animals showed an increased circulating neutrophil count, increased myeloperoxidase exocytosis but no improvement in phagocytosis or oxidative burst (Kimura et al., 2014). Subsequently, a number of studies have confirmed the increase in neutrophil counts as a result of PEG treatment (Hassfurther et al., 2015; Canning et al., 2017; McDougall et al.,

2017; Van Schyndel et al., 2018; Zinicola et al., 2018). At functional level, McDougall et al. (2017) also showed increased myeloperoxidase exocytosis but no difference in phagocytosis or oxidative burst, further confirming the results reported by Kimura et al (2014). Combining these results, it can be concluded that PEG treatment around parturition consistently increases neutrophil counts, and, at cell level, it appears to improve myeloperoxidase exocytosis. Thus, a fair hypothesis is that PEG treatment could help to prevent transition diseases, particularly those that are considered consequences of a dysfunctional immune response.

A number of trials have been conducted to test the effect of PEG treatment on health. Hassfurther et al. (2015) carried out a small-scale experiment (approximately 50 animals per treatment group) on dairy cows housed in a pen with dirt flooring, which was kept wet to maximize the incidence of CM. They used different dose regimens, reporting that, at 10 and 20 µg/kg, PEG treatment lowered the incidence of CM within 28 d in milk (DIM) by 50 (9/54) and 74% (5/53), respectively, compared to placebo controls (18/53). Under commercial conditions, Canning et al. (2017) performed a randomized trial with a total of 640 primiparous and multiparous cows from four herds, selected to represent the diversity of management systems in US dairy farms (Wisconsin, Colorado, California, and Washington). Overall, they reported that PEG treatment reduced the incidence of CM from 3 to 30 DIM by 35%. These authors did not perform statistical analyses by parity, likely due to sample size limitations. In a large-scale trial (n = 10,238 cows in total) in 17 Mexican dairy herds, PEG treatment reduced the incidence of CM during the first 30 DIM by 25%. Moreover, PEG treatment reduced the incidence of RP by 4.15%, while it increased the incidence of metritis by 17.1%. In this study, parity by treatment interaction was not reported. A higher incidence of metritis due to PEG was further reported in a small-scale study (n = 270) carried out on a single US dairy farm (Oliveira et al., 2020). In contrast, on a German dairy farm (Freick et al., 2018), PEG treatment reduced the incidence of acute puerperal metritis (i.e. fetid reddish-brown uterine discharge within the first 21 d postpartum and a rectal temperature > 39.5°C) in primiparous cows (n = 169 in total) by 48.3%. On a single commercial dairy farm with freestall housing (New York, US), with a total sample size of 830 primiparous and multiparous cows, PEG treatment did not affect the incidence of CM, RP or uterine disease, and increased the incidence of displaced abomasum and lameness (Zinicola et al., 2018). Van Schyndel et al. (2021) using a total of 1,607 dairy cows from 6 farms in Ontario and Quebec, Canada, reported

that PEG had no effects on the incidence of CM, RP, uterine diseases, or displaced abomasum. In conclusion, although a lower early lactation CM incidence during the first 30 DIM was reported in most studies, PEG treatment results ranged from preventive effects to an increased morbidity of early lactation clinical disease.

McDougall et al. (2017) hypothesized that the response to PEG may be modulated by the degree of NEB experienced during the transition, similar to the association of NEB with immune cell counts and functionality (Hachenberg et al., 2007; LeBlanc, 2020). However, they reported that the effect of PEG on neutrophil counts and their function was not altered by prepartum energy restriction. Interestingly, in their trial, both non-restricted and restricted groups presented a high frequency of cows with high NEFA concentrations (> 0.4 m*M*) one week before calving (56% vs 85%, respectively).

Some of the above reported field studies also reported on fertility and culling. Pegbovigrastim treatment reduced failure to return to estrus within 80 DIM (Canning et al., 2017) and increased the rate of insemination by 5.8% during the first 100 DIM (Ruiz et al., 2017). In contrast, Zinicola et al. (2018) reported a lack of PEG treatment effect on rate of insemination during the first 120 DIM and rate of pregnancy during the first 180 DIM. They also reported a lack of effect on the hazard of culling during the first 180 DIM. Van Schyndel et al. (2021) reported a lack of PEG treatment effect on the hazard of culling during the first 63 DIM, rate of first insemination during the first 150 DIM and rate of pregnancy during the first 250 DIM.

1.4. Knowledge gaps

Research on PEG treatment effects on the incidence of diseases has yielded inconsistent and sometimes contradictory results. In contrast to most studies (Hassfurther et al., 2015; Canning et al., 2017; Ruiz et al., 2017), Zinicola et al. (2018) concluded that PEG treatment was detrimental to cow health because it increased morbidity, while Van Schyndel et al. (2021) concluded that PEG treatment did not affect the incidence of disease. However, in the Zinicola et al. (2018) study, low and high BCS cows (BCS < 3 and > 3.75) were excluded from the study. Furthermore, the two studies with no or negative PEG treatment effects in randomly chosen subsets of cows reported low prepartum NEFA concentrations (Zinicola et al. 2018, Van Schyndel et al. 2021). These

two trials that appear to be in contradiction with previous reports would be biased toward a population of cows with no or minor metabolic challenge (Roche et al., 2009; Ospina et al., 2013), and thus minor immune dysfunction (Hachenberg et al., 2007; LeBlanc, 2020). Hence, we hypothesize that the metabolic status of dairy cows during the transition period could explain, at least in part, the disparity among studies.

Parity is an important factor determining the metabolic status during transition. In contrast to confined systems (Wathes et al., 2007), under grazing conditions primiparous cows were shown to have a more pronounced NEB (Meikle et al., 2004; Adrien et al., 2012). Moreover, parity is associated with early lactation clinical diseases such as CM and uterine disease (Steeneveld et al., 2008; De Vliegher et al., 2012; Toni et al., 2015; Sheldon et al., 2020). Hence, parity could be another factor that explains differences in PEG treatment effects on disease.

It has been suggested that under grazing conditions, early lactation disease is less frequent compared to high-producing cows under confinement conditions (Washburn et al., 2002). However, under grazing conditions, we recently reported a much higher incidence of early lactation CM (Cruz et al., 2021) compared to most of the studies of PEG treatment on early lactation clinical disease (Ruiz et al., 2017; Zinicola et al., 2018; Van Schyndel et al., 2021). Ribeiro et al. (2013) concluded that CM and uterine disease were highly prevalent in grazing dairy cows. Until now, no field trials to address the effect of PEG treatment on diseases have been carried out under grazing conditions.

Due to the link between early lactation clinical disease and fertility and culling, it may be hypothesized that early lactation clinical disease prevention could be reflected in improved fertility and reduced culling. As mentioned, the evidence of PEG treatment effect on fertility and culling is very limited. Only modest beneficial effects were reported on fertility (Canning et al., 2017; Ruiz et al., 2017). Zinicola et al. (2018) and Van Schyndel et al. (2021) reported a lack of effect on fertility and culling, which would be in line with the lack of effect on disease prevention that they reported. Moreover, there are no reports on the effect of PEG on fertility or culling as measured during a full lactation. This is of relevance as fertility and especially culling are particularly important later in lactation (Ribeiro et al., 2016; Carvalho et al., 2019).

Finally, an important question to answer is whether the economic benefits of preventing clinical disease associated with the transition period in a number of cows, by treating all cows with PEG around parturition, outweigh the cost of PEG and its application under field conditions (two doses). As far as I know, no economic studies on the use of PEG have been carried out.

1.5. Aims of this thesis

In this thesis, we aim to address the effect of PEG on health, fertility and longevity, and on the economic results during a full lactation in grazing dairy cows. In addition, we explore the effect of PEG treatment interactions with parity and the metabolic status of the transition dairy cow. In this research, while working on four commercial Uruguayan grazing dairy farms, we developed a close and growing cooperation between the Quantitative Veterinary Epidemiology group and the Business Economics Group from Wageningen University, The Netherlands, and the Animal Endocrine and Metabolism Laboratory at the Veterinary Faculty of the Universidad de la República, Uruguay. This cooperation gave us the unique opportunity to combine the expertise of dairy scientists from various disciplines to develop and carry out an interdisciplinary project, which combined metabolism, immunology, epidemiology, statistics, fertility and economics. The basis for this project consisted of an extensive longitudinal data collection of a large number of dairy cows. The data from these cows were combined to build up a large data set, which allowed testing our hypotheses under grazing conditions.

Figure 1.3 presents an outline of the experimental design and the objective of each chapter of this thesis.

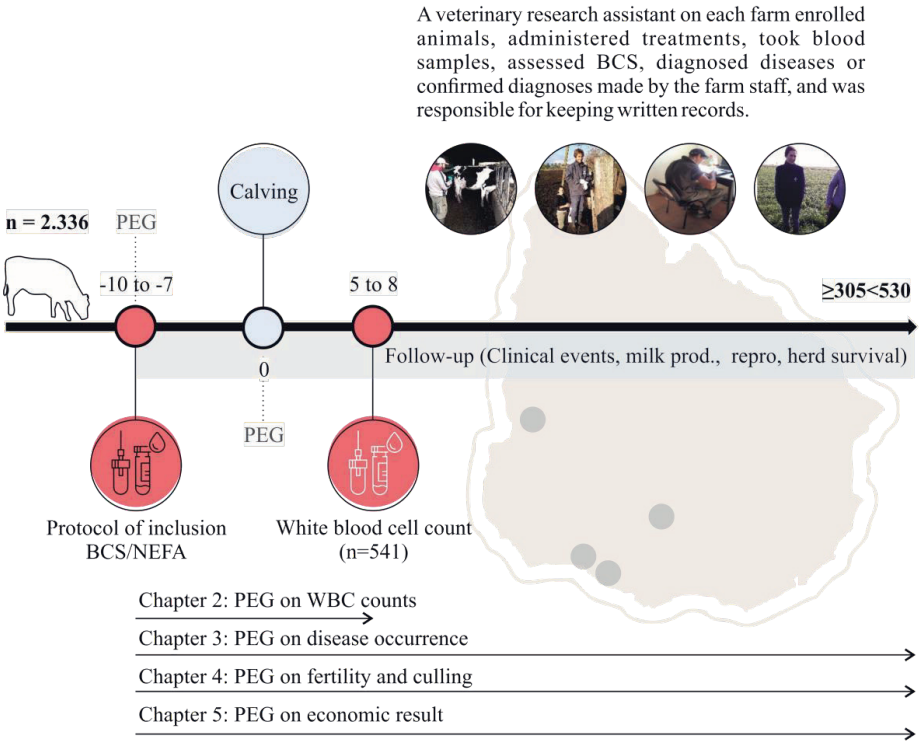

Figure 1.3. Outline of the experimental design and chapters' allocation.

In the current chapter, I have described the literature that led us to propose our experimental design. In chapter 2, as an approach to the understanding of the effect of PEG and its potential interactions with parity and the metabolic status of transition cows, we hypothesized that the response to PEG as measured by WBC counts may be associated with parity and the prepartum metabolic status of cows. Thus, we investigated whether PEG treatment affected postpartum WBC counts (5 to 8 DIM) and explored the effect of PEG treatment interactions with parity, prepartum BCS and prepartum NEFA concentration. In chapter 3, we hypothesized that PEG treatment may reduce disease occurrence in grazing dairy cows and that the response to treatment would be associated with parity and the prepartum metabolic status of cows. Thus, we investigated whether PEG treatment affected disease occurrence during a full lactation. We also explored the effect of PEG treatment interactions with parity, prepartum BCS, and prepartum NEFA on disease occurrence. In chapter 4, we hypothesized that PEG would increase fertility and decrease culling, and that the relationship between PEG treatment and fertility and

culling outcomes would interact with prepartum BCS and prepartum NEFA. Thus, we investigated whether PEG treatment affected fertility and culling during a full lactation in grazing dairy cows. We also explored the effect of PEG treatment interactions with parity, prepartum BCS, prepartum NEFA concentration and early lactation clinical disease on these outcomes. In chapter 5, we tested the hypothesis that PEG treatment may increase partial net return per cow as calculated from milk revenues and costs of feed, medical treatments, inseminations and culling during a full lactation, taking the interaction with prepartum BCS and prepartum NEFA concentration of cows into account. Thus, we investigated whether PEG treatment affected partial net return, milk revenues and costs for feed, medical treatments, insemination and culling during a full lactation. Finally, in chapter 6, we integrate the interpretation of results presented in each chapter, put in perspective the potential application of our findings under commercial farming conditions, and outline future research proposals.

CHAPTER 2

2

Increase in white blood cell counts by pegbovigrastim in primiparous and multiparous grazing dairy cows and the interaction with prepartum body condition score and nonesterified fatty acids concentration

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Abstract

The objective of this study was to determine if parity affected the effect of pegbovigrastim (PEG) treatment on white blood cell (WBC) counts in grazing dairy cows. Additionally, the association of prepartum body condition score (BCS) and nonesterified fatty acid (NEFA) concentration with WBC counts was investigated. The effect of early-lactation disease was included in the statistical analysis. A randomized controlled trial on four commercial grazing dairy farms was performed. Holstein primiparous (Control = 87, PEG = 89) and multiparous (Control = 181, PEG = 184) cows were randomly assigned to one of two treatments: first PEG dose 8 ± 5 (mean ± SD) days before the expected calving date and a second dose within 24 h after calving (PEG) compared to untreated controls (Control). Treatment effects were evaluated with mixed linear regression models. Treatment with PEG increased WBC, neutrophil, lymphocyte and monocyte counts at 6 ± 1 (mean ± SD) days in milk. Parity, BCS and their interactions with treatment were not associated with WBC counts. In control cows, prepartum NEFA concentration was associated with reduced WBC, neutrophil and lymphocyte counts and tended to be associated with reduced monocyte counts. Pegbovigrastim treatment reversed the negative association of prepartum NEFA concentration with neutrophil and monocyte counts and tended to reverse the negative association of prepartum NEFA concentration with WBC counts. In the PEG treated group, cows diagnosed with retained placenta or metritis showed lower neutrophil counts when compared to PEG treated cows without these clinical diseases. These data confirm that PEG treatment increases WBC, neutrophil, lymphocyte and monocyte counts in grazing dairy cows and that this effect is independent of parity. Pegbovigrastim treatment reversed the negative association of prepartum NEFA concentration with neutrophil and monocyte counts, and tended to reverse the negative association of prepartum NEFA concentration with WBC counts.

2.1. Introduction

Around 50% of dairy cows experience a metabolic or infectious disease or both during the first month of lactation. The risk for disease in early lactation has been associated with, among other causes, the negative energy balance (NEB) that takes place during the transition period (LeBlanc, 2010). Metabolites related to NEB such as nonesterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB), have been linked to immunosuppression and increased risk of infectious and clinical diseases (Hammon et al., 2006; Grinberg et al., 2008; Ingvartsen and Moyes, 2015). In early lactation, when NEB it is typically most profound (i.e. higher NEFA concentration), several studies have shown a decline in the neutrophil and lymphocyte counts as well as impaired function (i.e. reduced chemotaxis, phagocytosis and oxidative burst, and reduced proliferative capacity, respectively) (Kehrli and Goff, 1989; Kimura and Kehrli, 1999; Ingvartsen and Moyes, 2015). Although there are reports on the negative effect of NEB on neutrophil and lymphocyte function (Kimura and Kehrli, 1999; Hammon et al., 2006; Lacetera et al., 2005), there are few reports on the effect of NEB on the counts of these immune cells. It has been shown that cows with increased postpartum NEFA concentrations (> 0.5 mM) had decreased white blood cell (WBC) counts (Hachenberg et al., 2007). Conversely, in a controlled trial (McDougall et al., 2017), no significant effect of prepartum energy restriction on WBC count was found relative to non-restricted control cows. However, in this trial (McDougall et al., 2017), both groups presented a high frequency of cows with high (defined as > 0.4 m*M*) NEFA concentrations one week before calving (56% vs 85% non-restricted vs restricted, respectively). In vitro, it has been shown that NEFA concentrations markedly decreased the viability of neutrophils (Scalia et al., 2006). Over-conditioned cows mobilize more fat during the transition period (Roche et al., 2009) and this fat mobilization is associated with higher NEFA concentrations (Barletta et al., 2017). It has been suggested that under-conditioned cows tended to have decreased neutrophil counts (Roche et al., 2013).

Parity is an important factor determining health and metabolic events during transition to lactation. Compared with multiparous cows, primiparous cows before their first calving had a less pronounced NEB (in terms of NEFA concentration) in confined housing (Wathes et al., 2007). However, in pasture-based herds it has been reported that primiparous cows have a more pronounced NEB compared to multiparous animals (Meikle et al., 2004; Adrien et al., 2012). Parity also modifies the risk for disease:

Reinhardt et al. (2011), in a large number of US farms, reported that primiparous cows had a lower risk for hypocalcemia. Toni et al. (2015) reported that housed primiparous cows had a higher risk for metritis. In both confined and grazing primiparous cows, clinical mastitis (CM) incidence is typically higher during early lactation, particularly in the first week postpartum, while for the whole lactation it is lower than in multiparous cows (De Vliegher et al., 2012). Interestingly, an early study reported that older cows had impaired neutrophil function (Gilbert et al., 1993). In older cows, a decrease in lymphocyte types (γδT cell and B cells) has been shown, which might be associated with increased susceptibility to infection (Ohtsuka et al., 2009).

The use of tools to reduce disease incidence at the start of the lactation is of great interest. The treatment with a polyethylene glycolated form of recombinant bovine G-CSF (PEG, or Pegbovigrastim, marketed as Imrestor, Elanco Animal Health, Greenfield, IN) in periparturient dairy cows has been reported to be beneficial, as treatment increased the number of circulating WBC, neutrophil, lymphocyte and monocyte counts (Kimura et al., 2014; McDougall et al., 2017; Zinicola et al., 2018). It is currently not known whether parity affects the impact of PEG on WBC counts. Using gene expression data, it was suggested that PEG treatment improved migration, adhesion, and antimicrobial capacity and enhanced the inflammatory response regardless of parity (Lopreiato et al., 2020).

The response to PEG treatment maybe modulated by the metabolic status of the cow since metabolic markers (NEFA) affect the immune system. However, in a recent study, prepartum energy restriction did not affect the WBC count in response to PEG in comparison with controls (McDougall et al., 2017).

Thus, we hypothesized that parity affects the response to PEG as measured by WBC counts in grazing dairy cows, and that the response to treatment may be associated with prepartum BCS and NEFA concentration. Therefore, we investigated the effect of PEG treatment on postpartum (5 to 8 DIM) WBC counts in primiparous and multiparous grazing dairy cows. Additionally, the association of BCS and prepartum NEFA concentration with WBC counts was investigated. The effect of early-lactation disease was included in the statistical analysis.

2.2. Materials and methods

The experimental protocol (CEUAFVET-PI-162) was evaluated and approved by the Honorary Committee for Animal Experimentation in Uruguay (CHEA), University of Uruguay.

2.2.1. Experimental design

A subset of Holstein pregnant heifers (Primiparous, n = 194) and cows that were approaching their second or higher calving (Multiparous, n = 399) from a larger prospective controlled randomized trial was enrolled in this experiment. The trial was performed on 4 commercial grazing dairy farms in 3 different regions of Uruguay (San José, Florida, Rio Negro). Primiparous and multiparous cows on each farm were located in outdoor close-up paddocks around 3 weeks before the expected calving date (ECD) and remained in this paddock until calving. Both groups were followed for approximately 1 week postpartum (5 to 8 DIM), at which point blood sampling for WBC counts was performed.

Cows from farms 2, 3, and 4, were managed in separate groups based on parity, but under the same environmental conditions, including milking and feeding management. Farm 1 managed a single group after calving. Calving occurred from February 21st to July 24th of 2018. After calving, animals were kept on pasture and at least 40% of the dry matter intake (DMI) came directly from grazing. The diet was supplemented with a partial mixed ration. Exceptionally, when weather conditions did not allow grazing, cows were kept in outdoor paddocks. All cows were milked twice a day.

Figure 2.1 provides a diagram with the relevant time points and event measurements in the present study. Primiparous and multiparous cows in the close-up paddocks were assessed twice weekly. Cows that were between -10 to -7 days relative to the ECD were clinically examined to assess whether any of the exclusion criteria, i.e. clinical disease and/or fever (rectal temperature > 39.5°C) were present. When none of the exclusion criteria were met, BCS was assessed (Ferguson, 1994), blood samples were collected for NEFA determination and animals were assigned to one of two treatments. Cows with even national ear tag number were injected with PEG (Imrestor, Elanco Animal Health, Greenfield, IN) according to the product label (PEG) and animals with an odd national ear tag number remained as untreated controls (Control). Animals assigned to PEG

treatment received a second dose within 24 h after calving. Only cows that received both doses were included in the study. The included animals therefore represent the 'per protocol' inclusion rule.

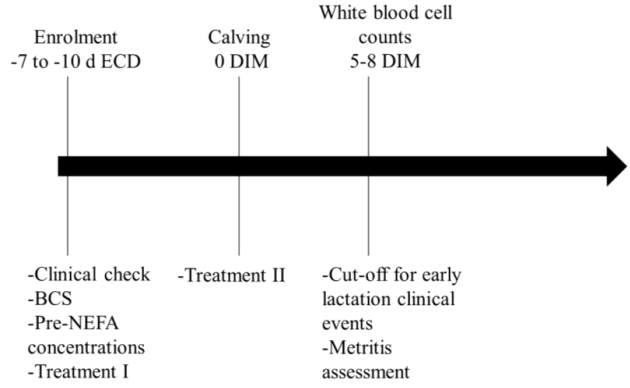

Figure 2.1. Diagram with time points and measurements taken. ECD: expected calving date, Pre-NEFA: prepartum NEFA concentration.

2.2.2. Clinical diagnoses and definitions

Clinical mastitis was diagnosed by trained farm personnel during forestripping at each milking and defined according to Pinzón-Sánchez and Ruegg (2011). Retained placenta (RP) was defined according to Ruiz et al. (2017). Puerperal metritis and clinical metritis were defined according to Sheldon et al. (2006). Fever was defined as a rectal temperature >39.5°C and >40.5°C during summer and when ambient temperature was higher than 30°C (Burfeind et al., 2012). In the present study, all CM cases, irrespective of severity, were defined as CM. Puerperal metritis and clinical metritis as defined according to Sheldon et al. (2006) were grouped and reported as metritis.

At the postpartum visit, at 5 to 8 DIM, all cows were carefully assessed to diagnose metritis. If metritis was diagnosed by the study personnel before this time point, it was also recorded and included in this disease category.

2.2.3. Nonesterified fatty acid and white blood cell count determination

Blood samples were collected from the coccygeal vessels (8.5-mL clot accelerator tubes, Becton Dickson, Franklin Lakes, NJ). Immediately, samples were centrifuged at 3000 x

g for 20 min and serum was stored frozen (-20°C) until further analysis. Serum was analyzed for NEFA concentration at the Animal Endocrine and Metabolism laboratory, Veterinary Faculty, Montevideo, Uruguay. Nonesterified fatty acid (NEFA) concentrations were measured by colorimetric assays on an A25 autoanalyzer (© Biosystems S.A., Barcelona, Spain) using commercial kits: Wako NEFA-HR (2), Wako Pure Chemical Industries Ltd., Osaka, Japan. The inter-assay coefficient of variation (CV) for commercial quality controls was less than 10%.

At approximately 1 week postpartum (5 to 8 DIM), a second blood sample was taken in K2- EDTA 6.5-mL tubes (Becton Dickson, Franklin Lakes, NJ) from the coccygeal vessels and sent immediately to the laboratory for total and differential WBC count. The total WBC count was determined using an automated haemocytometer (Sysmex XT1000, Roche Diagnostic, CA, USA) and cell morphology was assessed by microscopic examination of blood smears. The inter-assay CV for commercial quality controls was less than 5%.

2.2.4. Statistical analysis

Data were analyzed using the SAS software (SAS Institute Inc. 2018. SAS University Edition, Cary, North Carolina: SAS Institute Inc.). Descriptive statistics were performed using the t-test procedure (PROC TTEST) and chi-squared test (PROC FREQ) for continuous and discrete variables (i.e. occurrence of CM cases) respectively.

As the occurrence of inflammatory clinical diseases such as CM, RP, and metritis close to the time of measurement of WBC counts would likely impact these counts (Roland et al., 2014), we considered the occurrence of these diseases in the analysis of the WBC data. Clinical mastitis, RP, and metritis, occurring up until the time of blood sampling, were therefore included in the linear regression analyses. Frequencies of CM, RP, and metritis occurring up until the time of blood sampling (5 to 8 DIM) by treatment group were calculated using the frequency procedure (PROC FREQ). Values of NEFA and WBC counts were evaluated for normality and, where relevant, log10-transformed for inclusion in the statistical analyses.

Treatment effects on WBC counts were evaluated with mixed linear regression models (PROC MIXED). Fixed effects in the model included, as class variables, treatment

(Control/PEG), parity (primiparous/multiparous), BCS (under: < 3; proper: 3 to 3.5 and over: > 3.5; Roche et al., 2009) and calving month (February/March, April, May, June/July); Prepartum NEFA concentrations were log10-tranformed and included as a continuous variable. Two-way interactions with treatment and parity were checked for significance. Farm, as a class variable, was included as a random effect.

The initial statistical model looked like:

WBC count = intercept + treatment + parity + BCS + calving month + Log10 (prepartum NEFA) + two-way interactions + farm (random) + error

In a first model (Model 1) we considered only pre-treatment variables included in the modeling process (treatment, parity, BCS, calving month, prepartum NEFA, farm (random)). This model evaluates the full treatment effect of PEG, including the intermediate effect that treatment may have on reducing the incidence of clinical disease in early lactation.

In a second model (Model 2) the effect of disease occurrence on WBC count is addressed separately by introducing disease occurrence in the model. In this model, the treatment effect is now separated into a direct treatment effect and an indirect effect through disease occurrence (Gilbert et al., 1993). Clinical diseases (CM, RP or metritis) up until the time of blood sampling (5 to 8 DIM) and their two-way interactions with treatment were checked for significance in this second model.

After the initial full model lay-out, a backward stepwise selection process was performed. Given the design and objectives of this study, treatment and parity were always forced into the models. In the modeling process, only variables or interactions with a $P < 0.10$ remained in the model. Statistical tendency and statistical significance were decided at a $P < 0.10$ and a $P < 0.05$, respectively. The final model fit was evaluated using akaike's information criterion and the normality of the distribution of the final model residuals.

2.3. Results

2.3.1. Study population

A total of 593 animals was sampled of which 6 animals did not meet the per protocol inclusion rule: 5 animals in the PEG group were injected only once and 1 cow in the control group was injected erroneously. Ten animals (Control = 6 and PEG = 4) were excluded because of too early enrollment (≥ 47 and up to 113 days before calving), 7 cows (Control = 6 and PEG = 1) because of an excessive length of previous lactation (≥ 600 days in milk) and 1 PEG cow because of a very low daily milk production in the previous lactation (1.6 L/day). A total of 26 animals had no Prepartum NEFA determination (Control = 12, PEG = 14) and 2 Control cows had no WBC determination. Thus, 541 cows remained in the final analysis, representing the study population. The included cows according to parity and treatment were: 176 primiparous cows: Control = 87, PEG = 89 and 365 multiparous cows: Control = 181, PEG = 184.

In the study population, no differences ($P > 0.4$) where detected between treatment groups or between parity groups for the interval between enrollment and calving: mean and SD were 8 ± 5 days. Approximately 50% of the cows were enrolled within one week before calving, 43% within two weeks before calving, 5% within three weeks before calving and 2% more than three weeks before calving. Thus, 98% of the cows were enrolled within 21 days before calving.

Descriptive data of the previous lactation for the multiparous cows in the study population by treatment group are shown in Table 2.1. These descriptive data include parity, days open, previous milk production (kg/lactation), days in milk at dry off, daily milk production, somatic cell counts (log_{10}-transformed) at the last test day of the previous lactation, and occurrence of one or more clinical mastitis cases. No significant differences between treatment groups were found in any of these variables.

Table 2.1. Descriptive data from the previous lactation for multiparous cows. Control = 181; PEG = 184.

	Treatment (Mean ± SD)		
Item	**Control**	**PEG**	**P-value**
Lactation number at enrollment	2.1 ± 1.3	2.3 ± 1.3	0.19
Days open previous lactation	149 ± 102	143 ± 102	0.57
Previous lactation milk production*	7326 ± 2001	7374 ± 2202	0.83
Days in milk at dry-off	356 ± 87	347 ± 86	0.34
Daily milk production previous lactation**	21 ± 5	21 ± 5	0.36
Log_{10} SCC at dry-off	2.4 ± 2.3	2.4 ± 2.3	0.95
Occurrence of CM cases (%, n)***	41 (71/174)	40 (70/173)	0.95

* kg/lactation; ** kg/day; ***7 and 11 Control and PEG cows respectively have no previous data of CM.

No difference between treatment groups was found for BCS at enrollment (Control = 3.3 ± 0.4, PEG = 3.4 ± 0.4, $P = 0.17$). However, BCS was related to parity at enrollment (primiparous = 3.6 ± 0.3, multiparous = 3.3 ± 0.4, $P < 0.01$). After categorization by prepartum BCS, the number of animals by treatment group were: under-conditioned: Control = 34, PEG = 28, proper-conditioned: Control = 162, PEG = 172 and over-conditioned: Control = 72, PEG = 73.

No difference between treatment groups was found for prepartum NEFA concentration (Control = 0.5 ± 0.4, PEG = 0.5 ± 0.4, $P = 0.22$), but primiparous cows had higher prepartum NEFA concentrations than multiparous cows (0.6 ± 0.5 vs 0.5 ± 0.4 m*M*, $P < 0.01$).

In early lactation, up until the time of blood sampling, clinical disease occurrence was: CM: Control = 16, PEG = 15; RP: Control = 17, PEG =20; metritis: Control = 42, PEG = 53.

2.3.2. Effect of pegbovigrastim on white blood cell counts at 6 ± 1 days in milk in primiparous and multiparous cows

Overall, differences in least squares means (Tukey-Kramer adjustment) showed that treatment with PEG increased WBC count (Control = 12.2 ± 0.9, PEG = 21.7 ± 0.9 $x10^3/\mu L$; $P < 0.001$), neutrophil count (Control = 5.7 ± 0.6, PEG = 13.6 ± 0.6 $x10^3/\mu L$; $P < 0.001$), lymphocyte count (Control = 5.5 ± 0.3, PEG = 6.8 ± 0.3$x10^3/\mu L$; $P < 0.001$) and monocyte count (Control = 0.41 ± 0.05, PEG = 0.68 ± 0.05$x10^3/\mu L$; $P < 0.001$) at 6 ± 1 (mean ± SD) days in milk.

Table 2.2 shows the solutions for the final regression models. No parity effects were detected on WBC, neutrophil or monocyte counts. Primiparous cows tended to show lower lymphocyte counts ($P = 0.08$, Model 1), but this effect did not remain significant when early lactation disease was included in the analyses (Model 2). No BCS effects were detected for any of the cell types and there was no interaction of BCS with treatment.

Table 2.2. Solutions for the final regression models for white blood cell, neutrophil, lymphocyte and monocyte counts. Model 1 includes treatment and parity, model 2 also includes the effect of clinical disease on white blood cell counts.

		Model 1			Model 2		
Cell type	**Explanatory variable**	**Estimate**	**SE**	***P***	**Estimate**	**SE**	***P***
WBC	Intercept	8.8	1.3	0.007	8.4	1.2	0.006
	Treatment	14.2	1.4	<0.001	15.6	1.4	<0.001
	Parity	-0.9	0.7	0.21	-0.8	0.7	0.24
	Prepartum NEFA	-3.4	1.5	0.02	-4.0	1.4	0.005
	Calving month			0.02			0.01
	Prepartum NEFA x Trt	3.4	1.8	0.07	3.4	1.8	0.06
	Calving month x Trt			<0.001			<0.001
	Metritis				-0.2	1.3	0.89
	Metritis x Trt				-4.8	1.8	0.008
Neutrophils	Intercept	3.4	0.9	0.03	3.1	0.8	0.03
	Treatment	11.8	1.0	<0.001	12.8	1.0	<0.001
	Parity	-0.1	0.5	0.84	-0.09	0.5	0.86
	Prepartum NEFA	-1.8	1.0	0.08	-2.3	0.9	0.02
	Calving month			<0.001			<0.001
	Prepartum NEFA x Trt	3.0	1.3	0.03	3.0	1.3	0.02
	Calving month x Trt			<0.001			<0.001
	RP				-0.7	1.4	0.96
	Metritis				-0.05	1.0	0.96
	RP x Trt				-3.9	2.0	0.04
	Metritis x Trt				-2.7	1.3	0.04
Lymphocyte	Intercept	4.7	0.5	0.002	4.6	0.5	0.002
	Treatment	1.3	0.4	<0.001	1.6	0.4	<0.001
	Parity	-0.7	0.4	0.08	-0.6	0.4	0.11
	Prepartum NEFA	-2.0	0.5	<0.001	-2.0	0.5	<0.001
	Calving month			<0.001			<0.001
	Metritis				0.2	0.7	0.74
	Metritis x Trt				-2.0	1.0	0.03
Monocytes	Intercept	0.24	0.07	0.005	0.20	0.07	0.07
	Treatment	0.5	0.08	<0.001	0.40	0.08	<0.001
	Parity	-0.004	0.04	0.94	0.001	0.04	0.98
	Prepartum NEFA	-0.12	0.08	0.16	-0.20	0.08	0.08
	Calving month			0.009			0.005
	Prepartum NEFA x Trt	0.3	0.1	0.02	0.2	0.1	0.02
	Calving month x Trt			0.04			0.04
	Metritis				-0.04	0.08	0.63
	CM				0.2	0.1	0.04
	Metritis x Trt				-0.2	0.1	0.09

Primiparous: Control = 87, PEG = 89; multiparous: Control = 181, PEG = 184.
Model 2: Includes early lactation disease occurrence, up until the time of blood sampling: CM: Control = 16, PEG = 15; RP: Control = 17, PEG = 20; metritis: Control = 42, PEG = 53.

Reference groups: Month 3, Control group and multiparous cows. *White blood cells. Prepartum NEFA: prepartum NEFA concentrations, CM: clinical mastitis, RP: retained placenta, Trt: treatment.

As table 2.2 shows, there was an association of prepartum NEFA concentration with decreased WBC counts ($P = 0.02$ and $P = 0.005$, Model 1 and 2, respectively). Prepartum NEFA concentration tended to be associated with decreased neutrophil counts ($P = 0.08$, Model 1), which became significant when disease occurrence was included in the model ($P = 0.02$, Model 2). Moreover, prepartum NEFA concentration was associated with decreased lymphocyte counts ($P < 0.001$, Model 1 and 2) and tended to be associated with decreased monocyte counts ($P = 0.08$, Model 2).

Prepartum NEFA concentration tended to interact with treatment for WBC counts ($P = 0.07$ and $P = 0.06$, Model 1 and 2, respectively), and significantly interacted with treatment for neutrophil ($P = 0.03$ and $P = 0.02$, Model 1 and 2, respectively) and monocyte counts ($P = 0.02$, Model 1 and 2). Figure 2.2 presents observed values and prediction lines (model 2) for neutrophil counts by log10-transformed prepartum NEFA concentrations in control and PEG cows.

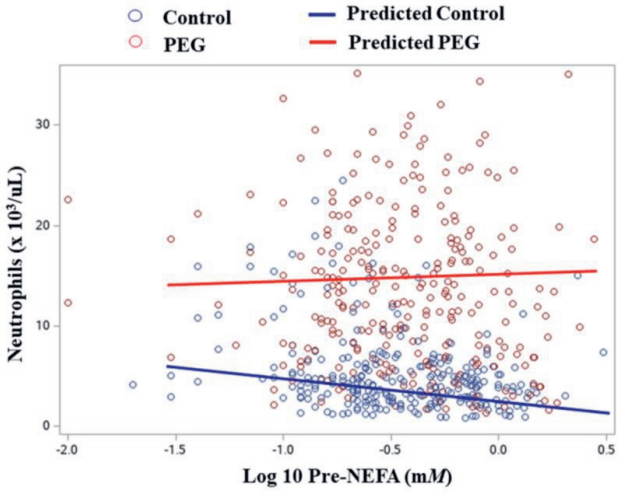

Figure 2.2. Observed values and prediction lines for neutrophil x $10^3/\mu L$ by log10-transformed Pre-NEFA (prepartum NEFA concentrations; m*M*). Control cows = 268; PEG cows = 273.

Model 2, that included the modifying effect of disease on cell counts (Table 2.2), showed that CM occurrence was associated with increased monocyte counts (P = 0.04). A treatment by RP interaction was observed for neutrophil counts (P = 0.04); in PEG treated cows, RP occurrence was associated with decreased neutrophil counts compared to PEG treated cows without RP. Similarly, a treatment by metritis interaction was shown for WBC, neutrophil and lymphocyte counts (P = 0.008, P = 0.04 and P = 0.03, respectively) and a tendency for this interaction was shown for monocyte counts (P = 0.09). In PEG treated cows, metritis occurrence was associated with decreased WBC, neutrophil and lymphocyte counts and tended to be associated with decreased monocyte counts compared to PEG treated cows without metritis.

Figure 2.3 shows least square means of neutrophil count differences (Tukey-Kramer adjustment) for RP (Panel A), and metritis (Panel B) in Control and PEG cows. In the PEG treated group, cows with RP and metritis showed lower neutrophil counts than PEG treated cows without these clinical diseases (P = 0.006 and P = 0.005, respectively).

Calving month showed an interaction with PEG treatment for WBC, neutrophil and monocyte count (Table 2.2).

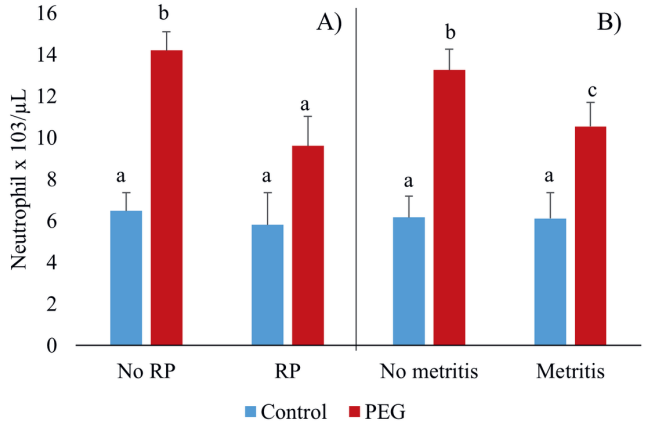

Fig 2.3. Blood neutrophil counts/µL at 6 ± 1 (mean ± SD) days in milk in cows diagnosed with: retained placenta (RP): Control = 17, PEG = 20, No RP: Control = 251, PEG = 253 (Panel A); metritis: Control = 42, PEG = 53, No metritis: Control = 226, PEG = 220 (Panel B). Different letters, P < 0.05.

2.4. Discussion

In this randomized controlled trial on four commercial grazing dairy farms, we tested the hypothesis that parity affects the WBC counts in response to PEG. Additionally, the association of prepartum BCS and prepartum NEFA concentration with WBC counts in control and PEG cows was investigated. Our data confirmed that PEG treatment increased WBC, neutrophil, lymphocyte and monocyte counts in grazing dairy cows and that this effect was independent of parity.

In the Control group, an increased prepartum NEFA concentration was associated with reduced WBC, neutrophil and lymphocyte counts and tended to be associated with reduced monocyte counts. Treatment with PEG increased neutrophil and monocyte counts independent of prepartum NEFA concentration. The effect of treatment on WBC counts also tended to be independent of prepartum NEFA concentration, thereby reverting the negative association of prepartum NEFA concentration with neutrophil and monocyte counts and tending to revert the negative association of prepartum NEFA with WBC counts.

A limitation of this study is that WBC counts were not measured before treatment. Nevertheless, as this was a controlled clinical trial with a large number of animals and a robust randomization as shown by the balance between treatment groups, it is unlikely that significant pre-treatment differences in WBC counts could bias the study results.

The observation that treatment with PEG resulted in higher WBC, neutrophil, lymphocyte and monocyte counts at 6 ± 1 (mean ± SD) days in milk was expected (McDougall et al., 2017; Zinicola et al., 2018). Although a lack of PEG effect on monocyte counts has previously been reported (Van Schyndel et al., 2018), our work is, on the whole, consistent with these previous studies (McDougall et al., 2017; Zinicola et al., 2018). The magnitude of the increase in neutrophil counts (2.5-fold) is similar to previous reports on commercial farms (Canning et al., 2017; Zinicola et al., 2018), but lower than reports from studies under more experimental conditions (6-fold change; McDougall et al., 2017). This could be due to variations in age, diet, body condition, disease occurrence or other variables (Roland et al., 2014) that may play a role under commercial conditions. Besides, calving month in our study had an effect on all cell type counts and interacted with treatment, which may be related to the importance of environmental and management conditions in grazing systems (Chilibroste, 2005); e.g., cows in late

gestation under overstocked conditions suffered stress as well as changes in energy metabolism compared to animals that were not overstocked (Huzzey et al., 2012).

The increase in WBC counts caused by PEG treatment was not affected by parity. Both groups were exposed to virtually the same environmental and management conditions. Our data is consistent with previous studies (Lopreiato et al., 2020) that suggested that PEG improves migration, adhesion and antimicrobial capacity and enhances the inflammatory response regardless of parity. Neither was the increase in WBC counts by PEG treatment affected by BCS. In this study BCS was measured only at enrollment. In other reports, BCS loss during transition has been associated with an increased NEFA concentration (Barletta et al., 2017; Sheehy et al., 2017).

Disease occurrence affected only monocyte counts; CM occurrence was associated with increased counts. Interestingly, when disease occurrence was included in the model the negative association between Prepartum NEFA concentrations with WBC, neutrophil and monocyte counts became more evident. When controlling for disease impact, Prepartum NEFA concentrations showed a profound negative effect on early lactation WBC counts. The difference between the two models would indicate that animals showing clinical signs of disease would have both higher NEFA and WBC values. Correcting for disease occurrence in the statistical modeling will then make the negative relationship between prepartum NEFA and WBC more visible.

Overall, our data is consistent with previous reports on the association between increased NEFA concentrations and the decline in WBC counts (Hachenberg et al., 2007). Neutropenia has been reported during the first and second week in lactation (Kehrli and Goff, 1989; Kimura et al., 2014), a period of time that has also been associated with low blood glucose and increased NEFA concentrations in modern high-producing dairy cows (Grummer, 1995). Glucose is required by neutrophils for proliferation, survival and differentiation (Ingvartsen and Moyes, 2015). Moreover, it has been reported that NEFA concentrations decreased the viability of neutrophils and increased necrosis of these cells (Scalia et al., 2006). It has also been reported that blood neutrophils shortly after parturition are more prone to apoptosis (Van Oostveldt et al., 2001).

This is the first study reporting the interaction of prepartum NEFA concentrations and PEG treatment with neutrophils and monocytes: PEG treatment reversed the decline of

neutrophil and monocyte counts in early lactation associated with high prepartum NEFA concentrations. Model results as shown in table 2 indicate that, with increasing prepartum NEFA concentrations, WBC counts decrease in control cows. However, this negative relationship between prepartum NEFA and WBC counts was not observed in PEG treated cows and there was even a slight increase in neutrophil counts. It was previously (McDougall et al., 2017) reported that the treatment effect of PEG was not affected by prepartum energy restriction, which is in accordance with our results. However, McDougall et al. (2017) did not observe a negative effect of prepartum NEFA on WBC counts in untreated control cows, which contrasts with our observations. In their trial (McDougall et al., 2017), a high percentage of cows had high prepartum NEFA concentrations in both the feed-restricted (85%) and the control group (56%), thereby making it more difficult to evaluate the relationship between high prepartum NEFA and PEG treatment.

Pegbovigrastim sharply increased the blood neutrophil counts in 24 hours due to the release from the bone marrow pools (Van Schyndel et al., 2018), and reportedly also increased the expression of genes related with cell survival (Heiser et al., 2018). Taking into account these results, we hypothesize that PEG treatment in cows with more pronounced NEB and decreased WBC counts would cause a restoration of the WBC count to levels observed in cows with better energy balance and thus, PEG treatment would have a stronger preventive effect against disease in high prepartum NEFA cows than in cows with normal values. Further studies relating the effect of PEG treatment with disease occurrence during transition and its association with metabolism are warranted.

Pegbovigrastim reversed the negative association of prepartum NEFA concentration with neutrophil counts and monocyte counts, and, likely as a consequence, tended to prevent the negative association of prepartum NEFA with WBC counts. However, this was not observed for lymphocyte counts. Among other differences, neutrophils and monocytes are both derived from myeloblasts while lymphocytes derive from lymphoid progenitors (Van Schyndel et al., 2018). It may be hypothesized that PEG could have different immune restoration mechanisms according to the cell type.

In PEG treated cows, an association of neutrophil count with clinical disease was detected. In the PEG group, animals diagnosed with RP or metritis showed lower neutrophil counts compared to treated cows without these clinical diseases, while this

reduction was not observed in PEG treated CM cows. However, Zinicola et al. (2018) reported that PEG treated cows diagnosed with both CM and metritis had lower WBC counts than PEG treated cows without the clinical event. These authors also reported that PEG treated metritis cows had higher neutrophil counts in the vagina than control cows with metritis. All these observations are consistent with the hypothesis of Ruiz et al. (2018) that PEG elicits a more robust (or longer lasting) intra-uterine migration of neutrophils.

2.5. Conclusions

Our data confirm that PEG treatment increases WBC, neutrophil, lymphocyte and monocyte counts in grazing dairy cows and that this effect is independent of parity. In control cows, prepartum NEFA concentration was associated with reduced WBC, neutrophil and lymphocyte counts and tended to be associated with reduced monocyte counts. In this study it was shown that PEG treatment reverted the negative association of prepartum NEFA concentration with neutrophil counts and monocyte counts, and tended to revert the negative association of prepartum NEFA concentration with total WBC count.

2.6. Acknowledgments

The cooperation of farmers and farm personnel is gratefully acknowledged.

CHAPTER 3

3

Effect of pegbovigrastim on clinical mastitis and uterine disease during a full lactation in grazing dairy cows

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Abstract

In this randomized controlled trial on four commercial grazing dairy farms, we investigated whether pegbovigrastim (PEG) treatment affects clinical mastitis (CM) and uterine disease (i.e. retained placenta (RP), metritis and endometritis) occurrence during a full lactation. The association of prepartum body condition score and prepartum nonesterified fatty acid (NEFA) concentration with disease occurrence was also evaluated. Holstein cows were randomly assigned to one of two treatments: first PEG dose approximately 7 d before the expected calving date and a second dose within 24 h after calving (PEG) compared to untreated controls (Control). In total, 2,153 animals were included in the study: 733 primiparous cows (Control = 391, PEG = 342) and 1420 multiparous cows (Control = 723, PEG = 697). Treatment effects were evaluated with generalized linear mixed models and Cox's proportional hazard models. Treatment with PEG reduced the occurrence of a first case of CM during the first 30 days in milk (DIM) by 24.6% and reduced the hazard of a first case and the rate of total cases of CM during the full lactation. All PEG treatment effects were independent of parity. Prepartum body condition score interacted with PEG treatment: in over-conditioned cows, PEG reduced the occurrence of a first case of CM during the first 30 DIM by 49.5%. The hazard analysis of a first case of CM during the full lactation suggested that the preventive effect of PEG disappeared with increasing DIM. Treatment with PEG did not affect the occurrence of RP or metritis. Pegbovigrastim treated cows with metritis subsequently showed a reduced occurrence of endometritis compared to control cows with metritis. Pegbovigrastim reduces the occurrence of CM particularly in cows at risk of elevated lipid mobilization, and PEG ameliorates the uterine healing process in cows that experienced metritis.

3.1. Introduction

The high prevalence of metabolic and infectious diseases that dairy cows experience during the first month of lactation is a main concern to the dairy industry. The risk for disease in early lactation has been associated, among other causes, with the negative energy balance (NEB) that takes place during the transition period (LeBlanc, 2010). Indeed, increased concentrations of nonesterified fatty acids (NEFA), related to the NEB, have been linked to immunosuppression and increased risk of mastitis, retained placenta (RP) and metritis (LeBlanc et al., 2004; Melendez et al., 2009; Galvão et al., 2010; Ospina et al., 2013; Ingvartsen and Moyes, 2015). The rate and extent of energy mobilization from fat tissues has been linked to an increased risk of periparturient metabolic and clinical disorders during the transition period (LeBlanc et al., 2004; Roche et al., 2009). Roche et al. (2009) reported that over-conditioned cows (BCS > 3.5) - that mobilize more fat reserves than cows with a moderate BCS (3 – 3.5) - had more mastitis, and suggested that high BCS is related to an impaired energy and lipid metabolism that may affect the immune response in these cows. Although the relationship between thin cows and the risk of periparturient metabolic disorders is less consistent (Roche et al., 2009), cows with a low BCS (BCS < 3) had more mastitis relative to cows with a moderate BCS, and low BCS has also been reported as a risk factor for uterine disease (Roche et al., 2009; Loker et al., 2012).

One of the latest developments in preventive tools is the use of a long-acting analogue of bovine granulocyte colony-stimulating factor (PEG, Pegbovigrastim, marketed as Imrestor® by Elanco Animal Health, Greenfield, IN). Pegbovigrastim increases circulating white blood cell (WBC) and neutrophil counts and myeloperoxidase exocytosis (Kimura et al., 2014; McDougall et al., 2017). Recently, we found that prepartum NEFA concentrations were associated with reduced neutrophil counts, and that PEG treatment reversed this negative association (Barca et al., 2021a). It has been reported that treatment with PEG reduced the occurrence of clinical mastitis (CM) during the first 30 days in milk (DIM). However, the percentage reduction in CM varied substantially, ranging from 23 % to 50 % (Canning et al., 2017; Ruiz et al., 2017). A more recent study found no effect on the occurrence of CM during the first 30 DIM (Zinicola et al., 2018). Evidence for the use of PEG to reduce metritis occurrence has also been inconsistent: Ruiz et al. (2017) reported an increase, Zinicola et al. (2018) no effect and Freick et al. (2018) a decrease.

Parity is a major factor that impacts the metabolic adaptation to lactation, as in primiparous cows the requirements for growth limit nutrient partitioning into milk. While NEFA, as a proxy of lipid mobilization, were lower in primiparous vs multiparous cows in confined systems (Rupretcher et al., 2018), a more pronounced NEB was observed in primiparous cows under grazing conditions (Meikle et al., 2004; Adrien et al., 2012). Under confined feeding conditions, the quantity and quality of nutrients for dairy cows can be controlled; however, when pasture is the main component of the diet, nutrient intake estimation is more complex (Chilibroste et al., 2012). Typically, primiparous cows have a higher risk of early lactation CM and metritis, although multiparous cows have more milk fever and CM throughout the full lactation (Reinhardt et al., 2011; De Vliegher et al., 2012; Toni et al., 2015). To our knowledge, the impact of PEG on disease occurrence by parity in grazing herds has not yet been reported.

The hypothesis for this study was that PEG treatment reduce disease occurrence in grazing dairy cows and that the response to treatment would be associated with parity, prepartum BCS and/or prepartum NEFA. Thus, we investigated whether PEG treatment affected disease occurrence during a full lactation in primiparous and multiparous grazing dairy cows, and evaluated the association of prepartum BCS and prepartum NEFA concentration with disease occurrence.

3.2. Materials and methods

The experimental protocol (CEUAFVET-PI-162) was evaluated and approved by the Honorary Committee for Animal Experimentation in Uruguay (CHEA), University of Uruguay.

3.2.1. Study design

Holstein primiparous and multiparous cows (n = 2,333) from 4 commercial grazing dairy farms in 3 different regions of Uruguay (San José, Florida, Rio Negro) were included in this prospective randomized controlled trial. Farms were selected for convenience; inclusion was based on excellent record keeping, a willingness to participate in the study and the ability to implement the research protocol. Farms 1, 3 and 4 had a seasonal calving system, with calving concentrated in autumn. These herds had a milking herd size

of approximately 1,000, 850 and 600 cows, respectively. Farm 2 had a continuous year-round calving system and a milking herd size of approximately 600 cows. Throughout this manuscript, primiparous animals are cows that were enrolled in the study shortly before their first calving. Multiparous animals are cows that were enrolled shortly before their second or higher calving. The follow-up period for all cows with regard to clinical events was a full lactation (305 DIM). Recorded reasons for exit from the study were: dry-off, culling, and death. In animals for which none of these exit reasons were recorded, 305 DIM was considered the (censored) end point of the study.

All farms used blanket antibiotic dry cow therapy, antibiotics to treat CM cases and antiseptic post-milking teat dips. The cows from all 4 farms were moved to outdoor close-up paddocks around 3 weeks before the expected calving date. Calving occurred in the same area or in a subdivision of the same paddock under the same conditions. Calving for cows enrolled in the study occurred between February 13th and September 30th of 2018. After calving, cows were kept on pasture at least one of the periods between the two daily milkings and at least 40 % of the dry matter intake came directly from the grazing sessions, supplemented with a partial mixed ration.

Three veterinary technicians were hired and trained as research assistants for this experiment. Technicians enrolled animals, administered experimental treatments, took blood samples, assessed BCS, diagnosed diseases or confirmed diagnoses made by the farm staff, and were responsible for keeping written records. Two farms had a full time veterinary technician per farm, while two farms shared one technician. On the latter two farms the technician was supported on a daily basis by the first author.

The time of enrollment in the study was between -10 to -7 days relative to the expected calving date (ECD). Animals that had fever (rectal temperature > 39.5°C) or any other clinical health disorder at the time of enrollment were excluded from the study. Animals that met the inclusion criteria were assigned to one of two treatments based on their national ear tag number. The national ear tag numbers are available from computer records but are independent of the large and easily visible ear tag number (cow ID) that is used for farm management. Animals with an even national ear tag number (randomization was carried out by a single flip of a coin before the start of the study) were injected with PEG (Imrestor®, Elanco Animal Health, Greenfield, IN) according to the product label (PEG). Briefly, periparturient dairy cows received a subcutaneous injection

of 15 mg of PEG approximately 7 d before their ECD and within 24 h of calving. Animals with an odd national ear tag number remained as untreated controls (Control). No placebo was used, as treatment allocation based on the national ear tag number provided sufficient blinding. Close-up paddocks were observed twice a week. Cows that were between -10 to -7 days relative to the expected calving date or were exhibiting clinical signs of calving such as swelling of the vulva and filling of the udder were clinically examined. Cows that met any of the exclusion criteria (i.e. fever or any other clinical disease) were excluded, while all other cows were included in the study. Only cows that received the two doses were included in the analyses. Blood samples were obtained from Control and PEG cows at -10 to -7 days relative to the expected calving date and within 24 h after calving (this second sample was taken for other purposes beyond the objectives of this study). The included animals therefore represent the 'per protocol' inclusion rule (Sargeant et al., 2010). Farm personnel (including milkers) and laboratory personnel were blinded to treatment status. Research technicians applied treatments and would therefore be aware of the treatment status. However, all animal observations, samplings and disease diagnoses were based on the visible on-farm cow ID number that was unrelated to the national ear tag number that was used for randomization.

Our objective was to enroll 2,400 animals. This number was based on a power calculation assuming a CM incidence during the first 30 DIM of 15 % and 11.25 % for Control and PEG treated animals respectively. Canning et al. (2017) reported a reduction in the incidence of CM of 35 %, and a field trial conducted for authorization of use of PEG in the European Union (EMA) showed a reduction in the incidence of CM of 26 %. We assumed an efficacy of 25 % to be on the safe side. Power calculations ($\alpha = 0.05$, $\beta = 0.20$) then showed that a sample size of 1,200 cows per treatment group was needed.

At day -10 to -7 from the expected calving date (enrollment), prepartum BCS was assessed and recorded by the veterinary technicians. At the same time, blood samples were collected from the coccygeal vessels (8.5-mL clot accelerator tubes, Becton Dickson, Franklin Lakes, NJ). Immediately, blood samples were centrifuged at 3,000 x g for 20 min and serum was stored frozen (-20°C) until further analysis for NEFA concentration.

3.2.2. Clinical diagnoses and definitions

Each veterinary technician was trained prior to the start of the study to assess BCS (1 to 5 score; Ferguson et al., 1994) and to diagnose clinical events including CM, RP, metritis, clinical endometritis, metabolic disorders (milk fever and ketosis) and lameness (see definitions below). All farm personnel were also trained in the recognition of these disorders and all diagnoses were ultimately confirmed by the trial technicians. At two post-partum visits, the first at 5 to 8 and the second at 27 to 30 d of lactation, all cows were carefully examined by the veterinary technician to diagnose metritis and endometritis respectively. The diagnosis was made by vaginal examination using a clean palpation glove. If metritis and clinical endometritis were diagnosed by the farm personnel at a different time point, this was also recorded and included into the disease categories described hereunder.

Clinical mastitis was diagnosed by trained farm personnel during forestripping prior to each milking. Clinical mastitis was scored according to Pinzón-Sánchez and Ruegg (2011) as mild (abnormal milk without other symptoms), moderate (abnormal milk and local symptoms in the udder), or severe (abnormal milk, local symptoms and also signs of systemic illness). Farm staff were instructed to record all treated cases of CM, irrespective of the time since a previous case; a single event per treatment protocol was recorded, irrespective of the number of affected quarters. All CM cases, irrespective of severity, were reported as CM. Retained placenta was defined as fetal membranes (placenta) still visibly hanging from the cow´s vulva 24 h after calving (Ruiz et al., 2017). Puerperal metritis was diagnosed if an animal showed a fetid watery red-brown uterine discharge, associated with signs of systemic illness (such as decreased milk yield, dullness or other signs of toxaemia) and fever (rectal temperature > 39.5°C or > 40.5°C during summer and when ambient temperature was higher than 30°C; Burfeind et al., 2006), within 21 days post-partum. Clinical metritis was defined as a purulent uterine discharge detectable in the vagina in the first 21 days after calving without systemic illness. Puerperal metritis and clinical metritis were combined and reported as metritis. Clinical endometritis was defined as the presence of purulent uterine discharge visible in the vagina 21 days or more post-partum, or mucopurulent discharge visible in the vagina more than 26 days post-partum. Manual vaginal examinations were performed using clean palpation gloves. All these uterine diseases were defined according to Sheldon et al. (2006). Milk fever was defined as either a standing animal showing mild ataxia,

excitability, muscle tremors and reduced ruminal motility or a recumbent cow (Kelton et al., 1998; Oetzel, 2011). Clinical ketosis was defined according to Kelton et al. (1998) as an animal with decreased appetite in the absence of another concurrent disease. Lameness was defined as animals with clinical signs of abnormal locomotion (Sprecher et al., 1997).

3.2.3. Nonesterified fatty acids determination

Nonesterified fatty acid concentrations were determined at the Animal Endocrine and Metabolism Laboratory, Veterinary Faculty, Montevideo, Uruguay. Colorimetric assays were performed on an A25 autoanalyzer (© Biosystems S.A., Barcelona, Spain) using commercial kits: Wako NEFA-HR (2), Wako Pure Chemical Industries Ltd., Osaka, Japan. The inter-assay coefficient of variation (CV) for commercial quality controls was less than 10%.

3.2.4. Statistical analysis

Data were analyzed using SAS software (SAS Institute Inc. 2018. SAS® University Edition, Cary, North Carolina: SAS Institute Inc.).

Descriptive statistics were performed using the t-test procedure (PROC TTEST) and chi-squared tests for continuous and categorical variables respectively. Categorical variables included last test day SCC of the previous lactation (low ≤ 200,000 cell/mL, high > 200,000 cell/mL), recorded as SCC at dry off, and occurrence of CM cases in the previous lactation (yes/no). The frequency procedure (PROC FREQ) was used to group cows by Prepartum BCS [under: < 3; acceptable: 3 to 3.5, and over: > 3.5; (Roche et al., 2009)] and Prepartum NEFAcategories (low ≤ 0.5 m*M,* high > 0.5 m*M*) by treatment group.

Logistic regressions to analyze occurrence of a first case of CM during the first 30 DIM and clinical uterine diseases (i.e. RP, metritis, and endometritis) were performed using the generalized linear mixed model procedure (PROC GLIMMIX). Fixed effects in the model included as class variables were: treatment (Control/PEG), parity (primiparous/multiparous), Prepartum BCS (under: < 3; acceptable: 3 to 3.5 and over: > 3.5), Prepartum NEFAcategory (low ≤ 0.5 m*M,* high > 0.5 m*M*) and calving month (1 to 6: February/March, April, May, June, July and August/September, respectively). Two-

way interactions with treatment were checked for significance. Farm, as a class variable, was included as a random effect. In the CM model, SCC at dry off was included as a class variable (low ≤ 200,000 cell/mL, high > 200,000 cell/mL, primiparous cows were coded as low) and the interaction with treatment was checked. In the metritis model, RP occurrence was included as a class variable. In the endometritis model, both RP and metritis were included as class variables. All class variables were coded in the class statement in the models.

The initial statistical model looked like:

Logit (disease) = intercept + parity + treatment + calving month + Prepartum BCS + Prepartum NEFA+ SCC at dry off (for CM) + RP (for metritis and endometritis) + metritis (for endometritis) + interactions + farm (random) + error.

After an initial full model lay-out, a backward stepwise selection process was performed.

Solutions for fixed effects are presented as comparisons with the reference groups specified in each table of results, and where relevant, estimated least squares means are presented. The overall treatment effect (type III tests of fixed effects) and estimated least squares means differences between Control and PEG cows are also presented.

A Cox's proportional hazard model was performed (PROC PHREG) to analyze the hazard of a first case of CM during a full lactation (305 DIM, right censored). This model included the same variables as the logistic regression described above. Additionally, a variable called Days-block (DIM blocked in 60 day intervals) was included, and the interaction with treatment was checked to assess a possible differential effect of treatment during the course of the lactation. A forward stepwise selection process was performed.

A Poisson regression model was performed to evaluate the effect of PEG on the total number of CM cases during the full lactation (PROC GLIMMIX). All cases of CM at cow level were included in the analyses, including those occurring within 14 days from a previous case as these early repeat cases did require antibiotic treatment and resulted in milk withhold. In the Poisson model, the number of CM cases during the full lactation was the outcome variable and the log number of days at risk was included as an offset. This model included the same variables as described above, with farm as a random effect. A forward stepwise selection process was performed.

In all analyses, treatment and parity were always forced into the models. All other variables or their two-way interaction with treatment with a $P \leq 0.10$ remained in the model during the variable selection process. Exceptionally, variables or their interaction term with treatment with a $P \leq 0.15$ remained in the model, but only when removal of the variable resulted in a considerable (> 20 %) change in the estimate of treatment. Such variables would be considered potential confounders. Statistical tendency and significance were set at $P \leq 0.10$ and $P \leq 0.05$ respectively.

3.3. Results

3.3.1. Study population

A total of 2,333 cows were initially enrolled, of which 116 did not meet the per protocol inclusion rule: 89 cows (Control = 30, PEG = 34 and no treatment data = 25) never calved and the farm veterinarian eventually diagnosed these cows as not pregnant; 5 cows (Control = 3 and PEG = 2) were enrolled twice and the second enrollment was removed; 5 cows (Control = 2, PEG = 2 and no treatment data = 1) did not have the date of inclusion recorded. In addition, PEG = 13 cows were injected at the time of inclusion but not injected at calving, and Control = 4 cows were erroneously injected at calving. Finally, 64 cows (Control = 30, PEG = 34) had no Prepartum NEFA determination. Thus, 2,153 cows were considered in the final analyses: 733 primiparous cows (Control = 391, PEG 342) and 1,420 multiparous cows (Control: 723, PEG = 697).

3.3.2. Balance between treatment groups

No differences between treatment groups were found for actual lactation number, with average values of 2.4 ± 1.5 and 2.5 ± 1.5 for Control and PEG cows respectively (P = 0.37). Table 3.1 presents descriptive data of the previous lactation for the enrolled multiparous cows by treatment group. It includes lactation number at enrollment, previous milk production, DIM at dry off, daily milk production, proportion of cows with high SCC at dry off, and proportion of cows with one or more clinical mastitis cases in previous lactation. No differences between treatment groups were found.

Table 3.1. Descriptive data from the lactation before enrollment in the study for multiparous cows.

	Treatment (Mean ± SD)		
Item	**Control**	**PEG**	**P-value**
Lactation number at enrollment	2.2 ± 1.4	2.2 ± 1.3	0.83
Previous lactation milk (kg)	7,636 ± 2,273	7,543 ± 2,197	0.43
Days in milk at dry-off	363 ± 102	354 ± 92	0.14
Daily milk (kg/day) previous lactation	21.4 ± 5.2	21.7 ± 5.4	0.31
Occurrence of CM cases (%, n)*	38 (276)	39 (269)	0.87
SCC at dry off (%, n)**	54 (388)	57 (398)	0.19

Control = 723; PEG = 697. *32 and 34 Control and PEG cows respectively have no previous data of CM. **proportion of cows with high (> 200,000 cell/mL) SCC at the last test day of previous lactation.

Figure 3.1 shows the number of cows by days between enrollment and calving for the two treatment groups. The mean values and SD were 9 ± 8 and 9 ± 10 days for Control and PEG cows respectively ($P = 0.42$). Primiparous Control and PEG cows were enrolled at 10 ± 10 and 10 ± 12 days before calving ($P = 0.90$) and multiparous Control and PEG cows at 9 ± 7 and 9 ± 9 days before calving ($P = 0.26$). Forty eight percent of the animals were enrolled within one week before calving, 41% between one and two weeks before calving and 7% between two and three weeks before calving, so that 96 % of the cows were enrolled within 21 days before calving.

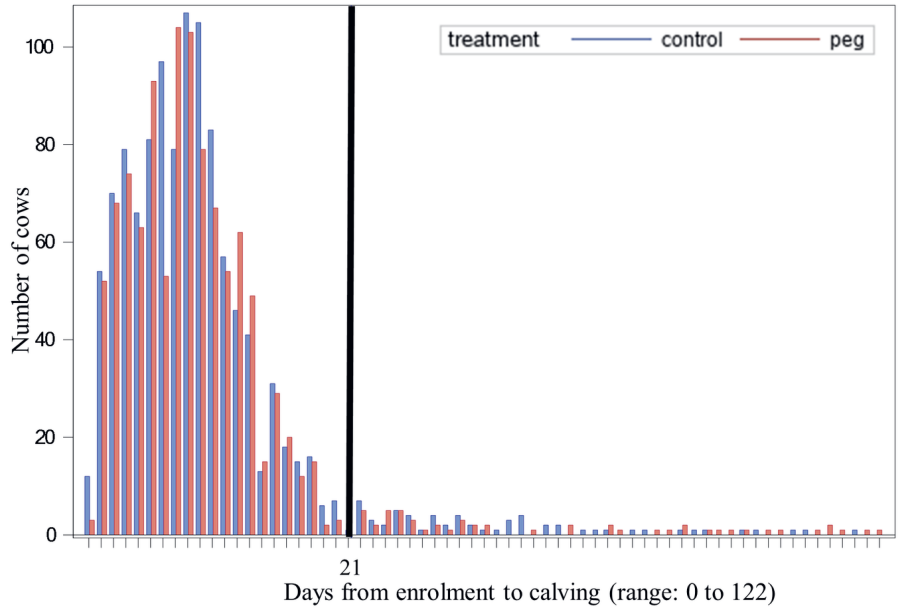

Figure 3.1. Number of cows by interval from enrollment to calving (day 0). Ninety six percent of the animals were enrolled within 21 days (black line) before calving. Control = 1,114; PEG = 1,039. Note: some values in the range have frequencies of 0 so are not represented in the x-axis.

No differences between treatment groups were found for BCS, with average scores of 3.4 ± 0.4 and 3.4 ± 0.4 for Control and PEG cows respectively ($P = 0.29$). The number of cows in each Prepartum BCS category was: Under: Control = 107; PEG = 99; Acceptable: Control = 702; PEG = 706 and Over: Control = 305; PEG = 234. No differences between treatment groups were found for Prepartum NEFAconcentration (0.50 ± 0.39 and 0.52 ± 0.42 m*M* for Control and PEG cows respectively, $P = 0.47$). The number of cows in each Prepartum NEFAcategory was: Low (≤ 0.5 m*M*): Control = 667; PEG = 621: High (> 0.5 m*M*): Control = 447 PEG = 418.

Differences between parity groups were found for Prepartum BCS, with scores of 3.5 ± 0.4 and 3.3 ± 0.4 for primiparous and multiparous cows respectively ($P < 0.001$) and for Prepartum NEFAconcentrations: 0.61 ± 0.42 and 0.46 ± 0.38 m*M* for primiparous and multiparous cows respectively ($P < 0.001$).

3.3.3. Disease occurrence

In total, 31 cows (Control = 17, PEG = 14) recorded milk fever and 6 cows (Control = 6, PEG = 0) clinical ketosis. A total of 223 (Control = 120, PEG = 103) cows recorded lameness. However only 45 (Control = 22, PEG = 23) had a case during the first 30 d of lactation. These clinical diseases were not further analyzed in regression analyses.

3.3.4. Effect of pegbovigrastim on clinical mastitis in primiparous and multiparous cows

A total of 2,005 (Control = 1,077, PEG = 928) cases of CM were recorded.
Regression analysis results for the occurrence of a first case of CM during the first 30 DIM are presented in Table 3.2. Treatment with PEG reduced the occurrence of a first case of CM during the first 30 DIM ($P = 0.002$). Occurrence of a first case of CM during the first 30 DIM was not associated with parity ($P = 0.50$) and no treatment by parity interaction was detected. Cows with an acceptable BCS had a lower occurrence of a first case of CM during the first 30 DIM compared with over-conditioned cows ($P = 0.02$). Prepartum BCS interacted with treatment: the preventive effect of PEG was not observed in cows with an acceptable Prepartum BCS ($P = 0.008$). Figure 3.2 presents least squares means differences of occurrence of a first case of CM during the first 30 DIM by Prepartum BCS in control and PEG cows. In over-conditioned cows, PEG reduced the occurrence of a first case of CM during the first 30 DIM by 49.5 % (Control = 20.8 %, PEG = 10.5 %; $P = 0.02$), while no significant reduction was detected in under-conditioned cows (Control = 20.4 %, PEG = 17.2 %; $P = 0.55$) and in cows with an acceptable BCS (Control = 14.4 %, PEG = 14.3 %; $P = 0.96$).

The Prepartum NEFA by treatment interaction did not reach significance ($P = 0.15$); however, removing this variable changed the estimate of treatment considerably (from -0.99 -as shown in table 2- to -0.70). When we analyzed least squares means differences, we found a differential effect of treatment with regard to prepartum NEFA. While no significant reduction due to PEG treatment was detected in low prepartum NEFA cows (Control = 16.5 %, PEG = 14.4 %; $P = 0.39$), treatment with PEG significantly reduced the occurrence of a first case of CM during the first 30 DIM (by 35.5 %) in high prepartum NEFA cows (Control = 20.3 %, PEG = 13.1 %; $P = 0.01$).

When testing the overall treatment effect (type III test), we found that treatment with PEG reduced the occurrence of a first case of CM during the first 30 DIM by 24.6 % (Control = 18.3 %, PEG = 13.8 %; $P = 0.03$).

Table 3.2. Occurrence of a first case of clinical mastitis during the first 30 days in milk.

Effect	Estimate	SE	*P* – value
Intercept	-1.36	0.30	0.02
Treatment	-0.99	0.32	0.002
Parity, 1 vs >1	-0.11	0.17	0.50
SCC at dry off	0.41	0.15	0.006
Prepartum BCS			
Under*	-0.03	0.30	0.92
Acceptable**	-0.44	0.20	0.02
Prepartum NEFA	-0.25	0.18	0.17
Prepartum BCS x Treatment			
Under*	0.60	0.44	0.17
Acceptable**	0.80	0.30	0.008
Prepartum NEFAx Treatment	-0.36	0.25	0.15

Reference groups: Control group, prepartum BCS: Over-conditioned cows (BCS > 3.5), Prepartum NEFA: high (> 0.5m*M*); SCC at dry off, 0 (< 200.000 cell/mL).
*Under-conditioned cows (BCS < 3), **Acceptable BCS cows (3 to 3.5).

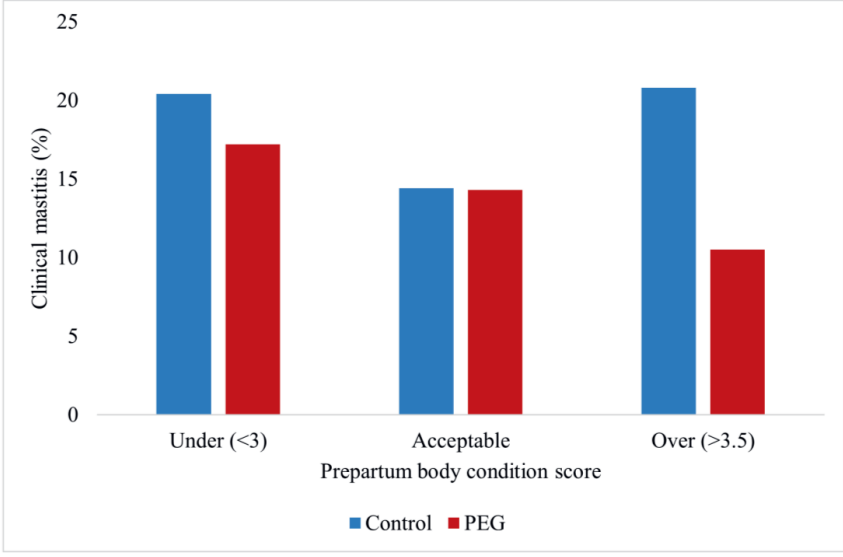

Figure 3.2. First case of clinical mastitis during the first 30 DIM by prepartum body condition score. Under-conditioned (< 3; Control = 107; PEG = 99); Acceptable-conditioned (3 – 3.5; Control = 702; PEG = 706); Over-conditioned (>3.5; Control = 305;

PEG = 234). In over-conditioned cows, occurrence of CM was lower in PEG treated cows ($P < 0.05$).

The hazard of a first case of CM during the full lactation is presented in Table 3.3. A significant treatment effect was detected: PEG cows had a 22% lower hazard of a first case of CM (HR = 0.78; $P = 0.008$). Parity was also significant: the hazard of a first case of CM was 24% lower in primiparous cows than in multiparous cows (HR = 0.76; $P = 0.003$). There was no treatment by parity interaction. The tendency towards significance of the interaction between treatment and Days-block ($P = 0.09$) suggested that the effect of treatment disappeared with increasing DIM (HR = 0.08 for each block of 60 DIM). Figure 3.3 shows the survival curves of time to first case of CM in control and PEG cows.

Table 3.3. Hazard of a first case of CM during a full lactation.

Effect	Estimate	SE	P - value	Hazard ratio
Treatment	-0.25	0.09	0.008	0.78
Parity, 1 vs >1	-0.28	0.09	0.003	0.76
SCC at dry off	0.37	0.08	< 0.001	1.45
Days-block* x Treatment	0.08	0.05	0.09	1.08

Reference group: Control group.
SCC at dry off 0 (< 200,000 cell/mL).
*Days-block were 60-day blocks for days of lactation.

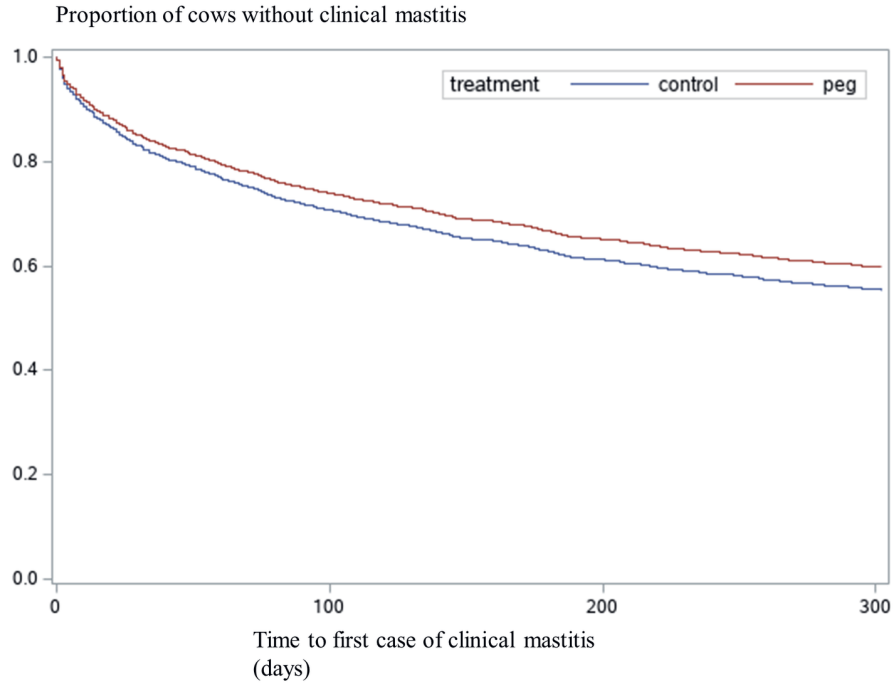

Figure 3.3. Survival curves of time to first case of clinical mastitis.
Control cows = 1,114, PEG cows = 1,039. Treatment *P* – value = 0.008 (Table 3).

The Poisson regression model (Table 3.4) showed a significant preventive effect of treatment on the rate of total cases of CM per cow-day at risk during the full lactation ($P = 0.02$). Parity was also significant, as primiparous cows had fewer total cases of CM ($P < 0.001$). No treatment by parity interaction was detected. Cows with an acceptable BCS had a lower rate of total cases of CM ($P = 0.04$).

Table 3.4. Rate of total cases of clinical mastitis per cow-day at risk during the full lactation.

Effect	Estimate	SE	P – value
Intercept	-5.77	0.13	<0.001
Treatment	-0.10	0.04	0.02
Parity, 1 vs >1	-0.34	0.06	<0.001
SCC at dry off	0.39	0.05	<0.001
Prepartum BCS			
Under*	-0.09	0.09	0.34
Acceptable**	-0.12	0.06	0.04
Calving month			< 0.001

Reference groups: Control group, prepartum BCS: Over-conditioned cows (BCS > 3.5), SCC at dry off 0 (< 200.000 cell/mL).
*Under-conditioned cows (BCS < 3), ** Acceptable BCS cows (3 to 3.5). Offset in the model was the natural log of the days-at-risk.

3.3.5. Effect of Pegbovigrastim on Clinical Uterine Diseases in Primiparous and Multiparous Cows

The results of the regression analysis for clinical uterine disease are presented in Table 3.5.

Parity was associated with the occurrence of RP, as multiparous cows showed a higher occurrence than primiparous cows ($P = 0.05$). Calving month showed a significant association ($P < 0.001$) and the calving month by treatment interaction tended to significance ($P = 0.09$). No overall treatment effect (type III test) was detected (Control = 8.1 %, PEG = 8.9 %; $P = 0.54$).

For metritis, there was no significant treatment effect ($P = 0.40$), while parity was associated with its incidence ($P < 0.001$); primiparous cows showed a higher incidence than multiparous cows. There was no treatment by parity interaction. No overall treatment effect (type III test) was detected (Control = 32.2 %, PEG = 34.5 %; $P = 0.37$).

For endometritis, model results showed no treatment ($P = 0.34$) or parity effects ($P = 0.90$). There was no treatment by parity interaction. Occurrence of RP ($P < 0.001$) and metritis ($P < 0.001$) increased the risk for endometritis. A significant interaction of treatment with the previous occurrence of metritis was detected ($P = 0.04$). Figure 4 presents endometritis occurrence in cows with and without a previous metritis case in

Control and PEG cows. In cows with metritis, PEG treatment reduced the occurrence of subsequent endometritis by 42.3 % (Control = 17.5 %, PEG = 10.1 %). No overall treatment effect (type III test) was detected (Control = 9.0 %, PEG = 7.6 %; *P* = 0.40).

Table 3.5. Regression analysis model results for uterine disease.

Uterine disease	Effect	Estimate	SE	*P* – value
Retained placenta	Intercept	-2.76	0.35	0.004
	Treatment	0.47	0.32	0.14
	Parity, 1 vs >1	-0.33	0.17	0.05
	Calving month			<0.001
	Calving month x Treatment			0.09
Metritis	Intercept	-1.99	0.16	0.001
	Treatment	0.10	0.12	0.40
	Parity, 1 vs >1	0.52	0.12	<0.001
	Retained placenta	1.93	0.16	<0.001
Endometritis	Intercept	-4.11	0.34	0.001
	Treatment	0.27	0.28	0.34
	Parity, 1 vs >1	0.03	0.23	0.90
	Calving month			0.01
	Retained placenta	0.99	0.27	<0.001
	Metritis	1.53	0.31	<0.001
	Metritis x Treatment	-0.90	0.44	0.04

Reference groups: Control group, month 3, no RP (retained placenta); no Metritis.

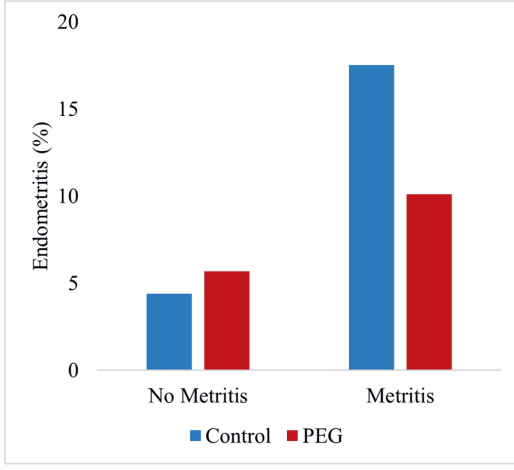

Figure 3.4. Cows with a diagnosis of endometritis (%) by previous metritis diagnosis. No metritis: Control = 837, PEG = 778; Metritis: Control = 177, PEG = 177.

There was a significant interaction between treatment and the previous occurrence of metritis *P* = 0.04.

3.4. Discussion

In this randomized controlled trial on four commercial grazing dairy farms, we investigated whether PEG treatment affected CM and uterine disease occurrence during a full lactation in primiparous and multiparous grazing dairy cows. We also evaluated the association of prepartum BCS and prepartum NEFA concentration and relevant interactions with disease occurrence. Treatment with PEG reduced the occurrence of a first case of CM during the first 30 DIM in grazing dairy cows and reduced the hazard of a first case and the rate of total cases of CM during the full lactation. The treatment effects were independent of parity. Also, for the first time, we showed that prepartum BCS interacts with PEG; in over-conditioned cows, PEG reduced the occurrence of a first case of CM by almost 50 % during the first 30 DIM. The hazard analysis of a first case of CM during a full lactation suggested that the preventive effect of PEG disappeared with increasing DIM. Finally, PEG treated cows with metritis subsequently showed a reduced occurrence of endometritis compared to control cows with metritis.

Treatment with PEG resulted in an overall reduction of almost 25 % in the occurrence of a first case of CM during the first 30 DIM. The beneficial effect of PEG decreased with increasing DIM (Table 3.2, Figure 3.2). Even so, PEG treated cows had fewer total cases of CM per day at risk (Table 3.4). To our knowledge, this study is the first that follows the treated animals during a full lactation. The preventive effect of PEG on CM during the first 30 DIM is in contrast with Zinicola et al. (2018), whose study included only cows with an acceptable BCS. Our results are compatible with others that included cows regardless of their BCS, in which a similar effect of PEG on CM was reported, for example the 34 % reduction reported by Canning et al. (2017) and the 25 % reduction reported by Ruiz et al. (2017). This transient beneficial effect of PEG is biologically plausible since immunosuppression is typically highest during early lactation (Kimura et al., 2014; Ingvartsen and Moyes, 2015) and the effect of PEG on WBC counts is transient (Van Schyndel et al., 2018). The postpartum beneficial effect is especially valuable since early lactation CM cases can be more severe compared with mid or late lactation cases (Burvenich et al., 2007). Also, early lactation CM increases the risk of subsequent CM cases (Hertl et al., 2018). The reduction in early lactation CM could be a possible explanation for the preventive effect of PEG on the total cases of CM during the full lactation. It could also be hypothesized that during early lactation PEG increases the cure rate after antibiotic treatment. Future analyses on these data will be done to evaluate this.

Indeed, the ability to recruit neutrophils into the mammary gland is essential for mastitis resolution (Schukken et al., 2011), and Powell et al. (2018) reported that PEG activated neutrophils for quick recruitment to the infected mammary gland, reduced severity of CM and lowered bacterial shedding in milk, suggesting an improved mastitis resolution. Early lactation CM impairs reproductive performance and increases the culling rate (Hertl et al., 2014; Hertl et al., 2018). Whether the preventive effect of PEG on the occurrence of CM during early lactation reported here could affect the reproductive performance and culling warrants further analysis of our data.

Our results showed that over-conditioned control cows had a higher occurrence of first cases of CM during the first 30 DIM and total cases of CM compared to cows with an acceptable BCS (Figure 3.2, Table 3.4). It has been reported before that cows with a high BCS had more mastitis relative to cows with a moderate BCS (Roche et al., 2009), and these authors suggest that this is related to an impaired energy and lipid metabolism that may affect the immune response. Over-conditioned cows mobilize more reserves than cows in an acceptable body condition (Roche et al., 2009), leading to increased postpartum concentrations of NEFA (Lacetera et al., 2005) and beta-hydroxybutyrate (Roche et al., 2009), both metabolites being associated with an impaired immune response during early lactation (Ingvartsen and Moyes, 2015). Interestingly, we found a prepartum BCS by treatment interaction, as PEG reduced the first cases of CM by almost 50% in over-conditioned animals, while no effect was observed in under-conditioned cows or cows with an acceptable BCS (Figure 3.2). These data could explain the lack of treatment effect found in the study reported by Zinicola et al. (2018), as they excluded low and high BCS cows (BCS < 3 and > 3.75).

The prepartum NEFA by treatment interaction did not reach statistical significance (P = 0.15; Table 2); however, the inclusion of this interaction in the final model changed the treatment estimate considerably. This suggests that PEG has a differential effect according to the prepartum NEFA category. We expected a stronger association based on our recent findings that PEG reverts the negative association between prepartum NEFA and neutrophil counts (Barca et al., 2021a). Analysis of the estimated least squares means differences by prepartum NEFA category shows that, in high prepartum NEFA cows, PEG treatment significantly reduced the occurrence of a first case of CM during the first 30 d of lactation by 35%. Melendez et al. (2009) used a very high cut-off (1.2 mM) of NEFA at calving to show an association with CM; however, as far as we know, no cut-

off for prepartum NEFA was described as a risk factor for CM. Based on the literature (Ospina et al., 2013) we performed our analyses using 0.3, 0.4 and 0.5 as cut-off points, and using a cut-off of 0.5 resulted in the smallest probability of committing a type I error. As mentioned before, the effect of PEG was strongly dependent on prepartum BCS, and prepartum BCS has been associated with peripartum NEFA concentrations (Roche et al., 2009; Barletta et al., 2017; Sheehy et al., 2017), which may partially explain our results. Overall, the beneficial effect of PEG treatment on the occurrence of a first case of CM during the first 30 DIM in over-conditioned cows suggests that PEG treatment reduces the potential negative effect of metabolic stress on the immune system, to demonstrate it, further studies measuring immune system markers are warranted.

Pegbovigrastim showed no effect on RP incidence. These results are in accordance with those reported by Zinicola et al. (2018). On the other hand, Ruiz et al. (2017) reported a reduction in the incidence of RP in PEG treated cows. A preventive effect of PEG on RP occurrence could be expected, since diminished neutrophil functional capacity is mentioned in the etiology of the disease (Kimura et al., 2002), and PEG improves neutrophil functional capacity (Kimura et al., 2014; McDougall et al., 2017; Heiser et al., 2018). Our data show that treatment interacted with calving month; nonetheless, we cannot make inferences about this finding since the present study was not designed to test the calving month effect and thus, further research will be needed to identify factors that could explain this relationship.

Metritis occurrence was not affected by treatment, which is in accordance with a previous report (Zinicola et al., 2018). Ruiz et al. (2017) reported a higher incidence of metritis in PEG treated cows. The study of Zinicola et al. (2018) and the present work were especially designed to assess all cows by trained technicians at the same time point (one week after calving approximately), while in the study of Ruiz et al. (2017) metritis diagnoses relied on trained field workers but without formal assessment protocols. A more robust vaginal discharge due to a greater intra-uterine neutrophil influx in PEG treated cows was suggested by Ruiz et al. (2017). This hypothesis is supported by the findings of lower blood neutrophil counts (Barca et al., 2021a) and higher neutrophil counts in the vagina in PEG cows with metritis compared to PEG cows without metritis (Zinicola et al., 2018). As expected (Toni et al., 2015), primiparous cows had a higher metritis occurrence, while no treatment by parity interaction was detected. A study using primiparous cows on one German farm reported that treatment with PEG reduced the

occurrence of acute puerperal metritis (Freick et al., 2018), but, due to the small sample size and the high incidence of the disease, these conclusions should be evaluated with caution.

Interestingly, treatment with PEG reduced (42.3 %) endometritis occurrence in animals that were diagnosed with metritis previously (Figure 3.4). High proportions of neutrophils in the endometrium soon after calving were reported as beneficial to uterine health and subsequent fertility (Gilbert and Santos, 2016). These authors reported that cows that are capable of recruiting large numbers of neutrophils rapidly to the uterus in the immediate postpartum period are less likely to suffer bacterial infections and more likely to have a healthy postpartum uterine involution. The lower endometritis occurrence in PEG treated cows with metritis would suggest that a higher neutrophil influx to the uterus due to PEG treatment improves the healing process. Endometritis on its own or as a consequence of metritis has a strong deleterious effect on reproductive performance and increases the chance to be culled for reproductive failure compared to cows without endometritis (LeBlanc et al., 2002; Toni et al., 2015). Whether our findings affect the reproductive performance and the risk of culling warrants further analysis.

3.5. Conclusions

Overall, treatment with PEG reduced the occurrence of a first case of CM during the first 30 DIM in grazing dairy cows and reduced the hazard of a first case and the rate of total cases of CM during the full lactation. We showed that these effects were independent of parity. Also, for the first time, we showed that prepartum BCS interacted with PEG; in over-conditioned cows, PEG strongly reduced the occurrence of a first case of CM during the first 30 DIM. The hazard analysis of first cases of CM during the full lactation suggested that the preventive effect of PEG disappeared with increasing DIM. Pegbovigrastim treated cows with metritis subsequently showed a reduced occurrence of endometritis compared to control cows with metritis. Pegbovigrastim reduces the occurrence of CM particularly in cows at risk of elevated lipid mobilization, and PEG ameliorates the uterine healing process in cows that experienced metritis.

3.6. Acknowledgments

The cooperation of farmers and farm personnel is gratefully acknowledged.

CHAPTER 4

Effect of pegbovigrastim on fertility and culling in grazing dairy cows and its association with prepartum nonesterified fatty acids

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Abstract

This randomized controlled trial on four commercial grazing dairy farms investigated whether treatment with pegbovigrastim (PEG) affected fertility and culling as measured during the full lactation. We also explored the effect of potential interactions of PEG treatment with parity, prepartum body condition score, prepartum nonesterified fatty acids concentration (prepartum NEFA) and early lactation clinical disease on these outcomes. Holstein cows were randomly assigned to one of two trial arms: a first PEG dose approximately 7 d before the expected calving date and a second dose within 24 h after calving (PEG: primiparous = 342; multiparous = 697) compared to untreated controls (Control: primiparous = 391; multiparous = 723). Cox's proportional hazards regression models were used to analyze rate of first insemination, rate of pregnancy [within 150 and 305 days in milk (DIM)] and hazard of culling. Additional analyses were performed on data that were stratified by parity group and prepartum NEFA class (Low ≤ 0.3; High > 0.3 m*M*). In High prepartum NEFA cows, PEG treatment increased the rate of first insemination [Hazard Ratio (HR) = 1.15]. Early lactation clinical mastitis (CM) and uterine disease (UD: retained placenta, metritis or both) were associated with a reduced rate of pregnancy within 150 DIM (HR= 0.49 and 0.78 respectively). Pegbovigrastim treatment in High prepartum NEFA cows with CM and UD increased the rate of pregnancy within 150 DIM (HR= 1.75 and 1.46 respectively). In High prepartum NEFA cows, PEG treatment resulted in a lower hazard of culling (HR= 0.79). No treatment effect was detected in Low prepartum NEFA cows. This study shows that the effect of PEG treatment on fertility and culling interacts with prepartum NEFA. In High prepartum NEFA cows, PEG treatment increased the rate of first insemination, counteracted the negative association of early lactation CM and UD with the rate of pregnancy and decreased the hazard of culling.

4.1. Introduction

The metabolic challenges that dairy cows experience around parturition (Grummer, 1995; Drackley, 1999) considerably impair their immune response (Trevisi et al., 2018; Gross and Bruckmaier, 2019). Metabolites related to negative energy balance (NEB), such as nonesterified fatty acids (NEFA) and β-hydroxybutyrate, have been identified as immunosuppressants (Ingvartsen and Moyes, 2015). Elevated NEFA concentrations were associated with decreased white blood cell (WBC) and neutrophil counts (Hachenberg et al., 2007; Barca et al., 2021a) and impaired neutrophil and lymphocyte function (Lacetera et al., 2005; LeBlanc, 2020). Moreover, epidemiological studies have shown that elevated NEFA concentrations are associated with increased risk of diseases such as mastitis, retained placenta (RP) and metritis (LeBlanc et al., 2004; Melendez et al., 2009; Galvão et al., 2010).

A recent meta-analysis (Dolecheck et al., 2019) suggested that time to first insemination and time to pregnancy in an animal with clinical mastitis (CM) before its first insemination is increased. Uterine diseases have been linked to impaired fertility, where RP, metritis and endometritis delayed time to first insemination and time to pregnancy (Fourichon et al., 2000; LeBlanc et al., 2002; Toni et al., 2015). Both CM and poor fertility are also major reasons for culling (Kossaibati and Esslemont, 1997; Bar et al., 2008; Hertl et al., 2018).

The use of a long-acting analogue of bovine granulocyte colony-stimulating factor, G-CSF (Pegbovigrastim or PEG, marketed as Imrestor by Elanco Animal Health) has been reported to be beneficial, as treatment reduced the incidence of early lactation CM (Canning et al., 2017; Ruiz et al., 2017; Barca et al., 2021b). However, Zinicola et al. (2018), including only cows with optimal body condition in late gestation, reported an absence of treatment effect on CM. More recently, Van Schyndel et al. (2021), including cows regardless of body condition, reported a lack of PEG treatment effect on the incidence of mastitis. Evidence for the use of PEG to improve uterine health has been inconsistent, since increases (Ruiz et al., 2017), decreases (Freick et al., 2018), or lack of effect (Zinicola et al., 2018; Van Schyndel et al., 2021) on metritis incidence were reported. We recently reported that treatment with PEG reduced the occurrence of endometritis in cows that had previous metritis (Barca et al., 2021b).

Treatment with PEG reduced failure to return to estrus within 80 days in milk (DIM) (Canning et al., 2017) and increased the rate of insemination by 5.8% during the first 100 DIM (Ruiz et al., 2017). In contrast, Zinicola et al. (2018) reported a lack of PEG treatment effect on rate of insemination during the first 120 DIM and rate of pregnancy during the first 180 DIM. They also reported a lack of effect of PEG on the hazard of culling during the first 180 DIM. Van Schyndel et al. (2021) reported a lack of PEG treatment effect on the hazard of culling during the first 63 DIM, rate of first insemination during the first 150 DIM and rate of pregnancy during the first 250 DIM. These studies were carried out under various management conditions and, to our knowledge, there are no reports on the effect of PEG treatment on fertility and culling under grazing conditions.

Recently, we showed that treatment with PEG prevented the negative association of prepartum NEFA concentration with postpartum neutrophil counts (Barca et al., 2021a). In addition, we found that PEG reduced the occurrence of a first case of CM during the first 30 DIM, particularly in cows with elevated prepartum NEFA concentrations and in cows with excessive prepartum body condition score (BCS) (Barca et al., 2021b).

As far as we know, there are no reports on the effect of PEG on fertility or culling as measured during a full lactation. This is of relevance as fertility and especially culling are particularly important later in lactation (Ribeiro et al., 2016; Carvalho et al., 2019). Hence, full lactation follow-up will provide a more complete picture of the effect of PEG treatment on fertility and herd life.

Therefore, we hypothesized that PEG would increase fertility and decrease culling, and that the relationship between PEG treatment and fertility and culling outcomes would interact with prepartum BCS and/or prepartum NEFA. Thus, we investigated whether PEG treatment affects fertility and culling during a full lactation in grazing dairy cows. We also explored the effect of PEG treatment interactions with parity, prepartum BCS, prepartum NEFA concentration and early lactation clinical disease on these outcomes.

4.2. Materials and methods

The experimental protocol (CEUAFVET-PI-162) was evaluated and approved by the Honorary Committee for Animal Experimentation in Uruguay, University of the Republic, Uruguay.

4.2.1. Study design

This randomized controlled trial was conducted on four commercial grazing dairy farms in three different regions of Uruguay. A total of 2,336 Holstein primiparous (animals that were enrolled in the study shortly before their first calving) and multiparous cows (animals that were enrolled shortly before their second or higher calving) were assessed for enrollment on the four farms. Farms 1, 3 and 4 had a seasonal calving system, with calving concentrated in autumn. These herds had a milking herd size of approximately 1,000, 850 and 600 cows, respectively. Farm 2 had a continuous year-round calving system and a milking herd size of approximately 600 cows. All farms used artificial insemination with estrus detection performed by trained farm personnel. Pregnancy diagnoses were performed by transrectal palpation or ultrasonography by the farm veterinarian.

All cows from each farm were located in outdoor close-up paddocks around 3 weeks before the expected calving date, where cows were fed a partial mixed ration (Table S 4.1) twice a day. Calving was in the same area or in a subdivision of the same paddock under the same conditions. Calving of cows included in the study occurred from February 13 to September 30 of 2018. After calving, cows were kept on pasture at least one of the periods between the two daily milkings and at least 40% of the dry matter intake (DMI) came directly from the grazing sessions, supplemented with a partial mixed ration.

Three veterinary technicians were hired and trained as research assistants for this experiment. One of these technicians was supported by the first author, and covered two dairy farms. The other two technicians each covered one of the remaining two farms. Technicians enrolled animals, administered experimental treatments, took blood samples, assessed BCS, diagnosed diseases or confirmed diagnoses made by the farm staff, and were responsible for keeping written records.

4.2.2. Enrollment and treatment allocation

The time of enrollment in the study was between -10 to -7 days relative to the expected calving date. Animals that had fever (rectal temperature > 39.5°C) or any other clinical health disorder at the time of enrollment were excluded from the study. Animals that met the inclusion criteria were assigned to either treatment or untreated control based on their national ear tag number. The national ear tag number is assigned to cattle at birth. The national ear tag numbers are available from computer records but are independent of the large and easily visible ear tag number (cow ID) that is used for on-farm management. Animals with an even national ear tag number were injected with 15 mg of pegbovigrastim (Imrestor®, Elanco Animal Health, Greenfield, IN) according to the product label (PEG) and animals with an odd national ear tag number remained as untreated controls (Control). The decision to treat the even-numbered cows was based on a single randomization procedure using the toss of a coin. No placebo was used, as treatment allocation based on the national ear tag number provided sufficient blinding, and Control and PEG cows were visited and blood sampled using exactly the same protocol, as explained below.

Research technicians applied treatments based on the national ear tag number and would therefore be aware of the treatment status. All animal observations, samplings and disease diagnoses were based on the visible on-farm cow ID. This on-farm cow ID was unrelated to the national ear tag number that was used for randomization. Farm personnel and veterinarians involved in disease diagnoses, estrus detection, insemination, pregnancy diagnoses and culling decisions were blinded to treatment status, and only used the visible on-farm cow ID. Electronic readers that might show both tags were not used on any of the farms at any time.

Close-up pens were observed two times a week. Cows that were between -10 to -7 days relative to the expected calving date or were exhibiting clinical signs of calving such as swelling of the vulva and filling of the udder were clinically examined to rule out exclusion criteria, i.e. fever or any other clinical disease.

Animals assigned to the PEG treatment received a second dose within 24 h after calving; only cows that received both doses were included in the study. The included animals therefore represent the ‘meet protocol’ inclusion rule (Sargeant et al., 2010).

Control and PEG cows remained in observation for at least 30 minutes to record any adverse event due to treatment or handling, both after enrollment and after calving.

4.2.3. Prepartum body condition score assessment, blood sampling and nonesterified Fatty Acids Determination

At day -10 to -7 from the expected calving date (enrollment), BCS was assessed (Ferguson et al., 1994) and recorded by each of the veterinary technicians. At the same time, blood samples were collected from the coccygeal vessel (8.5-mL clot accelerator tubes, Becton Dickson, Franklin Lakes, NJ). Control and PEG animals were blood sampled again within 24 h after calving (i.e. when PEG cows also received the second treatment), for further determinations beyond the aim of this report. Blood samples were centrifuged at 3000 x g for 20 min and serum was stored frozen (-20°C) until further analysis for NEFA concentrations at the Animal Endocrine and Metabolism Laboratory, Veterinary Faculty, Montevideo, Uruguay. Non esterified fatty acids concentrations were measured by colorimetric assays on an A25 autoanalyzer (Biosystems S.A., Barcelona, Spain) using commercial kits: Wako NEFA-HR (2) (Wako Pure Chemical Industries Ltd., Osaka, Japan), as reported before (Barca et al., 2021a; Barca et al., 2021b). Laboratory personnel were blinded to treatment status.

4.2.4. Clinical diagnoses and definitions

For the purpose of this study, the experimental unit was the cow; the follow-up period for all clinical diseases was limited from enrollment until 30 DIM. We only included early lactation clinical diseases as variables with potential interaction with PEG treatment, because immune stimulation due to PEG is transient (Kimura et al., 2014; McDougall et al., 2017; Van Schyndel et al., 2018) and clinical diseases later in lactation are logically associated with longer herd life, as animals need to be alive in the herd to be able to register disease.

Each veterinary technician was trained prior to the start of the study to diagnose CM, RP, metritis and clinical endometritis. At the same time, all farm personnel were trained in the recognition of these disorders and all diagnoses were ultimately confirmed by the trained trial technicians. At two postpartum visits, at 5 to 8 and 27 to 30 DIM, all cows were carefully assessed by the veterinary technician to diagnose metritis and clinical

endometritis respectively. If metritis and clinical endometritis were diagnosed by the farm personnel at a different time point, this was also recorded and included into the disease categories described hereunder. Clinical mastitis was diagnosed by trained farm personnel while forestripping all quarters of all cows at each milking. Clinical mastitis was scored according to Pinzón-Sánchez and Ruegg (2011) as mild (abnormal milk without other symptoms), moderate (abnormal milk and local symptoms in the udder), or severe (abnormal milk, local symptoms and also signs of systemic illness). All CM cases, irrespective of severity, were combined and reported as CM. Retained placenta was recognized when the fetal membranes (placenta) were visible hanging from the cow´s vulva at 24 h or more after calving (Ruiz et al., 2017). Puerperal metritis was diagnosed if an animal showed a fetid watery red-brown uterine discharge, associated with signs of systemic illness (such as decreased milk yield, dullness or other signs of toxemia) and fever (rectal temperature > 39.5°C or > 40.5°C during summer and when ambient temperature was higher than 30°C; Burfeind et al., 2012) within 21 days post-partum. Clinical metritis was defined as cows that were not ill, but that had a purulent uterine discharge detectable in the vagina within the first 21 days after calving. In the present study, puerperal metritis and clinical metritis were combined into one disease code and reported as metritis. Clinical endometritis was the presence of purulent uterine discharge detectable in the vagina 21 days or more post-partum, or mucopurulent discharge detectable in the vagina more than 26 days post-partum. Manual vaginal examinations were performed using clean palpation gloves. All uterine diseases were defined according to Sheldon et al. (2006).

4.2.5. Definition of fertility and culling outcomes

The full set of animals was considered at risk for breeding with two exceptions: 1) a cow removed during the voluntary waiting period, defined as a cow that was removed from the study during the first 30 DIM and that was not inseminated. All farms declared a voluntary waiting period of at least 30 DIM; however, if a cow was inseminated before 30 DIM by accident, it was still included in the analysis; 2) a ‘do not breed’ cow: a cow that was not inseminated within 305 DIM. We assumed that these animals were considered cows that were not going to be bred (Bewley et al., 2010).

Time to first insemination was defined as the interval in days from calving to the first insemination. We evaluated rate of first insemination with a censoring time of 305 DIM as explained above.

Time to pregnancy was defined as the interval in days from calving to the insemination that led to conception (last insemination before the pregnancy diagnosis). We evaluated rate of pregnancy with two censoring times. The first analysis used a censoring time of 150 DIM. This censoring policy was chosen since pregnancy status at 150 DIM was reported as a robust measure of the overall reproductive performance in commercial dairy farms (Caraviello et al., 2006). We also performed an analysis with a censoring time of 305 DIM.

Time to culling was defined as the interval in days from calving to removal from the herd (i.e. death or sales). The censored end point was either the day of dry-off or end of study, which was the 1st of August of 2019 (529 days from the first recorded calving in the study and 305 days from the last recorded calving in the study).

4.2.6. Statistical analysis

Data were analyzed using SAS software (SAS Institute Inc. 2018. SAS® University Edition, Cary, North Carolina: SAS Institute Inc.).

Descriptive statistics to evaluate balance between treatment groups with regard to prepartum BCS, prepartum NEFA concentration, interval between enrollment and calving, lactation number, previous lactation total milk production, days in milk at dry-off and previous lactation daily milk production were performed using the t-test procedure (PROC TTEST). The chi-square test (PROC FREQ) was used to evaluate balance between treatment groups with regard to season of enrollment, previous lactation CM (yes/no) and SCC at dry-off (high/low). Frequencies of treated and untreated animals grouped by lactation group (Lactation 1, Lactation 2 and Lactation 2+), prepartum BCS categories (under: < 3; acceptable: 3 to 3.5, and over: > 3.5; Roche et al., 2009), prepartum NEFA class (Low ≤ 0.3; High > 0.3 m*M*, Overton et al., 2017) and animals considered to be at risk for breeding were generated using the frequency procedure (PROC FREQ). The chi-square test was also used to assess whether treatment group was associated with the

frequency of a) cows excluded from the fertility analysis, b) cows removed from the herd during the voluntary waiting period and not inseminated, and c) "do not breed cows".

Rate of first insemination, rate of pregnancy and hazard of culling analyses were carried out using Cox's proportional hazards regression models (PROC PHREG). A first set of models (model 1) included as fixed effects only pre-treatment covariates. The following were considered as class variables: lactation (Lactation 1, Lactation 2 and Lactation 2+), prepartum NEFA, prepartum BCS, Treatment (Control/PEG) and calving month (6 classes: February/March, April, May, June, July and August/September). Farm, also as a class variable, was included as a random effect. Two-way interactions between covariates and treatment and the three-way interaction of lactation, prepartum NEFA and treatment were checked for significance.

The general model then looked like:

Hazard of (variable of interest) = baseline hazard + lactation + prepartum NEFA + prepartum BCS + treatment + calving month + interactions + farm (random)

A second set of models (model 2) was developed to evaluate the interaction of PEG treatment with clinical disease. Kaplan-Meier univariable analyses were performed to evaluate the association of CM, RP, metritis and endometritis with the outcomes of interest. Since RP and metritis occur sequentially and close together in time, and we have previously detected a strong association between these uterine diseases (Barca et al., 2021b), we also grouped them in a new category recorded as uterine disease (UD**,** i.e. a cow with a record of RP, metritis or both). In case that RP and metritis were simultaneously associated with an outcome of interest, we evaluated UD and, if it was also associated with the outcome of interest, we used UD only. The grouping methodology was identical to the one reported by Carvalho et al. (2019). This was done to avoid potential multicollinearity (correlated independent variables) in the models. Clinical events that were associated at a $P < 0.2$ were included in the multivariable modeling process.

The second general model then looked like:

Hazard of (interest variable) = baseline hazard + lactation + prepartum NEFA + prepartum BCS + treatment + calving month + CM + RP + metritis (or + UD) + endometritis + interactions + farm (random)

The interaction of treatment with a clinical event was always evaluated and two and three way interactions of lactation and prepartum NEFA with treatment were checked.

Because of our previous observation on the importance of prepartum NEFA concentration in PEG treated animals (Barca et al., 2021a; Barca et al., 2021b), the impact of parity (primiparous/multiparous), prepartum NEFA, and the potential clinical disease by treatment interaction were evaluated using stratified datasets. We stratified the data by parity (primiparous and multiparous) and prepartum NEFA.

With the exception of the stratified data by parity, lactation as a covariate was grouped in three categories (Lactation 1, Lactation 2 and Lactation 2+) because this categorization produced a better fit of models (smaller Akaike information criterion number) than using just two categories (Lactation 1 and Lactation 1+).

Modeling was done using a manual forward selection procedure and only variables or their interaction with a $P \leq 0.10$ were included in the model. Statistical tendency was defined at $P \leq 0.10$ and statistical significance at $P \leq 0.05$. The assumption of proportional hazards was evaluated using graphical assessment of observed and predicted survival. For ease of interpretation, in the results and discussion, the hazard rate is presented here as rate in case of time to first insemination and time to pregnancy and as hazard in case of time to culling. The outputs of the final models are presented and the hazard ratio (HR) for each variable or interaction provided. Compared with an unspecified baseline hazard function (HR with all covariates set to the reference groups), a HR > 1 means that an event occurs sooner, while a HR < 1 means that an event will occur later (Cox, 1972). Survival curves illustrating the most important findings are presented.

4.3. Results

4.3.1. Study population and balance between treatment groups

Initially 2,336 cows were assessed for enrollment on the four farms; out of those, 3 cows (Control = 2; PEG = 1) were excluded because they had fever (rectal temperature > 39.5°C) or another clinical health disorder at the time of enrollment. Out of the 2,333 initially enrolled cows, 2,153 (primiparous cows = 733; Control = 391; PEG 342 and multiparous cows = 1,420; Control: 723, PEG = 697) met the protocol inclusion rule (Barca et al., 2021b). No adverse events due to treatment or handling were recorded. Table 4.1 shows descriptive data for the enrolled cows by treatment group: season of enrollment, prepartum BCS, prepartum NEFA concentration, the interval in days between enrollment and calving (in case of PEG this is the interval between PEG doses), and lactation number of the enrolled cows after calving. For multiparous cows, descriptive data of the previous lactation included: lactation number at enrollment, previous total milk production, DIM at dry-off, daily milk production, proportion of cows with one or more CM cases, and proportion of cows with high somatic cell count (>200,000 cell/mL) at dry-off. No differences between treatment groups at the time of enrolment were found in any of these variables. No difference between treatment groups was found regarding prepartum NEFA concentration by prepartum NEFA class: Low: Control = 0.18 ± 0.07 m*M*; PEG = 0.18 ± 0.07 m*M*; $P = 0.50$; High: Control = 0.71 ± 0.37 m*M*; PEG = 0.73 ± 0.41 m*M*; $P = 0.26$; or in the number of cows in each prepartum NEFA class in each treatment group: Low: Control = 435; PEG = 408; High: Control = 679; PEG = 631; $P = 0.92$. In addition, no difference between treatment groups was found by prepartum NEFA class within each farm ($P \geq 0.45$). Figure 4.1 shows the total number of included animals in the control and PEG group by lactation group and prepartum NEFA class. Out of the 2,153 cows, 7.0% of the cows (Control = 3.5%; PEG = 3.5%; $P = 0.72$) were excluded from the fertility analysis because they were removed from the herd during the voluntary waiting period and not inseminated (Control = 4.2 %; PEG = 3.1 %; $P = 0.72$) or were defined as “do not breed cow” (Control = 2.9 %; PEG = 4.4 %; $P = 0.07$).

Prepartum BCS, as a class variable, was not associated with any outcome analyzed in this study and did not remain in the statistical models.

Table 4.1. Descriptive data (mean ± SD) for the cows enrolled in the trial by treatment group.

	Treatment						
	Control			PEG			
	All (n = 1.114)	Primiparous (n = 391)	Multiparous (n = 723)	All (n = 1,039)	Primiparous (n = 342)	Multiparaou (n = 697)	*P* – value[1]
Season of enrollment[2], %							0.27
February-April, n = 1,155	52	55	51	48	45	49	
May-June, n = 478	48	49	48	52	51	52	
July-September, n = 520	53	54	53	47	46	47	
Prepartum BCS	3.37 ± 0.43	3.54 ± 0.41	3.28 ± 0.41	3.35 ± 0.41	3.48 ± 0.39	3.29 ± 0.40	0.29
Prepartum NEFA[3], m*M*	0.50 ± 0.39	0.61 ± 0.44	0.45 ± 0.34	0.52 ± 0.42	0.62 ± 0.41	0.47 ± 0.42	0.47
Interval enrollment – calving, d[4]	9 ± 8	10 ± 10	10 ± 12	9 ± 10	10 ± 12	9 ± 9	0.42
Actual lactation[5]	2.4 ± 1.5	1.0 ± 0.0	3.2 ± 1.4	2.5 ± 1.5	1.0 ± 0.0	3.2 ± 1.3	0.37
Multiparous cows:							
Lactation number at enrollment			2.2 ± 1.4			2.2 ± 1.3	0.89
Previous lactation total milk, kg			7,636 ± 2,273			7,543 ± 2,197	0.43
Days in milk at dry-off			363 ± 102			354 ± 92	0.14
Previous lactation daily milk, kg/d			21.4 ± 5.2			21.7 ± 5.4	0.31
Previous lactation CM (yes), % (n)[6]			38 (276)			39 (269)	0.87
SCC at dry-off (high), % (n)[7]			54 (388)			57 (398)	0.19

[1]The *P*-value corresponds to statistical comparison of the two treatment groups.
[2]Each season correspond to approximately 1/3 of the duration (d) of the trial.
[3]NEFA = nonesterified fatty acids.
[4]In treated cows this corresponds to the interval between two doses.
[5]Lactation number of the enrolled cows after calving
[6]32 and 34 Control and PEG cows respectively have no previous data of CM.
[7]proportion of cows with high (> 200,000 cell/mL) SCC at the last test day of previous lactation.

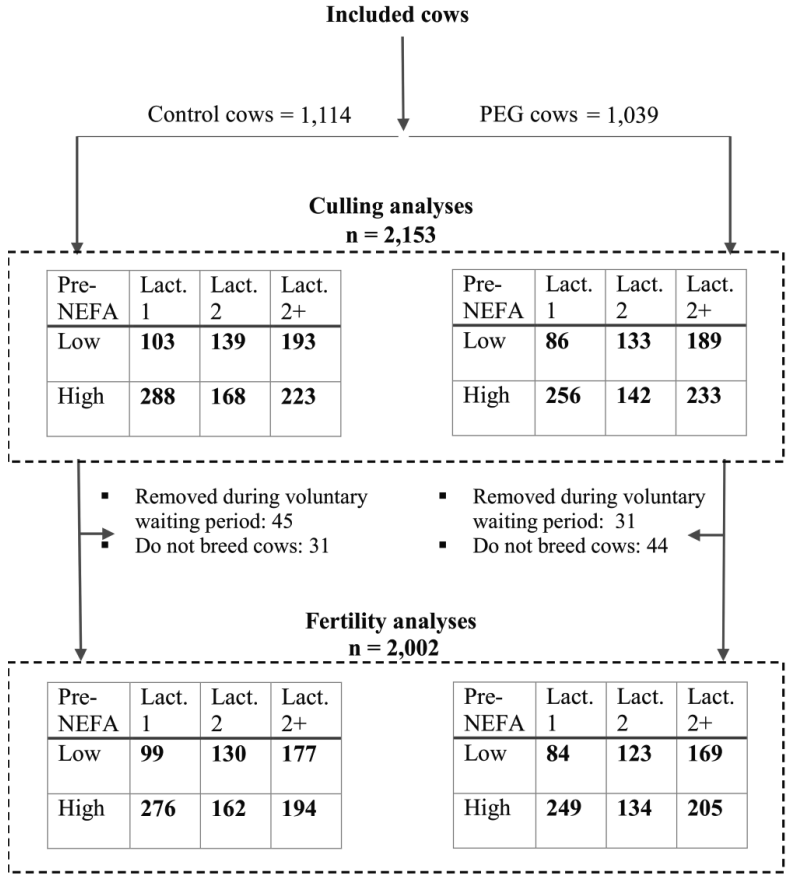

Figure 4.1. Total included cows and cows that were considered for fertility analyses. Low prepartum NEFA ≤ 0.3 m*M* > High prepartum NEFA.

4.3.2. Effect of treatment with Pegbovigrastim on rate of first insemination

In total, 1,835 out of 2,002 (91.7%) cows that were considered at risk for breeding were inseminated. The mean and standard deviation of time to first insemination was 80 ± 39 DIM and the range was 3 to 305 DIM. Out of these 1,835 cows, 14 (0.8%) cows were inseminated within 30 DIM. Kaplan-Meier analyses showed that CM and metritis were sufficiently associated ($P < 0.2$) with rate of first insemination to include in the multivariable modeling process.

The differences in PEG treatment results between Cox proportional hazards model 1 (only pre-treatment variables) and model 2 (pre-treatment variables + clinical disease) were minor, so we only present the outcome of model 2 in the main body of this chapter. Model 1 is presented in Table S 4.2.

In the Cox proportional hazards regression model for first insemination, we found an interaction between prepartum NEFA and PEG treatment, as High prepartum NEFA cows, when treated with PEG, showed an increased rate of first insemination (HR = 1.25, $P = 0.02$, Table 4.2, Figure 4.2). Clinical mastitis was associated with a decreased rate of first insemination (HR = 0.79, $P < 0.001$). Metritis by itself was not associated with the rate of first insemination but interacted with prepartum NEFA, as High prepartum NEFA cows with metritis showed a decreased rate of first insemination (HR = 0.75, $P = 0.03$).

Table 4.2. Cox proportional hazards regression model of first insemination including pre-treatment variables and clinical disease (Control = 1,038; PEG = 964).

	Estimate	SE	*P* - value	Hazard ratio
Lactation				
1	0.06	0.06	0.33	1.06
2+	-0.12	0.08	0.04	0.89
prepartum NEFA	-0.13	0.08	0.11	0.88
Treatment	-0.08	0.08	0.29	0.92
Calving month*			0.002	
Clinical Mastitis	-0.23	0.07	<0.001	0.79
Metritis	0.11	0.11	0.31	1.12
prepartum NEFA x Treatment	0.22	0.10	0.02	1.25
Metritis x prepartum NEFA	-0.29	0.13	0.03	0.75

Reference groups: Lactation 2, Low prepartum NEFA (≤ 0.3m*M*), Control, Calving month 4, No Clinical Mastitis and No metritis.
*Overall *P* – value for all calving month classes (type III test).

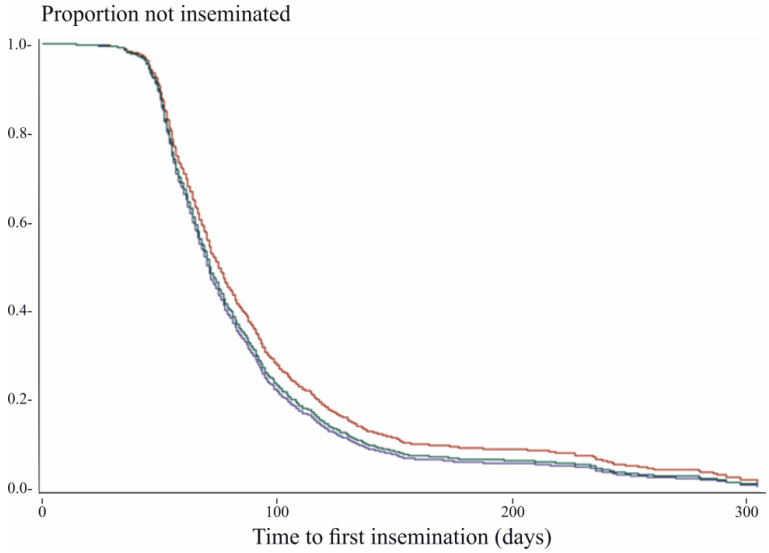

Figure 4.2. Survival curves of time to first insemination for: Low prepartum NEFA Control cows (n = 406, blue line); High prepartum NEFA Control cows (n = 632, red line) and High prepartum NEFA PEG cows (n = 588; green line). Low prepartum NEFA ≤ 0.3 m*M* > High prepartum NEFA.

4.3.3. Effect of treatment with Pegbovigrastim on rate of pregnancy

In total, 1,325 out of 2,002 (66.2%) cows that were considered at risk for breeding were identified as pregnant. Of these 1,325 cows, 35 (2.6%) became pregnant beyond 305 DIM, and were thus censored at 305 DIM in our analyses. At 150 DIM, 1,028 (51.3%) cows were pregnant while at 305 DIM this was 1,290 (64.4%). The mean and standard deviation of time to pregnancy in all 1,325 analyzed cows was 114 ± 56 DIM and the range was 17 to 305 DIM. When censoring either at 150 DIM or 305 DIM, Kaplan-Meier analyses showed that CM, RP, metritis, UD and endometritis were sufficiently associated ($P < 0.2$) with rate of pregnancy to include in the multivariable modeling process. As RP and metritis were both associated with rate of pregnancy, and UD was also associated with pregnancy, only the latter was used in the multivariable modeling process.

The Cox proportional hazards regression model 1 (pre-treatment variables only) for pregnancy, censored at 150 or 305 DIM, did not show treatment effects (Table S 4.2).

Table 4.3 presents the Cox proportional hazards regression model 2 (with pre-treatment variables and clinical disease) for pregnancy. Censoring at 150 DIM, we found a three-way interaction prepartum NEFA by Treatment by UD. High prepartum NEFA tended to be associated with decreased rate of pregnancy (HR = 0.87, $P = 0.09$) and UD was also associated with decreased rate of pregnancy (HR = 0.74, $P = 0.02$), while High prepartum NEFA PEG treated cows that subsequently recorded UD showed an increased rate of pregnancy (HR = 1.55, $P = 0.005$). Clinical mastitis was associated with decreased rate of pregnancy (HR = 0.69, $P < 0.001$) and endometritis tended to be associated with decreased rate of pregnancy (HR = 0.71, $P = 0.06$).

Within 305 DIM, the Cox proportional hazards regression model 2 for pregnancy (Table 4.3) showed that UD was associated with decreased rate of pregnancy (HR = 0.78, $P = 0.01$) and we found that PEG treated cows that subsequently recorded UD tended to show an increased rate of pregnancy (HR = 1.30, $P = 0.06$). Clinical mastitis and endometritis were associated with a decreased rate of pregnancy (HR = 0.75, $P < 0.001$ and HR = 0.74, $P = 0.05$, respectively). When endometritis was removed from the model, the treatment by UD interaction became significant (HR = 1.32, $P = 0.04$).

Table 4.3. Cox proportional hazards regression model of pregnancy including pre-treatment variables and clinical diseases (Control = 1,038; PEG = 964).

	Censored at 150 days in milk				Censored at 305 days in milk			
	Estimate	SE	*P* - value	Hazard ratio	Estimate	SE	*P* - value	Hazard ratio
Lactation								
1	0.16	0.08	0.04	1.18	0.13	0.07	0.06	1.14
2+	-0.22	0.08	0.006	0.80	-0.26	0.07	<0.001	0.77
Prepartum NEFA	-0.14	0.08	0.09	0.87				
Treatment	-0.10	0.07	0.14	0.90	-0.10	0.06	0.11	0.90
Clinical Mastitis	-0.38	0.10	<0.001	0.69	-0.28	0.09	<0.001	0.75
Uterine disease	-0.30	0.10	0.002	0.74	-0.25	0.10	0.01	0.78
Endometritis	-0.34	0.18	0.06	0.71	-0.29	0.15	0.05	0.74
Treatment x Uterine disease	-	-	-	-	0.26	0.14	0.06	1.30
Prepartum NEFA x Treatment x Uterine disease	0.44	0.16	0.005	1.55	-	-	-	-

Reference groups: Lactation 2, Low prepartum NEFA (≤ 0.3m*M*), Control, No Clinical Mastitis, No Uterine disease and No endometritis.

4.3.4. Effect of treatment with Pegbovigrastim on hazard of culling

During the study period, 425 out of 2,153 (19.7%) cows were removed from the herd (i.e. death or sales). The mean and standard deviation of time to removal was 187 ± 130 DIM and the range was 0 to 491 DIM. Out of these 425 cows, 88 cows (20.7%) were culled after 305 DIM. Kaplan-Meier analyses showed that CM and metritis were sufficiently associated ($P < 0.2$) with hazard of culling to include in the multivariable modeling process.

Since no differences were found in the treatment results between model 1 (pre-treatment variables only; Table S 4.2) and model 2 (pre-treatment variables and clinical diseases), we only present the latter.

Table 4.4 presents the Cox proportional hazards regression model 2 for culling. We found a tendency for prepartum NEFA by treatment interaction. High prepartum NEFA was associated with increased hazard of culling (HR = 1.42, $P = 0.02$) while High prepartum NEFA PEG treated cows tended to show a decreased hazard of culling (HR = 0.71, $P = 0.09$). Clinical mastitis and metritis were associated with increased hazard of culling (HR = 1.99, $P < 0.001$ and HR = 1.32, $P = 0.02$, respectively).

Table 4.4. Cox proportional hazards regression model of culling (i.e. death or sales) including pre-treatment variables and clinical diseases (Control = 1,114; PEG = 1,039).

	Estimate	SE	*P* - value	Hazard ratio
Lactation				
1	-0.06	0.15	0.67	0.94
2+	0.80	0.13	<0.001	2.23
prepartum NEFA	0.35	0.15	0.02	1.42
Treatment	0.10	0.16	0.53	1.10
Clinical Mastitis	0.69	0.11	<0.001	1.99
Metritis	0.28	0.12	0.02	1.32
prepartum NEFA x Treatment	-0.34	0.20	0.09	0.71

Reference groups: Lactation 2, low prepartum NEFA (≤ 0.3m*M*), Control, No Clinical Mastitis and No Metritis.

4.3.5. Effect of treatment with Pegbovigrastim on rate of first insemination, rate of pregnancy and hazard of culling stratified by prepartum NEFA class and parity

When performing the Cox proportional hazards regression model for first insemination on stratified data, in the stratum of High prepartum NEFA cows (Table 4.5), we found

that PEG increased the rate of first insemination (HR = 1.15, $P = 0.02$), while CM and metritis were associated with decreased rate of first insemination (HR = 0.77, $P = 0.003$ and HR = 0.85, $P = 0.03$, respectively). No treatment effect was detected in the stratum of Low prepartum NEFA cows (Table S 4.3). Stratifying by parity group (Table S 4.3), we found a tendency for prepartum NEFA by treatment interaction in multiparous cows, as multiparous High prepartum NEFA PEG treated cows tended to show an increased rate of first insemination (HR = 1.25, $P = 0.07$). No treatment effect was detected in primiparous cows.

Table 4.5. Cox proportional hazards regression model of first insemination, pregnancy (Control = 632; PEG = 588) and culling (i.e. death or sales); Control = 679; PEG = 631) in High prepartum NEFA (> 0.3 m*M*) cows, including pre-treatment variables and clinical disease.

Hazard of:	Effect	Estimate	SE	*P* – value	Hazard ratio
First insemination	Lactation				
	1	0.02	0.08	0.74	1.02
	2+	-0.12	0.08	0.15	0.89
	Treatment	0.14	0.06	0.02	1.15
	Clinical Mastitis	-0.26	0.09	0.003	0.77
	Metritis	-0.16	0.08	0.03	0.85
	Calving month*			0.01	
Pregnancy (150DIM)	Lactation				
	1	0.18	0.10	0.08	1.20
	2+	-0.21	0.12	0.07	0.81
	Treatment	-0.13	0.10	0.19	0.88
	Clinical Mastitis	-0.71	0.20	<0.001	0.49
	Uterine disease	-0.25	0.14	0.08	0.78
	Endometritis	-0.36	0.21	0.09	0.70
	Clinical Mastitis x Treatment	0.56	0.27	0.04	1.75
	Uterine disease x Treatment	0.37	0.20	0.06	1.45
Pregnancy (305DIM)	Lactation				
	1	0.20	0.09	0.03	1.22
	2+	-0.22	0.10	0.03	0.80
	Treatment	-0.08	0.09	0.38	0.92
	Clinical Mastitis	-0.34	0.11	0.003	0.71
	Uterine disease	-0.20	0.13	0.11	0.82
	Endometritis	-0.29	0.18	0.10	0.75
	Calving month*			0.02	
	Uterine disease x Treatment	0.31	0.17	0.07	1.36
Culling	Lactation				
	1	-0.07	0.18	0.69	0.93
	2+	0.82	0.17	<0.001	2.27
	Treatment	-0.24	0.12	0.05	0.79
	Clinical Mastitis	0.70	0.14	<0.001	2.02
	Metritis	0.28	0.14	0.05	1.32

Reference groups: Lactation 2, Control, No Clinical Mastitis, No Metritis, No Uterine disease, No endometritis and Calving month 4.
*Overall *P* – value for all calving month classes (type III test).

Cox proportional hazards regression model for pregnancy within 150 DIM showed that, in High prepartum NEFA cows (Table 4.5), CM was strongly associated with decreased rate of pregnancy (HR = 0.49. $P < 0.001$), whereas PEG treated cows that subsequently

recorded CM showed an increased rate of pregnancy (HR = 1.75, *P* = 0.04). Figure 4.3 (Panel A) illustrates the treatment by CM interaction in High prepartum NEFA cows. Uterine disease tended to be associated with decreased rate of pregnancy (HR = 0.78, *P* = 0.08) and PEG treated cows that subsequently recorded UD tended to show an increased rate of pregnancy (HR = 1.45, *P* = 0.06). Endometritis tended to be associated with decreased rate of pregnancy (HR = 0.70, P = 0.09). When endometritis was removed from the model, the negative association of UD with rate of pregnancy (HR = 0.75, *P* = 0.04) as well as the treatment by UD interaction (HR = 1.46, *P* = 0.05) became statistically significant. Figure 4.3 (Panel B) illustrates the treatment by UD interaction in High prepartum NEFA cows. No treatment effects were detected in Low prepartum NEFA cows or in separate parity groups (Table S 4.3).

For the model of pregnancy within 305 DIM, we found that High prepartum NEFA PEG treated cows that subsequently recorded UD tended to show an increased rate of pregnancy (HR = 1.36, *P* = 0.07) (Table 4.5). Clinical mastitis was associated with a decreased rate of pregnancy (HR = 0.71, P = 0.003) and endometritis tended to be associated with decreased rate of pregnancy (HR = 0.75, P = 0.10). No treatment effects were detected in Low prepartum NEFA cows (Table S 4.3). In multiparous cows, UD was associated with decreased rate of pregnancy (HR = 0.77, *P* = 0.05) and we found that multiparous cows treated with PEG that subsequently recorded UD tended to show an increased rate of pregnancy (HR = 1.38, *P* = 0.08). No treatment effects were detected in primiparous cows (Table S 4.3).

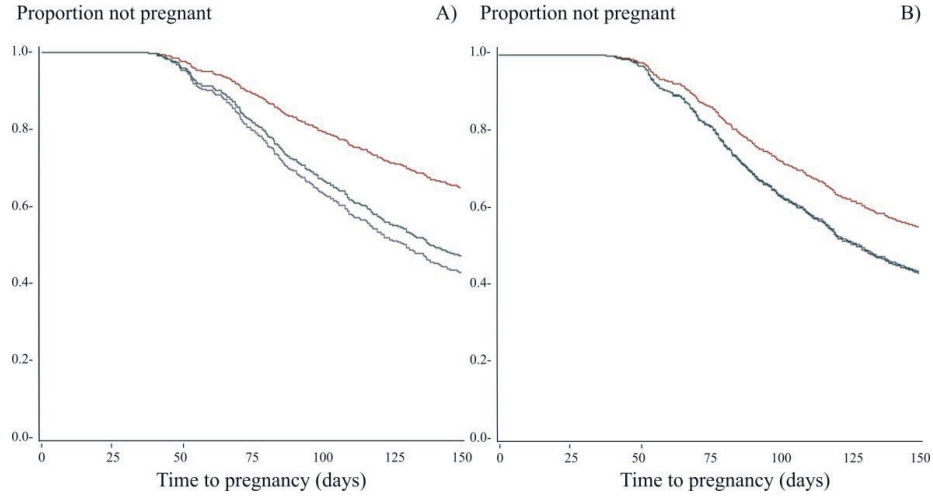

Figure 4.3. Survival curves of time to pregnancy. A) Low prepartum NEFA Control cows without clinical mastitis (n = 344; blue line); High prepartum NEFA Control cows with clinical mastitis (n = 105; red line) and High prepartum NEFA PEG cows with clinical mastitis (n = 90; green line). B) Low prepartum NEFA Control cows without Uterine disease (n = 333; blue line); High prepartum NEFA Control cows with Uterine disease (n = 149; red line) and High prepartum NEFA PEG cows with Uterine disease (n = 168; green line). Note: blue and green lines are virtually overlapping. Uterine disease: a cow with a record of retained placenta, metritis or both. Low prepartum NEFA ≤ 0.3 m*M* > High prepartum NEFA.

When performing the Cox proportional hazards regression model for culling on stratified data, in High prepartum NEFA cows (Table 4.5), we found that treatment with PEG decreased the hazard of culling (HR = 0.79, $P = 0.05$), whereas CM and metritis were associated with increased hazard of culling (HR = 2.02, $P < 0.001$ and HR = 1.32, P = 0.05, respectively). No treatment effects were found in Low prepartum NEFA cows (Table S 4.3). Figure 4.4 (panel A) illustrates the effect of PEG treatment on time to culling.

In multiparous cows, we found that PEG treatment tended to counteract the negative association between CM and hazard of culling. Clinical mastitis was strongly associated with increased hazard of culling (HR = 2.61, $P < 0.001$) while cows treated with PEG that subsequently recorded a CM case tended to show a decreased hazard of culling (HR =

0.62, $P = 0.06$, Figure 4.4 (panel B)). No treatment effects were detected in primiparous cows (Table S 4.3).

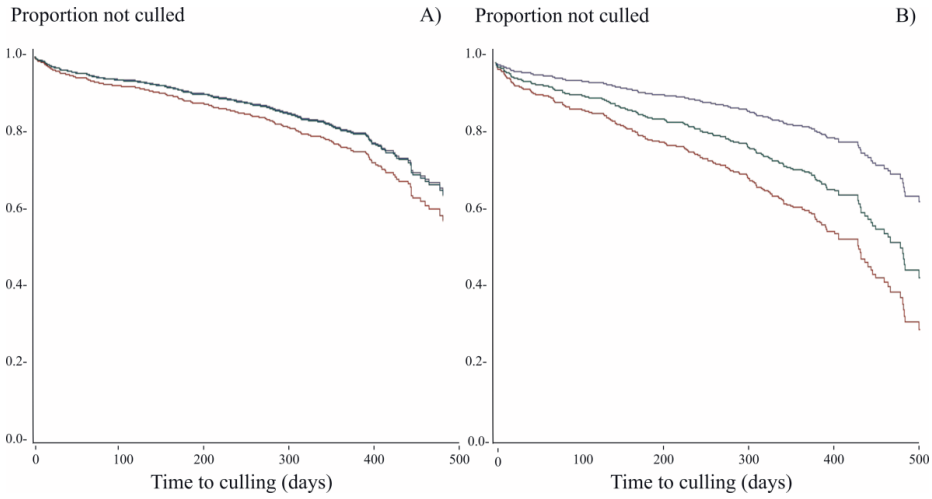

Figure 4.4. Survival curves of time to culling (i.e. death or sales). Panel A: Low prepartum NEFA Control cows (n = 435, blue line); High prepartum NEFA Control cows (n = 679, red line); High prepartum NEFA PEG cows (n = 631, green line). Low prepartum NEFA ≤ 0.3 m*M* > High prepartum NEFA. Note: blue and green lines are virtually overlapping. Panel B: Multiparous Control cows without clinical mastitis (n = 589, blue line); multiparous Control cows with clinical mastitis (n = 134, red line) and multiparous PEG cows with clinical mastitis (n = 111, green line).

4.4. Discussion

This study reports for the first time the effect of PEG on fertility and culling during a full lactation in grazing dairy cows. The main finding was the presence of an interaction between prepartum NEFA and treatment. In High prepartum NEFA cows, PEG increased the rate of first insemination and counteracted the negative association of early lactation CM and UD with the rate of pregnancy within 150 DIM. Similarly, in High prepartum NEFA cows, PEG treatment decreased the hazard of culling.

Treatment with PEG did not affect the overall rate of first insemination, but interacted with prepartum NEFA, as PEG increased the rate of first insemination only in High prepartum NEFA cows. Previous studies showed that PEG increased the rate of

insemination within 100 DIM by 5.8 % (Ruiz et al., 2017), while no effect was reported within 120 DIM when only cows in an optimal BCS were included (Zinicola et al., 2018), or within 150 DIM when including cows with no restrictions based on BCS (Van Schyndel et al., 2021). We are not aware of other studies showing that the effect of PEG on the rate of first insemination is associated with lipid mobilization in cows. As the prepartum NEFA by treatment interaction effect on rate of first insemination remained unchanged in the statistical models with or without clinical diseases, our data would suggest that reduction in occurrence of clinical disease (Barca et al., 2021b) is not the only mechanism by which PEG treatment increases the rate of first insemination in High prepartum NEFA cows. Treatment with PEG causes an increase in WBC counts during the critical period around calving (Kimura et al., 2014; McDougall et al., 2017; Van Schyndel et al., 2018), when NEB impairs the immune response (Trevisi et al., 2018; Gross and Bruckmaier, 2019). We recently reported that PEG treatment reversed the negative association of prepartum NEFA with postpartum neutrophil counts (Barca et al., 2021a). This could potentially decrease the impact of early lactation subclinical disease which is also negatively associated with fertility (Sheldon et al., 2006; Dolecheck et al., 2019). In a small study it was observed that PEG treatment was associated with lower postpartum NEFA concentration (Kimura et al., 2014). Lüttgenau et al. (2016) showed a negative association between postpartum NEFA concentration and rate of insemination. Therefore, the prepartum NEFA by treatment interaction identified in the model for rate of first insemination, which remained significant with and without clinical disease, could be explained by a reduction in subclinical disease or by a direct PEG effect on energy metabolism.

Treatment with PEG did not directly affect the rate of pregnancy within 150 DIM or 305 DIM, and there was no interaction of treatment with prepartum NEFA. Similarly, no effect of PEG on rate of pregnancy was reported in earlier studies (Zinicola et al., 2018; Van Schyndel et al., 2021). Interestingly, in High prepartum NEFA cows, PEG treatment counteracted the negative association of UD with rate of pregnancy within 150 DIM, as shown by the three-way interaction in the model 2, while for pregnancy within 305 DIM, PEG tended to counteract this negative association regardless of prepartum NEFA, as shown by the two-way interaction in the model 2. Further analyses on stratified data showed that this PEG treatment effect occurred predominantly in High prepartum NEFA cows. In High prepartum NEFA cows that subsequently recorded CM and UD, treatment

with PEG respectively increased and tended to increase, the rate of pregnancy within 150 DIM. As endometritis may be considered a consequence of previous uterine clinical events, we removed it from the model to estimate the total effect of UD on pregnancy (Toni et al., 2015). By removing endometritis from the model, the association of UD and the treatment by UD interaction became significant; therefore, we interpret endometritis as an intervening variable between UD and subsequent pregnancy. Consequently, we conclude that PEG significantly counteracted the negative association of UD with rate of pregnancy, which is consistent with our finding that PEG reduced endometritis occurrence in cows that had previous metritis (Barca et al., 2021b). Fourichon et al. (2000), in a meta-analysis, reported that RP was associated with a slight increase in days to first insemination and resulted in a more evident delay in days to pregnancy, while metritis consistently increased both days to first insemination and days to pregnancy. Carvalho et al. (2019) reported that, while UD was not associated with rate of first insemination, it was with rate of pregnancy and suggested that long-term effects of UD extend beyond the first postpartum insemination. These studies indicate that it is likely that early postpartum UD has a long-term impact on the uterine environment, resulting in lower conceptus viability. In High prepartum NEFA cows with CM, we only detected an effect of PEG treatment on the hazard of pregnancy within 150 DIM, and not for 305 DIM. At the same time, the data suggest that the interaction of PEG and UD also appears to be stronger for pregnancy within 150 DIM than within 305 DIM. This suggests that early improvement of the immune response due to PEG contributes to a rapid resolution of postpartum inflammatory processes, which has been proposed as a mechanism to achieve subsequent successful fertility (Bradford et al., 2015). Our data are consistent with our hypothesis that PEG treatment restores immune function in cows with elevated prepartum NEFA concentration (Barca et al., 2021a). The ability to recruit neutrophils into the mammary gland and into the endometrium soon after calving is essential for mastitis resolution, uterine involution and subsequent fertility (Schukken et al., 2011; Gilbert and Santos, 2016). Powell et al. (2018) showed that PEG primed neutrophils for quick recruitment to the infected mammary gland and Ruiz et al. (2017) suggested higher neutrophil influx into the uterus due to PEG treatment. In multiparous cows that subsequently recorded UD, PEG tended to increase the rate of pregnancy within 305 DIM 1.38 times, counteracting the negative association between UD and rate of pregnancy. Toni et al. (2015) found that metritis resulted in lower fertility only in multiparous cows and suggested that this was a consequence of lower immune function in these older cows.

Therefore, it is possible that treatment with PEG is more beneficial for cows with UD in their second and higher lactation.

Treatment with PEG did not affect the overall hazard of culling, but in the High prepartum NEFA stratum, PEG resulted in a lower hazard of culling, reinforcing the concept that PEG treatment is beneficial particularly in metabolically challenged cows. A lack of effect on the hazard of culling during the first 63 DIM and 180 DIM has been reported (Van Schyndel et al., 2021; Zinicola et al., 2018, respectively). In these studies, the prepartum NEFA concentration by treatment interaction was not assessed. Interestingly, both Zinicola et al. (2018) and Van Schyndel et al. (2021) in randomly chosen subsets of cows, reported low prepartum NEFA concentrations, well under 0.3 m*M*. In our data, prepartum NEFA concentration was ~ 0.50 ± 0.40 m*M,* while 61% of the cows had a prepartum NEFA concentration above 0.3 m*M*. Moreover, we found an incidence of CM during the first 30 DIM that was more than 4 times higher than in both cited studies. The elevated prepartum NEFA concentration, the relatively high incidence of CM and the follow-up during the full lactation, especially important for culling (Ribeiro et al., 2016; Carvalho et al., 2019), may explain apparent differences between the studies. As expected, CM was associated with increased hazard of culling (Kossaibati and Esslemont, 1997; Bar et al., 2008; Hertl et al., 2018). However, the reduction of CM in PEG treated cows, particularly in those with an elevated prepartum NEFA concentration (Barca et al., 2021b), may be only a partial explanation for the finding of a decreased hazard of culling in High prepartum NEFA PEG treated cows. Similar to our findings on the rate of insemination, the prepartum NEFA by treatment interaction remained present in the models with or without clinical disease, showing that PEG decreased the hazard of culling in High prepartum NEFA cows independently of a reduction in the occurrence of clinical diseases. Undoubtedly, a major reason for culling is reproductive failure (Kossaibati and Esslemont, 1997; LeBlanc et al., 2002) and we found that PEG treatment improved fertility in High prepartum NEFA cows (i.e. higher rate of first insemination and higher rate of pregnancy in either CM or UD cows). Thus, the data suggest that the reduction in hazard of culling in PEG treated cows may be a combination of a reduction in CM and improved fertility. Stratified analyses showed that, in multiparous cows that subsequently recorded CM, PEG treatment tended to result in a lower hazard of culling. Moreover our data suggests that CM impaired fertility, particularly in multiparous cows. It has been shown that multiparous cows respond more severely to the same intramammary challenge

(Vangroenweghe et al., 2004); thus, the suggested immune modulation due to PEG, decreasing the severity of CM (Powell et al., 2018), would be more beneficial for multiparous cows. The finding that PEG treatment decreased the hazard of culling is particularly important, since it is one of the main factors associated with the negative economic consequences of disease in dairy farming (Hogeveen et al., 2017).

We allocated treatment based on the national ear tag number. If there would be periodicity in the allocation of the national ears tags at birth, bias would have been introduced. However, these consecutively numbered tags are allocated to the calves in order of birth, which makes bias in relation to future performance unlikely. Moreover, the balance between treatment groups suggests that randomization was successful. A second potential limitation is the fact that research assistants who applied the treatment were aware of treatment status, and this could bias their objectivity when assessing BCS, metritis and endometritis. However, all cow observations were linked to the cow ID, an ear tag number completely different and unrelated to the national ear tag number. Moreover, all farm personnel and veterinarians that assessed the outcomes of interest in this study (estrus detection, insemination, pregnancy diagnoses and culling decisions) and laboratory personnel that determined prepartum NEFA concentrations were blinded with regard to treatment status.

We consider that these results contribute to a more efficient use of PEG treatment, since our data suggest that it may be used specifically in cows at risk for immune imbalances related to metabolic disturbances, as present in High prepartum NEFA cows. In this study we found a relatively high prevalence (61%) of animals with increased prepartum NEFA values, being apparently higher in primiparous cows (74%) than in multiparous cows (54%). This is consistent with previous reports from our research group in pasture-based herds (Meikle et al., 2004; Adrien et al., 2012). Despite major advances in diagnosis and management of NEB (Overton et al., 2017), excessive NEB still affects an important number of modern dairy cows (Ospina et al., 2013; Macrae et al., 2019). The challenge is to identify High prepartum NEFA cows easily and fast, as there is currently no cow-side test available (Overton et al., 2017). The emerging areas of big data, metabolomics and the use of automatic sensors to measure metabolic health (Overton et al., 2017) are promising technologies to identify cows that would benefit most from treatment with PEG. No association of prepartum BCS or its interaction with treatment was found with any of the outcomes considered. There is an association between prepartum BCS and

prepartum NEFA concentration (Roche et al., 2015), which could potentially introduce multicollinearity in the statistical models. However, since modeling was done using a manual forward selection procedure, variables such as prepartum BCS and prepartum NEFA were offered to the models one by one, preventing potential multicollinearity and overlapping effects. It has been shown that cows that lost body condition before calving had increased peripartum NEFA concentrations (Sheehy et al., 2017; Barletta et al., 2017). We only assessed BCS once, immediately before treatment. In future studies, BCS dynamics, which may now be performed using commercially available automated sensors (Mullins et al., 2019) would be more relevant than a one-time assessment and could give further insights in the use of PEG.

Our results suggest an important impact of PEG treatment on cow health (Barca et al., 2021b), fertility and longevity (this study). Further economic analyses will be necessary to evaluate the cost benefit ratio of PEG treatment.

4.5. Conclusions

This study shows that the effect of PEG treatment on fertility and culling interacts with prepartum NEFA. In Low prepartum NEFA cows, no treatment effect was detected. In High prepartum NEFA cows, PEG treatment increased the rate of first insemination, counteracted the negative association of early lactation CM and UD with the rate of pregnancy and decreased the hazard of culling.

4.6. Acknowledgments

The cooperation of farmers and farm personnel is gratefully acknowledged.

4.7. Supplemental materials

Table S 4.1. Prepartum partial mixed ration composition (kg) used in each farm.

	Farm 1	Farm 2	Farm 3	Farm 4
Corn silage	16	20		9
Sorghum silage	-	-	15	
Alfalfa silage				10
Wheat bran	3	-		
Wet grain corn	2	4		5.5
Soy expeller	0.5	1		2
Sorghum DDGS	-	2	3.7	4
Anionic salts	0.3	0.35	0.3	0.35
Barley hay	*Ad libitum*			
Bale of wheat		5	*Ad libitum*	

Table S 4.2. Cox proportional hazards regression model of first insemination, pregnancy (Control = 1038; PEG = 964) and culling (i.e. death or sales; Control = 1114; PEG = 1039) including pre-treatment variables only.

Hazard of:	Effect	Estimate	SE	P - value	Hazard ratio
Insemination	Lactation				
	1	0.06	0.06	0.29	1.07
	2+	-0.14	0.06	0.02	0.87
	Pre-NEFA	-0.18	0.08	0.02	0.84
	Treatment	-0.08	0.08	0.31	0.92
	Pre-NEFA x Treatment	0.21	0.10	0.03	1.23
Pregnancy (150DIM)	Lactation				
	1	0.14	0.08	0.08	1.14
	2+	-0.23	0.08	0.004	0.79
	Treatment	-0.03	0.06	0.63	0.97
Pregnancy (305DIM)	Lactation				
	1	0.13	0.07	0.06	1.14
	2+	-0.26	0.07	<0.001	0.77
	Treatment	-0.04	0.06	0.50	0.96
	Calving month*			0.04	
Culling	Lactation				
	1	-0.08	0.15	0.60	0.92
	2+	0.82	0.13	<0.001	2.27
	Pre-NEFA	0.36	0.15	0.02	1.43
	Treatment	0.10	0.16	0.54	1.10
	Pre-NEFA x Treatment	-0.35	0.20	0.08	0.70

Reference groups: Lactation 2, Low Pre-NEFA (≤ 0.3m*M*), Control, Calving month 4.
*Overall *P* – value for all calving month classes (type III test).

Table S 4.3. Cox proportional hazards regression model of first insemination, pregnancy and culling (i.e. deaths or sales), including pre-treatment variables and clinical diseases in Low Pre-NEFA cows, primiparous cows and multiparous cows.

		Low Pre-NEFA cows				Primiparous cows				Multiparous cows			
Hazard of:	Effect	Estimate	SE	P - value	Hazard ratio	Estimate	SE	P - value	Hazard ratio	Estimate	SE	P - value	Hazard ratio
Insemination	Lactation												
	1	0.14	0.10	0.18	1.15								
	2+	-0.11	0.09	0.22	0.90								
	Treatment	-0.06	0.08	0.45	0.94	0.11	0.08	0.16	1.12	-0.11	0.09	0.19	0.90
	Pre-NEFA					-0.23	0.12	0.05	0.79	-0.12	0.09	0.18	0.89
	Clinical Mastitis	-0.20	0.11	0.08	0.82					-0.32	0.08	<0.01	0.72
	Calving month			<0.001								<0.001	
	Pre-NEFA x Treatment									0.22	0.12	0.07	1.25
Pregnancy (150DIM)	Lactation												
	1	0.09	0.13	0.48	1.10								
	2+	-0.23	0.11	0.04	0.79								
	Treatment	-0.12	0.10	0.22	0.89	-0.04	0.10	0.70	0.96	-0.05	0.08	0.54	0.95
	Clinical Mastitis	-0.31	0.15	0.04	0.73					-0.47	0.13	<0.001	0.62
	Uterine disease	-0.35	0.14	0.01	0.71	-0.21	0.12	0.08	0.81				
	Endometritis									-0.47	0.25	0.06	0.62
Pregnancy (305DIM)	Lactation												
	1	0.06	0.12	0.62	1.06								
	2+	-0.30	0.10	0.002	0.74								
	Treatment	-0.10	0.08	0.23	0.90	-0.06	0.09	0.53	0.94	-0.11	0.08	0.16	0.90
	Clinical Mastitis	-0.23	0.13	0.07	0.79					-0.41	0.11	<0.001	0.66
	Uterine disease	-0.32	0.12	0.007	0.73					-0.26	0.14	0.05	0.77
	Endometritis									-0.36	0.21	0.09	0.70

	Calving month												0.02
	Uterine disease x Treatment									0.32	0.18	0.08	1.38
Culling	Lactation												
	1	0.03	0.27	0.92	1.03								
	2+	0.82	0.20	<0.001	2.27								
	Treatment	0.02	0.16	0.89	1.02	-0.26	0.20	0.21	0.77	0.09	0.13	0.51	1.09
	Clinical Mastitis	0.69	0.18	<0.001	2.00	0.62	0.24	0.01	1.86	0.96	0.16	<0.001	2.61
	Metritis					0.62	0.21	0.003	1.86				
	Clinical Mastitis x Treatment									-0.48	0.25	0.06	0.62

Reference groups: Lactation 2, Control group, No Clinical Mastitis, No metritis, No Uterine disease (i.e. a cow with record of retained placenta, metritis or both), No endometritis, Calving month 4.
Low Pre-NEFA < 0.03 m*M* > High Pre-NEFA.
*Overall *P* – value for all calving month classes (type III test).
Culling analyses: Low Pre-NEFA cows: Control = 435, PEG = 408, primiparous cows: Control = 391, PEG = 342, multiparous cows: Control = 723, PEG = 697.
Fertility analyses: Low Pre-NEFA cows: Control = 406, PEG = 376, primiparous cows: Control = 375, PEG = 333, multiparous cows: Control = 663, PEG = 631

CHAPTER 5

5

Pegbovigrastim treatment resulted in an economic benefit in a large randomized clinical trial in grazing dairy cows

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Abstract

This randomized controlled trial on four commercial grazing dairy farms investigated whether pegbovigrastim (PEG) treatment affected partial net return as calculated from milk revenues and costs for feed, medical treatments [clinical mastitis (CM), uterine disease (UD) and other diseases (i.e. any medical treatment that was not intended for CM or UD)], inseminations and culling during a full lactation in grazing dairy cows. We also explored the effect of potential interactions of PEG treatment with parity, prepartum body condition score and prepartum nonesterified fatty acids (NEFA) concentration on partial net return, milk revenues and the costs mentioned above. Holstein cows were randomly assigned to one of two trial arms: a first PEG dose 9.4 ± 0.3 [mean ± standard error (SE)] days before the calving date and a second dose within 24 h after calving (PEG: primiparous = 342; multiparous = 697) compared to untreated controls (Control: primiparous = 391; multiparous = 723). The effect of PEG treatment on the outcomes of interest expressed per year was tested using general linear mixed models. Results are presented as least squares means ± SE. Overall, PEG treatment increased the partial net return, resulting in an economic benefit per cow per year of US$ 210 ± 100. The cost of treatment of CM was lower for PEG treated cows compared to control cows (US$9 ± 3). The largest non-significant difference was seen for the cost of culling: PEG treatment numerically reduced the cost of culling by US$ 145 ± 77.

5.1. Introduction

Animal diseases associated with the transition period in dairy cows, such as clinical mastitis (CM), uterine disease, and metabolic diseases, have a negative effect on health, welfare and the economic performance of dairy farming (Overton and Fetrow, 2008; Hogeveen et al., 2019; Steeneveld et al., 2020). Production loss, discarded milk during the withdrawal period after medical treatment and the actual cost of treatments are important direct economic effects of early lactation diseases (Hogeveen et al., 2011; Pérez-Báez et al., 2021). Moreover, indirect effects of these diseases can be seen in impaired reproductive performance (Fourichon et al., 2000; LeBlanc et al., 2001; Dolecheck et al., 2019) and culling (Kossaibati and Esslemont, 1997; Bar et al., 2008; Carvalho et al., 2019). Multiple studies showed that culling makes an important contribution to the costs of clinical disease (Galligan, 2006; Overton and Fetrow, 2008; Heikkila et al., 2012).

Pegbovigrastim, a long-acting analogue of bovine granulocyte colony-stimulating factor (PEG, marketed as Imrestor by Elanco Animal Health), has been used as a tool to improve dairy cows' immune response during the transition period. Pegbovigrastim treatment consistently increases circulating white blood cell counts (McDougall et al., 2017; Van Schyndel et al., 2018; Barca et al., 2021a). Moreover, PEG treatment improves immune function at plasma cytokine profile and white blood cell gene expression level (Lopreiato et al., 2019; Lopreiato et al., 2020). Several field trials have evaluated the effect of PEG treatment, mainly on early lactation clinical disease. A number of these studies also report milk production, fertility and culling (Canning et al., 2017; Ruiz et al., 2017; Freick et al., 2018; Zinicola et al., 2018; Van Schyndel et al., 2021). A lower early lactation CM incidence due to PEG treatment was reported by Canning et al. (2017) and Ruiz et al. (2017), but two studies (Zinicola et al., 2018; Van Schyndel et al., 2021) reported no effects on early lactation CM incidence. Overall PEG treatment results ranged from a preventive effect for early lactation clinical disease (Canning et al., 2017; Freick et al., 2018) to increased morbidity (Zinicola et al., 2018), in particular for metritis (Ruiz et al., 2017). Recently, based upon results from a large clinical trial, we reported that PEG reduced the occurrence of a first case of CM during the first 30 DIM, particularly in cows with excessive prepartum body condition score (BCS), and in cows with elevated prepartum nonesterified fatty acids (NEFA) concentrations (Barca et al., 2021b). Moreover, PEG treatment reduced the occurrence of endometritis in cows that had a

previous case of metritis in the same lactation. In addition, in cows with elevated NEFA, PEG treatment increased the first insemination rate and counteracted the negative association of early lactation CM and uterine disease [UD, i.e. a cow with a record of retained fetal membranes (placenta), metritis or both] with the pregnancy rate. Ultimately, in cows with elevated NEFA, PEG treatment decreased the hazard of culling (Barca et al., 2022).

An important question to answer is whether the economic benefits of improved health, fertility and longevity due to PEG treatment outweigh the cost of PEG and its application under field conditions (two doses). Bio-economic simulation models are often used for this type of economic evaluations. In such bio-economic models, the available knowledge on the effect of an intervention is implemented in a simulation model of the relevant dairy cow disease(s), and the model is consequently used to mimic the use of the intervention and evaluate the economic effect of the intervention compared to a situation without it. This approach was used in recent literature to evaluate the economic impact of different durations of CM treatment (Pinzón-Sánchez et al., 2011; Steeneveld et al., 2011), the economic benefit of using nonsteroidal anti-inflammatory drugs in the treatment of CM (Van Soest et al., 2018) and the economic impact of implementing selective dry cow therapy (Hommels et al., 2021). However, this bio-economic simulation approach does not account for heterogeneity between cows. If sufficient longitudinal data are present, another possible approach is to study the economic performance of control and treated cows in a randomized clinical trial. In such longitudinal studies, the partial net return for each cow in the study may be determined (Burgers et al., 2022). In this report, the hypothesis to be tested is whether PEG treatment increases partial net return as calculated from milk revenues and costs for feed, medical treatments, inseminations and culling during a full lactation in grazing dairy cows, thereby taking the interaction with BCS and NEFA status of cows into account.

The objective of this study is therefore to investigate whether PEG treatment affects partial net return, milk revenues and costs for feed, medical treatments, insemination and culling during a full lactation. We also explored the effect of potential interactions of PEG treatment with parity, BCS and NEFA on these outcomes.

5.2. Materials and methods

The experimental protocol (CEUAFVET-PI-162) was evaluated and approved by the Honorary Committee for Animal Experimentation in Uruguay, University of the Republic, Uruguay.

5.2.1. Study Design

This randomized controlled trial was carried out on four commercial grazing dairy farms and has recently been described in companion publications (Barca et al., 2021b; Barca et al., 2022). In short, 2,153 (Farm 1 = 759; Farm 2 = 314; Farm 3 = 664; Farm 4 = 416) Holstein primiparous (animals that were enrolled in the study shortly before their first calving) and multiparous cows (animals that were enrolled shortly before their second or later calving) that calved from February 13 to September 30 of 2018 were included in the study. Twice a week, at -10 to -7 days relative to the expected calving date, cows were randomly assigned to either treatment group or untreated control group based on their unique national ear tag number, which is assigned to cattle at birth. Animals with an even national ear tag number were injected with 15 mg of PEG according to the product label (PEG) and animals with an odd national ear tag number remained as untreated controls (Control). Animals assigned to the PEG treatment received a second dose within 24 h after calving; only cows that received both doses were included in the study. The national ear tags used for treatment allocation are not related to the large visible ear tags that are used for on-farm identification and management decisions. Research assistants, who did not belong to the farm staff, applied treatments (Barca et al., 2021b; Barca et al., 2022).This meant that all people involved in daily farm management were blinded as to which cows had been treated. The resulting study population consisted of 733 primiparous cows (Control = 391; PEG 342) and 1,420 multiparous cows (Control: 723; PEG = 697). The interval in days between enrollment and calving (in case of PEG this is the interval between PEG doses), did not differ between treatment groups (Control = 9.1 ± 0.2; PEG = 9.4 ± 0.3; P = 0.42).

At the time of treatment assignment (-10 to -7 days relative to the expected calving date) BCS was assessed according to Ferguson et al. (1994) and blood was drawn for determination of NEFA (Barca et al., 2021b, Barca et al., 2022). Throughout the study,

milk yield and fat and protein concentration per cow were determined from monthly test-day samples (COLAVECO, Colonia, Uruguay). Clinical diseases were diagnosed and recorded as described before (Barca et al., 2021b, Barca et al., 2022), and all treatments were recorded. All farms used artificial insemination with estrus detection performed by trained farm personnel. Pregnancy diagnoses were performed by transrectal palpation or ultrasonography by the farm veterinarian. All inseminations and pregnancy diagnoses were recorded by farm personnel and/or by the farm veterinarian. Dry-off date and date of culling or on-farm death were also recorded. The study ended 305 d after the last recorded calving in the study; this was 529 d after the first recorded calving in the study.

5.2.2. Economic calculations, partial budgeting

The collected data were used to determine partial net return for each of the 2,153 cows in the trial. Consequently, the cow is the economic unit of interest. Partial net return is defined as combination of milk revenues where all relevant costs are subtracted for each cow *i* during the experimental days (ED) for cow *i*. Partial budgeting is widely used to evaluate the economic impact of interventions (for example Rowe et al. 2021) and partial net return as used here was previously defined by Burgers et al. (2022). Equal to a partial budget, the cow-level partial net return only takes into account economic factors that are, in theory, affected by the intervention of interest. Consequently, we based the partial net returns calculation on factors that could be potentially affected by PEG treatment. Briefly, milk revenues (R_i^{Milk}) were included as the income potentially affected by PEG treatment (Ruiz et al., 2017; Powell et al., 2018; Van Schyndel et al., 2021). Although, feed costs in relation to PEG has not been studied, feed intake in relation to PEG treatment has been studied preliminarily, in a CM challenge model, Powell et al. (2018), reported that postinfection, PEG treated cows consumed more feed than untreated control cows. Therefore costs for feed (C_i^{Feed}) were included in the partial net return. Pegbovigrastim treatment is associated with the occurrence of diseases and reproductive performance (Canning et al., 2017; Ruiz et al., 2017; Zinicola et al., 2018; Barca et al., 2022). Therefore, expenditures for treatment of CM (C_i^{CM}), UD (C_i^{UD}) and treatments for other diseases (C_i^{Other}; i.e. any medical treatment that was not intended for CM or UD) and cost of inseminations (C_i^{Ins}) were included. Finally, because diseases are known to be associated with hazard of culling (Carvalho et al., 2019), and that in cows with

elevated NEFA, we showed that PEG treatment decreased the hazard of culling (Barca et al., 2022), also cost of culling (C_i^{Cull}) was included. As far as we know, there are no indications that other cow-level costs are affected by PEG treatment. Consequently, the partial net return for each individual cow *i* was calculated as follows:

$$Partial\ net\ return_i = R_i^{Milk} - (C_i^{Feed} + C_i^{CM} + C_i^{UD} + C_i^{Other} + C_i^{Ins} + C_i^{Cull})$$

Because ED differed between cows, $Partial\ net\ return_i$ was standardized and expressed per cow per year (Burgers et al., 2022) as follows:

$$Partial\ net\ return_i^{\text{Year}} = \frac{Partial\ net\ return_i}{ED_i} \times 365$$

5.2.2.1. Milk Revenues and Costs Calculations

From the monthly test-day milk samples, milk returns were calculated per individual cow as follows: first, for each cow *i* and test-day *j*, the returns for milk (R_{ij}^{Milk}) were calculated based on the milk yield (MY_{ij}), percentage protein ($Perc_{ij}^{Prot}$) and fat ($Perc_{ij}^{Fat}$) and average prices for protein and fat for 2018 (P^{Prot} and P^{Fat}, respectively):

$$R_{ij}^{Milk} = MY_{ij}\ x\ Perc_{ij}^{Prot} x\ P^{Prot} + MY_{ij}\ x\ Perc_{ij}^{Fat} x\ P^{Fat}$$

Based on the returns per cow per test-day, the total milk returns per cow (R_i^{Milk}) were calculated for each cow *i* by multiplying the average milk returns in the period leading to the test-day by the number of days in that period as follows:

$$R_i^{Milk} = \frac{R_{i1}^{Milk}}{2} \times DIM_{i1} + \sum_{j=1}^{n} \frac{R_{ij}^{Milk} + R_{ij-1}^{Milk}}{2} \times (DIM_{ij} - DIM_{ij-1}) + R_{in}^{Milk} \times (ED_i - DIM_{in})$$

Where DIM_{ij} is the days in milk for test-days 1 to n_j (n_j is total number of test-days of cow *i*). We assumed a linear change of the daily milk returns between test-days. For the time until the first test-day of a cow, the milk return was taken as the average of the first test-day post partum (DIM_{i1}) and 0, thereby assuming that milk yield started at 0 kg. For the period after the last test-day (DIM_{in}), it was assumed that the milk returns were equal

to the milk returns at the last test-day with a period duration from the last test-day to exit from the study for each cow *i*.

Costs for feed supply were estimated per individual cow as follows:

$$C_i^{Feed} = \Big(ED_i \; x \; NE^{Maintenance} + FPCM_i \; x \; NE^{Milk} + \; NE_i^{Gestation} / \left(\frac{NE}{PMR(kg\ dm)}\right) \Big) x \; P^{PMR}$$

Where $NE^{Maintenance}$ is net energy for maintenance, considering a 20% increase for grazing activity in mixed systems (NRC, 2001); FPCM is fat and protein corrected milk, calculated as (Manzanilla Pech et al., 2014): 0.337 × total milk (kg) + 11.6 × total fat (kg) + 5.999 × total protein (kg); NE^{Milk} is net energy for milk production, calculated from milk composition as (NRC, 2001): NE^{Milk} (Mcal/kg) = 0.0929 × fat yield (%) + 0.0547 × protein yield (%) + 0.0395 × lactose (%; assuming lactose content of 4.9%); $NE^{Gestation}$ is the energy required for pregnancy (Mcal/d) calculated from 190 to 279 days of gestation as (NRC, 2001): (0.00318 x d - 0.0352) x (CBW/45)/0.218, where d is day of gestation and CBW is calf birth weight in kilograms (assuming here a CBW of 35 kg); NE/ partial mixed ration (PMR) kg dm is net energy (Mcal) per kg of dry matter of PMR and P^{PMR} is the price (US$/kg) of PMR (kg/DM).

The C_i^{CM} was calculated per individual cow similar to Steeneveld et al. (2011) as follows:

$$C_i^{CM} = CM_i \; x \; (C_i^{Milkwithheld} + \; C_i^{Medicine} \; + \; C_i^{Labor} \; - \; C_i^{Milkfedtocalves})$$

Where CM_i are number of CM cases for cow *i*.

$$C_i^{Milkwithheld} = \sum_{cc=0}^{n} D_c^{Withheld} \times (MY_{ic} \; x \; Perc_{ic}^{Prot} x \; P^{Prot} + \; MY_{ic} \; x \; Perc_{ic}^{Fat} x \; P^{Fat})$$

Where $D_c^{Milkwithheld}$ is the number of days of treatment and milk withhold, MY_{ic} is milk yield at the time of CM case *c* for cow *i*, $Perc_{ic}^{Prot}$ and $Perc_{ic}^{Fat}$ are percentage protein and fat at the time of a CM case *c* for cow *i,* respectively.

$C_i^{Medicine}$ is the price for medicine, C_i^{Labor} is the labor price for treatment application, and $C_i^{Milkfedtocalves}$ is MY_{ic} by the value of the milk replacer, all for cow *i*.

Similarly, C_i^{UD} and C_i^{Other} were calculated for each cow *i*.

Costs for insemination (C_i^{Ins}) were calculated per individual cow as follows:

$$C_i^{Ins} = N_i^{Ins} x\ P^{Ins}$$

Where N_i^{Ins} is the number of inseminations for each cow i and P^{Ins} is the price per insemination including costs of labor.

The C_i^{Cull} was calculated similar to Mostert et al. (2017) and Burgers et al. (2022) as:

$$C_i^{Cull} = ((P^{Heifer} - P^{Slaughter})/L^{Max}) \times (L^{Max} - L_i)$$

Where P^{Heifer} is the price of a replacement heifer, $P^{Slaughter}$ is the revenue of a culled cow at the slaughterhouse, assuming a weight of 550 kg (INALE, Montevideo, Uruguay). In case of a dead cow, the $P^{Slaughter}$ is equal to 0. L^{Max} is the lactation number of the oldest cow in the experiment and L_i is the actual lactation of a culled cow i. More than 5 lactations were considered as 6 lactations; thus L^{Max} is equal to 6 and L_i is an integer number from 1 to 6.

Costs of PEG and its application (two doses) were not included in the economic calculations.

5.2.2.2. Prices

Input values regarding price levels (Table 5.1) were based on prices for 2018 available at the governmental national institute for dairy production (INALE, Montevideo, Uruguay). Prices for medicines and milk replacer to feed calves were based on information supplied by one of the biggest medicine suppliers for dairy in Uruguay (PROLESA, Montevideo, Uruguay). Beef price at the slaughterhouse was based on available information at the Asociación de Consignatarios de Ganado (ACG, Montevideo, Uruguay). Prices were given in local currency and transformed to US$ using the average currency exchange rate of 2018 (INALE, Montevideo, Uruguay).

Table 5.1. Prices (US$) used to calculate the economic result of Control and pegbovigrastim treated cows in a randomized controlled trial on four commercial grazing dairy farms.

Variable		Reference
Milk solids		
Protein ($P^{Protein_N}$/kg)*	6.64	INALE, 2021
Min.	5.31	INALE, 2021
Max.	7.97	INALE, 2021
Fat (P^{Fat_T}/kg)**	2.65	INALE, 2021
Min.	2.12	INALE, 2021
Max.	3.18	INALE, 2021
Replacement Heifer (P^{Heifer})	1000	INALE, 2021
Max.	1200	INALE, 2021
Beef price		
$P^{Slaughter}$/kg	0.85	ACG, 2021
Min	0.65	ACG, 2021
Milk replacer (L)	0.22	PROLESA, 2021
Feed		
PMR (P^{PMR}/kg)***	135	INALE, 2021
Treatments clinical mastitis		
Products	Table S 5.1	PROLESA, 2021
Farm personnel labor**** / treatment	2.15	INALE, 2021
Veterinary labor**** / treatment	2.25	INALE, 2021
Treatments retained placenta/metritis		
Products	Table S 5.1	PROLESA, 2021
Farm personnel labor**** / treatment	2.15	INALE, 2021
Veterinary labor**** / treatment	2.25	INALE, 2021
Treatments other diseases		
Products	Table S 5.1	PROLESA, 2021
Farm personnel labor***** / treatment	2.9	INALE, 2021; Liang et., 2017
Veterinary labor***** / treatment	3	INALE, 2021
Insemination		
USD/insemination	25	Author's expertise

*Average national price paid to farmer, 2018;
**Average national price paid to farmer, 2018;
***Partially mixed ration (30% concentrate, 20% conserved forage, 50% fresh forage);
****15 minutes for farm personnel (adapted from Liang et al., 2017), 3 minutes for veterinarian.

*****20 minutes for farm personnel (adapted from Liang et al., 2017), 4 minutes for veterinarian. Minimum and maximum are the values assumed in sensitivity analyses to assess the effect of changes in revenues and costs on the partial net return per year.

5.2.3. Statistical Analysis

Data were analyzed using SAS software (SAS Institute Inc. 2018. SAS® University Edition, Cary, North Carolina: SAS Institute Inc.).

The effect of PEG treatment on partial net return, R^{Milk} and on the costs expressed per cow per year was tested using general linear mixed models (PROC MIXED). The following were considered as class variables: parity (primiparous/multiparous), prepartum BCS (Under: < 3; Acceptable: 3 to 3.5; Over: > 3.5; Roche et al., 2009), prepartum NEFA (Low ≤ 0.3; High > 0.3 m*M*, Overton et al., 2017), treatment (Control/PEG) and calving month (6 classes: February/March, April, May, June, July and August/September). Farm, also as a class variable, was included as a random effect. Two-way interactions between parity, prepartum BCS, prepartum NEFA and treatment were checked for significance.

The general model was then:

$Partial\ net\ return_i^{Year}$ = intercept + lactation$_i$ + prepartum BCS$_i$ + prepartum NEFA$_i$ + treatment$_i$ + calving month$_i$ + interactions + farm (random) + error

After the initial full model lay-out, a backward variable selection process was performed. Parity and treatment were forced into all models. All other variables or their two-way interaction with treatment remained in the model when $P \leq 0.10$ (significance level to stay, SLSTAY). Exceptionally, variables or an interaction term remained in the model when removal of the variable resulted in an important change in the treatment effect. Such variables would be considered potential confounders. Statistical significance was decided at a level of $P \leq 0.05$. The result of variables that remained in the final models are presented as least squares means ± standard error. All P-values of pair-wise comparisons of least squares means were corrected with the Tukey-Kramer-adjustment.

5.2.4. Sensitivity Analysis

To assess the effect of changes in revenues and costs on the partial net return per cow per year a sensitivity analysis was performed. Firstly, considering the variations observed in prices of protein and fat from 2012 to 2020 (INALE, Montevideo, Uruguay), milk revenues were based on either the average price for protein and fat for 2018, or on a 20% lower or higher price (Table 5.1). Secondly, it was assumed that milk withheld due to medical treatments would or would not be fed to calves. Thirdly, we assumed either the normal or the lowest beef price at slaughterhouse. The lowest beef price is the price paid for cows in an inferior health condition (ACG, Table 5.1). Fourthly, we varied the price of a replacement heifer from the normal price to a 20% higher price (INALE, Montevideo, Uruguay, Table 5.1).

5.3. Results

5.3.1. Descriptive Statistics

Table 5.2 presents an overview of ED, end-of-study status, productive performance and economic factors for Control and PEG cows. During the study, 4.4% of the cows died (Control = 4.6%; PEG = 4.2%), 14.5% were culled (Control = 15.1%; PEG =13.9%), 53.1% were dried-off being pregnant (Control = 53.3%; PEG = 53.0%), 10.5% were dried-off being not pregnant (assumed culled at that date; Control = 9.7%; PEG = 11.3%), 8.0% ended the study period being pregnant (Control = 8.9%; PEG =7.0%), and 9.4% ended the study period being not pregnant (assumed culled at the end-of-study date; Control = 8.4%; PEG = 10.5%).

Table 5.2. Experimental days, production performance, partial net return, milk revenues, and costs in Control and pegbovigrastim treated cows in a randomized controlled trial on four commercial grazing dairy farms.

	Control		PEG	
	N	Mean ± SEM	N	Mean ± SEM
Experimental days	1,102	348 ± 4	1,026	350 ± 4
Death	51	138 ± 18	43	107 ± 14
Cull	166	212 ± 10	143	230 ± 10
Dry pregnant	587	381 ± 2	544	380 ± 2
Dry not-pregnant*	107	292 ± 10	116	303 ± 10
End-date pregnant	98	500 ± 11	72	501 ± 12
End-date not-pregnant*	93	398 ± 6	108	400 ± 6
Milk yield (kg)	1,102	7,508 ± 99	1,026	7,552 ± 98
Protein yield (kg)	1,102	249 ± 4	1,026	250 ± 3
Fat yield (kg)	1,102	272 ± 4	1,026	275 ± 4
Economic factors (US$/cow/year)				
Partial net return	1,102	1,023 ± 57	1,026	1,100 ± 52
R^{Milk}	1,102	2,401 ± 27	1,026	2,429 ± 26
C^{Feed}	1,102	877 ± 6	1,026	883 ± 6
C^{Cull}	1,102	387 ± 44	1,026	340 ± 38
C^{CM}	1,102	40 ± 3	1,026	31 ± 2
C^{UD**}	1,102	8 ± 2	1,026	16 ± 4
$C^{Other***}$	1,102	18 ± 5	1,026	10 ± 3
C^{Ins}	1,102	48 ± 1	1,026	49 ± 1

*Cows that were not pregnant at the end-date were assumed to be culled at that date.
**Cost of treatment for retained placenta, metritis or both
***Cost of any other treatment recorded with exception of CM, retained placenta and metritis

Economic descriptive results, presented in Table 5.2, showed that R^{Milk} per year were US$ 2,401 ± 27 and US$ 2,429 ± 26, in Control and PEG treated cows respectively. The biggest cost was C^{Feed} per year, which represented 36.9% of the R^{Milk} per year in the Control group and 36.4% in PEG cows. The C^{Cull} per year represented 16.1% of the R^{Milk} per year in Control cows and 14.0% in PEG cows. A descriptive evaluation of C^{Cull} per year showed some extremely high values (right skewed) that would made statistical analysis difficult. Consequently, cows with a C^{Cull} per year > mean + 2 × SD were treated as outliers and not included in the final data set (Burgers et al., 2022) as presented in Table 5.2. Thus, Control = 12 (death = 11, cull = 1); PEG = 13 (death = 12; cull = 1) cows, which were defined as outliers, were not considered for the final statistical analyses, resulting in a number of remaining cows of Control = 1,102 and PEG = 1,026 (Table 5.2). Descriptive data of cows treated as outliers is provided in Supplemental Table S 5.2 For these cows, the ED were 6 ± 2 and 5 ± 1, and the C^{Cull} per year was US$ 72,333 ± 23,731

and US$ 42,446 ± 5,157, in Control and PEG cows, respectively. After removing these 25 cows, C^{Cull} per year still showed a relatively large variation (Table 5.2), but in the original data set, with a much higher mean value, this variation was considerably larger (Control = 1,162 ± 334; PEG = 867 ± 162). The C^{CM}, C^{UD}, C^{Other}, and C^{Ins} per year represented 2% or less of the R^{Milk} per year each.

5.3.2. Effect of Pegbovigrastim on Partial Net Return per Cow per Year

Final model results showed that PEG treatment affected partial net return per year (P = 0.036). Prepartum BCS was not associated with the partial net return per year (P = 0.24), but the BCS by treatment interaction remained in the model (P = 0.10). Table 5.3 presents treatment least squares means for partial net return per year, overall and by BCS class. Overall, PEG treatment increased the partial net return per year by US$ 210 ± 100 (P = 0.036). Figure 5.1 illustrates PEG treatment effect on the partial net return per year, overall and by BCS class. In addition, parity was associated (primiparous US$ 824 ± 281; multiparous US$ 1,261 ± 275; P < 0.001) and calving month remained in the model (P = 0.07).

Table 5.3. Partial net return, milk revenues, and costs per year (US$) in Control (n = 1,102) and pegbovigrastim treated (n = 1,026) cows in a randomized controlled trial on four commercial grazing dairy farms.

		Least squares means ± SE			
Variable	Class	Control	PEG	Treatment effect.	*P*-value***
Partial net return	Overall	938 ± 279	1,147 ± 280	210 ± 100	0.036
	Prepartum BCS*				
	Under	672 ± 323	1,110 ± 326	438 ± 242	0.46
	Acceptable	1,138 ± 278	1,106 ± 278	-33 ± 92	0.99
	Over	1,002 ± 289	1,226 ± 293	224 ± 150	0.67
C^{Cull}	Overall	467 ± 63	322 ± 66	-145 ± 77	0.061
	Prepartum BCS				
	Under	617 ± 139	299 ± 145	-318 ± 188	0.54
	Acceptable	346 ± 62	380 ± 61	34 ± 72	0.99
	Over	438 ± 289	287 ± 94	-151 ± 117	0.79
C^{CM}	Overall	40 ± 9	31 ± 9	-9 ± 3	0.008
C^{Other}	Prepartum NEFA**				
	Low	2 ± 7	10 ± 7	8 ± 10	0.85
	High	24 ± 5	6 ± 6	-19 ± 8	0.07
C^{UD}	Overall	10 ± 6	19 ± 5	9 ± 5	0.07
R^{Milk}	Overall	2,454 ± 285	2,485 ± 285	32 ± 29	0.28
C^{Feed}	Overall	885 ± 60	893 ± 60	8 ± 6	0.22
C^{Ins}	Overall	49 ± 4	50 ± 4	1 ± 2	0.58

*Prepartum body condition score: Under (< 3): Control = 106; PEG = 97; Acceptable (3 to 3.5): Control = 694; PEG = 696; Over (> 3.5): Control = 302; PEG = 233).
**Prepartum nonesterified fatty acids concentration: Low (≤ 0.3 mM): Control = 431; PEG = 401; High (> 0.3 m*M*): Control = 671; PEG = 625.
****P*-values of pair-wise comparisons of LSM corrected with the Tukey-Kramer-adjustment.

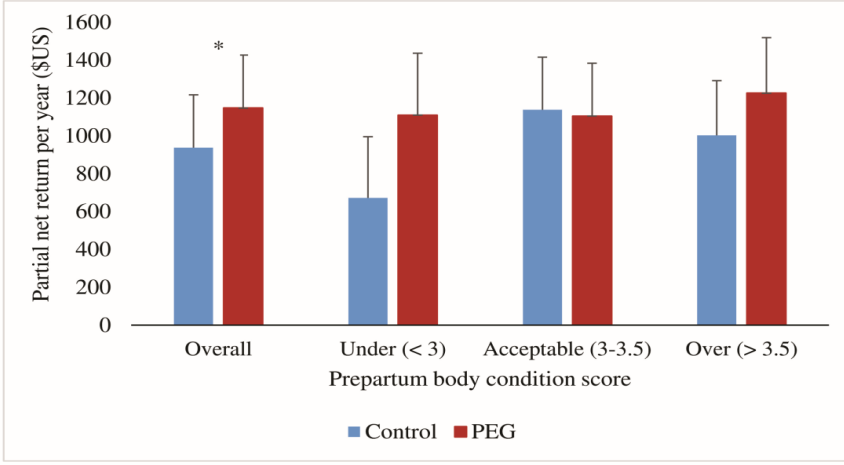

Figure 5.1. Least squares means (error bars represents SE) for the overall partial net return per year in Control (n = 1,102) and PEG treated (n = 1,026) cows and by prepartum body condition score class (Under: Control = 106; PEG = 97; Acceptable: Control = 694; PEG = 696; Over: Control = 302; PEG = 233) in a randomized controlled trial on four commercial grazing dairy farms. *Overall, PEG treatment increased the partial net return per year by US$ 210 ± 100 (Control = 938 ± 279, PEG = 1,147 ± 280; $P = 0.036$).

5.3.3. Effect of Pegbovigrastim on Milk Revenues and Costs per Cow per Year

Final model results of C^{Cull} per year showed that PEG treatment effect did not reach statistical significance ($P = 0.061$). Prepartum BCS was not associated with the C^{Cull} per year ($P = 0.65$), while the removal of the BCS by treatment interaction ($P = 0.12$) resulted in an important change in the treatment effect and it was therefore maintained in the model. Table 5.3 presents treatment least squares means for C^{Cull} per year, overall and by BCS class. Overall, PEG treatment numerically reduced the C^{Cull} per year by US$ 145 ± 77, but this effect did not reach statistical significance ($P = 0.061$). Differentiated by parity, the C^{Cull} per year was US$ 453 ± 68 and 336 ± 53 in primiparous and multiparous cows, respectively ($P = 0.07$).

Pegbovigrastim treatment reduced the C^{CM} per year by US$ 9 ± 3 (P = 0.008; Table 5.3). Parity was associated with C^{CM} (primiparous US$ 26 ± 9; multiparous US$ 45 ± 9; P < 0.001). In addition, BCS was associated with C^{CM} (P = 0.01): Over BCS cows presented a higher C^{CM} per year compared to acceptable BCS cows (P = 0.01) (Under BCS US$ 29 ± 10; Acceptable BCS US$ 32 ± 9; Over BCS US$ 45 ± 9). Finally, calving month (P = 0.003) was associated with the C^{CM} per year.

Pegbovigrastim treatment did not affect the C^{Other} per year (P = 0.37) and NEFA was not associated with the C^{Other} per year (P = 0.16); however, NEFA interacted with treatment (P = 0.03). Table 5.3 presents treatment least squares means by NEFA class. In addition, parity was associated (primiparous US$ 4 ± 5; multiparous US$ 17 ± 4; P = 0.05) with the C^{Other} per year.

Pegbovigrastim treatment did not affect the C^{UD} per year (P = 0.07; Table 5.3). Parity was not associated (primiparous US$ 16 ± 6; multiparous US$ 13 ± 5; P = 68), and calving month was associated with the C^{UD} per year (P < 0.001).

Pegbovigrastim treatment did not affect the R^{Milk} per year (P = 0.28; Table 5.3). Parity was associated with R^{Milk} per year (primiparous US$ 2,257 ± 285; multiparous US$ 2,682 ± 284; P < 0.001), and so was BCS (P = 0.01): Under BCS cows presented a lower R^{Milk} per year compared to over BCS (P = 0.004) cows (Under BCS US$ 2,368 ± 288; Acceptable BCS US$ 2,485 ± 284; Over BCS US$ 2,555 ± 285). Prepartum NEFA remained in the model (Low US$ 2,500 ± 285; High US$ 2,439 ± 285; P = 0.10).

Pegbovigrastim treatment did not affect C^{Feed} per year (P = 0.22; Table 5.3). There was an association with parity (primiparous US$ 851 ± 60; multiparous US$ 926 ± 60; P < 0.001) and BCS (P = 0.003): Under BCS cows presented a lower C^{Feed} per year compared to acceptable BCS (P = 0.02) cows and compared to over BCS (P = 0.002) cows (Under US$ 863 ± 61; Acceptable US$ 894 ± 60; Over US$ 910 ± 60).

Pegbovigrastim treatment did not affect C^{Ins} per year (P = 0.58; Table 5.3). Parity was not associated (primiparous US$ 48 ± 4; multiparous US$ 50 ± 4; P = 0.38), and calving month (P < 0.001) was associated with C^{Ins} per year.

5.3.4. Sensitivity Analysis

Supplemental Table S 5.3 provides an overview of the final regression models for partial net return per year assuming different input levels as used for the sensitivity analyses.

Least squares means and the treatment effect on partial net return per year by different variable levels as used in the sensitivity analyses are presented in Table 5.4. Compared with the price levels assumed in this study, a 20% lower price for milk protein and fat resulted in a 7% lower economic benefit of PEG treatment, while a 20% higher price showed a 6% higher economic benefit. Assuming that milk withheld due to medical treatments would not be fed to calves resulted in a 2% higher economic benefit of PEG treatment. Assuming the lowest beef price at slaughter resulted in a 10% higher economic benefit of PEG treatment. Assuming a 20% higher price of a replacement heifer showed a 22% higher economic benefit of PEG treatment.

Table 5.4. Relative change of partial net return per year (US$) by different variable levels as used for sensitivity analyses, in Control (n = 1,102) and pegbovigrastim treated (n = 1,026) cows in a randomized controlled trial on four commercial grazing dairy farms.

	Least squares means ± SEM			
Variable	Control	PEG	Treatment effect.	*P*-value**
Milk fat and protein – min.	464 ± 223	660 ± 224	196 ± 95	0.040
Milk fat and protein – max.	1,427 ± 316	1,650 ± 317	223 ± 105	0.034
Milk withheld discarded*	915 ± 257	1,130 ± 258	214 ± 100	0.032
Beef price – min.	891 ± 263	1,122 ± 264	230 ± 106	0.030
Replacement heifer	788 ± 292	1,045 ± 293	257 ± 120	0.032

*Assuming that milk withheld due to medical treatments would not be fed to calves.
** *P*-values of pair-wise comparisons of LSM corrected with the Tukey-Kramer-adjustment.

5.4. Discussion

In this study, we report for the first time the economic result of using PEG in dairy cows. Based on the cows' economic performance during a full lactation, we determined the partial net return per cow per year. The main finding was that PEG treatment resulted in an overall economic benefit, as it increased the partial net return per cow per year. Interestingly, although numerical differences could be noticed in the descriptive statistics of the underlying economic factors of the partial net return, only the C^{CM} per year was individually statistically significant, while all other factors represented in the partial net return (i.e. R_i^{Milk}, C_i^{Feed}, C_i^{UD}, C_i^{Other}, C_i^{Ins} and C^{Cull} per year) were not statistically significant. However, the total effect of all these economic factors, represented in the partial net return per year, resulted in a statistically significant economic benefit for PEG treated compared to Control cows.

The method we used is relatively novel. Previous studies on the economics of cow-level health interventions used bio-economic simulation models (e.g. working on Meloxicam; van Soest et al., 2018 or working on Cabergoline; Steeneveld et al., 2019). In such bio-economic simulation modeling studies, specific decisions need to be taken on what treatment effects need to be considered. Effects of treatment on occurrence of udder health were derived from clinical trials. In contrast, effects on reproduction or culling were modelled mechanistically using other studies than clinical trials. Such bio-economic simulation studies do not allow to account for heterogeneity between cows. In this study, we utilized longitudinal data from each enrolled cow during the full lactation. It allowed us to evaluate the combined effect of all cow production factors that may influence the economic performance of that cow. By using the data of all individual cows, we could evaluate the overall economic effect of PEG treatment, accounting for heterogeneity between cows. We consider our current approach as a more reliable method of evaluating the true economic effects of cow-level interventions.

Almost 70% of the increased partial net return per cow per year due to PEG treatment was explained by the reduced C^{Cull} per cow per year. Culling has previously been identified as one of the main contributors to the cost of clinical disease (Galligan, 2006; Overton and Fetrow, 2008; Heikkila et al., 2012). Rollin et al. (2015) attributed more than 40% of the cost of CM during the first 30 DIM to costs of culling and replacement. Van Soest et al. (2018) reported that reducing the proportion of culling had the highest

economic impact on the net economic benefit of using nonsteroidal anti-inflammatory drugs in the treatment of CM. In a novel approach, Denis-Robichaud et al. (2021) reported that PEG treatment as an adjunct therapy increased survival after 30 days in cows with severe CM. It may therefore be hypothesized that PEG treatment reduces early culling as a consequence of disease, which would be particularly expensive due to the short time that those cows remain in lactation. In our trial, a first case of CM during the first 30 DIM was associated with an almost two-fold increase in the hazard of culling (Barca et al., 2022). Pegbovigrastim treatment reduced the occurrence of a first case of CM during the first 30 DIM (Barca et al., 2021b) and, in multiparous cows, counteract the negative association of a first case of CM during the first 30 DIM with the hazard of culling (Barca et al., 2022). We also reported that PEG treatment reduced the hazard of a first case of CM and the rate of total cases of CM (Barca et al., 2021b). Hertl et al. (2018) reported that more CM cases in early lactation resulted in an increased rate of CM cases during the cow's lifetime and that these CM cases also increased the hazard of culling. Thus, the reduced C^{Cull} per year in PEG treated cows may be explained by the preventive effect on CM in combination with a lower rate of repeat CM cases in cows that had a first case of CM during the first 30 DIM.

For partial net return per year, the BCS by treatment interaction remained in the final model, suggesting that the economic benefit of using PEG depends on BCS. Again, this would be mainly explained by the C^{Cull} per year. For the C^{Cull} per year, the removal of the BCS by treatment interaction resulted in an important change in the PEG treatment effect. Therefore, BCS acted as a modifier for the effect of PEG treatment on the C^{Cull} per year. Numerically, PEG treatment increased partial net return per year in under and over BCS cows, but not in cows with an acceptable BCS. Simultaneously, PEG treatment numerically reduced C^{Cull} per year in under and over BCS cows and not in cows with an acceptable BCS. These results are in line with our earlier results: PEG treatment reduced the occurrence of a first case of CM during the first 30 DIM in under (only numerically) and over BCS cows and not in cows with an acceptable BCS (Barca et al., 2021b). It is relevant that, in our study, most of the cows that classified as under BCS were multiparous cows (data not shown). In multiparous cows, a first case of CM during the first 30 DIM was associated with a more than 2.5 fold increase in the hazard of culling. As mentioned, in multiparous cows, PEG treatment counteracted the negative association between a first

case of CM during the first 30 DIM and the hazard of culling (Barca et al., 2022). This contributes to explain the numerical differences particularly in under BCS cows.

Pegbovigrastim treatment reduced the C^{CM} per year. This further supports the findings related to the reduced C^{Cull} per year in PEG treated cows. As mentioned, PEG treatment reduced the occurrence of a first case of CM during the first 30 DIM (Barca et al., 2021b), consistent with previous reports (Canning et al., 2017; Ruiz et al., 2017). Moreover, we reported that PEG treatment reduced the hazard of a first case and the rate of total cases of CM during the full lactation (Barca et al., 2021b). All these effects explain the reduction of the C^{CM} per year. Additionally, Powell et al. (2018) reported that PEG treatment reduced the severity of a CM case. In an experimental mastitis challenge, it was reported that PEG treated cows exhibited a less severe milk yield drop (Powell et al., 2018). A less pronounced milk drop would result in an economic benefit. Moreover, reduced severity of a CM case would affect $C^{Medicine}$ and C^{Labour} per year for a CM treatment. To study whether PEG treatment affects severity of CM or whether the CM cases in Control and PEG treated cows differ (e.g. causal pathogen, duration, bacteriological cure, etc.) warrants further research.

For the C^{Other} per year, NEFA interacted with treatment, in High NEFA cows, PEG treatment numerically reduced the C^{Other} per year. In this trial, all treatments were recorded. However, we did not evaluate the effect of PEG on the occurrence of diseases other than CM and uterine disease. Further research analyzing the effect of PEG on the occurrence of other diseases would add information about the effect of PEG treatment on the C^{Other} per year. Elevated prepartum NEFA concentrations were associated with an increased risk of disease (Ospina et al., 2013). Previously, we reported that PEG treatment reverted the negative association between prepartum NEFA concentration and postpartum circulating neutrophil counts (Barca et al., 2021a), potentially improving the immune responsiveness in PEG treated cows.

We decided to perform our analyses parametrically, as, despite the usual skewness in the distribution of costs, in pragmatic randomized trials like this one, it is the arithmetic mean that is the most informative measure, and comparisons between treatment groups are reliable if skewness is not too extreme (Thompson and Barber, 2000). We excluded 12 Control and 13 PEG cows, due to their extremely high C^{Cull} per year that made statistical analyses using parametric linear models difficult, if not impossible. The extremely high

C^{Cull} per year was a consequence of the very few ED (≤ 15 DIM) of these cows. This same approach with regard to outliers in C^{Cull} per year calculations was recently reported by Burgers et al. (2022). Because of the calculation method and the need to express the economic performance of cows during an equal time period (a year in this study), extremely high costs or returns per year may occur when cows have very few ED. For these 25 cows, the C^{Cull} per year was considerably lower in PEG treated cows compared to Control cows. Hence, including these cows in the analyses would only further favor the economic outcome toward the PEG treatment. We acknowledge that our method to calculate the C^{Cull} per year has shortcomings, as it resulted in some extremely high values which were excluded from the final analyses. However, we believe that this method allows us to achieve a realistic estimation in most cows, accounting for the future value of a cow, as it calculates the C^{Cull} of each cow according to its actual lactation while accounting for the ED that each cow remained in the experiment. We consider this to be the best approach with the available data, as any revenue or cost of each cow should be spread out over the ED of each cow.

Sensitivity analyses showed that different variable levels did not have large effects on the economic consequences of PEG treatment. This indicates that the results are robust and not particularly sensitive with regard to differences in inputs (mainly price levels). The most influential variable with regard to the economic benefit of using PEG was the price of a replacement heifer. In this study, we used a conservative price, since in most scenarios prices of replacement heifer are higher (INALE, Montevideo, Uruguay).

As mentioned, costs of PEG and its application (two doses) were not included in the economic calculations. From an economic point of view, the calculated differences between control and PEG treated cows may be considered as the maximum amount of money that can be spent on PEG treatment. The costs of PEG treatment consist of the expenditure on the product, as well as the time it takes to administer treatments as part of farm transition cow management. It would consist of the time to identify and restrain the cow, and administer PEG. This may be highly dependent on the cost of labor and the ease with which cows can be identified and restrained. Thus, it could vary in different regions or herds with different management and facilities.

Overall PEG treatment resulted in a net economic benefit per cow per year. However, PEG treatment appears to be particularly beneficial in cows that are metabolically challenged (Barca et al., 2021a, b; Barca et al., 2022). Our results suggest that targeting PEG treatment to cows at a higher risk of disease due to a metabolic challenge and lower immune competence (Roche et al., 2009; Ingvartsen and Moyes, 2015; Roche et al., 2015) would be the more efficient option from an economic point of view. Thus, it would be of great value to develop predictive models to identify high-risk animals to further target the use of PEG. Loss of BCS would be a reliable indicator of metabolic challenge (Sheehy et al., 2017; Barletta et al., 2017). Under and over BCS cows may be identified by routine BCS scoring protocols or by using commercially available automated sensors (e.g. Mullins et al., 2019), while big data, metabolomics and the use of automatic sensors to predict metabolic health (Overton et al., 2017) are promising technologies to identify cows that would benefit most from PEG treatment.

5.5. Conclusions

Based on data from a large randomized clinical trial, we conclude that PEG treatment resulted in an overall economic benefit, as it increased the partial net return per cow per year by US$ 210 ± 100. Although the difference detected in the partial net return was statistically significant, the individual components of partial net return were not significantly different between control and PEG treated cows. Only the cost of treatment of CM was significantly lower for PEG treated cows compared to control cows (US$9 ± 3). The largest numerical difference was seen for the cost of culling: PEG treatment reduced the cost of culling by US$ 145 ± 77.

5.6. Acknowledgments

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5.7. Supplemental materials

Supplementary Table S 5.1. Prices for medicines used to calculate the economic result of Control and pegbovigrastim treated cows in a randomized controlled trial in four commercial grazing dairy farms.

Product name*	Pack size	Price ($US)	Dose/day**	$US/day of treatment
ALIVIOS	100 mL	37	24 mL	9
ARTRALGIN	50 mL	7	12 mL	1.7
BANAMINE	50 mL	57	24 mL	27
BUSCAPINA COMPOSITUM	50 mL	17	25 mL	9
CALCIUM FAST GEL	450 g	9	450 g	9
CEFALEXINA 200	100 mL	17	20 mL	3.4
CEFTIOMAX	250 mL	42	11 mL	1.8
CEFTIOZUR INY.	250 mL	39	17 mL	2.6
CLAMOXIL LA	100 mL	21	55 mL	11.6
CLAVAMOX JERINGA	unit	2	2	4
COBACTAN 2,5 %	100 mL	62	22 mL	13.6
COBACTAN 2,5 %	100 mL	62	22 mL	13.6
COBACTAN LC	unit	4	2	8
DALMAPROST	20 mL	12	2 mL	1.2
DELMOR	90 mL	29	11 mL	3.5
DEXA	50 mL	4	15 mL	1.2
DIFRAN FCO	50 mL	9	11 mL	2
DIPIRONA 50 R	100 mL	9	60 mL	5.4
DIUREX	50 mL	11	22 mL	5
DODICILE	100 mL	13	10 mL	1.3
EMEMAST PLUS	unit	2	2	4
ENERGOL	500 mL	8	500 mL	8
ESPES	250 mL	27	22 mL	2.4
ESPES	250 mL	27	22 mL	2.4
ESTRADIOL	20 mL	5	10 mL	2.5
EXCEDE CATTLE 200mg	100 mL	174	18 mL	31.3
EXCENEL RTU	100 mL	58	25 mL	14.5
FATROCORTIN	100 mL	7	10 mL	0.7
FATROXIMIN BOLOS	unit	2	2	4
FLUMEXINE	100 mL	14	49 mL	7
FLUNAZINE	100 mL	19	24 mL	4.6
GENTAMOX	100 mL	32	40 mL	13
GLANDINEX	20 mL	30	2 mL	3
GLUCAFOS	1000 mL	26	600 mL	15.6
INDIGEST	100 mL	16	37 mL	6
INMODULEN	100 mL	23	10 mL	2.3
L-300	250 mL	73	18 mL	5.3
LEVAC	1000 mL	10	1000 mL	10
MASTIJET FORT POMO	unit	2	1	2

METRICURE JERINGA	unit	9	1	9
PEN-STREP	250 mL	26	22 mL	2.3
PIRSUE JERINGA	unit	5	1	5
PROCREATIN	500 g	6	10 g	0.1
PRO-PEN G	250 mL	15	12 mL	0.7
REPEN	250 mL	34	28 mL	3.8
REVIVA	15 kg	188	1 kg	12.5
RILEXINE 150	100 mL	21	37 mL	7.7
ROBORANTE	100 mL	10	25 mL	2.5
ROMAGEL	unit	2	1	2
SUERO GLUC.ISOT	1000 mL	3	1000 mL	3
TERRAMICINA S.I	250 mL	18	20 mL	1.4
TOLFEDINE CS	250 mL	67	28 mL	7.5
TYLAN 200	250 mL	42	28 mL	4.7
UBROLEXIN	unit	4	1	4
VITAMINA B1-B12 FUERTE	50 mL	9	10 mL	1.8
YODIMASPEN	15 mL	12	15 mL	12

*Product name as marketed in Uruguay.
**Dose per day was calculated based on the product label and assuming a cow weight of 550 kg (INALE, 2021).

Supplementary Table S 5.2. Descriptive data of cows treated as outliers in a randomized controlled trial on four commercial grazing dairy farms.

Farm	Cow id	Actual lactation	Caving date	end-of-study date	exit study	Treatment	Cost of culling	Experimental days	Cost of culling per year
1	890	4	24-2-2018	25-2-2018	death	Control	333	2	60,833
1	3585	5	22-4-2018	24-4-2018	death	Control	167	3	20,278
1	4115	2	30-3-2018	8-4-2018	death	Control	667	10	24,333
1	6104	1	21-8-2018	4-9-2018	death	Control	833	15	20,278
1	4504	2	5-7-2018	7-7-2018	death	PEG	667	3	81,111
1	4568	2	24-5-2018	28-5-2018	death	PEG	667	5	48,667
1	4829	1	19-7-2018	25-7-2018	death	PEG	833	7	43,452
1	6275	1	28-8-2018	1-9-2018	death	PEG	833	5	60,833
2	3586	2	21-5-2018	22-5-2018	death	Control	667	2	121,667
2	3810	1	30-5-2018	13-6-2018	death	Control	833	15	20,278
2	3105	3	15-3-2018	20-3-2018	death	PEG	500	6	30,417
2	3446	2	17-6-2018	20-6-2018	death	PEG	667	4	60,833
2	3861	1	25-4-2018	8-5-2018	death	PEG	833	14	21,726
3	4229	4	1-7-2018	1-7-2018	death	Control	333	1	121,667
3	5309	3	6-5-2018	9-5-2018	dry	Control	266	4	24,295
3	6019	2	27-7-2018	30-7-2018	death	Control	667	4	60,833
3	6262	1	21-3-2018	21-3-2018	death	Control	833	1	304,167
3	4363	5	26-4-2018	26-4-2018	cull	PEG	89	1	32,394
3	4485	4	17-5-2018	20-5-2018	death	PEG	333	4	30,417
3	4961	4	2-4-2018	5-4-2018	death	PEG	333	4	30,417
3	5291	2	4-4-2018	7-4-2018	death	PEG	667	4	60,833
4	3077	2	30-5-2018	9-6-2018	death	Control	667	12	20,279
4	3233	1	11-5-2018	14-5-2018	death	Control	833	4	69,087
4	2468	4	3-6-2018	7-6-2018	death	PEG	333	6	20,280
4	2621	4	6-5-2018	8-5-2018	death	PEG	333	4	30,422

Supplementary Table S 5.3. Overview for final regression models for partial net return per year assuming different input levels as used for the sensitivity analyses.

	P-value					
Variable	Treatment	Prepartum BCS*	Treatment x Prepartum BCS	Parity	Prepartum NEFA**	Calving month
Milk fat and protein – min.	0.040	0.28	0.10	<0.001	NS	0.09
Milk fat and protein – max.	0.034	0.21	0.11	<0.001	0.10	0.07
Milk withheld discarded*	0.032	0.28	0.12	<0.001	0.10	0.06
Beef price – min.	0.030	0.26	0.09	<0.001	0.10	0.07
Replacement heifer	0.032	0.27	0.09	<0.001	NS	0.07

Prepartum body condition score: Under (< 3): Control = 106; PEG = 97; Acceptable (3 to 3.5): Control = 694; PEG = 696; Over (> 3.5): Control = 302; PEG = 233).
**Prepartum nonesterified fatty acids concentration: Low (≤ 0.3 mM): Control = 431; PEG = 401; High (> 0.3 m*M*): Control = 671; PEG = 625.

CHAPTER 6

6

General discussion

6.1. Introduction

Early lactation clinical diseases, which affect up to 50% of modern dairy cows (Ingvartsen et al., 2003; Leblanc, 2010; Galvao, 2013), are associated with impaired productive and reproductive performance, reduced longevity, and have an important impact on the economic performance of dairy farming (Hogeveen et al., 2017). Around parturition, immune dysfunction and metabolic challenge, a major factor associated with the immune dysfunction (Hachenberg et al., 2007; Ingvartsen and Moyes, 2015), have been linked to early lactation clinical disease (LeBlanc et al., 2004; Melendez et al., 2009; Galvão et al., 2010; Pomeroy, et al., 2017).

Immune stimulation therapies may be an innovative development that would mitigate this problem. Recently, a long-lasting analogue of G-CSF has been developed, a pegylated form of G-CSF (pegbovigrastim; PEG), that is injected once a week. Experimentally, when injected around parturition, PEG treatment substantially increased neutrophil counts (Kimura et al., 2014). At the time the research described in this thesis was designed, only a few field studies had been carried out, showing that PEG treatment reduced the incidence of early lactation CM (EMA, 2015; Hassfurther et al., 2015). Subsequently, several field studies have been conducted (Canning et al., 2017; Ruiz et al., 2017; Freick et al., 2018; Zinicola et al., 2018; Van Schyndel et al., 2021). However, up to the present, the long-term effect of PEG on the incidence of clinical diseases, fertility, culling and the potential interactions of treatment with the metabolic status of the transition dairy cow have not been studied. In addition, no economic evaluation of using PEG treatment has been carried out.

The overall objective of this thesis was therefore to evaluate the use of PEG under commercial conditions in grazing dairy farms. In addition, we evaluated the potential interaction of PEG treatment with parity and the metabolic status of the transition dairy cow.

Chapter 2 focuses on the effect of PEG treatment on postpartum (5 to 8 DIM) WBC counts. We showed that PEG treatment reversed the negative association of prepartum NEFA concentration with neutrophil and monocyte counts, and tended to reverse the negative association of prepartum NEFA concentration with WBC counts. In the PEG treated group, cows diagnosed with RP or metritis showed lower neutrophil counts when compared to PEG treated cows without these clinical diseases. In chapter 3, we showed

that PEG treatment reduced the occurrence of a first case of CM during the first 30 DIM, particularly in cows at risk of elevated lipid mobilization. Moreover, PEG treatment reduced the hazard of a first case and the rate of total cases of CM during the full lactation. In addition, PEG treatment improved the uterine healing process in cows that experienced metritis. In chapter 4, we investigated the effects of PEG on long-term fertility and culling, and we showed that the effect of PEG treatment on fertility and culling interacts with prepartum NEFA concentration. In cows with low prepartum NEFA concentration, no treatment effect was detected. In cows with elevated prepartum NEFA concentration, PEG treatment increased the rate of first insemination and counteracted the negative association of a first case of CM during the first 30 DIM and uterine disease (UD: RP, metritis or both) with the rate of pregnancy. Ultimately, in cows with elevated prepartum NEFA concentration, PEG treatment decreased the hazard of culling. In multiparous cows with a first case of CM during the first 30 DIM, PEG treatment tended to reduce the hazard of culling. In chapter 5, we concluded that PEG treatment resulted in an overall economic benefit, mostly explained by a reduced cost of culling in PEG treated cows.

The main finding of this thesis, observed throughout all chapters, was that the beneficial effects of PEG treatment were dependent on the prepartum metabolic status of transition dairy cows. Depending on the evaluated outcome, the effect of PEG treatment was associated with prepartum BCS (Chapter 3, 5) and/or prepartum NEFA concentration (Chapter 2, 3, 4). Regarding the potential association of PEG treatment with parity, the only evidence detected was that PEG treatment tended to result in a lower hazard of culling in multiparous cows that recorded a first case of CM during the first 30 DIM. This treatment effect was not detected for primiparous cows. Thus, we conclude that PEG treatment effects were associated with the metabolic status of transition dairy cows, while no associations with parity were detected. Based on our data, I also suggest that PEG treatment appears to be associated with improved clinical disease outcomes.

In this final chapter, I will integrate the results, and put them in context by comparing our findings with results from other studies. Based upon our results, I will develop a number of hypotheses that may contribute to the understanding of the biological mechanisms underlying PEG treatment and to the fine-tuning of its practical application, as well as suggest lines for future research.

6.2. Pegbovigrastim treatment restores the immune system, particularly in cows that are metabolically challenged.

The results from this field study show that PEG treatment restores the immune response in prepartum cows with a potentially challenged immune system due to metabolic stress (based on BCS and/or NEFA concentration). The evidence for this is summarized in four main arguments I want to discuss in detail and put in perspective.

6.2.1. Pegbovigrastim treatment reversed the negative association of prepartum NEFA concentration with neutrophil and monocyte counts

The novelty of chapter 2 is that PEG treatment reverted the negative association of prepartum NEFA concentration with neutrophil and monocyte counts, and tended to revert the negative association of prepartum NEFA concentration with WBC counts. As discussed in chapter 2, McDougall et al. (2017) reported that WBC counts in response to PEG treatment were not affected by restricted prepartum energy, which is somehow similar to our findings; the effect of PEG on WBC counts was the same, unrelated to the prepartum NEFA concentrations (Chapter 2). The difference between our study and the study of McDougall et al. (2017) lies in the control groups. We found a negative association between prepartum NEFA concentration, with reduced WBC, neutrophil and lymphocyte counts, while McDougall et al. (2017), did not find any association between energy balance and WBC counts. This apparent contradiction can be explained because, in the trial of McDougall et al. (2017), a high percentage of cows had elevated prepartum NEFA concentrations (i.e. > 0.4m*M*) in both the feed-restricted (85%) and the full feeding group (56%), thereby making it more difficult to evaluate the relationship between prepartum energy balance and WBC counts. The negative association between prepartum NEFA concentrations and neutrophil counts, the primary outcome of interest in chapter 2, constitutes an original finding in itself. Although it has been shown that cows with increased postpartum NEFA concentrations (> 0.5 m*M*) had decreased WBC counts (Hachenberg et al., 2007), most of the studies on the relationship between energy balance and neutrophils have assessed the (*in vitro*) functionality, rather than the blood circulating counts (LeBlanc, 2020). Despite the paucity of *in vivo* studies, our data is supported by previous literature. It has been reported that high NEFA concentrations decreased the viability of neutrophils *in vitro* and increased necrosis of these cells (Scalia et al., 2006). Scalia et al. (2006) suggested that, despite a substantial resistance of bovine neutrophils to an overload of NEFA, greater intensity of lipid mobilization might be associated with

down-regulated neutrophil functions. Van Schyndel et al. (2018) reported a more than 4-fold increase in neutrophil counts due to PEG as soon as 24 h after a first prepartum injection, reaching a 10-fold increase after a second injection at calving. Bone marrow appears to be an impressive reservoir of hematopoietic progenitors with a great capacity for proliferation, which enables a boost in the production of neutrophils in response to inflammatory stimuli such as G-CFS (Mayadas et al., 2014). Moreover, PEG increased the expression of genes related with cell survival (Heiser et al., 2018), while inflammatory stimuli generally delay neutrophil apoptosis (Bassel and Caswell, 2018). At WBC gene expression level, Lopreiato et al. (2020) reported improved migration, adhesion, and antimicrobial ability and an enhanced inflammatory response, which suggests cell activation. Thus, the bone marrow reserves and the potentially increased functionality at cell level would support the effect of PEG treatment on neutrophil counts, despite the presence of peripartum NEB.

As measured by WBC counts, our results support the hypothesis that the effect of PEG treatment on disease occurrence could be associated with the metabolic status of the transition dairy cow; PEG treatment would have a stronger preventive effect against disease in cows with elevated prepartum NEFA concentration than in cows with normal values.

6.2.2. Pegbovigrastim reduced the occurrence of a first case of early lactation clinical mastitis, particularly in cows with excessive prepartum BCS and in cows with elevated prepartum NEFA concentrations

Overall, PEG treatment reduced the occurrence of a first case of CM during the first 30 DIM, in line with previous field studies (Canning et al., 2017; Ruiz et al., 2017). Contrarily, two field studies reported a lack of PEG treatment effect on the occurrence of a first case of CM during the first 30 DIM (Zinicola et al., 2018; Van Schyndel et al., 2021). The main novelty in chapter 3 was that the preventive effect of PEG on the occurrence of a first case of CM during the first 30 DIM was associated with the prepartum metabolic status; PEG treatment reduced the occurrence of a first case of CM during the first 30 DIM particularly in cows with excessive prepartum BCS (i.e. > 3.5) and in cows with elevated prepartum NEFA concentrations (i.e. > 0.5m*M*).

Our data explain the lack of PEG treatment effect on the occurrence of a first case of CM during the first 30 DIM reported by Zinicola et al. (2018), as they excluded low and high BCS cows (i.e. BCS < 3 and > 3.75). Over-conditioned cows mobilize more reserves than cows in an acceptable body condition, and present a higher metabolic challenge, lower immune competence and higher risk of disease (Roche et al., 2009; Sordillo and Raphael, 2013; Ingvartsen and Moyes, 2015). Apparently in contrast with this explanation, Van Schyndel et al. (2021) in a Canadian study (freestall or tiestall housing), in which 40% of the cows were over-conditioned at enrolment (i.e. > 3.5), still reported a lack of PEG treatment effect on the occurrence of a first case of CM during the first 30 DIM. Even the prepartum BCS by treatment interaction was not significant. Interestingly, a considerable number of studies has found that early lactation grazing cows, compared to cows in confined housing, consistently lose more live weight and body condition, and present higher blood concentrations of NEFA and β-hydroxybutyrate, consistent with a more limited energy supply (Kolver and Muller, 1998; Fontaneli et al., 2005; Arnott et al., 2016). A previous study from our research group (Astessiano et al., 2015) evaluated the metabolic status of early lactation cows fed either total mixed rations (TMR; without grazing) or diets combining TMR and grazing (similar to the feed strategy used on the four participating farms in this thesis). They found higher concentrations of NEFA and β-hydroxybutyrate when cows grazed. Thus, under grazing conditions, transition cows may undergo a deeper and/or longer-lasting NEB because of low dry matter intake (Kolver and Muller, 1998; Bargo et al., 2003; Astessiano et al., 2015). The lower dry matter intake, added to increased energy requirements for grazing activity (NRC, 2001), increases the risk of excessive fat mobilization in comparison to confined cows. Ribeiro et al. (2013), under grazing conditions, showed that cows that calved with a BCS of 3 to 3.25 experienced minor BCS loss during early lactation and presented the lowest incidence of diseases. Thus, I hypothesize that compared to housed cows (e.g. Van Schyndel et al., 2021), grazing cows that calve in an over-conditioned state experience an excessive peripartum fat mobilization (Roche et al., 2009) associated with a greater immune dysfunction (Sordillo and Raphael, 2013; Ingvartsen and Moyes, 2015). This difference in fat mobilization in grazing versus confined management systems may explain apparent contradictions between our trial and the Canadian trial (Van Schyndel et al., 2021).

The low metabolic challenge, or its absence, in both the Zinicola et al. (2018) and Van Schyndel et al. (2021) studies is further supported by the fact that, in randomly chosen

subsets of cows, both reported low prepartum NEFA concentrations, well under 0.3 m*M*. In our study, prepartum NEFA concentration was on average 0.50 ± 0.40 m*M*, while 61% of the cows had a prepartum NEFA concentration above 0.3 m*M*. The preventive effect of PEG, particularly in cows with elevated prepartum NEFA concentration, appears to be in line with our results as reported in chapter 2, further supporting the hypothesis that PEG treatment would have a stronger preventive effect against disease in cows with elevated prepartum NEFA concentration than in cows with normal values.

Taken together, the data show that the immune stimulation due to PEG treatment would particularly benefit cows at risk of elevated lipid mobilization, such as the transition cows under a limited energy supply, which appears to be a frequent characteristic of grazing systems (Kolver and Muller, 1998; Bargo et al., 2003; Astessiano et al., 2015).

6.2.3. In cows with elevated prepartum NEFA concentration, Pegbovigrastim treatment increased the rate of first insemination, counteracted the negative association of early lactation clinical mastitis and uterine disease with the rate of pregnancy, and decreased the hazard of culling.

In chapter 4, we assess for the first time the effect of PEG on fertility and culling during a full lactation. This is of relevance, as the negative association of the incidence of early lactation clinical diseases such as CM and uterine disease with fertility and culling have been demonstrated throughout the lactation. Culling in particular would be more important late in lactation (Santos et al., 2004; Ribeiro et al., 2016; Carvalho et al., 2019). Previous studies have assessed the effect of PEG on fertility and culling based on much shorter follow-up periods (Canning et al., 2017; Ruiz et al., 2017; Zinicola et al., 2018; Van Schyndel et al., 2021).

Similar to our findings regarding WBC counts (Chapter 2) and disease occurrence (Chapter 3), we found that PEG treatment effect on fertility and culling interacted with prepartum NEFA concentration. In cows with elevated NEFA concentration (i.e. > 0.3m*M*), PEG treatment increased the rate of first insemination. In addition, PEG treatment in cows with elevated prepartum NEFA concentrations with a first case of CM during the first 30 DIM or UD increased the rate of pregnancy within 150 DIM, compared to untreated cows with the same set of risk factors. Ultimately, in cows with elevated NEFA concentration, PEG treatment resulted in a lower hazard of culling. No treatment

effects were detected in cows with lower NEFA concentration. Previously, a reduced failure to return to estrus within 80 DIM (Canning et al., 2017) and a modest increase in the rate of insemination (5.8%) during the first 100 DIM (Ruiz et al., 2017) were reported. Zinicola et al. (2018) reported a lack of PEG treatment effect on rate of insemination during the first 120 DIM, rate of pregnancy and hazard of culling during the first 180 DIM. Van Schyndel et al. (2021) reported a lack of PEG treatment effect on the hazard of culling during the first 63 DIM, rate of first insemination during the first 150 DIM and rate of pregnancy during the first 250 DIM. Due to differences in the follow-up periods and the prepartum NEFA concentration by treatment interaction, our results are difficult to compare with previous reports. However, the lack of PEG treatment effect on fertility and culling reported by Zinicola et al. (2018) and Van Schyndel et al. (2021) does not come as a surprise, considering the low metabolic challenge, the lack of PEG treatment effects on disease occurrence, and the shorter follow-up periods in their studies. Indeed, our primary hypothesis was that PEG treatment would prevent early lactation clinical diseases and that this treatment effect would be reflected in fertility and culling.

To analyze the effect of PEG on fertility and culling (Chapter 4), we proposed two sets of models, one with and one without early lactation clinical diseases as covariates. We included early lactation clinical disease in the models to assess whether early lactation clinical disease is an intermediate variable in the causative pathway between PEG treatment and the evaluated outcome. In our study, PEG treatment increased the rate of first insemination only in cows with elevated prepartum NEFA concentrations. Subsequently, we saw that the PEG treatment effect on rate of first insemination remained unchanged in the statistical models with or without clinical diseases. The occurrence of a first case of CM during the first 30 DIM was associated with a reduced rate of first insemination. As indicated, when including the occurrence of a first case of CM during the first 30 DIM in the model, the effect of PEG treatment remained unchanged. Hence, the reduction in the incidence of a first case of CM during the first 30 DIM (Chapter 3) is not the only mechanism by which PEG treatment increases the rate of first insemination in cows with elevated prepartum NEFA concentration. I hypothesize that this additional PEG treatment effect may be explained by two factors. Firstly, PEG treatment, and particularly the effect of reverting the negative association of prepartum NEFA with postpartum neutrophil counts (Chapter 2), could potentially limit the impact of early lactation subclinical diseases that are negatively associated with fertility (Sheldon et al.,

2006; Dolecheck et al., 2019). Secondly, PEG treatment could improve the metabolic status and inflammatory response postpartum. Elevated NEFA concentrations postpartum have been associated with reduced estrous cyclicity (Ribeiro et al., 2013) and reduced rate of insemination (Lüttgenau et al. 2015). In a small-scale study, it was observed that PEG treatment was associated with a lower postpartum NEFA concentration (Kimura et al., 2014). However, Van Schyndel et al. (2021) reported that PEG treatment was associated with slightly increased postpartum concentrations of NEFA and β-hydroxybutyrate. Immune activation implies a high cost of energy (Kvidera et al., 2017). Recently, it was proposed that a more pronounced NEB could be a mere sign of immune activation (Horst et al., 2021), as disease increases nutrient requirements in order to mount an immune response while inducing hypophagia (Brown and Bradford et al., 2021). A more effective immune response due to PEG treatment would reduce the NEB due to immune activation in diseased animals. This would be particularly relevant in a population of cows that are at a high risk of metabolic stress and immune dysfunction, such as cows with elevated prepartum NEFA concentrations (Sordillo and Raphael, 2013; Ingvartsen and Moyes, 2015).

The increased availability of circulating neutrophils due to PEG treatment could improve physiological processes such as uterine involution, which would reduce postpartum inflammation (LeBlanc, 2014; Gilbert and Santos, 2016; Sheldon et al., 2020). Inflammation directly affects the metabolic status of transition cows (Bradford et al., 2015), and it has been shown that pro-inflammatory cytokines inhibit feed intake and increase insulin resistance, worsening the metabolic status of the affected animal (Brown and Bradford, 2021). In summary, I hypothesize that PEG could improve the postpartum metabolic status by limiting the effect of disease and/or by mitigating the inflammation process commonly seen postpartum. These subjects warrant further research.

As mentioned, in chapter 4 we found that, in cows with an elevated prepartum NEFA concentration, PEG treatment counteracted the negative association of early lactation CM and UD with the rate of pregnancy. The ability to recruit neutrophils into the mammary gland and into the endometrium soon after calving is essential for mastitis resolution, uterine involution and subsequent fertility (Schukken et al., 2011; Gilbert and Santos, 2016). Furthermore, there are two key clinical findings that support the concept that PEG treatment improves the subsequent outcomes of CM and UD. In chapter 3, we reported that PEG treatment reduced the rate of total cases of CM during the full lactation. This

explains at least in part the benefit of PEG on the rate of pregnancy, as CM close in time (pre- or post-) to insemination negatively affects the rate of pregnancy (Dolecheck et al., 2019). Regarding the beneficial effect of PEG on rate of pregnancy in cows that recorded UD, in chapter 3 we reported that, in cows that recorded metritis, PEG reduced the incidence of subsequent endometritis. Van Schyndel (2018) reported in her master thesis that, in cows that experienced RP, PEG reduced the incidence of subsequent metritis, although this was not reported in her peer-reviewed paper (Van Schyndel et al., 2021). Altogether, our results suggest that PEG treatment supports the uterine healing process, which results in improved fertility (Gilbert and Santos, 2016). Both aforementioned key clinical findings (i.e. a reduced rate of total cases of CM during the full lactation and, in cows that recorded metritis, a reduced incidence of subsequent endometritis) were not associated with prepartum NEFA concentrations. However, it would be reasonable to hypothesize that, in cows with an elevated prepartum NEFA concentration, the effect of PEG treatment would be stronger and easier to detect. These cows with elevated NEFA concentrations are at higher risk of immune dysfunction (Sordillo and Raphael, 2013; Ingvartsen and Moyes, 2015; Chapter 2 this thesis), and are potentially less able to respond adequately to CM and UD (Schukken et al., 2011; Gilbert and Santos, 2016; Chapter 2 this thesis). The finding that PEG counteracted the negative effects of clinical disease on long-term outcomes such as pregnancy leads us to think that PEG might be useful as an adjunct treatment for disease and not only as a preventive treatment. The concept of PEG as an adjunct treatment will be developed later in this chapter.

Ultimately, PEG treatment did not affect the overall hazard of culling, but in cows with elevated prepartum NEFA concentration, PEG resulted in a lower hazard of culling. This reinforces the concept that PEG treatment is particularly beneficial in metabolically challenged cows. A lack of effect on the hazard of culling during the first 63 DIM and 180 DIM has been reported in previous field studies (Van Schyndel et al., 2021; Zinicola et al., 2018, respectively). Once more, the lack of PEG treatment on hazard of culling in these two studies may be due to a population under low metabolic stress and the lack of PEG treatment effect on disease as reported by them. In addition, the positive association between the incidence of early lactation clinical disease and culling would be more important late in lactation (Santos et al., 2004; Ribeiro et al., 2016; Carvalho et al., 2019). Carvalho et al. (2019) reported that the proportion of cows culled by 305 DIM increased from 23% in cows without clinical disease during the first 21 DIM, to 36% and 54% in

those diagnosed with a single and multiple clinical diseases, respectively. Thus, a longer follow-up period is important to observe treatment effects on culling.

As expected, the occurrence of a first case of CM during the first 30 DIM was associated with increased hazard of culling (Bar et al., 2008; Hertl et al., 2018). However, the reduction in the occurrence of a first case of CM during the first 30 DIM in PEG treated cows, particularly in those with an elevated prepartum NEFA concentration (Chapter 3), may be only a partial explanation for the finding of a decreased hazard of culling in PEG treated cows with an elevated prepartum NEFA concentration. Similar to the findings regarding the rate of insemination, the prepartum NEFA concentration by treatment interaction remained in the hazard of culling models with or without clinical disease, indicating that PEG treatment decreased the hazard of culling in cows with elevated NEFA concentration independently of a reduction in the occurrence of clinical diseases. Undoubtedly, a major reason for culling is reproductive failure (Kossaibati and Esslemont, 1997; LeBlanc et al., 2002) and we found that PEG treatment improved fertility in cows with elevated prepartum NEFA concentration (i.e. higher rate of first insemination, and higher rate of pregnancy in cows with a first case of CM during the first 30 DIM or UD; chapter 4). As mentioned, we also reported that PEG treatment reduced the hazard of a first case of CM and the rate of total cases of CM (Chapter 3). Hertl et al. (2018) reported that more CM cases in early lactation resulted in an increased rate of CM cases during the cow's lifetime and that these CM cases also increased the hazard of culling.

6.2.4. Pegbovigrastim treatment resulted in an overall economic benefit, which was associated with prepartum body condition score

In chapter 5, we study the economic impact of PEG treatment. This is a novelty, because the economic impact of PEG treatment has not been considered in any research published so far. Moreover, the methodology we used to perform the economic evaluation is of interest. We did not perform a bio-economic simulation (an alternative to our approach) but utilized real longitudinal data from each enrolled cow during a full lactation. This allowed accounting for heterogeneity between cows. Moreover, it allowed us to evaluate all effects of PEG treatment. For instance, small, not statistically significant effects of PEG treatment on disease occurrence may still add to an effect on milk revenues. By

using the data of all individual cows, we could evaluate the overall economic effect of PEG treatment. In contrast, in bio-economic simulation modeling studies, specific decisions need to be taken on what treatment effects need to be considered. Moreover, in such simulation studies, the effect of treatment on disease occurrence as well as the effect of disease on cow performance need to be parameterized, which is always a complex task.

To study the economic impact of PEG treatment, we defined the partial net return as the sum of milk revenues and costs of feed, medical treatments, insemination as well as the costs for culling. We also explored the effect of potential interactions of PEG treatment with parity, prepartum BCS and prepartum NEFA concentration on these outcomes.

The main finding in chapter 5 was that PEG treatment increased the partial net return per cow per year by US$ 210 ± 100. A reduced cost of culling per cow per year explained most (70%) but not all of this economic benefit.

Interestingly, only the C^{CM} per year was individually statistically significant, while all other factors represented in the partial net return (i.e. R_i^{Milk}, C_i^{Feed}, C_i^{UD}, C_i^{Other}, C_i^{Ins} and C^{Cull} per year) were not statistically significant. However, all these single economic factors combined, represented in the partial net return, contributed significantly to the economic benefit in PEG treated compared to Control cows. This illustrates how our approach accounts for all PEG treatment effects regardless of the magnitude or the statistical significance.

The resulting economic benefit of PEG relative to the partial net return was 22% per cow per year, which clearly is significant improvement. However, most of the economic benefit was explained by a reduced cost of culling, which can be seen as an important fraction of the failure cost of disease (Hogeveen et al., 2011). Quite frequently dairy farmers underestimate the failure costs of diseases (Hogeveen et al., 2017). Consequently, it may happen that dairy farmers do not see the economic benefit of PEG treatment. Raising awareness in the farmer about the (economic) consequences of disease and the efficacy of an intended intervention to mitigate its effects are critical points to its implementation (Jansen and Lam, 2012; Ritter et al., 2017). On the other hand, it is also known that economic benefits is only a part of the reason dairy farmers may choose for certain intervention (Lam et al., 2017). Veterinary practitioners are key intermediaries to show the farmer the consequences of disease and the benefits of an intervention (Jansen

and Lam, 2012; Ritter et al., 2017). Thus, I believe that farmers and advisors will need support to assess the benefits of the use of PEG, which are not easily visible and could be underestimated.

The partial net return and cost of culling per cow per year were associated with prepartum BCS. Numerically, the increase in partial net return and the reduction in the cost of culling were seen in under- and over-conditioned cows, but not in cows in an acceptable condition. From an economic point of view, the logical consequence of this observation would be to target the use of PEG to under- and over-conditioned cows. Using PEG only in under- and over-conditioned cows would save almost two thirds of the PEG doses, as two thirds (65%) of the cows in this study classified as cows in an acceptable condition (Chapter 3). Obviously, this implies the need to perform BCS assessments in all cows, for instance by using routine BCS scoring protocols. Despite the somewhat subjective nature of the variable, it has been suggested that after training of farm staff, BCS assessment is accurate and consistent (Roche et al., 2009). Nowadays, BCS assessment may even be performed using commercially available automated sensors (e.g. Mullins et al., 2019). Thus, based on my data, to implement the use of PEG in commercial dairy farms, I suggest implementing prepartum BCS assessment protocols and targeting the use of PEG to under- and over-conditioned cows. However, in the Canadian trial (Van Schyndel et al., 2021), prepartum BCS was not a useful indicator to target the use of PEG. Although a one-time BCS assessment allows the identification of cows at higher risk of elevated lipid mobilization (Roche et al., 2009), it certainly does not provide evidence of how much body reserves a cow has mobilized or will mobilize. Assessing BCS loss, which has been consistently associated with elevated peripartum NEFA concentrations, starting at least 21 days before the expected calving date, would provide more reliable information than a single assessment closer to calving (Barletta et al., 2017; Sheehy et al., 2017). So, to fine-tune even further, I hypothesize that both in housed cows (TMR fed cows) and cows under grazing conditions, targeting PEG treatment to cows that lose BCS during prepartum would be more efficient than to target PEG based on a single assessment at one week before calving.

6.3. Pegbovigrastim treatment appeared to be associated with better clinical disease outcomes

A finding that was not a primary hypothesis in this thesis was that PEG treatment was associated with better CM and uterine disease outcomes. This is suggested by the reduced rate of total cases of CM during the full lactation (Chapter 3), the increased rate of pregnancy in cows with elevated prepartum NEFA concentrations with a first case of CM during the first 30 DIM, and the tendency to reduced hazard of culling in multiparous cows with a first case of CM during the first 30 DIM (Chapter 4). Concerning uterine disease, PEG treated cows with metritis subsequently showed a reduced occurrence of endometritis (Chapter 3). Moreover, PEG treated cows with elevated prepartum NEFA concentrations that recorded UD had an increased pregnancy rate compared to control cows with the same set of risk factors (Chapter 4). Thus, PEG appears to be effective at restoring an affected mammary gland and an affected uterus.

To our knowledge, this is the first study to report that PEG treatment was associated with a reduced rate of total cases of CM during the full lactation. The preventive effect on the occurrence of a first case of CM during the first 30 DIM could be a possible explanation for the reduced rate of total cases of CM during the full lactation in PEG treated cows. More CM cases in early lactation result in an increased rate of CM cases during the cow's lifetime (Hertl et al., 2018). However, in contrast with the preventive effect of PEG on the occurrence of a first case of CM during the first 30 DIM, the effect of PEG treatment on the rate of total cases of CM during the full lactation was not associated with the prepartum BCS. This suggests that the reduced rate of total cases of CM during the full lactation relies on a reduced recurrence of CM cases rather on a preventive effect of a first case of CM during the first 30 DIM. It could be hypothesized that, during early lactation, PEG treatment increases the cure rate of CM after antibiotic treatment, as the ability to recruit neutrophils into the mammary gland is essential for mastitis resolution (Schukken et al., 2011). Powell et al. (2018), using PEG treatment prior to an experimental mastitis challenge, reported an improved mastitis resolution. Ruiz et al. (2017) reported that PEG treated multiparous cows that subsequently recorded CM during the first 30 DIM produced more milk compared to their control counterparts.

The improvements in long-term outcomes such as the increased pregnancy rate in cows with elevated prepartum NEFA concentration with a first CM case during the first 30

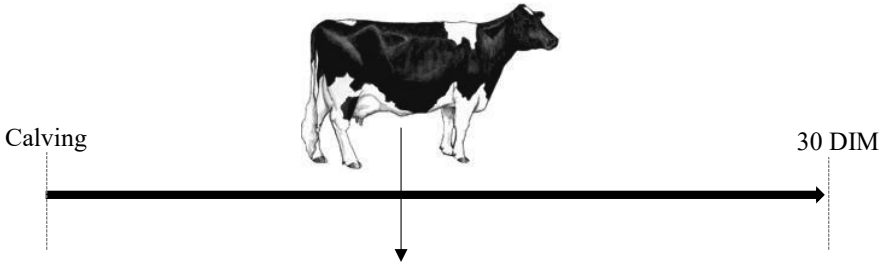
DIM and the tendency to reduction in the hazard of culling in multiparous cows with a first CM case during the first 30 DIM (Chapter 4) further support the association of PEG with improved outcomes after disease. In a novel approach, Denis-Robichaud et al. (2021) used PEG not as a preventive tool but as a treatment. They reported that cows treated with PEG as an adjunct therapy at the time that a naturally occurring case of severe CM was diagnosed had a better survival than control cows. Interestingly, severe CM may be associated with metabolic alterations and NEB (Burvenich et al., 2003). Thus, it could be speculated that severe CM may particularly benefit from the immune restoration that PEG exerts in cows under metabolic stress (this thesis).

Concerning uterine disease, we found that PEG treated cows with metritis subsequently showed a reduced occurrence of endometritis (Chapter 3). As mentioned above, Van Schyndel (2018) reported in her master thesis that in cows that experienced RP, PEG reduced the incidence of subsequent metritis. High proportions of neutrophils in the endometrium soon after calving were reported as beneficial to uterine health and subsequent fertility (Gilbert and Santos, 2016). These authors reported that cows that are capable of recruiting large numbers of neutrophils rapidly to the uterus in the immediate postpartum period are less likely to suffer bacterial infections and more likely to have a healthy postpartum uterine involution. Thus, the lower endometritis occurrence in PEG treated cows with metritis may be related to a higher neutrophil influx to the uterus due to PEG treatment. Previously, Ruiz et al. (2017) and Oliveira et al. (2020) reported an increased incidence of metritis due to PEG treatment. Ruiz et al. (2017) reported that PEG treated cows that subsequently recorded metritis produced more milk compared to their control counterparts. Oliveira et al. (2020) found no production differences between control and PEG groups. In our study, no effects of PEG treatment on the incidence of metritis was detected (Chapter 3). Mechanistically, physical damage and bacterial contamination associated with uterine clinical events such as RP and metritis will demand a great influx of neutrophils to the uterus (LeBlanc, 2014; Gilbert and Santos, 2016; Sheldon et al., 2020). The up to 10-fold increase of circulating neutrophils due to PEG treatment (Van Schyndel et al., 2018) would provide the neutrophils for a more robust and longer-lasting vaginal discharge. This is further supported by our findings that cows that recorded RP or metritis showed reduced circulating neutrophil counts compared to cows without these clinical events (Chapter 2). In addition, Zinicola et al. (2018) reported that PEG treated cows that recorded metritis had higher neutrophil counts in the vagina

compared to control cows with metritis. The increased rate of pregnancy in PEG treated cows with elevated prepartum NEFA concentration and UD when compared to control cows with the same set of risk factors (Chapter 4) further supports the association of PEG with a better outcome after uterine clinical disease.

Based on the results presented in this thesis, which are supported by the literature (Ruiz et al., 2017; Powell et al., 2018; Denis-Robichaud et al. 2021), and the patho-biology of mastitis and uterine disease in dairy cows (Schukken et al., 2011; Gilbert and Santos, 2016), I hypothesize that PEG treatment could be valuable as an adjunct treatment, when an early lactation clinical disease is diagnosed. Interestingly, this implies that the use of PEG would shift from a blanket use in all cows to the prevention/mitigation of disease in only a proportion of those cows, to treatment only the diseased cows, this would be relevant from an economic point of view.

I propose to carry out a randomized clinical trial to evaluate the effect of PEG as an adjunct treatment for early lactation CM and uterine disease. The outcomes of this trial would be the subsequent health, long-term fertility and longevity and ultimately the economic performance of enrolled cows. Moreover, I would study the effect of PEG on antibiotic usage and welfare of cows. The hypothesis to test is that the effect of PEG as a treatment for early lactation CM and uterine disease depends on the metabolic status of the cows at the time of the diagnosis. To assess the postpartum metabolic status of a cow, a β-hydroxybutyrate measurement can be performed. Beta-hydroxybutyrate after calving is a metabolite widely used to monitor the metabolic status of cows. This parameter is a valid predictor of health, milk production and fertility outcomes (Ospina et al., 2013; Overton et al., 2017). Beta-hydroxybutyrate measurements can be easily performed on blood, urine and milk using cow-side handheld devices. A potential approach to implementation at farm level could be the use of strips to determine β-hydroxybutyrate concentration in milk (Keto-test, Elanco Animal Health). In short, the proposed trial would diagnose CM and uterine disease, determine β-hydroxybutyrate concentration and randomize the affected cows to one of two trial arms: conventional treatment (control) or conventional treatment + PEG. The concentration of β-hydroxybutyrate would then be used as a covariate in the analyses of the data. A schematic representation for this trial is provided in figure 6.1.



1- Clinical diagnose
- Clinical mastitis
- Retained placenta
- Metritis
- Endometritis

2- β-hydroxybutyrate estimation

3- Treatment randomization
-Control: Conventional treatment
-PEG: Conventional treatment + PEG

Figure 6.1. Schematic representation of a trial to evaluate the effect of PEG as an adjunct treatment for early lactation CM and uterine clinical disease.

Another potential approach could be to target the use of PEG to cows that present risk factors for subsequent early lactation clinical disease. Events occurring during the previous lactation and during the dry period have a significant effect on the incidence of CM in the subsequent lactation. Among others, it has been reported that previous-lactation factors such as the length of lactation (Pinedo et al., 2012), the average SCC, high peak milk production (Niemi et al., 2021), clinical mastitis cases, SCC status across the dry period (Pantoja et al., 2009), milk leakage near dry-off (Schukken et al., 1993) and milk production at dry-off (Rajala-Schultz et al., 2005) were associated with early lactation CM. In the same line, induction of parturition, dystocia, stillbirths, twins, male calves, RP, or metritis were associated with subsequent uterine disease (Sheldon et al., 2020). I propose that these risk factors for early lactation CM and uterine disease could be used to target the use of PEG.

Interestingly, Zandkarimi et al. (2018) were able to predict early lactation CM occurrence by evaluating serum metabolomics phenotypes. Thus, the development of metabolomics could be a promising technology to associate with the use of PEG.

6.4. Synthesis of the main findings

The results from this field study show that PEG is effective in restoring the immune system in prepartum cows with a potentially stressed immune system due to metabolic challenges (based on BCS and/or NEFA). Pegbovigrastim treatment reversed the negative association of prepartum NEFA concentration with postpartum neutrophil counts (Chapter 2). In prepartum over-conditioned cows and in cows with elevated prepartum NEFA concentration, PEG treatment reduced the occurrence of a first case of CM during the first 30 (Chapter 3). In addition, in cows with an elevated prepartum NEFA concentration, PEG treatment increased the rate of first insemination, counteracted the negative association of early lactation CM and UD with the rate of pregnancy and decreased the hazard of culling (Chapter 4). Ultimately, PEG treatment resulted in an economic benefit per cow per year (Chapter 5).

Moreover, our data show that PEG treatment appeared to be associated with better clinical disease outcomes. Pegbovigrastim treatment reduced the rate of total cases of CM during the full lactation, and in cows with metritis subsequently reduced the occurrence of endometritis (Chapter 3). In cows with elevated prepartum NEFA concentration, PEG treatment counteracted the negative association of early lactation CM and UD with the rate of pregnancy and tended to reduce the hazard of culling in multiparous cows with a first case of CM during the first 30 DIM (Chapter 4), always compared to control cows with the same set of risk factors. Figure 6.2 provides a schematic diagram with the main findings of this thesis.

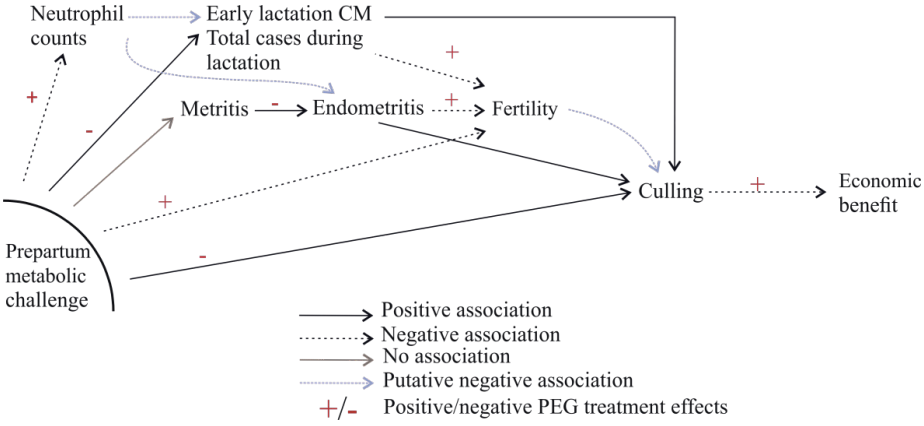


Figure 6.2. Schematic diagram with the main findings of this thesis. Metabolic challenge based on prepartum excessive body condition score and/or elevated nonesterified fatty acids concentration. A + on an arrow indicates a positive (beneficial) Pegbovigrastim treatment effect, a – on an arrow indicates a negative (still beneficial, as it reduced unwanted outcomes such as early lactation CM and hazard of culling) Pegbovigrastim treatment effect.

6.5. Contextualization of the impact of farm conditions on Pegbovigrastim treatment results

There are two underlying factors related to farm that need to be discussed to interpret the results and put in perspective the generalizability of the trial findings.

Firstly, the participating farms presented a high degree of metabolic challenge. This is an important point given the disparity of results between similar studies [i.e. this thesis vs Zinicola et al (2018) and Van Schyndel et al. (2021)] and the main finding of this thesis: PEG interacts with the metabolic status of cows. An important question to answer is whether the participating farms are representative of a greater population, at least in terms of prevalence of elevated prepartum NEFA concentration. The high prevalence of cows with elevated prepartum NEFA concentration is consistent with previous reports from our research group in pasture-based herds (Meikle et al., 2004; Adrien et al., 2012; Meikle et al., 2013; Astessiano et al., 2017; Garcia-Roche et al., 2021). A large field study carried out under different conditions in the US reported that 34% of the cows showed elevated prepartum NEFA concentrations (Ospina et al., 2010). Macrae et al. (2019) reported that

excessive NEB affected 52% of UK dairy cows in late gestation, with NEFA values as high as 0.5 m*M* or more in 26% of the cows. Interestingly, Spaans et al. (2022) described the metabolism during transition in a database consisting of 2,610 cow lactations of low to moderate-yielding grazing dairy cows in New Zealand. They conclude that metabolic stress in moderate-yielding grazing cows or high-yielding housed cows has a similar magnitude and appears to be a natural or hard-wired feature during the peripartum period. Overall, the data suggest that, despite major improvements in the diagnosis and management of periparturient metabolic stress (Overton et al., 2017), excessive metabolic stress still affects an important number of modern dairy cows. At cow level, the challenge would be to identify cows with elevated prepartum NEFA concentrations easily and fast, as there is currently no cow-side test available (Overton et al., 2017). Prepartum, it has been shown that lying time is associated with NEFA concentration (Menichetti et al., 2020). Thus, the use of devices to monitoring behavioural activities could be a promising approach to target the use of PEG.

At herd level, higher probability of disease, decreased milk production and decreased reproduction performance can be seen when more than 15% to 25% of the individual cows (given an appropriate sample size) show elevated prepartum NEFA concentrations (Ospina et al., 2013). Thus, a potential innovative approach for the use of PEG on dairy farms would be to select on a herd level basis rather than on a cow level basis. The hypothesis is that herds with a high proportion of cows with elevated prepartum NEFA concentrations would benefit the most from PEG treatment.

The second underlying factor related to farm is that the participant farms had a high incidence of early lactation clinical disease. In our study, the incidence of CM during the first 30 DIM was almost 4-fold higher than in most other field studies that evaluated the effect of PEG (18.3% vs < 5% from Ruiz et al., 2017; Zinicola et al., 2018; Van Schyndel et al., 2021). Out of these, only Ruiz et al. (2017) reported a preventive effect of PEG treatment on the incidence of CM by 25%. When a higher incidence of CM during the first 30 DIM was reported [23.1%; Canning et al. (2017)], the preventive effect of PEG treatment was a 35% reduction in incidence. Even Hassfurther et al. (2015), in a small-scale experiment using cows housed in a pen with a dirt floor that was kept wet to maximize the incidence of CM, reported that PEG treatment lowered the incidence of CM within 28 DIM by 50% and 74% depending on the dosage regimen.

Along the same lines, the incidence of metritis was higher in our study (32.2% vs < 21% from Ruiz et al., 2017; Zinicola et al., 2018; Van Schyndel et al., 2021). The high incidence of early lactation clinical diseases in the framework of this study is important with regard to the observed results at least from two points of view. First, as introduced in chapter 1, the high incidence of early lactation CM and uterine disease is linked to metabolic stress, immune dysfunction, deficient hygiene, etc. (Smith et al., 1985; Melendez et al., 2009; Giuliodori et al., 2013; Sheldon et al., 2020). Thus, a high incidence of early lactation clinical disease indicates shortcomings in transition cow management that would probably provide favourable conditions for PEG use. On the other hand, there would be little or no room for improvement using immune stimulation provided by PEG in well-managed dairy herds with a very low clinical disease incidence (e.g. Van Schyndel et al., 2021).

Secondly, as discussed earlier in this chapter, PEG not only showed preventive effects on clinical disease (Chapter 3). Pegbovigrastim treatment also appeared to be associated with better outcomes of clinical disease and appeared able to restore a mammary gland and uterus affected by clinical disease (Chapter 3; 4). Thus, in herds with a high incidence of clinical disease, PEG treatment effects such as disease prevention, better outcomes of clinical diseases and improved restoration of affected organs, would be beneficial for a greater proportion of cows, which would positively affect the economic balance of PEG treatment. Again, a potentially innovative approach to the use of PEG treatment could be to select herds, based on the hypothesis that herds with a high incidence of early lactation clinical disease would benefit the most from PEG treatment.

Considering that, the effect of PEG depends on the periparturient metabolic status of the cows (this thesis) and, potentially, on the incidence of clinical disease, PEG may be employed strategically. Based on known disease conditions, but also season, environmental conditions, feeding regimen and climate, herd managers may be able to target PEG use to certain moments. To estimate nutrient intake in terms of quantity and quality under grazing conditions is complex (Chilibroste, 2005). Moreover, in these systems, dairy cows are exposed to environmental conditions such as heat stress that are associated with immune dysfunction (Lacetera et al., 2005, Lacetera et al., 2006). Rain and mud affect the hygiene of cows and complicate feed management, so that grazing dairy cows may alternate between excellent environmental conditions and very adverse situations. Figure 6.3 illustrates two different environmental situations in the close-up

pens on one of the participating farms recorded during this trial. For this reason, calving month was consistently used as a covariate in the statistical models. Calving month was associated with WBC counts (Chapter 2), rate of total cases of CM during the full lactation, RP, endometritis (Chapter 3), rate of first insemination (Chapter 4) and ultimately tended to be associated with the partial net return per cow per year (Chapter 5). Hence, I hypothesize that environmental conditions in the study herds represent risk factors associated with immune dysfunction and with pathogen burden. These risk factors could be also associated with the effect of PEG treatment.

Figure 6.3. Two different environmental situations in the close-up pens in one of the participant farms recorded during this trial. Panel A: an excellent environmental condition, Panel B: very adverse situation.

Coming to the end of this thesis, I would like to underline three robust points of our trial: it relied on an appropriate sample size and research protocol compliance was ensured with the aid of research assistants fully dedicated to the field trial. As noted by Van Schyndel (2018), to ask owners or herd managers of large commercial herds to comply with specific research protocols (e.g. disease diagnoses and accurate recording) is unrealistic, and could result in misclassification bias. For the research reported in this thesis, three veterinary technicians were employed as research assistants, who covered the four farms seven days a week. Thus, treatment application, blood sampling, disease diagnosis or confirmation of diagnoses made by farm personnel, as well as record keeping were performed by specifically employed and well trained veterinary technicians. I believe that this is relevant in terms of protocol compliance, particularly in this study, in which longitudinal data were recorded during a long period of time (18 months). Thirdly, the most novel finding from our research, the fact that PEG treatment effect depends on the prepartum

metabolic status of cows, is supported by the literature on biology and epidemiology of the transition dairy cow.

On the other hand, this is the first study that enrolled herds with a large proportion of cows that showed a high degree of metabolic challenge and a high incidence of early lactation clinical disease. I consider that these results need to be confirmed on a greater population of herds. I propose to validate the use of PEG in terms of health, fertility, longevity and economic performance in a large multicenter field study, enrolling herds that could benefit most from using PEG as discussed and proposed here. These herds should present a high degree of metabolic challenge, within a production system that potentially exposes cows to adverse environmental conditions, and as a result show a high incidence of early lactation clinical disease. It appears that all these features are potentially more representative of some grazing productive systems rather that well-managed housing systems. South American countries such as Argentina, Brazil, Chile and Uruguay, which have more than 20 million dairy cows (Dairy Global, 2019; Lazzarini et al., 2019; DIEA, 2020; ODEPA, 2021), have production systems that are based at least partially on grazing (FAO, 2022). Some studies carried out in this region indicate that early lactation CM and uterine disease affect an unacceptably high number of cows (Melendez et al., 2009; Giuliodori et al., 2013; Oliveira et al., 2015; Sepúlveda-Varas et al., 2015; Cruz et al., 2021; this thesis). Thus, to carry out a multicenter field study under these conditions could validate the use of PEG as a tool with a high implementation potential. Such a trial will also provide further knowledge on the use of a BCS profile rather than a one-time assessment for decision-making at cow level and at herd level. Such a trial will provide farmers with robust information about a tool that could greatly enhance productivity, economic performance and the sustainability of their farms. I propose to also evaluate the effects of PEG on animal welfare and antimicrobial usage, as both outcomes are strongly associated with disease in dairy cows (Pol and Ruegg, 2007; Petersson-Wolfe et al., 2018).

Conclusions

In this thesis we evaluated the effect of PEG on circulating WBC counts, health, fertility and longevity and on the economic results during a full lactation in grazing dairy cows. In addition, we explored the effect of PEG treatment interactions with parity and the metabolic status of the transition dairy cow. We combined metabolic, immunological, epidemiological, fertility and economic data to build a large data set, which allowed testing our hypotheses. The main novelty in this study is that the metabolic status, measured as prepartum BCS and/or prepartum NEFA concentration, interacts with the effect of PEG on postpartum WBC counts, health, long-term fertility and hazard of culling, and ultimately on the economic performance of cows. The beneficial effect of PEG treatment was only observed in cows that were metabolically challenged.

The effect of PEG on postpartum WBC counts provided a first insight in the interaction between metabolism and the effect of PEG. Prepartum NEFA concentration was associated with reduced WBC, neutrophil and lymphocyte counts and tended to be associated with reduced monocyte counts. Pegbovigrastim treatment reversed the negative association of prepartum NEFA concentration with neutrophil and monocyte counts and tended to reverse the negative association of prepartum NEFA concentration with WBC counts. These findings supported our hypothesis that PEG treatment would have a stronger preventive effect in cows undergoing metabolic challenge and immune dysfunction.

The effect of PEG on health confirmed our previous hypothesis. Pegbovigrastim treatment reduced the occurrence of a first case of CM during the first 30 DIM particularly in cows with excessive prepartum BCS and in cows with elevated prepartum NEFA concentrations. Moreover, PEG treatment reduced the rate of total cases of CM during the full lactation.

To evaluate the effect of PEG on fertility and culling, we used the longitudinal data of each cow gathered during a full lactation. This approach was different from other field studies evaluating PEG, in which the observation of control and treated animals was limited to a much shorter follow-up period. We showed that the effect of PEG treatment on fertility and culling interacts with prepartum NEFA concentration. No treatment effect was detected in cows with low prepartum NEFA concentration, whereas PEG treatment in cows with elevated prepartum NEFA concentration showed an increased rate of first

insemination, counteracted the negative association of early lactation CM and UD with the rate of pregnancy and decreased the hazard of culling.

To evaluate the economic effect of using PEG, we defined the partial net return per cow per year as the combination of milk revenues and costs for feed, medical treatments, inseminations and culling during the full lactation. We showed that PEG treatment resulted in an overall economic benefit, as it increased the partial net return per cow per year by US$ 210 ± 100. This economic benefit was mostly explained by the tendency to reduce cost of culling in PEG treated cows.

Integrating results, PEG treatment appeared to be associated with better disease outcomes. This is suggested by the reduced rate of total cases of CM during the full lactation, the increased rate of pregnancy in cows with elevated prepartum NEFA concentrations with a first case of CM during the first 30 DIM, and the reduced hazard of culling in multiparous cows with a first case of CM during the first 30 DIM. Concerning uterine disease, PEG treated cows with metritis subsequently showed a reduced occurrence of endometritis, PEG treated cows with elevated prepartum NEFA concentrations that recorded UD (i.e. RP, metritis or both) had an increased rate of pregnancy.

Altogether, we showed that the beneficial effect of PEG treatment depends on the metabolic status of transition dairy cows, and that PEG treatment was particularly beneficial for cows undergoing prepartum metabolic challenge.

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Summary

The last decades have seen major improvements in the incidence of clinical disease during the transition from late gestation to early lactation (Ingvartsen et al., 2003, LeBlanc, 2010; Overton et al., 2017). However, this critical period in the productive life of the dairy cow, remains one of the biggest challenges for the dairy sector in many regions of the world (Kay et al., 2015; Overton et al., 2017; Gross and Bruckmaier, 2019). Early lactation clinical diseases affect up to 50% of modern dairy cows (Ingvartsen et al., 2003; Leblanc, 2010; Galvao, 2013), and these early lactation diseases are associated with impaired productive and reproductive performance a shortened longevity and have an important impact on the economic performance of dairy farming (Hogeveen et al., 2017).

A dysfunctional immune response has been linked to these early lactation clinical diseases (Waller, 2000; LeBlanc, 2014; Pomeroy, et al., 2017). Around parturition, dairy cows experience a decreased immune response capacity (Kehrli et al., 1989; Sordillo et al., 1995; Kimura et al., 1999). Among other causes, the metabolic burden to cope with milk production and the resulting negative energy balance (NEB) has been identified as a major factor associated with immune dysfunctionality (Galvao et al., 2010; Ingvartsen and Moyes, 2015). Metabolites related to NEB, such as nonesterified fatty acids (NEFA) and β-hydroxybutyrate, have been identified as immunosuppressants (Ingvartsen and Moyes, 2015). Elevated NEFA concentrations were associated with decreased white blood cell (WBC) counts (Hachenberg et al., 2007). Epidemiological studies have shown that elevated NEFA concentrations are associated with increased risk of diseases such as clinical mastitis (CM), retained placenta (RP) and metritis (LeBlanc et al., 2004; Melendez et al., 2009; Galvão et al., 2010).

Immune stimulation therapies such us the use of bovine granulocyte colony-stimulating factor (G-CSF) may be an innovative development that would mitigate this problem. Peer-reviewed studies evaluated the use of daily injections of recombinant bovine granulocyte colony-stimulating factor (G-CSF; Kehrli et al., 1991; Cai et al., 1994; Mitchell et al., 2003) on neutrophil counts and functional capacities. More recently, a long-lasting analogue of G-CSF has been developed, a pegylated form of G-CSF (pegbovigrastim; PEG) that is injected approximately 7 d before calving and again within 24 h after calving. Experimentally, when injected around parturition, PEG treatment substantially increased neutrophil counts (Kimura et al., 2014). At the time the research

described in this thesis was designed, just a few field studies had been carried out, showing that PEG treatment reduce the incidence of early lactation CM (EMA, 2015; Hassfurther et al., 2015). Subsequently, several field studies were conducted (Canning et al., 2017; Ruiz et al., 2017; Freick et al., 2018; Zinicola et al., 2018; Van Schyndel et al., 2021). However, up to date, the long-term effect of PEG on the incidence of clinical diseases, fertility, culling and the potential interactions of treatment with the metabolic status of the transition dairy cow has not been studied. In addition, no economic evaluation of using PEG treatment has been carried out.

In this thesis, we aim to address the effect of PEG on health, fertility and longevity, and on the economic results during a full lactation in grazing dairy cows. In addition, we explore the effect of PEG treatment interactions with parity and the metabolic status of the transition dairy cow. In this research, while working on four commercial Uruguayan grazing dairy farms, we developed a close and growing cooperation between the Quantitative Veterinary Epidemiology group and the Business Economics Group from Wageningen University, The Netherlands, and the Animal Endocrine and Metabolism Laboratory at the Veterinary Faculty of the Universidad de la República, Uruguay. This cooperation gave us the unique opportunity to combine the expertise of dairy scientists from various disciplines to develop and carry out an interdisciplinary project, which combined metabolism, immunology, epidemiology, statistics, fertility and economics. The basis for this project consisted of an extensive longitudinal data collection of a large number of dairy cows. The data from these cows were combined to build up a large data set, which allowed testing our hypotheses under grazing conditions.

Chapter 2 focuses on the effect of PEG treatment on postpartum (5 to 8 DIM) WBC counts. We showed that PEG treatment reversed the negative association of prepartum NEFA concentration with neutrophil and monocyte counts and tended to reverse the negative association of prepartum NEFA concentration with WBC counts. In the PEG treated group, cows diagnosed with RP or metritis showed lower neutrophil counts when compared to PEG treated cows without these clinical diseases. In chapter 3, we showed that PEG treatment reduced the occurrence of a first case of CM during the first 30 DIM, particularly in cows at risk of elevated lipid mobilization. Moreover, PEG treatment reduced the hazard of a first case and the rate of total cases of CM during the full lactation. In addition, PEG treatment improved the uterine healing process in cows that experienced metritis. In chapter 4, we investigated the effects of PEG on long-term fertility and

culling, and we showed that the effect of PEG treatment on fertility and culling interacts with prepartum NEFA concentration. In cows with low prepartum NEFA concentration, no treatment effect was detected. In cows with elevated prepartum NEFA concentration, PEG treatment increased the rate of first insemination, counteracted the negative association of a first case of CM during the first 30 DIM and uterine disease (UD: RP, metritis or both) with the rate of pregnancy. Ultimately, in cows with elevated prepartum NEFA concentration, PEG treatment decreased the hazard of culling. In multiparous cows with a first case of CM during the first 30 DIM, PEG treatment tended to reduce the hazard of culling. In chapter 5, we concluded that PEG treatment resulted in an overall economic benefit, mostly explained by a reduced cost of culling in PEG treated cows.

In chapter 6, integrating results, I discuss that the main finding of this thesis, observed throughout all chapters, was that beneficial effects of PEG treatment were dependent on the prepartum metabolic status of transition dairy cows. Depending on the evaluated outcome, the effect of PEG treatment was associated with prepartum BCS (Chapter 3, 5) and prepartum NEFA concentration (Chapter 2, 3, 4). Regarding the potential association of PEG treatment with parity, the only evidence detected was that, in multiparous cows that subsequently recorded a first case of CM during the first 30 DIM, PEG treatment tended to result in a lower hazard of culling. This treatment effect was not detected for primiparous cows. On the other hand, I discuss that PEG treatment appeared to be associated with better disease outcomes. This is suggested by the reduced rate of total cases of CM during the full lactation, the increased rate of pregnancy in cows with elevated prepartum NEFA concentrations with a first case of CM during the first 30 DIM, and the reduced hazard of culling in multiparous cows with a first case of CM during the first 30 DIM. Concerning uterine disease, PEG treated cows with metritis subsequently showed a reduced occurrence of endometritis, PEG treated cows with elevated prepartum NEFA concentrations that recorded UD (i.e. RP, metritis or both) had an increased rate of pregnancy. While discussing future research opportunities, I propose to carry out a randomized clinical trial to evaluate the effects of PEG as an adjunct treatment for early lactation CM and uterine disease. In addition, accounting for all our results, I hypothesize that herds with a high degree of metabolic challenge and a high incidence of early lactation clinical disease could benefit the most from using PEG. I propose to validate the use of PEG in terms of health, fertility, longevity and economic performance in a large multicenter field study, enrolling herds with these features.

To conclude, in this thesis we showed that the beneficial effect of PEG treatment depends on the metabolic status of transition dairy cows, where PEG treatment was particularly beneficial for cows undergoing prepartum metabolic challenge. No associations with parity were detected. Based on our data, I also propose that PEG treatment appears to be associated with better clinical disease outcomes.

Curriculum vitae

About the author

Publications

Training and education

About the author

Joaquín Barca was born on the 4th of November of 1981 in Montevideo, Uruguay. Right from his childhood he felt affinity with animals, and he was always drawn to biology, chemistry and physical sciences. In 2012, he graduated as doctor in veterinary science at the Facultad de Veterinaria de la Universidad de la República, Uruguay. Since then, he has an assistant lecturer position at the Dairy Science and Technology department. In 2021, he also obtained an assistant lecturer position at the Preventive medicine and Epidemiology department. His first intention was to work in the dairy processing industry, but then he became interested in primary milk production, in particular the health of dairy cows and whether health affects milk quality. In 2014, he started his master in animal production at the same university, obtaining his master's degree in 2016. His vocation to work in the health of dairy cows, with special focus on udder health, led him to develop ties with Wageningen University, the Netherlands. In 2022, he obtained his PhD degree at the Quantitative Veterinary Epidemiology group in Wageningen University. His research subject was immunology of the dairy cow, especially around calving, with focus on udder and uterine health, and its impact on production, fertility, longevity and profitability. He has developed strong skills in the field of epidemiology, statistics and data analysis. Joaquín has combined his academic career with work in the dairy industry at both industrial and farm level. He enjoys the combination of research and teaching. As a researcher and as an udder health advisor, he considers data analysis to be the cornerstone of a work routine. Moreover, translating up-to-date scientific knowledge into training courses and workshops for veterinarians, dairy herd owners and farm personnel has always been one of his strengths.

Publications

Barca, J., Y.H. Schukken, A. Meikle, P. Chilibroste. M. Bouman, and H. Hogeven. 2022. Pegbovigrastim treatment resulted in an economic benefit in a large randomized clinical trial in grazing dairy cows. J. Dairy Sci. In review.

Barca, J., A. Meikle, M. Bouman, and Y.H. Schukken. 2022. Effect of pegbovigrastim on fertility and culling in grazing dairy cows and its association with prepartum nonesterified fatty acids. J. Dairy Sci. 105:710–725.

Barca, J., A. Meikle, M. Bouman, G. Gnemmi, R. Ruiz, and Y.H. Schukken. 2021. Effect of pegbovigrastim on clinical mastitis and uterine disease during a full lactation in grazing dairy cows. PLoS ONE 16(5): e0252418.

Barca, J., Y.H. Schukken, and A. Meikle. 2021. Increase in white blood cell counts by pegbovigrastim in primiparous and multiparous grazing dairy cows and the interaction with prepartum body condition score and non-esterified fatty acids concentration. PLoS ONE 16(1): e0245149.

Cruz, I., I. Pereira, G. Ruprechter, **J. Barca**, A. Meikle, and A. Larriestra. 2021. Clinical disease incidence during early lactation, risk factors and association with fertility and culling in grazing dairy cows in Uruguay. Prev. Vet. Med. 191, 105359.

Ruprechter, G, M. Noro, O. Meotti, C. Batista, M de L. Adrien, **J. Barca**, and A. Meikle. 2020. Endocrine and reproductive parameters in sick and healthy primiparous and multiparous dairy cows. Theriogenology. 141:173–179.

Barca, J. M. Carriquiry, L. Olazabal, M. Fajardo, P. Chilibroste, A. Meikle. 2018. Milk fatty acid profile from cows fed with mixed rations and different access time to pastureland during early lactation. Journal of Animal Physiology and Animal Nutrition, 102(3), 620-629.

Training and education

The Basic Package (2 credits)	
WIAS introduction day	2016
Course on philosophy of science and/or ethics	2016

Disciplinary Competences (30 credits)	
Research proposal	2016
Applied mixed models in the analysis of experiments in animal production (UY)	2016
Advanced statistics course - design of experiments	2016
Economic principles and concepts for the veterinary sciences	2016
Dairy nutrition under grazing (UY)	2015
Applied economic modelling for the veterinary sciences	2016
Lactation biology (UY)	2017
Epidemiology: concepts and its application	2017
Udder health, epidemiology and management (UY)	2017
Veterinary epidemiology	2019
Epidemiology. Module I: Principles. Observational Studies design. Module II: Cluster data analysis (UY)	2019

Professional Competences (3 credits)	
Organization of a seminar (Visit of Prof. Dr. Ynte Schukken to Uruguay)	2017
WIAS course The Final Touch	2019

Presentation Skills (4 credits)	
National Mastitis Council (Poster)	2019

ICAR 2020 (Abstract)	2020
ICAR 2022 (Poster)	2022
Elanco Animal Health. Follow-up seminars (Oral)	2019-2022

Teaching competences (6 credits)	
Assistant Prof. Department of Dairy Science and Technology/Department of Preventive medicine and epidemiology. Facultad de veterinaria, Uruguay.	2016-2022
Tutorship of a BSc. Thesis (2)	2017
Tutorship of a MSc. Thesis (1)	2020

Total credits	45

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Ana, we have been working together for the last nine years and I am fully convinced we will be working together for much more time. I believe this thesis is the evidence of the success of our process together and the person you represent to me. In 2013, I reached out to you looking for a tutor and I found one. You encouraged me to develop my skills and introduced me to science, but to this day, what impresses me most are your exceptional ethic principles and your drive to help people develop themselves as scientists, but even more importantly, as well-rounded persons. I love the way you continuously challenge me to give more, pushing me to grow, and I admire your fascination when I, in turn, challenge you. My acknowledgments to this healthy, challenging and beautiful symbiosis.

Ynte, working with you has been one of the greatest pleasures and privileges of my professional career. You have welcomed me right from the start, and your support and the positive atmosphere you create permeate our research and our relationship. I thank you for the opportunity to let me learn a bit about your most rigorous methodology, and be a witness to your impressive capacity for work. Our interactions always end with solved problems, new knowledge, new ideas, and, during the entire journey, there are plenty of jokes and laughs. Thank you also for introducing our families to each other.

Henk, I enjoy your easy-going ways and the seemingly effortless manner you bring to our work. I clearly remember the much-appreciated interactions I have had with you. From the economic courses you invited me to take to the several meetings we have had in your office, you have consistently taught me to put animal health in an economic perspective. I consider this as a positive and robust distinguishing mark of my PhD formation. I believe the pandemic interrupted many of our plans, such as your visit to my home country. To this day, I have not had an opportunity to attend one of your world-famous PhD barbecues! If things move in the direction I want them to, and they will, we will have much more work and barbecues together.

Mette, my honorary promoter, you have taught me a lot about udder health. Your passion and unimpeachable rigour to give professional assistance to dairy farmers and to veterinary colleagues is contagious. I love the challenge that represents to work at your side. However, what is even much more remarkable, is the close friends we became suddenly. Friendliness is a meaningful word for me, it involves mutual affection and trust, and this is just what I feel we have.

Mi familia ha sido determinante en este proceso, y más aun, ha sido quien define lo que soy hoy. Mamá, Papá, ustedes son los responsables directos de la persona que soy. El apoyo incondicional que siempre me han dado, tiene como resultado que hoy sea una persona íntegra, lleno de confianza en mí mismo y lleno de amor propio. Ustedes dos han forjado mis ansias de superación y el carácter para recorrer el camino. Además han logrado repetir esto tres veces, porque estos rasgos personales son también muy evidentes en mis dos hermanos. Diego, Lucía, me han acompañado en este y en todos los procesos que me han tocado vivir. Somos partícipes de una hermandad excepcional, cómplices en las buenas y más aun en las malas. Muy frecuentemente buscamos nuestra complicidad y ahí estamos, siempre. Cada vez que pienso en ustedes me invade la sensación de fortaleza, parecería que nuestra unión es eso.

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