BIOMARKERS FOR BOVINE MAMMARY GLAND INVOLUTION AND DISEASE

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PUBLIC ABSTRACT

Dairy cows go through various cycles during the milk production cycle: 1.) give birth and begin to produce milk, 2.) cows produce copious amounts of milk and are bred again to become pregnant, 3.) about 2 months before they give birth, farmers stop milking cows and they rest until they give birth and begin the cycle anew. The period when cows are not being milked is called the dry period. During the dry period, the mammary gland is repaired which is essential for mammary gland health and milk production. However, the mammary gland can become infected or not properly repaired during the dry period, compromising milk production and cow welfare. Markers in the milk and blood can improve our knowledge of why the mammary glands of some cows become infected or are not fully repaired during the dry period, while others remain healthy. In this dissertation, I explore the association between blood and milk markers with the early mammary gland repair process during the first 2 weeks of the dry period.

ABSTRACT

Bovine mammary gland involution occurs after the abrupt cessation of lactation and involves a complex sequence of inflammatory processes, morphology changes, and mammary epithelial cell turnover. The mammary gland is vulnerable to bacterial infections in the first 3 weeks after dry off, especially if milk leakage occurs. Increased risk of milk leakage and infection are associated with high milk production at the end of lactation. Therefore, as advances in dairy cow genetics and management continue to increase milk production- more cows will be at risk for infection during early mammary involution. Many aspects of mammary gland involution remain unknown. Biomarkers can be used to predict disease, such as with plasma non-esterified fatty acids in close up dry cows. Biomarkers can also be used to monitor the effect of treatment or intervention. There is not strong evidence that many of the current biomarkers for mammary gland involution, such as lactoferrin, are associated with disease risk or improved involution. Thus, this dissertation is centered around the association of certain biomarkers (fat soluble vitamins and lipid mediators) with mammary gland involution, oxidative stress, and diseases in dairy cows.

I dedicate this dissertation to my loving family, to my best friends- Mom and Jen, my wonderful dogs- Bear and Thelma, and to my love, Bernard. Thank you for your love, laughter, and support.

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LIST OF ABBREVIATIONS

IMI	Intramammary infection
CMT	California mastitis test
MEC	Mammary epithelial cell
RER	Rough endoplasmic reticulum
TPH1	Tryptophan hydroxylase-1
BMB	Blood milk barrier
βCN1-28	β-casein fragment1-28
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitors of matrix metalloproteinase
TNF-α	Tumor necrosis factor-α
SCC	Somatic cell count
PMN	Polymorphonuclear cell
rBST	Recombinant bovine somatotropin
IHC	Immunohistochemistry
RT-PCR	Reverse transcriptase polymerase chain reaction
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
IgG	Immunoglobulin G
SAA	Serum amyloid A
DIM	Days in milk
BSA	Bovine serum albumin
MY	Milk yield

DO	Dry off
CU	Close up
C+7	7 days post-calving
TMR	Total mixed ration
SEM	Standard error of the mean
NEL	Net energy of lactation
CP	Crude protein
NFC	Non-fiber content
AOP	Antioxidant potential
ROS	Reactive oxidant species
LC/MS	Liquid chromatography mass spectrometry
ABTS	Monocation, 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfric acid)
CarrU	Carratelli units
LC-MS/MS	Liquid chromatography tandem mass spectrometry
HPLC	High-performance liquid chromatography
BAEC	Bovine aortic endothelial cells
AAPH	2,2'-azobis-2-methyl-propanimidamide, dihydrochloride
ECIS	Endothelial Cell-substrate Impedance Sensing
VDR	Vitamin D receptor
COX	Cyclooxygenase
LOX	Lipoxygenase
CYP	Cytochrome P450
PUFA	Polyunsaturated fatty acids

PEG	Pegbovigrastim
BLV	Bovine leukosis virus
BHT	Butylated hydroxytoluene
ARA	Antioxidant reducing agent
TPP	Triohenylphosphine
PGF2α	Prostaglandin F2α
HETE	Hydroxyeicosatetraenoic acid
DHET	Dihydroxyeicosatrienoic acid
DiHOME	Dihydroxyoctadecenoic acid
oxoETE	Oxoeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
5-IPFαVI	5-iso-prostaglandin FαVI
ATRA	All-trans retinoic acid
9cisRA	9-cis retinoic acid
13cisRA	13-cis retinoic acid
RA	Retinoic acid
8,12iPGF2 α VI	8,12-iso-prostaglandin-F2α VI
8iPGA2	8-iso-prostaglandin A2
AA	Arachidonic acid
ALA	α-Linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid

LA	Linoleic acid
Dihomo γ LA	Dihomo-γ-linolenic acid

INTRODUCTION

Dairy cow mammary health management has advanced significantly during the last several decades, contributing to increased milk production and improved animal welfare. However, approximately 25% of dairy cows are diagnosed with clinical mastitis every year, and of those, about 25% of cows are sold or die because of treatment failure. Therefore, further research on mastitis prevention is essential, particularly as new obstacles arise from the unintended consequences of increased milk production. The risk for intramammary infection is greatest between dry off through around the 3-4 weeks post-calving. The early dry period (dry off – 4 weeks post-dry off) is increasingly being recognized as a pivotal juncture for mammary gland health. Indeed, one of the unintended consequences of increased milk production is that greater milk production at the time of dry off increases the risk for new intramammary infections resulting in mastitis after dry off. Together, these factors demonstrate the importance of studying the physiological processes occurring in the mammary gland during the transition from lactating to nonlactating, also known as mammary gland involution.

Mammary gland involution is triggered by the abrupt cessation of lactation at dry off and involves alterations in immune function, metabolism, biochemical, and mammary gland morphology. Additionally, milk-synthesizing mammary epithelial cells damaged during lactation are removed and replaced by new cells, enhancing future milk production. Optimizing mammary gland involution could improve mammary gland health and enhance milk production; however, many aspects of involution are poorly understood. In this dissertation, I performed experiments to determine the role of biomarkers representing physiological responses to involution and intramammary infection during the critical transition period.

CHAPTER 1: BIOMARKERS FOR MAMMARY GLAND INVOLUTION IN DAIRY COWS

ABSTRACT

After dry off, the process by which the bovine mammary gland transitions from lactating to non-lactating is referred to as mammary gland involution. Mammary gland involution in dairy cows is a complex process with implications for mammary gland health, milk production, and cow welfare. For example, when shortened dry periods do not provide enough time for completion of involution, cows will produce significantly less milk in the next lactation. Decades of studies centered around optimizing dry cow management have led to advances in dry cow health and welfare. However, many aspects of mechanisms involved in mammary gland involution are still poorly understood. As a result, it is difficult the fully assess differences between a mammary gland that has completed optimum involution and another with inadequate involution. The exception is the measure of direct outcomes, such as mastitis risk, future milk yield, and behavioral changes, which can be more easily measured. However, it is important for investigators to understand what mechanisms are affected when applying various treatments to optimize mammary gland involution. Therefore, the objective of this review is to identify biomarkers of mammary gland involution and to relate these biomarkers to specific mechanisms or outcomes of involution. By integrating various techniques and standardizing methods for assessing mammary gland involution, progress toward improving dairy cow health and welfare may be accelerated.

INTRODUCTION

The bovine mammary gland is an incredibly adaptable organ that undergoes many morphological, immunological, histological, and biochemical changes as the cow transitions through different life and lactational stages. Despite the adaptable nature of the mammary gland,

it is relatively vulnerable to disease. Intramammary infection (IMI) is the most common clinical disease reported by dairy farmers affecting nearly 25% of cows, is among the top 3 reasons for culling, and accounts for approximately 13% of deaths in adult cows (NAHMS, 2016). The stages of lactation when the mammary gland is most vulnerable to new IMI are the transitions from lactating to non-lactating (at dry off) and the transition from non-lactating to lactating in the periparturient period (Dingwell et al., 2003). The periparturient period accounts for the greatest percentage of new IMI (Dingwell et al., 2003). However, physiological events occurring during the transition from lactating to non-lactating can have significant effects on disease risk at later stages, including early lactation (Dingwell et al., 2003). Thus, optimizing conditions for cows around the time of dry off may help reduce rates of IMI identified in the transition period. Consequently, the transition period from a lactating state to a non-lactating state ("mammary gland involution"), is increasingly recognized as a critical juncture for determining milk production and mammary gland health in subsequent lactations in dairy cows (Zhao et al., 2019, Capuco and Choudhary, 2020, Ouellet et al., 2020).

The dry period is a manufactured phase of the dairy cow lactation cycle when milk collection ceases for the last 6-8 weeks of pregnancy and has been widely adopted since the early 1900's (van Knegsel et al., 2013). While it was clear that a dry period prior to parturition was essential to ensure adequate milk production, an understanding of the mechanisms during the dry period promoting milk production and mammary health was not well discerned. Although our knowledge regarding bovine mammary gland involution has advanced substantially, a significant proportion of primary mechanistic studies that serve as the foundation for current research were completed more than 20 years ago (Hurley, 1989, Zhao et al., 2019). The importance of this earlier research cannot be understated; however cows have changed greatly in recent decades

with genetic and nutritional advances increasing milk production (Miglior et al., 2017). Indeed, milk yield per cow has increased 13% over the last decade (NASS, 2020). In 2000, typical milk production for a North American dairy cow at the time of dry off was around 16 kg/d, whereas today, current milk production at dry off is closer to 28 kg/d with some cows drying off while still producing >75 kg/d (Dingwell et al., 2001, Olagaray et al., 2020). Previous researchers have concluded that complete mammary gland involution requires 21-28 d (Sordillo and Nickerson, 1988). With current milk production however, there is evidence that cows with high milk production at dry off may need more time (a longer dry period) for mammary gland involution to be completed (Olagaray et al., 2020). Alternatively, reducing milk production prior to dry off or accelerating mammary gland involution may enhance the transition from lactation to non-lactation in high producing cows. Thus, a better understanding of methods to optimize mammary gland involution in high producing cows is essential.

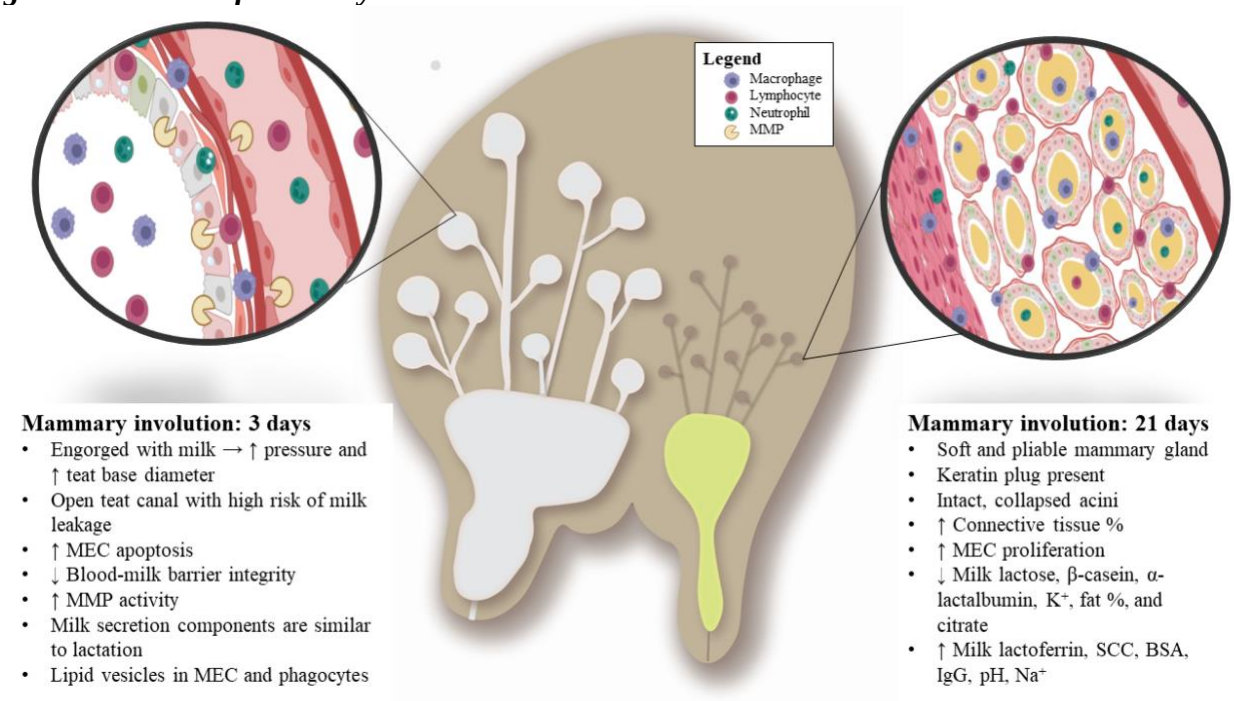
Mammary gland involution studies have utilized biomarkers to quantify the involution process – often used comparing a treatment to control group. Biomarkers are physiological measurements that indicate biochemical, morphological, and physiological changes. For example, milk synthesis decreases quickly in the days after dry off resulting in decreased lactose concentrations in milk secretions during early involution. Lactose is uniquely produced by milk synthesizing mammary epithelial cells (MEC). Thus, decreasing lactose concentrations in milk appear to be biomarkers for the decline in MEC milk synthesis during early mammary gland involution. However, MEC activity changes are just one component of mammary involution and so measuring multiple biomarkers representing other aspects of involution should be included as well. To study mammary gland involution properly utilizing biomarkers, one must be knowledgeable in the complex processes involved.

Mammary gland involution: morphology and mechanisms

Mammary gland involution is the process through which a lactating mammary gland transitions to a nonlactating state. There are 3 different types of mammary gland involution: 1) gradual involution, which occurs as milk production decreases during a normal lactation, 2) active involution, which occurs after abrupt cessation of lactation at dry off; and 3) senile involution which, occurs at the end of an animal's reproductive life (Hurley, 1989). Abrupt cessation of milk removal at dry off invokes a cascade of hormonal, physiological, and morphological changes in the mammary gland that are referred to as active involution Figure 1, (Piantoni et al., 2010). When compared to other commonly studied animals (such as rodents), the process of mammary gland involution in dairy cows is unique (Capuco and Ellis, 2013). Dairy cows are pregnant during involution and factors related to pregnancy likely account for the speed and degree of morphological changes occurring in the mammary gland (Capuco et al., 2002). Mice are most common animal model used to study mammary gland involution and after weaning there is a complete collapse of alveolar structures and removal of cells associated with milk production (Lund et al., 1996). After approximately 2 weeks of involution, the mouse mammary gland resembles that of a virgin mouse; thus, mammary epithelial cells undergo massive apoptosis resulting in a loss of alveolar structure (Lund et al., 1996). However, mice and most other species are not pregnant during mammary gland involution, unlike the dairy cow which undergo mammary gland involution with a 'parallel pregnancy' (Hughes, 2021). When pregnant mice were weaned the degree of cell death has been shown to be reduced which resembled the parallel pregnancy signature of dairy cow mammary gland involution (Capuco et al., 2002, Hughes and Watson, 2018). Non-pregnant cows are generally not dried off, thus less is known about the course of involution without a parallel pregnancy in cows (Hughes, 2021).

Interestingly, non-pregnant cows that had been non-lactating for 6 years maintained mammary alveolar structure, although they were collapsed (Finot et al., 2019). Furthermore, the non-pregnant dry cows had the same proportion of mature epithelial cells expressing casein and α-lactalbumin genes as did cows in mid-lactation (Finot et al., 2019). Dry cows have been shown to have an increase in putative mammary progenitor cells compared to lactating cows which could be future targets for improved mammary tissue repair during involution (Capuco et al., 2012, Finot et al., 2019).

Figure 1.1: ***Diagram of physiological processes occurring in the dairy cow as the mammary gland transitions from early to late active involution***



MEC = mammary epithelial cell, MMP = matrix metalloproteinase, SCC = somatic cell count, IgG = immunoglobulin G

Morphometric changes in the udder and teats occur during the first 7 d after dry off as a result of mammary gland engorgement resulting from cessation of milk removal. The engorged mammary gland can be characterized by increased pressure, swollen quarters with increased surface area, and distended teats (Bertulat et al., 2017, de Campos et al., 2020, Larsen et al., 2021). The engorgement period generally occurs during the first 3 d after cessation of milking and is more

severe in cows with greater milk yields (Bertulat et al., 2013, Bertulat et al., 2017). Moreover, the severity of mammary gland engorgement after abrupt milk cessation in high producing cows is associated with increased stress and behaviors indicating pain (Bertulat et al., 2013, Zobel et al., 2013a).

The teat is often ignored in studies concerning involution, but teat end integrity is an essential factor in maintaining mammary gland health (Dingwell et al., 2003). During lactation, teat characteristics such as presence of hyperkeratosis, width of teat end, or width of the teat barrel are associated with increased risk for intramammary infection (IMI) (Chrystal et al., 1999, Guarín et al., 2017). Furthermore, the teat cistern diameter, as measured by ultrasound, was positively associated with CMT score (Seker et al., 2009). Interestingly, the width of the teat apex of the front, but not the rear, teats has also been associated with increased risk for IMI in lactating cows (Guarín and Ruegg, 2016, Guarín et al., 2017). Whether or not the interaction of teat morphology and position on the udder also applies to IMI risk the dry period has not been fully investigated. The teat goes through morphological changes during the dry period and, like the mammary gland itself, becomes more resistant to bacterial infection (Dingwell et al., 2003). At dry off, teat characteristics associated with risk of new IMI during the dry period include teat end condition and formation of the keratin plug (Dingwell et al., 2004). However, after dry off the teat and teat canal progresses through morphological and biochemical changes throughout the entire dry period and the role of those changes relative to risk of IMI is unknown (Oldham et al., 1991, Tóth et al., 2019). For example, in the first week of the dry period, the teat canal becomes shorter and wider (Comalli et al., 1984, Oldham et al., 1991). The degree of shortening and widening of the teat canal during the early dry period may increase risk of new IMI because those 2 factors are associated with higher rates of mastitis (Klein et al., 2005). The change in teat

canal morphology in the first week after dry off may be associated with increased pressure in the mammary gland after cessation of milking. In support of this theory, nearly half of the teat sphincters of cows producing more than 21 kg/day of milk at dry off were still open 6 weeks after dry off (Dingwell et al., 2003). At the same time point, only 19% of teat sphincters from cows producing less than 21 kg/day of milk at dry off remained open (Dingwell et al., 2003). In a different study, cows producing greater than 20 kg/day milk at dry off, had increased mammary pressure through 9 days after dry off and were 5.1 times more likely to leak milk than lower producing cows (Bertulat et al., 2013). Consequently, cows that leak milk after dry off are 4 times more likely to develop a new IMI during the dry period (Schukken et al., 1993). Putting this together, Larsen et al. (2021) conducted a study of effects and interactions among different treatments aimed at decreasing milk production before dry off (reduced energy intake, reduced milking frequency, injection of prolactin inhibitor) on changes in teat perimeter and milk leakage after dry off in high producing cows (25.9 +/- 5.8 kg/d MY) (Larsen et al., 2021). Treatment groups had reduced milk yields prior to dry off, smaller teat perimeters during the week before dry off, and their teat perimeters increased less after dry off as compared to controls (Larsen et al., 2021). However, this study did not directly evaluate the relationship between teat perimeter and milk leakage after dry off and cows in almost all treatment groups had milk leakage after dry off, regardless of teat perimeter (Larsen et al., 2021). Future researchers should investigate the interaction between teat characteristics and teat characteristic changes during the early involution period in relation to risk of IMI.

The keratin plug is comprised of epithelial cells, lipids, and proteins that forms a physical barrier preventing entry of pathogens into the mammary gland between milk collections. Keratin originates from keratinocytes lining the teat cistern which are eventually sloughed into the teat

canal (Paulrud, 2005). The keratin plug also contains fatty acids that may provide some additional bacteriostatic factor (Hogan et al., 1987). The fatty acid profile of the keratin plug changes after dry off with an increase in proportion of cholesterol and decreased triglyceride content (Bright et al., 1990). Barrier function is presumably improved during the dry period as the mass of the keratin plug is twice that of lactation, but any potential benefits of the shift in fatty acid profile against intramammary infection IMI remain unknown (Bright et al., 1990). Formation of the keratin plug is the most important quarter-level factor associated with decreased risk for IMI during the dry period (Dingwell et al., 2003). During the first 3 days after dry off, the teat canal becomes shorter and wider which may impede keratin plug formation (Oldham et al., 1991). Keratin plug formation usually occurs by 16-40 d post dry off (Comalli et al., 1984, Williamson et al., 1995). Lack of keratin plug formation may occur in approximately 23% of quarters and is associated with increased milk production at dry off (Dingwell et al., 2004). Interestingly, increased keratin plug formation within 4 weeks of dry off has been positively associated with use of dry cow antibiotic therapy (Williamson et al., 1995).

Despite maintaining its' alveolar structure, throughout the dry period the bovine mammary gland progresses through extreme physiological and morphological changes. Historically, the morphological changes occurring in the mammary gland during the non-lactating period have been divided into 3 stages: 1) active involution which begins after dry off and is completed within approximately 21-30 d, 2.) the steady state period which follows active involution during and occurs when the mammary gland is fully involuted, and 3.) the redevelopment stage in which mammary gland tissues begin producing colostrum approximately 14 d before parturition (Oliver and Sordillo, 1989). Active involution is characterized by turn-over of inferior milk producing epithelial cells, an infiltration of leukocytes (especially lymphocytes and

macrophages) into the mammary gland, increased permeability in tight junctions of epithelial and endothelial cells, and alterations in the extracellular matrix of the mammary gland (Sordillo and Nickerson, 1988). During the early dry period the mammary gland is particularly vulnerable to bacterial infections (Oliver and Sordillo, 1989). In contrast, during the steady state, the mammary gland is fairly resistant to IMI, hence shortening the duration of active involution could help reduce risk of IMI during the dry period (Dingwell et al., 2003). The final phase of the dry period is lactogenesis, a phase that is characterized by rapid mammary epithelial cell (MEC) proliferation, infiltration of lymphocytes and macrophages into alveolar epithelium and acinar lumen, and increased number and size of organelles important in milk production such as the rough endoplasmic reticulum (RER) and Golgi apparatus (Sordillo and Nickerson, 1988, Capuco et al., 1997).

The categorization of the involution process into phases has been considered as an effective way to compare involution in the cow to other species (such as mice or humans), which have vastly different processes. However, Capuco et al., (1997) conducted a study on morphometric and metabolic changes in the non-lactating, bovine mammary gland and concluded that the term "involution" and its 3 stages mischaracterize the physiological processes occurring between dry off and parturition. Rather, Capuco et al. (1997) argued that the bovine mammary gland during the non-lactating period is never static and removal of senescent MEC and accelerated MEC cellular division that occurs throughout the dry period are more accurately described by the term "regenerative involution" (Capuco et al., 1997). Insights about MEC population dynamics have informed our understanding of why the dry period is essential for future milk production (Capuco and Choudhary, 2019). During the early involution period, the rate of apoptosis in MEC is greater than during later stages (Sorensen et al., 2006). Conversely, MEC proliferation

increases during the dry period and this balance results in no actual changes in the number of MEC during involution (Capuco et al., 1997, Sorensen et al., 2006). This turnover of MEC during the dry period replaces nonfunctional MEC with newly synthesized MEC resulting in greater milk production capacity. Dry periods of <30 d have been shown to reduce milk yield by 3.2-13.2% and omission of the dry period has been estimated to reduce milk yield in the subsequent lactation by 9-29% (van Knegsel et al., 2013). Similarly, after 25 d of mammary gland involution, conventionally dried mammary gland halves had greater milk synthesizing epithelial cell proliferation than the mammary gland half milked continuously through the dry period (Capuco et al., 1997). Additionally, there is evidence that older cows experience greater negative effects of short dry periods, including reduced capacity for milk synthesis and compromised mammary health (Pattamanont et al., 2021). Although the term "regenerative involution" aptly describes MEC turnover, the nomenclature of 3 involution phases is still useful in describing remodeling of alveoli, parenchyma, and ECM of the mammary gland that occurs during the non-lactating period.

The exact prompt for initiation of involution in dairy cattle is not as well understood. In mice, mammary gland involution is initiated by weaning and increased pressure that occurs with the abrupt cessation of daily milk harvest (Marti et al., 1997). Mechanical stretching of MEC in-vitro induces expression of the serotonin producing enzyme, tryptophan hydroxylase-1 (TPH1). The activated TPH1 increases serotonin concentrations in milk, which can occur within 10-18 hrs of delayed milking, mirroring the initial udder engorgement occurring at that time (Horseman and Collier, 2014). Furthermore, in humans and mice, infusion of serotonin causes mammary gland involution (Marshall et al., 2010). Serum serotonin concentrations have been shown to be highly associated with increased albumin concentrations in milk in post-partum cows demonstrating a

relationship between serum serotonin concentrations and impairment of the blood milk barrier (BMB); a process that also occurs during early involution (Kessler et al., 2019). Another potential mechanism for initiation of mammary gland involution involves increased cleavage of β-casein in milk by plasmin at the end of lactation or as a result of stress, begetting increased β-casein fragment (βCN1-28) concentrations (Silanikove et al., 2000). The βCN1-28 inhibits milk production and enhances cellular matrix degradation by closing potassium channels on the apical side of MEC (Silanikove et al., 2009). The resulting decrease in potassium output into milk lessens osmotic pressure and decreases overall milk yield (Holt, 1993). Currently, the precise triggering mechanism that initiates mammary gland involution is unknown, but if the variety of methods used to hasten involution can be used as evidence, multiple pathways likely exist (Zhao et al., 2019).

The extensive extracellular matrix (ECM) remodeling during mammary gland involution decreases alveolar space and increases stromal area (Sordillo and Nickerson, 1988). The ECM remodeling is facilitated by activity of matrix metalloproteinases (MMP), tissue inhibitors of metalloproteinases (TIMP), and plasminogen activator system (Rabot et al., 2007). Plasmin is the proteolytic enzyme that is activated form of plasminogen and its activity increases in milk secretions during involution (Athie et al., 1997). Both plasminogen activators and inhibitors can be found in mammary gland tissue, but in-vitro studies have shown that neutrophils activated by TNF-α can also activate plasminogen (Chou et al., 2009, Zhao et al., 2019). The MMP are a family of zinc-containing enzymes that play a large role in the process of involution. During involution, MMP-1 and MMP-2 are upregulated in epithelial and endothelial cells, respectively suggesting that different MMP may have different roles (Rabot et al., 2007). When neutrophils are activated by certain cytokines (such as TNF- α), they produce and store MMP-9 in granules

which are then released via degranulation (Yu et al., 2012). During early mammary gland involution, MMP-9 concentrations and activity in milk secretions increase rapidly (Zhao et al., 2019). The concentration of TIMP-1 and -2 mRNA are least during the middle of the dry period and increase closer to parturition (Rabot et al., 2007). Concentrations of TIMP regulate activation of MMP and a decrease in the TIMP:MMP ratio could increase the speed and degree of ECM remodeling (Talhouk et al., 1992).

The BMB is comprised of MEC and the basal membrane, connective tissue, and endothelial cell lining of the blood vessels supplying blood to mammary alveoli. The MEC junctional integrity has the largest impact on BMB permeability (Wellnitz and Bruckmaier, 2021). During early involution, increased protease activity degrades tight junctions between MEC. Impaired tight junctions result in mingling of components of the milk alveoli with components normally found only in blood or interstitial fluid and vice versa (Stelwagen et al., 1997). This blending of compartmental components can serve as biomarkers for BMB integrity. For example, lactose, is only synthesized in MEC, but can be found in increasing concentrations in blood and urine during periods when tight junctions are impaired (such as during involution or as a result of mastitis) (Stelwagen et al., 1997). The alteration of BMB integrity can occur early in mammary gland involution. For example, integrins are proteins that make up a part of MEC junctional complexes which are downregulated after only 18-24 hours of milk stasis (Singh et al., 2005). During early involution, increased concentrations of serum albumin, immunoglobulins, and sodium can be found in milk secretion (Sordillo et al., 1987, Ollier et al., 2013). Concurrently, the pH of milk secretions increases during this time period which is likely the result of an influx of buffering components from interstitial fluid (Hurley, 1987).

During involution, leukocytes leave circulation and infiltrate the mammary gland leading to an increase in SCC (Nickerson, 1989). Macrophages and lymphocytes are the primary leukocyte in the healthy involuting mammary gland, but infected mammary glands have a greater number of polymononuclear cells (PMN) (Nickerson, 1989). Both macrophages and PMN aid in clearing lipid droplets and milk proteins (Sordillo and Nickerson, 1988, Nickerson, 1989). Phagocytes are also essential to clear apoptotic MEC that accumulate during involution (Akhtar et al., 2016). Infiltrating leukocytes are insufficient to clear all senescent MEC so healthy MEC become phagocytic, engulfing their neighbors in a similar fashion as PMN (Hughes, 2021). This phenomenon is known as efferocytosis (which means 'to bring to the grave') is essential for normal involution in the mouse and has been described in ovine mammary involution (Tatarczuch et al., 1997, Hughes, 2021). It is not known what role efferocytosis may play in bovine mammary gland involution.

The paramount role of adipose tissue in homeostasis, inflammation, and disease in dairy cattle has only recently been recognized (Bradford et al., 2015, Contreras et al., 2017). The growing field of adipose research regarding the potential role of adipose tissue in the mammary gland of adult cows is lacking (Depreester et al., 2018). In contrast, adipose tissue dynamics are considered essential in humans and mice during mammary gland involution (Kothari et al., 2020). For example, in mice, lipids derived from MEC are taken up by surrounding adipocytes resulting in adipocyte hypertrophy during involution (Zwick et al., 2018). Additionally, Zwick et al. (2018) demonstrated that mice altered to be absent of mammary gland adipocytes had hampered indicators of MEC proliferation and apoptosis and milk fat remained in the alveoli long after fat was removed from the alveoli of control mice (Zwick et al., 2018). During the dry period, adipocytes are dynamic and make up a significant portion of the ECM of the mammary

gland (Sordillo and Nickerson, 1988). The removal of milk fat in early involution is important in cows because milk fat globules can impede involution and decrease leukocyte function (Nickerson, 1989). Adipocytes in the bovine mammary fat pad secrete leptin and may promote MEC apoptosis during involution through increased phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Feuermann et al., 2006, Palin et al., 2017). Thus, in addition to effects on immune function, mammary adipose tissue may affect MEC regeneration. The complex structural and physiological processes occurring during mammary gland involution affect IMI risk, milk production, and cow welfare. Various aspects of mammary gland involution can result in alterations in biomarkers that can be quantified. The changes in biomarkers may be able to demonstrate the duration and magnitude of mammary gland involution processes. However, the association of these biomarkers and mammary gland involution outcomes (IMI, milk production, welfare) is poorly understood. A better understanding of the association of biomarkers with mammary gland involution will facilitate investigation of treatments intended to improve involution outcomes.

Biomarkers of mammary gland involution: measurements of mechanisms

Mammary gland involution is a complex and dynamic process which isn't always successful. The negative consequences of incomplete mammary gland involution have led researchers to study methods which may optimize involution (Zhao et al., 2019). Involution failure refers to delays or absence of essential mechanisms in mammary gland involution which results in the impaired milk production, health, or welfare of the cow. Thus, studies with the objective of accelerating or optimizing involution to avoid involution failure typically use various biomarkers as indicators of the timing and degree of mammary gland involution. These biomarkers of involution are generally indicators of mechanisms involved in mammary gland involution. While

measuring failure of involution can be straight forward, evaluating the timing and degree of involution is not well defined. Biomarkers for disease in the early fresh period have been widely adopted for predicting disease risk and have prompted the study of mechanisms for disease prevention (Chapinal et al., 2011, Bradford et al., 2015, Wisnieski et al., 2019). However, it is not known if specific biomarkers can be used to predict involution failure or effects of treatments used to hasten involution.

Studies of mammary gland involution are highly variable and based on diverse objectives, they vary in type and frequency of sample collection, number and type of biomarkers assessed, and duration of sample collection. Objectives of most studies can be classified as addressing one of 4 broad categories: 1) mechanism of involution (Table 1), 2) methods used to accelerate involution (Table 2), 3) impact on cow welfare (Table 3), and 4) impact on cow health (Table 4). Studies of mechanisms of involution are generally observational studies, but some include defined treatments (Table 1).

Table 1.1: ***Mechanistic studies evaluating various physiological processes that occur in the mammary gland during mammary gland involution***

Authors	Biomarkers	Outcome
Comalli et al. 1984	Teats microscopy and bacterial culture	• Dilation of teat lumen D0 to D+7 • Ketatin plugs visible D+16.
Nonnecke,et al. 1984	Milk involution biomarkers and bacteria culture	• pH increased 6.7 to 7.6 on D+15. • Milk BSA, IgG, leukocytes, lactoferrin increased

Table 1.1 (cont'd)

Holst et al. 1987	Surgical biopsy	• D0-Alveolar lumen is largest % of space • D+21-30 most alveoli collapsed • Overall samples followed a similar pattern but varied greatly even within cow
Hurley et al. 1987	Milk fatty acids	• The largest decrease in short and medium chain fatty acids was in the first 3 d post D0 • Fatty acid changes consistent with decreased de novo fat synthesis
Oldham et al. 1991	Impressions of teat canals measured, bacterial culture, and SCC	• Teat canal shortened from D0-D+3 and then increased through dry period • Cross sectional area decreased D+14-D+21.
Bittman et al. 1992	Milk fatty acids	• Free fatty acids, free cholesterol, cholesterol esters percent highest D+20 • Changes in fatty acids consistent with impaired BMB
Hurley et al. 1993	Mammary biopsy – IHC, explant tissue culture	• Lactoferrin located at MEC basal surface post D0 • Explant tissue from lactating cows synthesized less lactoferrin than involuting mammary tissue
Park et al. 1991	Blood and milk- flow cytometry	• Ratio of CD4:CD8 T-lymphocytes in blood was lowest during non-lactating period • Activated CD+8 higher in milk in non-lactation than 48 hr post-partum.
Capuco et al. 1997	MG weight, DNA, RNA, hydroxyproline, 3H-thymadine and -uridine incorporation,	• Epithelial cell proliferation was increased in dry cows than cows milked through dry period D+25-7 DIM
Stelwagen et al.1997	MY, MG volume	• 1x reduced MY 28-37% • Udder volume increased in 1x cows on D+8 compared to 2x (likely byproduct of MG shape change)
Wild et al. 1997	MG histology, mRNA, DNA	• Alpha casein decreased 85% in 1 wk. • Lactoferrin was increased 20 fold in involuting cows • 2 fold apoptotic cells in involuting cows.
Cooper et al. 2004	MG PCR and western blotting	• Expression of zonula occludens-1 expression decreased by 36 hrs post D0 and continued to decreased through D+8

Table 1.1 (cont'd)

Ondensten et al. 2005	Blood biomarkers of metabolism. Rumen: pH, NH3. Milk components	• Decreased energy diet increased NEFA D+1-D+8 • Diet had no effect on MY
Wall et al 2005	Mammary biospy tissue proliferation - DABS-E, DNA conc, 3H-thymidine uptake, apoptosis - TUNEL, PCR,	• Short day photoperiod during the dry period increased MY by 3 kg/d • Cows with short day photoperiod had increased cell proliferation and increased IGF-II expression compared to long day cows
Wall et al. 2005	Mammary tissue biopsies- PCR SOCS-1, SOCS-2, SOCS3, and CIS mRNA expression	• Suppressor of cytokine signaling (SOCS-1 and -3) increased during dry in short day photoperiod cows • SOCS expression may enhance prolactin signaling
Puvogel et al. 2005	Milk and plasma retinol and retinol metabolites, lactoferrin, IGF-1 and -3. Mammary tissue for lactoferrin and IGF-1 mRNA. MY.	• At parturition, increased apoptotic cells in high vit A. • High vit A had lower MY at wk 12 and 13 in milk. • High vit A had lower milk fat.
Annen et al. 2007	MG biopsy: MEC proliferation, apoptosis, ultrastructure, PCR, MY, milk components	• Continuously milked MG halves had lower MY(10.6 vs. 22.2 kg/d) • MEC proliferation reduced in CM on d-8 • Apoptosis MEC higher in d+1 and +7 in con cows.
Rabot et al. 2007	mRNA and IHC in mammary tissue for certain MMP, TIMP, and PA	• MMP-2, -14, -19 and TIMP-1 and -2 increased expression at I3 than I2 • In ductal epithelium very high increase in staining on MMP-1 and moderate increase in TIMP-2. • In MEC, very high staining of MMP-2
Norgaard et al. 2008	Mammary gland IHC and PCR	• MEC proliferation increased from early to late dry period • MEC apoptosis was highest D+29
Velasco et al. 2008	MY, plasma PRL, DMI	• Short photo period average 3.5 kg/d more milk • Short PP had higher DMI -6, -5, -4, -1, +1 and +5 wk relative to calving. • Long PP had higher plasma prolactin -21d and -6d - +4d relative to calving..

Table 1.1 (cont'd)

Singh et al. 2008	Mammary tissue collected to create cDNA microarrays. Western blotting for lf, beta1-integrin alpha-lactalbumin and beta-lactoglobin.	• Most changes related to apoptosis, oxidative stress, and immune function. • Antioxitant related genes had greatest increase fold change and included metallothionine, super oxide dismutase, and glutathione peroxidase
Newman et al. 2009	Milk lactoferrin, bacterial culture, SCC	• Intermittent milking prior to D0 increased milk lactoferrin • Increased SCC at D0 was associated with decreased lactoferrin
De Vries et al. 2010	Mammary gland biopsies for histology, H&E, IHC	• Activated fibroblasts greater at d+7 than 1 wk pre-calving. • Percent of stroma was greater at d+7 than 1 wk pre-c. • TGF-beta1 decreased 15% from LL to d+7
Dallard et al. 2011	Milk bacterial culture, biopsies from euthanized cows d+7-IHC,RT-PCR	• Ginsing tx had sig more % stroma, BAX and casp-3 staining. • GS had increased bax expression and increased bax:bcl-2 (apoptosis) expression, TUNEL, increased apoptosis in MEC
Tao et al. 2011	Mammary gland biopsies	• Heat stress decreased MEC proliferation 26 d after D0 • Heat stress decreased MY after calving
Bertulat et al. 2012	Dynamometer vs manual palpation	• Excellent repeatability of measurements between observers
Yu et al. 2012	Milk SCC, MMP-9 activity, immuno-blotting	• MMP-9 increased during study • SCC: highest percentage was neutrophil which increased to D+17 and macrophage % increased D0-D+10
Boggs et al. 2015	Quantitiative proteomics - gel electrophoresis-LC-MS/MS. Western blotting of select proteins.	• Albumin, lactoferrin, IgG1, and polymetric immunoglobulin receptor only of 51 identified that changed at least 2 fold
Biet et al. 2016	MG tissue IHC	• Primary cilia on MEC was decreased by D+28

Table 1.1 (cont'd)

Singh et al. 2016	MG tissue collected post-mortem: IHC, western blot, qRT-PCR	• Activation of STAT5 and STAT3 occurs at the onset of mammary gland involution • The regulation of IGF1-Akt signaling suggests that by 192 h postmilking in dairy cows, the involution process is still in the reversible phase
Singh et al. 2017	MG in-situ hybridization, northern and western blot, IHC	• Variation in lactoferrin staining within the same MG indicated that alveoli involute at variable rates • Wide variation in expression of genes essential for MG involution between cows indicating a high degree of variation between even healthy cows
Dado-Senn et al. 2018	RNA Sequencing on mammary tissue -3, 3, 7, 14, 25 d from DO	• Heat stressed cows had down regulated branching morphogenesis, inflammatory response, and cell death • In all cows the greatest down-regulated genes were related to milk synthesis at D+3 • CL cows tended to have increased milk production over 9 weeks (4.8 kg).
Putman et al. 2018	Blood ROS, AOP, Ca, NEFA, BHB, albumin, haptoglobin, cortisol, isoprostanes, leukocyte differentials	• Altered metabolism: Ca^{+2} peaked D+2,NEFA Peaked D+1 • Oxidative stress: ROS and AOP tended to increase except from D+2. • 15F-isoprostane peaked D+2 whereas 8-isoprostane-A1 increased on D+12.
Dado-Senn et al. 2019	Mammary biopsies (post parturition) – IHC	• Heat stress cows colostrum production (3.72 vs. 7.79 kg), and lower milk yield ~3 kg. • Heat stress cows had fewer alveoli, but no diff in size, Alveoli number pos corr with MEC number (r= 0.89). • Heat stress cows had higher % connective tissue
Finot et al. 2019	Mammary gland flow cytometry, PCR, IHC	• Putative mammary stem cells were present in cows that had been dry for 6 years • Even after 6 years, the structure of alvoli and ducts was intact
Putman et al. 2019	Plasma PUFA, MUFA, SFA, and oxylipids	• PUFA such as arachidonic acid, alpha-linolenic acid and linoleic acid peak D+2 • EPA peaked D+1 while DHA and DPA peaked D+2 • Oxylipids varied over sampling period - both pro- and anti-inflammatory.

Table 1.1 (cont'd)

Putz et al. 2019	Milk RNA isolation and sequencing	• Found 816 known and 80 novel miRNA, 46 miRNA changed over sampling period • Within the 46, miRNAs associated with pregnancy, lactation, and inflammation
Suarez-Trujillo et al. 2019	MG and hypothalmus tissue collected post slaughter: MG RT-qPCR and IHC.	• IHC found that all subtypes were in lactating MEC, 5 were in vascular endothelial cells, and 2 were in myoepithelial cells • Dry cows MEC expressed same subtypes,
Mezzetti et al. 2019	BW, BCS, DMI, Rumen boluses for body temp and rumination, blood metabolites	• High MY had higher DMI from -1 wk to +4 wk around D0 • High MY had increased haptoglobin, k+, Ca+, nitrite at 2 and 7 week after D0
Toth et al. 2019	Sonoscape A6 ultrasound with 5-7 MHz linear transducer with water bath method.	• Compared to D-1, streak canal shortened 8.3% by D+6 • Area of teat end decreased 14.6% from D-1 to D+6
Fabris et al. 2019	MG biopsies: gene expression and histology - IHC	• Early DP HT- down regulation of autophogy-related genes • IHC- early HT had decreased apoptosis (TUNEL) and proliferation (Ki67) in MEC, stromal, and overall than CL. • HT in early dry period had more negative effects than HT in late dry period
Bonelli et al.2020	Ultrasonic measure of gland cistern area - UC images processed with ImageJ	• Longitudinal and cross section areas correlated with time • UC changed from wide arborized pattern to elongated round shape - more prominent in fore quarters. • D+38 to colostrogenesis UC margins were blurred
Cattaneo et al. 2021	Blood: qPCR in leukocytes	• mRNA inflammation: Il-18 higher in high MY at D-7 and D+7 • IL-8 higher in high MY than low MY from D-7 to D+34. • Recognition and antimicrobial: CD16 decreased D+7, TLR2 higher in high MY on D-7 then decreased on D+7
Suzuki et al. 2021	MG IHC, Milk PCR and immunoblotting	• MEC produced chemerin during dry period and chemerin was detected in MG tissue and milk • Chemerin increased in MG with mastitis

Studies investigating acceleration of involution include clinical trials in which a defined treatment was used in a group to accelerate the onset, shorten the duration, or increase the intensity of mammary gland involution as compared to non-treated cows (Table 2).

Table 1.2: ***Studies with the objective to accelerate mammary gland involution***

Authors	Biomarkers	Outcome
Breau et al. 1985	Milk fluid volume, SCC, pH, BSA, IgG, citrate, lactoferrin,	• PHA had decreased MY by 24 and 48 hr post tx. • Both tx halves had 50% volume of con at 7d post DO. • No differences in MY or components at 7 or 14 DIM.
Lacy-Hulbert et al. 1999	SCC, MY, Fat, casein, lactose in milk, IgG, BSA	• Milk pH, fat, protein, casein, IgG, BSA, Na^+, and Ca^+ higher in 1x milking • Milk in low feed cows had higher protein, fat, SCC, pH, casein, whey, IgG, Na^+, Ca^+, Na^+:K^+
Shamay et al. 2003	MY, milk total protein, whey protein, total casein, proteose peptone, bsa, lf, IgG, activities of PA, plasmin, plasminogen, lactose, Na, K, Ca, Mg, P, and S	• Milk volume decreased rapidly and on d 3 of tx, 100-300 ml vs 2.5 L in con. • Lactose concentrations at D-1 in tx were similar to con at d+10
Silanikove et al. 2005	Milk pH, chloride, xo activity, uric acid, LPO activity, hydrogen peroxide, thiocyanate, nitrite, nitrosothiols, nitrate, beta-carotene oxidation, rate of DTNB formation, bacterial culture inhibition.	• CH tx increased pH for 3 D before D0 and remained elevated • XO increased D-2 in CH and con, CH was higher than con D-2 - D10 • Uric acid higher, hydrogen peroxide lower in CH.
Raja-Schultz et al. 2005	MY, bacterial culture	• Every 5 kg increase over 12.5 kg/d MY at D0 resulted in 1.8x higher odds of IMI at calving • IMI associated with decreased MY in early lactation.

Table 1.2 (cont'd)

Ollier et al. 2015	Serum: prolatin Milk: MY, SCC, BSA, lactoferrin, citrate, Na+, K+, gelatinase activity	• PRL decreased serum prolactin d-3 to d+3 • MY decreased D-3 and -2. MY reduced d-3, d-2 (decreased about 5 kg from about 21 kg/d)
Domenech et al. 2014	Milk MMP-2 and -9, SCC, fat, protein.	• MMP-9 activity higher in SAA D+1-3 • SAA increased % fat D+2 and 3.
Ollier et al. 2014	Milk Yield, IMI, SCC, . Blood and milk PRL. Milk Na+, K+, BSA, citrate, lactoferrin, gelatinase activity. Blood NEFA, BHB, amino acids, glucose, and mononuclear cell proliferation and IL-4 production (ex vivo).	• Both tx decreased MY before dry off with tx 1: 24.8 kg/d, 2: 17.9 kg/d, 10.1 kd/d on D0 • At D0, lactoferrin higher in 3 and 2 and remained higher after D0 • MMP 9 increased after D0 in all groups
Ponchon et al. 2014	Milk: lactoferrin, SCC, BSA, Na+, K+, MMP-2 and -9	• EGTA increased SCC, BSA, lactoferrin • MMP2 activity increased in all tx to D+7 • MMP9 increased 2-3x on D+1 and all tx increased through D+14.
Boutinaud et al. 2016	Milk SCC, milkk fat, true protein, lactose, citrate, alpha lactalbumin, lactoferrin	• Lactose significantly lower in CAB on D+1 • Lactoferrin 51% higher in CAB SCC 1.4jfold higher in cab and sig on d1, 2, and 3.
Boutinaud et al. 2017	Milk Na+, K+. MEC con, MEC viability, MMP-2 and -9 activity, blood lactose, Mammary biopsies	• Lactose in plasma increased D0 to D2 then decreased D4-D14. • Lacteal MEC- MEC increased in conc d0-14. MEC greater in CAB. • MEC conc peaked in CAB on d8 and CON on d14.
Lanctot et al. 2017	Milk SCC, BSA, lactate dehydrogenase, lf, mammary inflammation score (swelling), SCC flow cytometry, bacterial culture, PCR on SCC	• 2.5 and 5 mL low-viscosity chitosan tx had higher inflammation scores than control from 0-24 hr • No tx effects on lactoferrin, LDH, SCC, or BSA or flow cytometry

Table 1.2 (cont'd)

Maynou et al. 2018	MY before d0, udder pressure, milk leakage, lying behavior, subsequent lact MY. Serum P, pH, Ca, prolactin, NEFA, β-OH-butyrate	• 1: 0 bolus: -0.23 kg/d milk, 1: -1,15 kg/d, 2: -2.56 kg/d 2 d post bolus • Bolus lower udder pressure • Tx increased lying time (85 min) in 24 hr post D0
Gifre-Renom et al. 2020	Milk SCC, WBC components of SCC, BSA, lf, Na, K, endogenous MMP-9	• SCC, BSA, lf and Na:K ratio increased by all doses on at least d+1 post d0 • rMMP-9 not often different from vehicle.
Thiago et al. 2020	Mammary biopsies D-3, 3, 7, 14, 25 d relative to DO. immunohistochemistry for proliferation (Ki67), apoptosis (dUTP), Alveoli size on H&E. Connective tissue with Masson's trichome. Tissue PCR. Plasma PRL.	• Early DP HT vs CL: decreased apoptosis on d3, 7, 14 in HT, decreased proliferation in HT d3 and d14 • On d25, HTHT and HTCL had higher %connective tissue than CLCL. • d14 HT had more alveoli than CL. On d25, HTHT had larger alveoli than CLCL.
Ouellet et al. 2021	Mammary biopsies d3 and 35 from DO. Ex vivo tissue culture in varying concentrations of PRL and eatrodiol, qPCR	• Late lactation heat stress resulted in decreased expression of prolactin and estrogen receptors
Pares et al. 2021	Milk: SCC, MMP activity, BSA, lactoferrin, Na+, and K+	• MMP activity higher in rMMP tx on d+1 than control • IB had higher lactoferrin on d+1 and d+3 • . BSA higher in IB on D+1, +2, +3.

Studies investigating cow welfare have assessed different aspects of involution or dry cow management on dairy cow behavior and physiological measures of well-being such as stress (Table 3).

Table 1.3: ***Studies investigating the effects of mammary gland involution on dairy cow welfare***

Authors	Biomarkers	Outcome
Leitner et al. 2007	Manual mammary palpation, laying time	• CH cows had lower mammary gland pressure d1-d4 dry • Con cows took more steps in early dry period than CH.
Ondensten et al. 2007	Clinical signs, bacterial culture, milk plasma cortisol	• No change in MY between groups but both decreased from ~17 to ~9 to ~3.5 kg for pre-dry, d3 and d5. • Straw diet increased cortisol d 3 and 4 • Cortisol increased in all cows at d0
Tucker et al. 2009	Mammary gland pressure, behavior	• Feed restriction prior to D0 reduced mastitis • Feed restriction prior to D0 increased lying time and vocalization • Decreased milking prior to D0 did not affect behavior
Zobel et al. 2013	Lying time/behavior, milk leakage, bacterial culture, MY	• All cows had reduced lying time, lower lying bouts, increased lying bout duration, and increased feeding time after D0 • Abrupt cows had increased time standing at gate (desire to be milked) • MY at dry off inversely related to lying time after DO
Bertulat et al. 2013	fecal 11,17-dioxoandrostane (11,17-DOA), MY, udder pressure with dynamometer	• Udder pressure peaked on D+2 for all groups, only high milk cows had udder pressure greater than baseline • Odds of milk leakage 5.1x higher in high MY • Fecal DOA were different at baseline based on MY and udder pressure and increased in med and high MY D+2 and +3
Bach et al. 2015	Milk leakage, udder engorgement quantified with digital algometer, Acceleration data.	• D+1 CAB tx decreased pressure (43 vs. 50) D+2 CAB cows increased in udder engorgement and were similar to con thereafter • No effect of parity for engorgement • Con cows spent 1.5 fewer hours laying

Table 1.3 (cont'd)

Bertulat et al. 2017	Mammary gland pressure, teat to teat distance, milk leakage, pain	• Total teat distance increased from D0 to D+2 and was increased in multiparous cows and cows with increased 305 d MY • Cabergoline treatment reduced pain incidents after D0 • 1st lactation cows were 2.45 times more likely to have mammary gland pain response
Raja-Schultz et al. 2018	Daily lying time and steps taken per day	• Gradual dry off had decreased MY at D0 than abrupt (14.3 kg vs. 22.7 kg) • Lying behavior most affected by parity x MY: primiparous cows decreased lying time 0.31 hr/d for every 5 kg increase in MY at D0.
Blau et al. 2019	Dynamometer, and intra-arterial pressure monitor in the teat canal	• Coerrelation of 0.67 for internal pressure and dynomometer • Increased IMP lasted in control cows 18-72 hrs from
Larsen et al. 2021	DMI, MY, milk leakage, udder engorgement, teat perimeter	• Cows given CAB, 2xM, and RED had greatest negative energy balance the week before D0 • Con feed and 2xM cows had greatest distance between rear quarters D+1 and +2 after D0 • Con feed and 2xM had great teat perimeter D+1, +2, and +4
Franchi et al. 2021	Behavior, eye temperature by infrared	• Cows more likely to eat within 5 min on d+1 and 2 • Eye temps higher on D0 and D+2.
Franchi et al. 2022	Udder firmness and soreness; image-based hock-hock distance	• After the last milking, REDU cows displayed shorter hock-hock distance compared with NORM cows • CAB cows showed lower odds of having a firm udder, shorter hock-hock distance, and lower degree of udder fill than SAL cows • Udder soreness (kicking etc) on D+2 and + 5 highest in norm 2xM and CAB

Researchers assessing cow health usually evaluated effects of a treatment on mammary gland health or the effect of mastitis on mammary gland involution (Table 4).

Table 1.4: ***Studies evaluating the association between mammary gland involution and mastitis***

Authors	Biomarkers	Outcome
Oliver et al. 1982	Milk: bacterial culture	• Tx quarters infection (number of isolations) reduced 50% from D0 through +7 • No difference in periparturient period.
Bushe et al. 1987	milk SCC, citrate, whey protein, LF, IgG, BSA, bacterial growth	• Decrease 26.8 and 69.2% MY in 1x and 1x+feed restriction • Lactoferrin higher D-3-D+4 in (1x+feed restriction) • Better E. coli inhibition on D0 and D+4 and Klebsiella on D-3 for 1x+feed restriction
Sordillo et al. 1987	SCC, milk leukograms, bacterial counts, lactoferrin, citrate, fat, protein, IgG, pH, serum alpha lactalbumin	• SCC higher in infected quarters C-7-C+7 • Quarters infected with major pathogen had lower lactoferrin and fat, higher pH and protein C0-C14
Sordillo et al. 1988	MG Biopsies and larger tissue samples when cows slughtered on CO. Histology and electron microgram.	• Non-infected quarters had greater lumen and lower stroma % than infected during dry period • Non-infected had lower percentage of active epithelial cells • Infected quarters had more neutrophils (1.22 vs. 6.05 ~5x more)
Nickerson et al. 1993	Milk secretions -SCC, leukogram, histology	• IL tx had higher SCC and highest number of neutrophils on D+1-4 compared to con • IL2 had lowest epithelial and lumen %, and largest stroma and leukocytosis • IgG2 in IL2 > IL1
Rejman et al. 1995	Milk secretions- milk fat, total protein, SNF, lactose, LF, citrate, alpha alctalbumin, BSA.	• Milk lactoferrin higher in ILB on D+14 and higher in MG tissue D+7 and +14 than control • Milk lactose lowest in IL2 by D+4
Williamson et al. 1995	Milk SCC, bacterial culture, teat end closure, udder palpation	• DCT had higher proportion of closed teat ends up to 49 D post dry off • 83% of new IMI occurred in first 21 d post D0 • When infection identified, 97% of those teats were open.

Table 1.4 (cont'd)

Kelly et al 1998	SCC and MY. Milk fat and protein, PMN, plasmin and plasminogen activity, albumin, alpha-lasctalbumin	• Milk SCC and PMN levels were increased in reduced milking, and, at day 15, the increase was not linked to decreased milk yield. • Milk lactose decreased and pH, α-lactalbumin levels, plasmin activity and plasminogen activity significantly increased by reducing milking frequency
Dingwell et aL. 2004	MY, teat-end integrity, prescence of keratin plug, and bacteriological culture	• Odds of open teat canal 1.8x higher with high MY • 23% of teat ends never closed • New IMI increased with high MY at D0
Summers et al. 2004	Milk bacterial culture, SCC, keratin plug samples, BCS, BW	• 2x the amount of infections for quarters still open at D+21 • 2.5x more keratin collected on D+14 than pre-D0 • Closed teats had 70% more keratin than open
Ondensten et al. 2007	MY, SCC, blood: NEFA, insulin, glucose, BHB, urea, visual - milk leakage, teat end closure, teat end condition.	• Only breed difference was lower SCC in Red and Whites before and after D0 • Low MY had fewer IMI in post-partum period and had fewer open teat ends at week 2 and 3 after D0 • No effect of MY on SCC or IMI 28 DIM.
Wantanabe et al. 2012	SCC, IL-8, leukocyte elastase, lactoferrin, lactoferrin derived peptides (LDP)	• Intramammary IL-8 increased milk leukocyte elastase • IL-8 suppressed lactoferrin for 28 d post D0 • Il-8 increased LDP
Silanikove et al. 2013	MY, milk components, SCC and leukogram. Milk *E. coli* growth inhibition	• Cows that had rapid drop off in MY naturally prior to D0 (ANI) had higher lactoferrin, BSA, lactate dehydrogenase, SCC and higher proportion of leukocytes • ANI had superior bacterial growth inhibition • Higher CD4 and CD8 T lymphocytes in ANI D+1-+3
Ollier et al. 2015	PRL concentrations, milk yield at DO, Blood BHB, glucose, NEFA. Milk: SCC, bacterial culture	• MY decreased in prolactin-inhibitor (PRL) and reduced energy diet 21.7 to 10 and 12 kg/d • Bacterial colonies lowest in PRL on D+8 and +14. • Percent infected quarters lowest in PRL on D+14 percent infected in PRL, reduced energy, control: 17.2%, 33.7%, and 57.5%.

Table 1.4 (cont'd)

Gott et al. 2016	Milk bacterial culture, MY, leakage	• 1x milking prior to D0 decreased IMI risk in 1st parity cows (risk 3.5 times higher for abrupt dry off) • 1x milking increased risk for IMI in multiparous cows (2.8 x)
Anderotti et al. 2017	Mammary gland tissue IHC	• Cows infected with Staph. aureus during mammary gland involution had increased TLR2, IL-a, and CD14 staining • Staph. aureus cows had decreased lactoferrin staining
Renna et al. 2019	Milk lf, NO2, cytokines IL-1beta, -6, and -4, IgG1 and 2, in-vitro functionality of ex-vivo macrophages from milk secretions.	• IL-1β, IL-6 and IL-4 levels were increased in Staph. aureus • Milk lactoferrin nearly 60 fold greater in con vs SA on D+7, +14 and +21 • Total IgG and specific IgG1 levels decreased in SA D+7-D+21
Reinhardt et al. 2020	Milk peptide chromatography and mass spectroscopy, bacterial culture in d21 secretions	• D+21 upregulated genes included inflammation, lipid transport and metabolism, and proteolysis functions • Neither lactoferrin nor lactoferrin:citrate in D+21 dry secretions was not correlated with any antibacterial action • D21 milk inhibited bacterial growth ~40% compared to d0 except for SA and SU which had very little inhibition after 8 hr.
Britten et al. 2021	Milk- bacterial culture, SCC, BSA, lf (ELISA), udder half MY	• No effect of casein hydrosylate (CH) on SCC • Lactoferrin higher in CH than CH+TS, CH+DC, and control
Beccaria et al. 2021	Milk: bacterial culture, gelatin zymography MG tissue biopsy IHC - MMP-2, -9, TIMP-1, -2,	• Protein expression of MMP-2 and -9 was increased in chronically S. aureus-infected mammary glands D+14 and D+21 • The MMPs/TIMPs ratio was increased in chronically S. aureus-infected glands D+14 • The MMP-2 and -9 activities were increased in mammary secretions of infected glands
Putman et al. 2022	Plasma oxylipids and isoprostanes by LC-MS/MS	• 7 differed plasma oxylipids during early dry period differed between healthy and cows diagnosed with a disease in the first 30 DIM • 9-oxoODE higher in healthy D-6, D+2, D+6. • Diseased had higher 13-oxoODE at every timepoint D-6 to C+7 • 8-isoP higher on D-6- -14D from calving

Studies vary greatly in collection periods and often have small sample sizes resulting in limited power to detect differences among treatment groups (Tables 1-4). These issues, along with others, contribute to challenges in developing diagnostic criteria for biomarkers of mammary gland involution.

Collection of samples during the non-lactating period is difficult and creates numerous challenges for researchers. Mammary gland biopsies can provide insight on alterations in morphology, cellular ultrastructure, and gene expression (De Vries et al., 2010, Dado-Senn et al., 2018). Collection of surgical biopsies is the best technique for collecting intended mammary gland structures and is beneficial when greater amounts of tissue are needed (Knight et al., 1992). Surgical biopsies require feed withdrawal for 16-24 hour before collection because cows require deep sedation (Knight et al., 1992). Feed withdrawal decreases milk yield for approximately 48 hours which may affect results especially if biopsies are collected serially (Knight et al., 1992). Needle biopsies are less invasive, but in one study, 15% of biopsy samples did not include any secretory structures (the structure of interest) (de Lima et al., 2016). The increased risk for not collecting the tissue of interest could affect the power level during analysis and must be considered in the experimental design. All biopsy collection methods require anesthetic and a skilled veterinarian because even the needle biopsy technique can cause mammary hematomas, blood in milk, edema, fibrosis, and mastitis (de Lima et al., 2016). Mammary gland samples collected after euthanasia can improve collection of samples containing desired mammary gland structures but aren't useful for tracking changes over time. Researchers collecting post-mortem tissue samples have the benefit of collecting large samples with accuracy and to account for variations among cows by weighing the udder and its components such as parenchyma and stroma (Capuco et al., 1997). Correcting for tissue weight aids in interpretation

of results. For example, on histological examination parenchyma tissue appears to decrease, however one study found that parenchyma mass did not change over 45 d of involution (Sordillo and Nickerson, 1988, Capuco et al., 1997). Alternatively, Capuco et al. (1997) found that hydroxyproline content increased at 18 d of involution corresponding with increased stroma percentage on histological investigation. This suggests that synthesis of stroma tissue is increased during involution. Researchers utilizing post-mortem sample collection can produce unique findings that cannot be easily achieved with other methods. However, in many instances, cows included in euthanasia studies may not represent the general population because they may be non-pregnant, have low milk production, and may be an earlier stage of lactation than most cows at dry off (Singh et al., 2008, Dallard et al., 2011).

Mammary gland tissue samples can be especially useful for identifying characteristics and functions of specific cell types. Immunohistochemistry (IHC) utilizes labeled antibodies that bind to specific antigens in cells to identify cellular processes such as apoptosis and proliferation to quantify MEC turnover (Dallard et al., 2011). Cell signaling pathways, gene expression, proteins such as lactoferrin, and cellular functions can be quantified in specific cell types with IHC using light microscopes, electron microscopy, and autoradiography. The results of IHC can be quantified through a manual scoring system or with computer software image analyses (Duraiyan et al., 2012). The IHC technique is used in involution studies to evaluate MEC apoptosis and proliferation, protease activity, hormone receptors, and MEC cell signaling (Singh et al., 2016, Suárez-Trujillo et al., 2019, Beccaria et al., 2021). Cellular activity is assessed by incubating tissue with radiochemicals and incorporation of these compounds is quantified using autoradiography (Duraiyan et al., 2012). For example, titrated thymidine is incorporated into DNA during cell synthesis and division, so it is a good marker of cell proliferation (Capuco et

al., 1997). Traditional hematoxylin and eosin staining is widely used and beneficial for identifying tissue structures, evaluating cell contents, and classifying infiltration of leukocytes into mammary gland tissue during involution (Holst et al., 1987, Sordillo and Nickerson, 1988). Electron microscopy is useful for characterizing functional appearance of organelles especially those involved in milk synthesis and secretion such as the Golgi apparatus (Sordillo and Nickerson, 1988). The Golgi apparatus produces lactose and transports lactose and milk proteins produced by the RER in budding vesicles to the cell membrane for secretion into milk (Neville, 2009). The Golgi apparatus regresses and secretory vesicles diminish in number during the first 7 d of involution and then reemerge 14-7 d pre-parturition during lactogenesis (Sordillo and Nickerson, 1988). Thus, electron microscopy is a useful technique for analysis of MEC function. Tissue samples are often analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) to quantify changes in gene expression during mammary gland involution. High throughput cDNA microarray is a tool for analyzing and making connections among multiple genes and varying mRNA concentrations. Analysis of gene expression demonstrated the inhibitory effects of prolactin inhibitor injection on MEC milk production as well as decreased prolactin receptor expression in mammary tissue (Boutinaud et al., 2017). Thus, PCR analysis of mammary gland tissue can demonstrate associations between multiple cell processes that can be further investigated. Characterization of mammary tissue in early involution by cDNA microarray identified alterations in gene expression related to apoptosis and immune function and gene expression for antioxidant enzymes such as glutathione peroxidase increased through 192 hr of involution (Singh et al., 2008).

The composition of lacteal secretions changes from milk to mammary secretion during the dry period. Collection and analysis of milk secretions is a direct and relatively noninvasive method

for detecting changes in MEC function. Collection of milk secretion during the dry period can increase the risk for false conclusions, however, because the collection process can stimulate milk production in MEC and alter secretory cell and alveolar structure (Akers and Heald, 1978). Indeed, researchers have shown that lactation can be reinitiated as late as 11 d after dry off complicating analysis of milk secretions in the early dry period (Noble and Hurley, 1999). Another consideration when sampling milk secretions is the study design, particularly regarding intramammary treatments. Some studies have used quarters or mammary gland halves from the same cow as controls for treatments designed to accelerate involution (Rejman et al., 1995b, Lanctôt et al., 2017, Britten et al., 2021). Results from these studies did not investigate the effect of treatment on adjacent non-treated quarters. However, the lack of treatment effects between treatment and control quarters for parameters such as SCC indicate that there could have been inflammatory effects on adjacent control quarters (Lanctôt et al., 2017, Britten et al., 2021). Indeed, the mammary gland quarters are not as physiologically independent as was previously believed. Milk samples collected from quarters adjacent to a quarter with naturally occurring clinical mastitis had decreased protein and lactose and increased SCC compared to healthy controls (Paixão et al., 2017). This suggests that treatment in one quarter could affect the involution process in an adjacent quarter. Nickerson et al. (1993) examined the effects of intramammary injections of IL-1 and -2 at dry off on mammary gland involution. Treatment of IL-1 or -2 accelerated the speed of involution and induced an equivalent degree of leukocyte infiltration in both adjacent and treated quarters compared to an untreated cow (Nickerson et al., 1993). Although this study had a small sample size, the effects of treatment on control quarters or mammary gland halves should be further investigated.

Bacterial culture of milk secretion samples is essential in studies evaluating mammary gland health and milk quality. Bacterial culture is the most used method for identification of IMI in involution studies (Table 2). Pairing culture results with other parameters such as clinical signs can be useful for diagnosis of clinical mastitis (Flöck and Winter, 2006). Complimenting bacterial culture with other diagnostic tools such as matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) which is fast, accurate, and useful to verify culture results. Identification of proteins separated by gel-electrophoresis from milk secretions such as lactoferrin, immunoglobulin G (IgG), and serum amyloid A (SAA) is another use for MALDI-TOF (Boggs et al., 2015). Lactoferrin, IgG, and SAA are common biomarkers for immune and inflammatory processes. Therefore, using MALDI-TOF to identify mastitis pathogens and to characterize the inflammatory profile could be useful in assessing mammary gland health during involution.

Concentrations of SCC are increased in milk secretions during the early involution period which makes detection of mastitis using SCC more difficult. Sordillo et al. (1987) evaluated differences in biomarkers of involution between healthy and infected cows from dry off to 14 DIM and observed that SCC was often not different between groups. In this study, pathogens were divided into major (*Streptococci* spp and *Staphyloccus aureus*) and minor pathogens (coagulase-negative *Staphylococci* and *Cornynebacterium* bovis) (Sordillo et al., 1987). At 7 d post dry off, uninfected quarters had higher SCC than cows infected with minor pathogens and SCC of cows infected with major pathogens were significantly greater 7 d prior to calving (Sordillo et al., 1987). At the other 4 sampling points from 14 d post dry off to 2 wk prior to calving, there were no differences in SCC between groups (Sordillo et al., 1987). It's not clear whether SCC is a reliable biomarker for involution failure during early involution although it is a commonly used

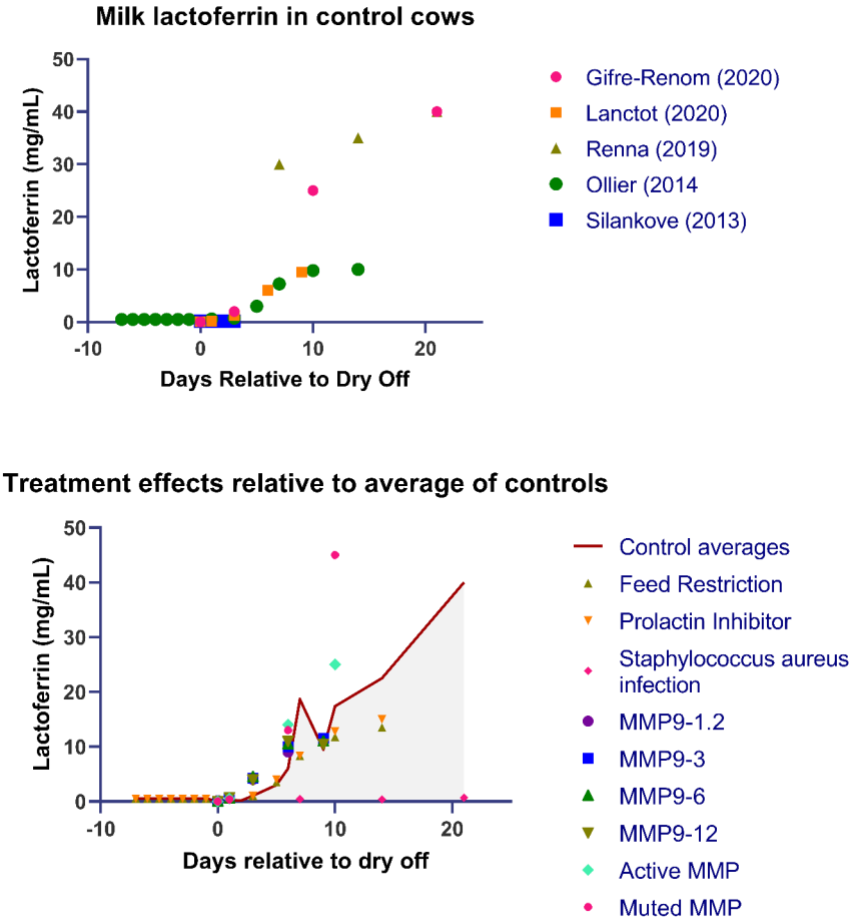
biomarker indicating accelerated involution (Zhao et al., 2019). Kelly et al. (1998) tested effects of an increased milking interval (milking every 24 hr for 7 d, then every 48 hr for 7 d) in the 2 wk prior to dry off altered milk components compared to conventionally dried cows. As expected, milk production was less in the treatment group at 7 and 15 d into the study, however the increase in SCC was not proportional to milk production compared to the control group (Kelly et al., 1998). The change in ratio of SCC to milk yield was greater in the control group from 7 to 15 d of the study than the treatment group (Kelly et al., 1998). This study did not investigate the association between SCC and mammary gland health and milk production in the next lactation. The implications of varying SCC during early mammary gland involution for involution failure are not well defined.

One of the earliest measurable mechanistic alterations occurring during the first week of involution is alterations in MEC milk and secretion products (Hurley, 1989). During lactation, MEC produce sugar, fats, and proteins that are secreted into milk that which can serve as direct or indirect measures of MEC activity. These substances include lactose, α-lactalbumin, citrate, fatty acids, caseins, ad β-lactoglobulin (Hurley and Rejman, 1986, Sordillo et al., 1987, Annen et al., 2004, Costa et al., 2020). Production of milk components decline with decreasing milking frequency, decreased energy consumption, and during early involution (Sordillo et al., 1987, Lacy-Hulbert et al., 1999). Production of milk fats and proteins varies between cows so baseline samples prior to dry off are therefore important to consider when evaluating MEC function (Ziv and Gordin, 1973).

Lactoferrin is an iron-sequestering protein that can inhibit bacterial growth and increases in concentration of mammary secretions during early mammary gland involution (Bishop et al., 1976). Despite the rapid decrease in MEC protein synthesis during the early dry period,

production of lactoferrin increases and the concentration in milk secretions has been shown to increase for up to 30 d after dry off (Hurley and Rejman, 1993). Lactoferrin is a common biomarker of involution and concentrations can be affected by different acceleration techniques (Figure 2). Decreasing the frequency of milking 1 week prior to dry off increased lactoferrin concentrations in milk secretions after dry off (Newman, 2008). Lactoferrin concentrations in milk secretions were increased by 7 and 14 d after dry off in cows treated with intramammary interleukin-1β at dry off (Rejman et al., 1995a). Concentrations of lactoferrin in milk are also increased in response to inflammation (such as that caused from infusion of endotoxin) (Shuster et al., 1991). Lactoferrin (in coordination with IgG) has inhibitory effects against growth of coliform bacteria in the mammary gland (Sordillo et al., 1997). Lactoferrin has been shown to have bacteriostatic effects, particularly against *E. coli* (Smith and Schanbacher, 1977, Nonnecke and Smith, 1984b, Sordillo et al., 1997). Lactoferrin decreases free iron in the mammary gland which may reduce risk of oxidative stress as well as enhance phagocytic activity by leukocytes (Sordillo et al., 1997). Lactoferrin has been shown to be increased in quarters in response to IMI (Harmon et al., 1975, Cheng et al., 2008a) but recent studies have not found correlations between milk secretion of lactoferrin concentrations and mammary gland health (Reinhardt and Lippolis, 2020). Although Reinhardt et al. (2020) did not find a correlation between lactoferrin concentration and inhibition of bacteria growth, they did find that mammary secretions from one cow effectively inhibited the growth of several different pathogens while secretions from other cows were more variable. This experiment was repeated in the same cow the following year and her milk secretions inhibited bacterial growth similarly to the year before. This suggests that there are some yet unidentified factor or combination of factors in milk secretions that effectively protect the mammary gland from infection.

Figure 1.2: ***Milk lactoferrin concentrations during mammary gland involution from select studies***



Milk lactoferrin concentrations during early mammary gland involution. A: Lactoferrin concentrations in milk secretions from untreated cows. Studies were included if they were conducted in the last 10 years, used Holstein cows, and analyzed lactoferrin by enzyme-linked immunoassay. B: Lactoferrin concentrations were averaged from untreated control cows in panel A. Milk lactoferrin concentrations from treated or unhealthy cows from the same studies to illustrate treatment effects compared to expected concentrations. Feed restriction and prolactin inhibitor are from Ollier et al. (2014), Staphylococcus aureus infection is from Renna et al. (2019), all MMP treatments are from Gifre-Renom et al. (2020). The number behind MMP9 refers to the dose of MMP9 (12 mg). Active MMP contained 12 mg of MMP9 while muted MMP was inactivated to test the effect of nanoparticle carrier.

Citrate has been used as a biomarker for MEC activity during involution because it is involved in de novo fat synthesis in the mammary gland and greater than 99% of milk citrate is produced and secreted from MEC (rather than being transferred from blood) (Faulkner and Peaker, 1982). The concentration of citrate declines quickly in milk secretions and reaches its nadir around 7 to 14 d post dry off (Oliver and Smith, 1982, Rejman et al., 1995a). Citrate concentrations in milk can be

influenced by diet, dry matter intake, overall energy balance, stage of lactation, and casein and calcium concentrations in milk (Faulkner and Peaker, 1982, Xu et al., 2020). Milk citrate concentrations have been suggested as a marker for energy balance in periparturient cows. Milk citrate concentrations have a large, negative correlation with energy balance and are positively correlated with milk fat yield in early lactation cows (Xu et al., 2020). Energy restriction used to reduce milk production in the 7-14 d prior to dry off can put late lactation cows into a transient, yet significant, negative energy balance (Vilar and Rajala-Schultz, 2020, Larsen et al., 2021). In fact, cows fed restricted energy diets the week before dry off had 50-100% greater milk citrate concentrations than cows fed a control ration (Bushe and Oliver, 1987, Ollier et al., 2014). The metabolic, hormonal, and inflammatory conditions are vastly different between periparturient and late lactation cows and more research exploring associations between energy balance and milk citrate concentrations is needed. However, based on available data, citrate may be a useful indicator of energy balance in studies using energy restriction prior to dry off. Minimizing alterations in energy balance before dry off is important because even short bouts of negative energy balance can affect the immune response to pathogens in the mammary gland (Moyes et al., 2010).

In addition to serving as a marker of MEC activity, citrate has been assessed as an indicator of the mammary gland's potential defense capabilities against IMI (Oliver and Sordillo, 1989). Citrate concentrations decrease in mammary secretions during the first 2 wk of involution as MEC activities decline (Sordillo et al., 1987). In vitro studies have demonstrated that citrate decreased growth inhibition of lactoferrin for certain coliform bacteria (Bishop et al., 1976, Nonnecke and Smith, 1984a). Indeed, in-vitro studies demonstrated that citrate forms complexes with free iron and promotes the uptake of iron by certain bacteria such as *Escherichia coli*

(Bishop et al., 1976). Therefore, citrate concentrations in milk secretions are often reported with lactoferrin as a citrate:lactoferrin molar ratio (Sordillo et al., 1987, Boutinaud et al., 2016). One study demonstrated that in response to intramammary LPS challenge, early lactation cows had a higher ratio of milk citrate:lactoferrin than late lactation cows for up to 72 hrs (Hyvönen et al., 2010). The authors hypothesized that the elevated citrate:lactoferrin ratio may be a factor that could increase susceptibility to clinical mastitis in post-partum cows (Hyvönen et al., 2010).. However, the citrate:lactoferrin isn't an ideal biomarker for IMI risk during involution because so many other factors contribute to IMI risk (Dingwell et al., 2003).

α-Lactalbumin makes up about 2.5% of milk protein and plays a role in lactose synthesis as part of the lactose synthase enzyme complex which gives it a unique role in both protein and carbohydrate metabolism in the mammary gland (McFadden et al., 1987). During the early dry period, α-lactalbumin concentrations decrease rapidly in milk secretions, but nearly triple in serum concentrations during the same time period (Sordillo et al., 1987). The increase of α-lactalbumin concentrations in serum is considered to be a biomarker for the impairment of BMB tight junctions similar to lactose (Stelwagen et al., 1997). Serum α-lactalbumin concentrations were higher in cows milked twice daily when compared to those milked trice daily (McFadden et al., 1987). Additionally, after intramammary infusion of endotoxin, serum α-lactalbumin concentrations increased nearly 24-fold when mammary gland inflammation would lead to impaired epithelial and endothelial tight junctions (McFadden et al., 1988, Nguyen and Neville, 1998). Serum α-lactalbumin is hypothesized to be a good biomarker for subclinical mastitis as it is highly correlated with SCC (McFadden et al., 1988). Serum α-lactalbumin concentrations also increase during times of increased MEC proliferation such as during mammary development in

the last 100 d of pregnancy in heifers and during lactogenesis in multiparous cows (McFadden et al., 1987, Sordillo et al., 1987).

Lactose is a disaccharide of glucose and galactose produced by MEC and is the main determinant of milk osmolarity and thus the main driver of milk volume (Holt, 1983). The percentage of milk lactose tends to remain quite constant in healthy cows even as milk production decreases in late lactation (Miglior et al., 2006). Lactose percentage is hypothesized to be a good biomarker for mammary gland health and longevity as decreasing lactose percentages are highly associated with early culling, poor milk persistency, and inferior mammary morphology (Miglior et al., 2006, Costa et al., 2020). As mammary involution progresses, there is a rapid decline of lactose in milk secretions as MEC activity declines (Sordillo et al., 1987). Lactose concentrations could be used to estimate milk secretion volume as well as to adjust for any effects of dilution on other components (Silanikove et al., 2013). The osmotic properties of lactose could make it a useful biomarker for estimating risk for milk leakage, mammary gland pressure, and to evaluate changes in milk secretion volume.

Lactose is produced exclusively by MEC and so blood lactose concentrations are a useful biomarker for the integrity of the blood-milk barrier. Transient decreased concentrations of lactose can be observed in milk when severe local inflammation is initiated in the mammary gland from infusion of endotoxin (Shuster et al., 1991). Decreased milk lactose concentrations are coupled with increased urinary excretion of lactose demonstrating that there is increased 'leakiness' between the milk alveoli and surrounding blood vessels (Shuster et al., 1991). Blood lactose concentrations in healthy cows are <20 μl/mL but can increase to >120 μl/mL from mammary gland engorgement when milking is delayed more than 18 hr in early lactation (Stelwagen et al., 1997). Moreover, when mammary involution is induced with injections of 17β-

estradiol, urine lactose concentrations increase 7-fold and are highly correlated with plasma lactose concentrations (Agenäs et al., 2019). Therefore, blood or urine lactose may be a useful biomarker for mammary involution and milk lactose percentage in the subsequent lactation could be a useful biomarker for determining quality of MEC turnover that occurred during the previous dry period. Blood lactose concentrations can be increased with IMI, however, so concurrent bacterial culture of milk secretions may aid in accurate interpretation (Stelwagen et al., 1997). The mammary gland is impermeable to certain ions such as sodium and potassium during lactation. During lactation, milk has low concentrations of sodium and high levels of potassium because the BMB effectively controls the transfer of molecules in the mammary gland into circulation and extracellular space (Wellnitz and Bruckmaier, 2021). During mammary gland involution, the integrity of the BMB is reduced allowing sodium to flow into the mammary gland and for potassium to flow out (Linzell and Peaker, 1971, Boutinaud et al., 2017). Thus sodium:potassium ratio in milk secretions is used by researchers as a measure of tight junction permeability (Boutinaud et al., 2016). Bovine serum albumin (BSA) increases in milk secretions during the first 2-3 wk of involution indicating an increasingly impaired BMB. Other biomarkers for BMB integrity include lactate dehydrogenase and IgG transfer into milk which can differ between stimuli independent of SCC (Wellnitz et al., 2011). The of amalgamation of intra and extra-alveolar components alters the pH of milk. The pH of milk secretions increases from approximately 6.8 at dry off to 7.25 in healthy cows or 7.5 in cows with mastitis 7 d after dry off (Sordillo et al., 1987). Copper et al. (2004) analyzed the integrity of the BMB directly by measuring the expression of a component of MEC tight junctions (zonula occludens-1). The expression of zonula occludens-1 decreased by 50% 8 d after the abrupt cessation of lactation indicating that the BMB became impaired (Cooper et al., 2004). The flux of BMB integrity

during mammary gland involution is an important component of the immune response and influences infiltration of leukocytes into mammary gland tissue (Wellnitz and Bruckmaier, 2021). However, excessive infiltration of leukocytes can cause tissue damage during transmigration or from oxidative stress resulting from production of free radicals (Zhao and Lacasse, 2008, Sordillo and Aitken, 2009). Thus, the consequences of the degree of BMB impairment during involution on mammary gland health should be further investigated.

The proteolytic enzymes, MMP, are essential for mammary gland involution and can be analyzed in both milk secretions and tissue samples (Rabot et al., 2007, Zhao et al., 2019). The concentrations as well as enzymatic activity can be increased with treatment such as prolactin antagonists (Boutinaud et al., 2017). The ability to quantify the concentration and activity of MMP demonstrates the gross degradation potential in mammary gland secretions (Cheng et al., 2008b). The inhibitors of MMP, TIMP, should also be quantified to assess the net potential tissue degradation in a sample (Cheng et al., 2008b). Increased MMP:TIMP ratio has been associated with mammary glands chronically infected with *S. aureus* during involution (Beccaria et al., 2021). An imbalance between MMP and TIMP may cause tissue damage even in healthy mammary glands. Gifre-Renom et al. (2020) treated cows with nanoparticles imbedded with recombinant MMP-9. Treated cows had greater concentrations of serum albumin in milk secretions 1 and 6 d post dry off which suggests that increased MMP-9 increased the rate of ECM breakdown, but TIMP concentrations were not investigated in this study (Gifre-Renom et al., 2020). Relationship between increased MMP:TIMP, alterations in the ECM and subsequent milk production in cows is not well understood.

Udder engorgement that results from abrupt cessation of milking causes discomfort in cows (Vilar and Rajala-Schultz, 2020). This discomfort is apparent by decreased lying time and

increased vocalization in cows with greater mammary gland engorgement. Mammary gland engorgement is difficult to measure objectively but several methods to estimate engorgement have been reported (Table 1 and 4). Manual mammary gland palpation can be used and is non-invasive (Franchi et al., 2021a) but is subjective, and has poor repeatability (Bertulat et al., 2012). More objective measures have included use of a dynamometer, which is a tool that was developed to measure the firmness of fruit and has been adapted to use on mammary glands (Bertulat et al., 2012). The dynamometer is a repeatable, objective method for udder engorgement, but it can have poor correlation with manual palpation (Rees et al., 2014). The dynamometer was also compared to internal teat pressure measurements. A catheter used for evaluating intra-arterial blood pressure in humans was inserted through the teat canal to the level of the teat base and pressure was recorded for 72 hr post dry off (Blau et al., 2019). Control cows had elevated mammary gland pressure compared to pre-dry off concentrations (greater than double) and remained elevated throughout the duration of the sample collection period (18-72 hr) (Blau et al., 2019). Feed restriction reduced intramammary pressure and intramammary pressure results from all cows significantly correlated with dynamometer results ($r = 0.67$) (Blau et al., 2019).

Mammary engorgement can also be measured using alternative methods. Stelwagen et al., (1997) were particularly creative in their approach to assessing effects of once daily milking on udder volume in late lactation cows. Contralateral quarters were assigned to either twice or once daily milking for the last 3 weeks of lactation (Stelwagen and Knight, 1997). The contralateral quarter volumes were assessed by painting the deviations between right, left, front, and rear quarters before applying a quick-set spray on foam to create a cast of the udder (Stelwagen and Knight, 1997). The painted lines were then used to place cardboard at the correct deviations between

quarters and volume was measured by filling the cast with barley of a defined specific gravity (Stelwagen and Knight, 1997). Interestingly, by using this technique it appeared that once daily milking for 3 weeks increased the volume of contralateral quarters by 1.8 liters compared to controls. As the authors noted, the foam cast method likely could not account for the increased flaccidity of the involuting quarters and thus would not be appropriate for measuring morphometric changes in the early dry period. However, as methods for quantifying the morphometric changes in mammary gland involution advance, accounting for potential effects of changes in mammary gland shape, fill, and texture on measurements will be essential.

Another method for capturing and quantifying morphological changes in the mammary gland is with the use of a 3D Scanner. A 3D Scanner was compared to measurements used to measure the changes in surface area of the rear quarters in involuting dairy cows (de Campos et al., 2020). The surface area was calculated from the images with 3-Matic software and the percent change was compared to measurements taken with a metric tape (de Campos et al., 2020). The 3D scanner found that the surface area increased from baseline at dry off 125% at 2 d dry, returned to baseline by 7 d dry, and then no significant change occurred by 14 d dry, which was similar to the metric tape results (de Campos et al., 2020). Mammary engorgement was also measured by evaluating the increase in distance between hocks after dry off (Franchi et al., 2022) and the total distance between all 4 teats (Bertulat et al., 2017). Larsen et al. (2021) combined the distance between rear mammary gland quarters as well as teat perimeter to evaluate mammary engorgement (Larsen et al., 2021). The association between mammary gland engorgement and involution failure should be further investigated.

Use of ultrasound to identify alterations in mammary gland tissue is noninvasive and could be useful for evaluating mammary gland involution. Use of ultrasound with an 5.0 MHz linear array

transducer is particularly effective for examination of the teat canal (Franz et al., 2009). In particular, the content of the keratin in the stratum corneum can be evaluated as different levels of echogenicity, which suggests that integrity of the keratin plug could possibly be evaluated by ultrasound (Franz et al., 2001). Measurements collected by ultrasound discerned that teat canals in healthy cows were longer and narrower than teat canals associated with infected mammary glands during lactation (Klein et al., 2005). Thus, ultrasound may be a useful tool to measure changes in teat canal morphology after dry off. Ultrasound images of teats were used to evaluate teat congestion caused by various levels of milking vacuum, b-phase duration, and liner compression (Spanu et al., 2008). The change in teat wall thickness were compared to baseline values before milking over 4 hours after milking (Spanu et al., 2008). Use of ultrasound to measure teat wall thickness was effective to test differences in teat congestion and recovery between treatments and could be applied to investigation of the effects of post-dry off teat swelling on teat morphology.

Imaging with ultrasound can be effective in evaluating structural changes within the mammary gland. Ultrasound images were useful in characterization of clinical mastitis of varying grades and different pathogens/etiologic agents (Flöck and Winter, 2006). Using both a convex and linear transducer, investigators were able to visualize pathologies in mammary tissue such as edema, abscesses, hematomas, gas bubbles, and inflamed lactiferous ducts (Flöck and Winter, 2006). Ultrasound images were not specific enough to replace milk culture for diagnosis of an etiologic agent with perhaps the exception of *Trueperella pyogenes* which in this study was associated with round hypoechoic areas with a hyperechoic center (Flöck and Winter, 2006). The relevant finding from this study for this review was that indicators of tissue inflammation and udder swelling could be visualized with ultrasound and therefore ultrasound could be useful and

non-invasive tool to characterize the involuting mammary gland. For example, mammary parenchyma was evaluated at the center of each quarter. The computerized image analysis of echotextural of mammary parenchyma increase in pixel heterogeneity after milking compared to before milking (Schwarz et al., 2020). Interestingly, the pixel heterogeneity of mammary gland parenchyma was negatively correlated with milk components including casein and lactose (Schwarz et al., 2020). The changes in pixel heterogeneity may be related to mammary gland milk volume and pressure and therefore may provide an objective measure of mammary gland engorgement after dry off. Highly detailed 3D images of the mammary gland parenchyma, gland cistern, and teat anatomy can be produced with 3D ultrasonography which could provide more specific morphometric changes during involution than conventional ultrasonography (Franz et al., 2004).

Changes in blood flow to the mammary gland may also be examined during mammary gland involution. Although measurements were not compared in the same cows over time, blood flow velocity analyzed with color doppler in the milk and musculophrenic vein measurements differed between dry and lactating cows (Braun and Forster, 2012). Blood flow into the mammary gland is an essential factor in milk production and is regulated in part by integrity of MEC tight junctions and the main components affecting osmotic pressure of fluid into the mammary gland: lactose, Na^+, K^+, and Cl^- (Cai et al., 2018). Concentrations of lactose, Na^+, and K^+ changes in milk secretions during the first week of the dry period could therefore be associated with for changes in blood flow to the mammary gland (Lacy-Hulbert et al., 1999, Shamay et al., 2003). Thus, doppler analysis of the blood flow to the mammary gland could be a non-invasive method to measure involution.

Advances in technology can be useful in identifying new biomarkers of mammary gland involution or less invasive measurement techniques of current biomarkers. Improved analytical techniques such as liquid chromatography tandem mass spectrometry may be useful to analyze and differentiate biomarkers that have small endogenous concentrations such as oxylipids and isoprostanes. Oxylipids are essential mediators for the inflammatory response and isoprostanes are stable indicators of oxidative stress and both are associated with disease risk in dairy cows (Mavangira and Sordillo, 2018). Seven plasma oxylipid concentrations were different during early mammary gland involution for cows that were healthy or experienced clinical disease between 1 and 39 DIM (Putman et al., 2022). This suggests that certain oxylipids could be excellent biomarkers for mammary gland involution failure. In addition to lipid mediators measured by liquid chromatography tandem mass spectrometry, some new technologies can be applied directly to the cow. Accelerometers, for example, provide valuable behavioral data such as decreased lying time after dry off that can indicate discomfort associated with mammary gland engorgement. Infrared technologies for measurement of skin level temperature were found to accurately reflect the severity of clinical mastitis infection in one study and could be a useful tool in assess mammary gland engorgement after dry off (Colak et al., 2008). Additionally, eye temperature measured by infrared technology found variations in cow eye temperature in the first 2 d post dry off and could be a non-invasive measure of stress (Franchi et al., 2021b). Other technologies such as “omics” and microbiome studies are still in their infancy. Proteomics and microRNA analysis of milk secretions identified dozens of potential biomarkers for mammary gland involution but their utility as biomarkers remain unknown (Boggs et al., 2015, Putz et al., 2019, Reinhardt and Lippolis, 2020). Currently, there is no consensus to the relevance of the mammary gland microbiome or whether a core commensal microbial community in the bovine

mammary gland exists (Ruegg, 2022). Thus, the potential for the mammary gland microbiome to serve as a biomarker for mammary gland involution should be investigated as microbiome sampling and analysis techniques improve. Biomarkers for mammary gland involution can represent the series of physiological processes required for successful involution. Improving our understanding of the relationships between biomarkers and outcomes of mammary gland involution will enhance our ability to assess the speed and magnitude of active involution. Thus, researchers will be more effective in designing, executing, and analyzing studies aimed at mammary involution enhancement.

CONCLUSION

Mammary gland involution is an essential lactation stage for ensuring mammary gland health and milk production. Cows with high milk yield at dry off may be at greater risk for mammary gland involution failure. Advances in genetics, nutrition, and fertility can all contribute to increasing the proportion of cows with high milk yield at dry off and therefore risk of involution failure is likely to be increasingly common. Strategies to reduce involution failure are needed to ensure dairy cow health and welfare in the future. However, many involution biomarkers commonly used are not specific to precise mammary gland functions or risk of involution failure. Future research should use specific and objective outcomes so that involution biomarkers will be more useful in assessing risk for involution failure.

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CHAPTER 2: SERUM VITAMINS AS BIOMARKERS FOR DISEASE IN PERIPARURIENT COWS

ABSTRACT

The effectiveness of using serum vitamin concentrations as biomarkers to predict diseases in dairy cows during the transition period are currently not well known. Thus, the objective of this study was to evaluate the association of serum vitamin A, β-carotene, and vitamin E concentrations and periparturient cow diseases on commercial dairies. In the current study, serum vitamin concentrations at dry off (DO) and during the close up (CU), and early-lactation periods (approximately 7 d post calving) (C+7) were measured and their association to clinical diseases in the first 30 d in milk were examined. Diseases were diagnosed by trained personnel and recorded using computer database software. Blood samples were taken from 353 cows from 5 different farms over a 3 yr period. Blood samples were analyzed for β-carotene, retinol, α-tocopherol, and cholesterol. Separate mixed logistic regression models were built for each disease outcome: hyperketonuria, lameness, mastitis, uterine diseases (retained placenta and/or metritis), and an aggregate outcome. For the aggregate outcome, a cow was considered positive if she had one or more of the following: hyperketonuria, lameness, mastitis, uterine diseases, pneumonia, milk fever, and displaced abomasum. Concentrations of all 3 fat-soluble vitamins decreased significantly at C+7, relative to either prepartum sampling time. Serum retinol concentrations at CU and C+7 were negatively associated with odds of post-partum hyperketonuria. At C+7, cows with uterine disease had lower serum retinol concentrations than cows that did not have uterine disease. Similarly, lower serum retinol concentrations were associated with greater odds of having disease in the periparturient period. Milk production was positively correlated with increased serum α-tocopherol and negatively correlated with β-carotene concentrations. This study demonstrates the potential for serum vitamins to serve as

biomarkers for disease risk as well as solidifies the importance for more rigorous research investigating ideal serum vitamin concentrations for promoting health in dairy cows.

Key Words: vitamin A, vitamin E, b-carotene, antioxidant

INTRODUCTION

Vitamins A and vitamin E are essential nutrients in the diets of dairy cows. Deficiencies of these essential vitamins can result in a range of health aberrations including loss of vision, cardiac muscle degeneration, and an overall increase in morbidity and mortality (Frye et al., 1991, Herdt and Stowe, 1991). β-carotene is a pro-vitamin and serves as a precursor to vitamin A, however, for simplicity it will not be distinguished from vitamins for the remainder of this manuscript (D'Ambrosio et al., 2011). Serum β-carotene, retinol, and α-tocopherol also are known to contribute to important antioxidant defense mechanisms and optimal immune functions (Sordillo, 2016). Vitamin requirements for immune or antioxidant functions, however, were shown to be higher than those for milk production (Weiss, 1998). Increased milk production escalates production of reactive oxygen species, which can result in elevated disease risk if antioxidant capacities are overwhelmed (Sordillo, 2005, Abuelo et al., 2015). Milk production continues to increase in the United States and therefore, continued investigations of the potential link between serum vitamins concentrations and disease are warranted in order to decrease disease risk in the modern cow (NASS, 2019).

Serum concentrations of < 3 μg/mL β-carotene, <150 ng/mL retinol, and <0.2 μg/mL α-tocopherol are unusual in healthy cattle and generally considered evidence of deficiency (Herdt and Stowe, 1991). However, the precise serum concentrations of these vitamins required to obtain optimum health are not well understood currently, particularly during the periparturient period when disease risk increases. Serum retinol and α-tocopherol were shown to decrease

through the peripartum period with the nadir occurring 1-3 d post calving (Goff et al., 2002). Conversely, β-carotene remains depressed several days into early lactation (Goff et al., 2002). The dynamics of serum vitamin concentrations might influence disease risk during this vulnerable period. The decrease in serum β-carotene, retinol and α-tocopherol could be a result of decreased feed intake, increased losses from secretion into colostrum, increased use as antioxidant defense, or lack of transport molecules to move them through circulation (Ingvartsen and Andersen, 2000). During the transition period there is an increased demand for micronutrients, including vitamins, to provide adequate antioxidant defenses against the metabolic stress associated with the periparturient period (Sordillo and Aitken, 2009). Thus, ensuring that vitamins can be delivered to tissues in adequate supply is important for cow health, but is complicated by a number of factors during the periparturient period.

There is extensive literature regarding the health benefits of vitamin E supplementation in dairy cows. The best known function of vitamin E is as an antioxidant, but its role in enhancing the immune function through improved neutrophil function also was demonstrated in cattle (Higuchi and Nagahata, 2000, Qu et al., 2018). Injectable vitamin E supplementation (1,000 IU) given weekly for 3 wk prepartum decreased retained fetal membranes, decreased incidence of stillbirth, and tended to decrease death rates by 200 d postpartum (Pontes et al., 2015). Additionally, serum α-tocopherol concentrations >3 µg/mL at calving dramatically reduced the risk of mastitis in the periparturient period (Weiss et al., 1997). Conversely, abundant knowledge gaps remain regarding the potential health effects and serum concentration responses associated with dietary vitamin A and β-carotene supplements in dairy cattle; however, studies investigating their effects on the immune system have been promising. Dietary vitamin A supplementation hastened immune system maturation in pre-weaned calves and improved humoral immunity in vaccinated

feedlot calves (Nonnecke et al., 1999, Jee et al., 2013). Supplementation with β-carotene in peripartum cows enhanced lymphocyte and phagocyte activity and resulted in a decrease in incidence of metritis and retained placenta (Michal et al., 1994).

Despite being the subject of many reviews, the concentrations of circulating fat-soluble vitamins that promote health in dairy cows at different stages of lactation are not established (Spears and Weiss, 2008, Abuelo et al., 2015, Sordillo, 2016). The objectives of this study were 2-fold. The first objective was to determine the concentrations of serum β-carotene, retinol, and α-tocopherol from dry off through the first week of calving. The second objective was to explore the association between these serum vitamin concentrations and incidence of disease during the transition period. We hypothesized that serum vitamin concentrations would decrease in the peripartum period in all cows, and that this decrease would be greater among cattle that developed disease. We performed a longitudinal, herd-based epidemiologic investigation of serum β-carotene, retinol, and α-tocopherol concentrations in dairy cattle on 5 commercial farms at 3 specific time points through the nonlactating and early lactating period.

MATERIALS AND METHODS

Animals

A total of 353 cows from 5 commercial dairy herds in Michigan were enrolled and samples were collected over a 3 yr period. Cohorts consisted of approximately 15 cows/cohort (5 nulliparous cows <25 mo old, 5 primiparous cows, and 5 multiparous cows). Cohorts were formed by selecting cows from the pen that were to be dried off that week, or for heifers, the equivalent length of time from estimated calving date. At the time of dry off, cows were approximately 200 to 230 d pregnant and <380 DIM. All cows were bred by artificial insemination, which provided more accurate estimations of impending calving dates, relative to natural breeding.

Health Monitoring

The health status of each cow was monitored from dry off through 30 d post-calving and recorded utilizing either Dairy COMP305 (Alta Genetics Inc., Watertown, WI) or PCDART (CentralStar Michigan Lab, Grand Ledge, MI) on-farm software. On each farm trained individuals were responsible for monitoring health of cows daily from calving through 30 DIM. Herd veterinarians confirmed disease diagnosis when needed. Negative health outcomes of interest included milk fever, mastitis, metritis, retained placenta, lameness, displaced abomasum, hyperketonuria, and pneumonia. Case definitions for this study have been described previously and will be briefly described here (Wisnieski et al., 2020). Milk fever was diagnosed by presence of clinical signs including muscle weakness, nervousness, muscle tremors, cold ears, and the inability to rise. Mastitis was diagnosed by the appearance of abnormal milk such as the presence of flakes or clots. In this study, metritis was defined as the presence of abnormal vaginal discharge and a flaccid uterus that was found by transrectal palpation to not have involuted normally within the first 21 DIM. A cow would be diagnosed with retained placenta when fetal membranes were not expelled within 24 hr post calving. Lameness was diagnosed by presence of an abnormal gait equivalent to a score of ≥3 on a 1-5 scale (Sprecher et al., 1997). Displaced abomasum was diagnosed by auscultation of the area between the 9^{th} and 12^{th} rib and detecting a ping when thumping the body wall. Hyperketonuria was diagnosed by presence of urine acetoacetate concentrations ≥1.5mmol/L (Ketostix, Bayer AG, Leverkusen, Germany), during daily health assessments of fresh cows 2-10 d postpartum by trained farm personnel. In this study, pneumonia was diagnosed by presence of abnormal lung sounds on auscultation of the thorax, altered breathing patterns, and usually with a body temperature exceeding 39.44°C. Cows with other negative health outcomes such as abortion and death were excluded from the analysis.

Each farm fed a TMR supplemented with vitamin A and vitamin E (Table 1). Parental vitamin supplementation was not used in adult cows on any of the farms included in the study. The Animal Use and Care Committee at Michigan State University (East Lansing) approved this study and all animal protocols.

Table 2.1: ***Ration summary from study farms***

Item (% of dry matter unless noted)	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5		
	Far-Off Dry	Close Up Dry	Fresh cow	Far-Off Dry	Close Up Dry	Fresh cow	Far-Off Dry	Close Up Dry	Fresh cow	Far-Off Dry	Close Up Dry	Fresh cow	Far-Off Dry	Close Up Dry	Fresh cow
NEL (Mcal/kg DM)	1.27	1.31	1.52	1.28	1.34	1.69	1.35	1.55	1.81	1.5	1.45	1.76	1.47	1.45	1.69
CP	11	13.2	18.1	11.13	13.02	16.75	15.37	15.37	17.94	11.9	14.5	16	14.34	14.94	17.67
Fat	2.3				2.9	4.06	3.02	2.79	4.04						
NFC	17	24.1	35.9	20.83	24.23	29.8	26	14.86	29.7	24.3	23.8	34.3	9.91	13.49	29.3
Calcium	0.51	1.05	1.25	0	1.33	1.01	0.3	0.3	0.94	0.65	0.61	0.73	0.75	1.59	0.78
Phosphorous	0.36	0.36	0.39	0	0.36	0.46	0.31	0.31	0.47	0.25	0.44	0.47	0.28	0.41	0.39
Magnesium	0.31	0.35	0.42	0.37	0.48	0.38	0.42	0.42	0.42	0.41	0.8	0.33	0.39	0.5	0.31
Potassium	2.5	1.34	1.48	1.33	1.05	1.27	1.28	1.28	1.5	1.62	1.84	1.61	1.95	0.96	1.34
Sodium	0.34	0.107	0.49	0.07	0.11	0.19	0.09	0.09	0.45	0.211	0.27	0.79	0.14	0.11	0.39
Chloride	0.99	0.92	0.57	0.32	0.85	0.36	0.35	0.35	0.44	0.41	0.63	0.51	0.28	0.47	0.53
Sulfur	0.18	0.43	0.28	0.31	0.41	0.27	0.27	0.27	0.27	0.2	0.67	0.28	0.14	0.45	0.24
Selenium (mg/kg DM)	0.48	0.55	0.39	0.56	0.61	0.49	0.58	0.58	0.39				0.61	0.7	0.32
Vitamin A (KIU/kg DM)	3.50	6.14	1.45	2.80	5.80	0.91	2.52	3.36	1.96				1.40	3.62	2.09
Vitamin E (IU/kg DM)	22.00	20.45	9.24	20.12	32.44	9.85	8.17	33.4	39.58				24.84	33.43	8.71

Vitamin Analysis

Blood samples were collected from coccygeal vessels into blood tubes without anticoagulants. The samples were collected at dry off (DO, -48±12d pre-calving), close up (CU, -17±7d pre-calving), and C+7 (7±3d post-calving). Serum was separated by centrifugation at 1,449 x *g* for 15 min at 4°C and was stored at -20°C until analysis. All samples were analyzed within 6 mo of

collection. Retinol, α-tocopherol, and β-carotene have been found to be stable for at least 6 mo when stored at -20°C (Comstock et al., 1993). The serum concentrations for retinol, α-tocopherol, and β-carotene were analyzed at the Michigan State University Veterinary Diagnostic Laboratory (Lansing, MI 48910) by using UPLC by their routine method (Arnaud et al., 1991). Briefly, 5-point calibration curves were constructed using stock β-carotene standard (Sigma Aldrich, St. Louis, MO) (absorbance of 1.29 AU at 450nm), retinol standard (Sigma Aldrich, St. Louis, MO) (absorbance of 0.5153 AU at 325nm), and α-tocopherol (Sigma Aldrich, St. Louis, MO) solution (absorbance of 1.29 AU at 292nm). For the internal standard, 12 µM apocarotenal (Sigma Aldrich, St. Louis, MO) was added to standards and samples. Samples were analyzed chromatographically using a Waters Acquity system and Waters Empower Pro Chromatography Manager software (Waters Corporation, Milford, MA 01757). All peaks were reviewed manually by trained laboratory employees after initial autointegration.

Cholesterol analysis

The concentration of α-tocopherol measured in serum is dependent on the concentration of lipoproteins in circulation as well as the concentration of α-tocopherol within each lipoprotein particle (Herdt and Smith, 1996). Similar to α-tocopherol, lipoproteins are the serum transport system for β-carotene as well. Therefore, cholesterol was included as an independent variable in our disease models, alone and as an interaction term with both α-tocopherol and β-carotene, in order to adjust for the influence of lipoprotein concentration on serum α-tocopherol and β-carotene concentrations. Cholesterol was analyzed at the Michigan State University Veterinary Diagnostic Laboratory using a validated protocol. Briefly, cholesterol (Cholesterol, Beckman Coulter Inc., Brea, CA) was analyzed with a commercially available assay by colorimetric measurement on an autoanalyzer (Olympus AU 640e, Beckman Coulter Inc.).

Statistical analysis

Graphpad Prism version 6 was used to create a table of mean concentrations of biomarkers (retinol, α-tocopherol, β-carotene) for cows with each disease outcome (mastitis, uterine disease, hyperketonuria, lameness, and the aggregate outcome) or without disease in the first 30 DIM. Mann-Whitney U, two-tailed test was used to test for significance at each time point (DO, CU, and C+7). Serum vitamin concentrations are reported as mean ± standard error of the mean (SEM).

The *pwcorr* command in Stata version 14.2 was used to perform Pearson correlation analyses to assess the correlation between each biomarker (β-carotene, α-tocopherol, retinol and cholesterol) at each sample point (DO, CU, and C+7). Biomarker values that did not meet normality assumptions were transformed (i.e. log transformation). Scatterplots were used to verify a linear relationship between each pair of biomarkers prior to calculation of a correlation coefficient. Linear mixed models were built in SAS 9.4 using the *PROC MIXED* command. A separate linear mixed model was built for each of the vitamin biomarkers (β-carotene, retinol, and α-tocopherol). The explanatory candidate variables for entry into the models included parity (1^{st}, 2^{nd}, or 3^{rd} and greater lactation), season, and time point. Seasons were defined as spring (March-May), Summer (June-August), Fall (September-November), and Winter (December-February). A parity and time point interaction term was also included in order to investigate trends in biomarker concentrations over time in different parity groups. Backwards selection was performed at an alpha of 0.05 to select the variables in the final model. Candidate variables that were not significant were removed from the model. A random intercept for cow in the REPEATED statement was used to account for the correlation between samples that were collected from the same cow over time. A spatial covariance matrix was used to adjust for

unequal spacing between time points. Random intercepts for the farm and cohort level were included unless the variance estimates estimated to be 0 in order to reflect the structure of the data. A solution for random effects matrix was used to determine differences in variables between farms. Multicollinearity was carefully monitored throughout the model building by assessing if there were inflated standard errors and large changes in coefficients after removing a variable. If multicollinearity was present, the problematic variable was removed. Heteroskedasticity of residuals across levels of the categorical variables in the final models were assessed via box plots and Levene's test. If heteroskedasticity was present, variances were estimated separately by the categorical variable and degrees of freedom were estimated using the Satterthwaite approximation. Normality of residuals was assessed via quartile-quartile (Q-Q) plots and histograms. Variables were transformed to meet normality assumptions if necessary. All statistical comparisons between levels of categorical variables present in the models were performed using a Bonferroni adjustment for multiple comparisons.

A linear mixed model was built to assess the association between the β- carotene, retinol and α-tocopherol with milk production (reported as ME305 estimated from first milk test). Parity, season, time point, cholesterol, interaction terms between each biomarker and time point, interaction terms between each biomarker and parity, and interaction terms between cholesterol and both α-tocopherol and β- carotene were candidate variables. The final variables were selected using a backwards selection procedure using an alpha of 0.05. Random intercepts for cohort and farm were included using the RANDOM statement. A random intercept for cow was included in the REPEATED statement to account for repeated samples from the same cow. Heteroskedasticity and normality of residuals were tested using the methods described above for

the descriptive models. A Bonferroni adjustment was used to adjust for multiple comparisons for comparisons between levels of categorical variables.

Mixed logistic regression models were built in SAS 9.4 using the *PROC GLIMMIX* command with a binomial distribution. A separate logistic regression model was built for each disease outcome: hyperketonuria, lameness, mastitis, uterine diseases (RP and/or metritis), and an aggregate outcome (hyperketonuria, lameness, mastitis, uterine diseases, pneumonia, milk fever, and displaced abomasum). The variables eligible for entry into each model included retinol, β-carotene, α-tocopherol, parity, cholesterol, and season. Two-way interactions between each biomarker and time point, between each biomarker and lactation group, and between cholesterol and both α-tocopherol and β- carotene were included in variable selection if the number of events per variable (EPV) was at least 5 (Vittinghoff and McCulloch, 2007). Multivariate modeling involved a backward selection procedure using an alpha of 0.05. A random intercept for cow in the RANDOM statement was used to account for observations from the same cow. The default variance components covariance structure was used due to convergence issues with the spatial covariance structure. Farm and cohort level random intercept were included, unless the variance estimates were estimated to be 0. The assumption of linearity of the association between each continuous variable and the outcome on the log odds scale was assessed via a lowess smoothing curve. Continuous variables that violated this assumption were either transformed (i.e. square root, log) or were made into a categorical variable with 4 levels based on the distribution of that variable. All comparisons between categorical variables present in the models were performed using a Bonferroni adjustment for multiple comparisons.

RESULTS

Study sample

Our total sample included 353 cows from 28 cohorts. However, 4 cows were excluded from disease models because they were lost to follow up. Among the 349 cows that were followed through calving, 27 had hyperketonuria (8%), 19 had mastitis (5%), 40 had metritis and/or RP (11%), and 23 had lameness (7%). In total, 111 cows (32%) had one or more clinical disease (hyperketonuria, lameness, mastitis, uterine diseases, pneumonia, milk fever, and displaced abomasum). Data from rations from all 5 farms can be found in Table 1. Summary statistics for the biomarkers used in this study are presented in Table 2. The correlation between each biomarker at each sample point is shown in Table 3.

Table 2.2: ***Summary statistics of biomarkers (by time point) and ME305***

Biomarker	Time point	N	Mean	SEM	Min	25th percentile	Median	75th percentile	Max
α-tocopherol (ug/mL)	DO	351	4.88	0.13	0.84	2.95	4.49	6.49	13.11
	CU	321	3.13	0.09	0.41	1.91	2.79	4.13	11.06
	C+7	302	1.51	0.06	0.001	0.001	1.32	1.93	7.09
Cholesterol (mg/dL)	DO	353	183.8	3.3	70	130	189	227	397
	CU	310	99.8	1.4	50	84	96	113	191
	C+7	293	81.3	1.7	25	66	78	92	310
β-carotene (ug/mL)	DO	350	4.9	0.19	0.21	2.65	3.99	5.9	23.96
	CU	321	3.36	0.13	0.1	1.81	2.81	4.53	14.23
	C+7	303	1.55	0.07	0.1	0.69	1.11	1.83	6.39
Retinol (ng/mL)	DO	351	298.02	4.15	102	239	294	351	528
	CU	321	267.57	4.85	76	203	263	322	598
	C+7	301	233.95	6.69	53	141	217	298	764
ME305		319	26113.2	316.34	2281	23155	26010	29079	45690

Table 2.3: ***Correlation matrix of biomarkers by sample period***

	α-tocopherol[1]	Cholesterol[1]	β-carotene[1]	Retinol
Dry off				
α-tocopherol[1]	1	-		
Cholesterol[1]	0.70**	1		
β-carotene[1]	0.17**	0.33**	1	
Retinol	0.17**	0.28**	0.36**	1
Close Up				
α-tocopherol[1]	1	-		
Cholesterol[1]	0.40**	1		
β-carotene[1]	0.31**	0.04	1	
Retinol	0.35**	-0.06	0.18**	1
C+7				
α-tocopherol[1]	1			
Cholesterol[1]	0.48**	1		
β-carotene[1]	0.22**	0.32**	1	
Retinol	0.25**	0.39**	0.57**	1

** denotes statistical significance at alpha = 0.01, * denotes statistical significance at alpha = 0.05.
[1]Transformed by the log (base 10) function to meet linearity assumptions.

Descriptive analyses

Linear mixed model results for β-carotene are shown in Table 4. The final model included time point (P<0.01), parity group (P<0.01), season (P<0.01), and a parity and time point interaction term (P<0.01). Concentrations of β-carotene significantly decreased from DO to CU (P<0.01) (LSM±SE, 3.85±1.21 µg/mL to 2.68±1.21 µg/mL) and from CU to C+7 (P<0.01) (LSM±SE, 2.68±1.21 µg/mL to 1.22±1.21 µg/mL). The β-carotene concentrations were greater in 2 and 3+ parity cows compared with 1st parity cows (P<0.01) (LSM±SE, 2.52±1.21 µg/mL, 2.69±1.21 µg/mL, 1.85±1.21 µg/mL respectively). However, concentrations were not significantly different between those of 2nd parity and those of 3+ parity (P= 0.12). Concentrations of β-carotene were significantly higher in summer compared with spring (P<0.01) and winter (P=0.04) (LSM±SE, 2.69±1.22 µg/mL, 2.2±1.22 µg/mL, 2.16±1.22 µg/mL respectively). The interaction term for time point by parity group indicated that cows in the 2 and 3+ parity group had smaller declines in β-carotene concentrations from CU to C+7, compared with 1st parity cows. Interestingly, β-

carotene serum concentrations were significantly different between farms (P<0.05), which may have reflected differences in forage characteristics; supplemental β-carotene was not fed on any of the farms.

Table 2.4: ***Linear mixed model results for β-carotene***

Variable	Coefficient	SE	LSM[1]	SE for LSM	t Value	P-value
Time point						<0.01
DO	(ref)		3.85	1.21		
CU	-0.16	0.03	2.68	1.21	-4.59	<0.01
C+7	-0.41	0.04	1.22	1.21	-11.35	<0.01
Parity group						<0.01
1st	(ref)		1.85	1.21		
2nd	0.17	0.03	2.52	1.21	5.70	<0.01
3rd and greater	0.22	0.03	2.69	1.21	7.43	<0.01
Season						<0.01
Spring	(ref)		2.20	1.22		
Summer	0.09	0.03	2.69	1.22	3.34	<0.01
Fall	0.01	0.03	2.27	1.22	0.41	0.68
Winter	-0.01	0.03	2.16	1.22	-0.30	0.76
Time point and parity group interaction						<0.01
DO and 1st parity	(ref)					
CU and 2nd parity	0.01	0.04			0.19	0.85
CU and 3+ parity	-0.001	0.04			-0.02	0.99
C+7 and 2nd parity	-0.11	0.04			-2.58	0.01
C+7 and 3+ parity	-0.15	0.04			-3.67	<0.01
Intercept	0.43	0.09			4.92	<0.01

[1] β-carotene log (base 10) transformed to meet linearity assumptions. LSM back transformed from log scale. Adjusted for heterogenous variances by parity

Random effect variance estimates: Farm= 0.03; Cohort= 0.03; Cow (SP POW): 1st parity= 0.96, 2nd parity= 0.54, 3+ parity = 0.98.

Table 2.5: ***Linear mixed model results for retinol***

Variable	Coefficient	SE	LSM	SE for LSM	t Value	P-value
Time point						<0.01
DO	(ref)		312.74	16.15		
CU	5.62	9.92	283.68	16.20	0.57	0.57
C+7	-14.01	10.26	250.50	16.26	-1.37	0.17
Parity group						<0.01
1st	(ref)		283.24	16.27		
2nd	40.50	9.44	294.63	16.25	4.29	<0.01
3rd and greater	39.61	8.96	269.04	16.16	4.42	<0.01
Time point and parity group interaction						<0.01
DO and 1st parity	(ref)					
CU and 2nd parity	-40.30	13.53			-2.98	0.003
CU and 3+ parity	-63.73	12.78			-4.99	<0.01
C+7 and 2nd parity	-47.02	13.86			-3.39	<0.01
C+7 and 3+ parity	-97.68	13.04			-7.49	<0.01
Intercept	286.04	17.13			16.70	<0.01

Adjusted for heterogenous variances by parity.
Random effect estimates: Farm: 315.97; Cohort:4753.91; Cow (SP POW): 1st parity= 0.81, 2nd parity= 0.97, 3+ parity = 0.98

Linear mixed model results for retinol are presented in Table 6. The variables in the final model included time point, parity, and a time point by parity interaction term. Retinol concentrations significantly increased from DO to CU (P<0.01) (LSM±SE, 312.74±16.15 ng/mL to 283..68±16.20 ng/mL) and then significantly decreased from CU to C+7 (P<0.01) (LSM±SE, 283±16.20 ng/mL to 250.50±16.26 ng/mL). Retinol concentrations were significantly higher among cows in the 2nd parity group compared with the 3+ parity group (P<0.01)) (LSM±SE, 294±16.25 ng/mL and 269.04±16.16 ng/mL, respectively). Retinol concentrations decreased from DO to CU and from CU to C+7 among 3+ parity cows. In 2nd parity cows, serum retinol decreased from DO to CU then increased from CU to C+7. Retinol concentrations remained relatively steady from DO to CU and from CU to C+7 among 1st parity cows. Concentrations of serum retinol were not different between farms (P>0.05).

Table 2.6: ***Linear mixed model results for α-tocopherol***

Variable	Coefficient	SE	LSM	SE for LSM	t Value	P-value
Time point						<0.01
DO	(ref)		4.69	1.09		
CU	-0.13	0.02	3.00	1.09	-5.14	<0.01
C+7	-0.4	0.03	1.44	1.09	-15.62	<0.01
Parity group						<0.01
1st	(ref)		2.46	1.09		
2nd	0.19	0.02	3.03	1.09	7.56	<0.01
3rd and greater	0.13	0.03	2.72	1.09	5.05	<0.01
Time point and parity group interaction						<0.01
DO and 1st parity	(ref)					
CU and 2nd parity	-0.13	0.04			-3.56	<0.01
CU and 3+ parity	-0.08	0.04			-2.08	0.04
C+7 and 2nd parity	-0.16	0.04			-4.46	<0.01
C+7 and 3+ parity	-0.18	0.04			-4.84	<0.01
Intercept	0.57	0.04			14.47	<0.01

[1]α-tocopherol log (base 10) transformed to meet linearity assumptions. LSM back transformed from log scale. Random effect variance estimates: Cohort: 0.03; Cow (SP POW): 0.94.

Linear mixed model results for the α-tocopherol are shown in Table 5. The final model included time point (P<0.01), parity (P<0.01), and a time point by parity interaction term (P<0.01). From DO to CU, α- tocopherol significantly decreased (P<0.01) (LSM±SE, 4.69±1.09 µg/mL to 3.00±1.09 µg/mL) and then further decreased from CU to C+7 (P<0.01) (LSM±SE, 3.00±1.09 µg/mL to 1.44±1.09 µg/mL). Overall, α- tocopherol was highest in 2nd parity cows, followed by 3+ parity cows, and lowest in 1st parity cows (LSM±SE, 3.03±1.09 µg/mL, 2.72±1.09 µg/mL, 2.46±1.09 µg/mL respectively). The time point by parity interaction indicated α- tocopherol concentrations were significantly lower in 1st parity cows at DO compared with both 2nd parity and 3+ parity cows but were not significantly different from 2nd parity and 3+ parity at CU or C+7. Serum α- tocopherol concentrations did not differ between farms (P>0.05).

Milk production model

The final model for first-test ME305 is presented in Table 7. The default variance components covariance structure was used due to convergence issues with the spatial covariance matrix. ME305 was significantly lower among 1st parity cows compared to 2nd and 3+ parity cows (P < 0.01) (LSM±SE, 24,162±1,029.19, 26,481±1,026.94, 27,531±1,031.14, respectively). Higher α-tocopherol concentrations were associated with greater ME305, especially among 1st parity cows. Lower β-carotene concentrations were associated with higher ME305. ME305 was significantly different between farms (P<0.05).

Table 2.7: ***Linear mixed model results for ME305[1]***

Variable	Coefficient	SE	LSM	SE for LSM	t Value	P-value
α-tocopherol[2]	2369.63	663.28	-	-	3.57	<0.01
β-carotene[2]	-1681.83	678.32	-	-	-2.48	0.01
Parity group						<0.01
1st	(ref)		24162	1029.19		
2nd	2318.81	416.23	26481	1026.94	5.57	<0.01
3rd and greater	3368.72	433.47	27531	1031.14	7.77	<0.01
Intercept	23880.00	1043.68	-	-	22.88	<0.01

[1]3 cattle missing ME305 data.
[2]Transformed by the log (base 10) function to meet linearity associations.
Random effect estimates: Farm: 4566744; Cohort:1036697; Cow (SP POW): 25456699.

Disease models

There were no vitamins significantly associated with lameness or mastitis in the multivariable models, therefore, these results were omitted.The final logistic regression model for hyperketonuria is shown in Table 8. The variables in the final model included parity (P<0.01) and retinol (P=0.03). Higher retinol concentrations were associated with lower odds of hyperketonuria (OR = 0.997; 95% CI = 0.994- 0.9997). The OR can be interpreted as: for every 100 ng/mL increase of retinol, the odds of kyperketonuria decreased by 0.3. Cows with hyperketonuria in the first 30 DIM had mean retinol concentration s of 230.8±14.4 ng/mL and

145.9±13.3 ng/mL at CU and C+7, whereas cows that did not have hyperketonuria had mean retinol concentrations of 270.8±5.1 ng/mL and 240.9±7.0 ng/mL during the same time periods (P<0.05 and P<0.0001 respectively). Cows in the 2nd and 3+ parity group had significantly higher risk of hyperketonuria compared with cows in the 1st parity group (P=0.04 and P<0.01, respectively).

Table 2.8: ***Logistic regression model for hyperketonuria[1]***

Variable	OR	SE for OR	Lower 95% CI for OR	Upper 95% for OR	t Value	P-value
Retinol	0.997	1.00	0.994	0.999	-2.20	0.03
Parity						<0.01
1st	(ref)					
2nd	1.71	1.29	1.01	2.82	2.47	0.01
3rd and greater	3.07	1.27	1.91	4.93	4.82	<0.01
Intercept	0.03	2.05	0.01	0.25	-4.92	<0.01

[1]4 cattle excluded due to missing data.
Random effect estimates: Farm: 0.83, Cohort: 3.03: Cow (VC): 0.49.

The logistic regression model for uterine disorders is presented in Table 9. The final model included parity, retinol, time point, and an interaction term between retinol and timepoint. Cows in the 3+ parity group had a significantly higher risk of uterine disorders compared to 2nd parity cows (P = 0.03). The interaction term indicated that higher serum retinol concentrations at C+7 were associated with reduced odds of uterine diseases. Cows with uterine disease had a mean serum retinol concentration of 154.3 ng/mL at C+7 whereas cows that did not have uterine disease had mean retinol concentrations of 243.4 ng/mL at C+7 (P<0.0001).

Table 2.9: ***Logistic regression model for uterine disorders[1]***

Variable	OR	SE for OR	Lower 95% CI for OR	Upper 95% CI for OR	t Value	P-value
Parity						0.03
1st	(ref)					
2nd	0.68	1.31	0.40	1.15	-1.42	0.16
3rd or greater	1.33	1.27	0.83	2.13	1.23	0.22
Time point						0.03
DO	(ref)					
CU	1.16	2.48	0.20	6.90	-0.08	0.93
C+7	3.79	2.26	0.76	18.80	1.87	0.06
Retinol	0.997	1.00	0.99	1.00	-1.11	0.27
Retinol and time point interaction term						0.01
DO	(ref)					
CU	0.989	1.00	0.40	1.15	-0.28	0.78
C+7	0.997	1.00	0.83	2.13	-2.84	0.005
Intercept	0.26	2.03	0.04	1.88	-1.93	0.13

[1]4 cattle excluded due to missing data.
Random effect estimates: Farm: 0.06; Cohort: 0.32, Cow(VC): 0.86.

Variables remaining in logistic regression model for the aggregate risk of disease are presented in Table 10 and included β-carotene, retinol, α- tocopherol, parity, a β-carotene by parity interaction term, and an α- tocopherol by parity interaction term. Higher serum retinol concentrations were associated with a lower risk of disease (P<0.01). Cows of 3+ parity had significantly higher risk of disease compared with 1st parity and 2nd parity cows (P<0.01 and P<0.01, respectively). Higher β-carotene concentrations were positively associated with the aggregate disease risk in 1st parity cows but were not significantly associated with the aggregate disease risk in 2nd and 3+ parity cows. Higher α- tocopherol concentrations were associated with a lower risk of disease among 1st parity but were associated with a higher risk of disease among 2nd parity cows.

Table 2.10: ***Logistic regression model for aggregate outcome[1]***

Variable		OR	SE for OR	Lower 95% CI for OR	Upper 95% CI for OR	t Value	P-value
α-tocopherol		0.84	1.09	0.71	0.99	-2.12	0.03
β-carotene		1.24	1.06	1.10	1.40	3.43	<0.01
Parity group							<0.01
1st		(ref)					
2nd		1.35	1.42	0.68	2.67	0.85	0.39
3rd and greater		3.38	1.42	1.71	6.69	3.50	<0.01
Retinol		0.996	1.00	0.994	0.998	-40	<0.01
β-carotene and parity interaction term							<0.01
1st	(ref)						
2nd		0.78	1.08	0.66	0.91	-3.11	<0.01
3rd and greater		0.79	1.07	0.69	0.91	-3.35	<0.01
α-tocopherol and parity interaction term							<0.01
1st	(ref)						
2nd		1.35	1.10	1.14	1.62	3.09	<0.01
3rd and greater		1.18	1.10	0.97	1.44	1.69	0.09
Intercept		0.72	1.48	0.32	1.60	-0.85	0.40

[1]4 cattle excluded due to missing data.
Random effect estimates: Cohort: 0.59, Cow(VC): 0.95.

DISCUSSION

In the current study, decreased serum retinol was associated with increased risk for periparturient diseases. Although the cows in the current study were supplemented at or above current NRC recommendations, >110 IU vitamin A/kg body weight, 28% of the cows became deficient in circulating concentrations of retinol (<150 ng/mL retinol) at C+7 (Herdt and Stowe, 1991, NRC, 2001). Indeed, cows that were diagnosed with uterine disease had a mean serum retinol concentration that was 100 ng/mL lower than their herd mates that did not have metritis or retained placenta at the C+7 time point. This suggests an inadequate supply of circulating retinol may have increased the risk for retained placenta and metritis. This may reflect the substantial role of vitamin A in multiple facets of immunity (Duriancik et al., 2010, Sirisinha, 2015, Larange

and Cheroutre, 2016). These include a role for retinol in neutrophil function, which may be directly related to placental retention (Cai et al., 1994, Kimura et al., 2002). Consistent with our observations, Michal et al. (1994) observed that increased serum retinol concentrations associated with β-carotene supplementation resulted in a reduced incidence of retained placenta and metritis, relative to non-supplemented animals. Results of that experiment were confounded, however, by large changes in serum β-carotene concentrations, which may have influenced uterine disease risk directly (Michal et al., 1994). Alternatively, the low serum retinol concentrations might have been an effect, rather than the cause, of metritis and its associated inflammation. Serum retinol concentrations are dependent on the presence of retinol binding protein (RBP), a short half-life protein produced by hepatocytes and adipocytes (Contreras et al., 2017). Synthesis and secretion of RBP is necessary for the mobilization of retinol from hepatic stores and RBP is a "negative acute phase" protein (Smith et al., 1973a, Bertoni et al., 2008), indicating that its synthesis and secretion are suppressed by generalized inflammation; thus, through this mechanism serum retinol concentrations might be depressed by inflammation resulting from metritis.

Hyperketonuria was significantly associated with decreased serum retinol concentrations at both the CU and C+7 time points. Hyperketonuria results from hyperketonemia, which is a well-established risk factor for early culling, periparturient morbidity, and decreased reproductive performance (LeBlanc, 2010, Probo et al., 2018). Hyperketonemia results from negative energy balance, rapid adipose mobilization, and a high glucose demand. In association with these metabolic conditions, cows in the first two weeks of lactation are, on the average, in substantial negative protein balance, which is created by the amino acid demands for gluconeogenesis as well as for milk production (Bell, 1995). This demand for amino acids could limit the synthesis

of RBP, resulting in low serum retinol concentrations(Smith et al., 1973a, Smith et al., 1973b, Shenkin et al., 1996). Thus, the association between serum retinol and disease conditions as observed in this study may result from a complex interplay of nutrition, metabolism, and inflammation.

Changes in serum retinol concentration between time points varied significantly between 1st and 2nd or greater parities. These results differ from previous reports on serum retinol concentrations during the transition period in that not all cows, specifically 1st parity cows, experience a decrease in serum retinol concentrations (Johnston and Chew, 1984, Goff et al., 2002, LeBlanc et al., 2004). It was clear, however, that 3+ parity cows were at greater risk of low serum retinol concentrations than 1st or 2nd parity cows.

The reduction in serum α-tocopherol from DO to CU and from CU to C+7 was observed in previous studies (Herdt and Smith, 1996, Goff et al., 2002). In the aggregate disease model, greater serum α-tocopherol concentrations were associated with decreased disease risk in 1st, but not 2nd parity cows. These parity differences suggest the need for investigating parity-specific serum vitamin concentration recommendations. Additionally, excess α-tocopherol may be detrimental to the health of cows and in this study 2nd parity cows had higher mean α-tocopherol serum concentrations than 1st parity cows. Bouwstra et al. (2008) demonstrated that 1st parity cows at calving with mean serum α-tocopherol concentrations of approximately 3.8 µg/mL had increased markers of oxidative stress than cows with α-tocopherol concentrations of 1.9 µg/mL (Bouwstra et al., 2008). This same group later proposed that α-tocopherol-induced oxidative stress increased risk of diseases such as mastitis (Bouwstra et al., 2010). In the current study, 1st and 2nd parity cows averaged 3.0 and 3.6 µg/mL α-tocopherol over the study period, respectively.

Serum concentrations of β-carotene decreased from DO to CU and from CU to C+7 and were greater in 2nd and 3+ parity cows when compared to 1st parity cows. This was a similar trend that was observed in periparturient cows previously (Goff et al., 2002, Rosendo et al., 2010). Higher serum β-carotene concentrations were associated with decreased milk production. Serum β-carotene concentrations may be decreased in higher producing cows because of its increased consumption as a free radical scavenger. High producing cows would generate more free radicals through a greater need for energy production than lower producing cows and thus may utilize an increased amount of serum antioxidants such as β-carotene (Sordillo, 2016). Alternatively, both serum β-carotene concentrations and ME305 were significantly different between farms and it is possible that farms with lower ME305 were feeding more β-carotene-containing ingredients such as forages in their diets as opposed to concentrates.

The present observational study was a broad investigation of the association between serum concentrations of β-carotene, retinol, and α-tocopherol with animal health. As such, broad conclusions as to the potential mechanisms involved between serum vitamins and disease could not be determined. In the case of uterine disease, for example, blood samples were collected at 7±3 DIM and metritis and retained placenta were diagnosed at 5.5±3.6 and 1.1±1 DIM, respectively. Therefore, in this case, serum retinol concentrations reflected the circulating retinol status approximately at the time of disease occurrence. Cows with hyperketonuria, however, had significantly lower serum retinol at the time of their move to a CU pen. The CU sample was taken an average of 24±10 days prior to diagnosis of hyperketonuria and thus temporal association was present in this case (data not shown). Indeed, the relationship between the time of sample collection and disease occurrence in this study could have biased our results to capture only associations between serum vitamins and diseases that occur immediately after calving. For

example, lameness and mastitis may have been diagnosed 2-3 wk post sample collection and therefore our analyzed sample did not accurately represent vitamin metabolism at the time of disease.

CONCLUSIONS

The findings of the current study demonstrate the complex nature of the association between serum fat-soluble vitamins and health in dairy cows. Although serum vitamin concentrations historically were used to determine supplementation status of a herd, in the case of well managed herds such as those in the present study, they more likely serve as biomarkers for much more complex processes such as inflammation and hepatic function. Future studies should ideally investigate hepatic vitamin stores in addition to circulating concentrations in relation to disease risk during the transition period.

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CHAPTER 3: SERUM VITAMIN D IS ASSOCIATED WITH ANTIOXIDANT POETENTIAL IN PERI-PARTURIENT COWS

ABSTRACT

Dairy cows have increased risk for oxidative stress during periods of transition such as at the cessation of lactation and around the periparturient period, increasing disease risk. Despite routine supplementation of transition cow diets with certain vitamins in the dairy industry to mitigate oxidative stress, there is no currently available data directly linking vitamin supplementation with antioxidant potential (AOP) in transitions cows. The objective of this study was to determine the association between serum vitamins and biomarkers of oxidative stress in healthy cows. Blood samples were collected from 240 cows at dry off (DO), close up (CU), and 2-10 days post-calving (DIM2-10). Blood samples were analyzed for vitamins (A, D, E), β-carotene, reactive oxygen species (ROS), and AOP. Spearman correlations and mixed linear regression models were used to assess associations between vitamins and measures of oxidants status. Vitamin D concentrations were positively associated with AOP at the CU and DIM2-10. Based on the positive association with AOP, additional in-vitro studies were conducted that showed vitamin D mitigated barrier integrity loss in endothelial cells during oxidative stress. These results indicate for the first time that vitamin D may have a role in promoting antioxidant potential in transition dairy cows.

INTRODUCTION

Dairy cattle are particularly susceptible to oxidative stress during the peripartum period because of metabolic and endocrine challenges occurring during parturition, lactogenesis, and external stressors such as pen and dietary changes (Sordillo and Mavangira, 2014). Oxidative stress is caused from an imbalance of reactive oxygen species (ROS) and antioxidant potential (AOP) (Sordillo and Aitken, 2009b). ROS are oxygen-containing compounds that donate electrons to

molecules in cells causing damage and dysfunction, whereas AOP encompasses a variety of enzymes and compounds that neutralize radicals and provide protection for cells (Sies, 2015). Oxidative stress is the term that refers to the damaged proteins, DNA, or lipids when ROS overwhelm AOP's neutralizing capabilities (Sies, 1997). Oxidative stress is increased in dairy cattle prior to and during periparturient disease and may play a role in the pathogenesis of periparturient diseases (Sordillo and Aitken, 2009a, Wisnieski et al., 2019, Tsuchiya et al., 2020). Indeed, there is increasing focus on supplementing vitamins and minerals to dairy cows in order to prevent oxidative stress and thus decrease the risk for periparturient diseases (Abuelo et al., 2015).

Decades of research have demonstrated the benefits of feeding antioxidant supplements such as vitamin E and selenium to dairy cows (Sordillo and Raphael, 2013, Weiss, 2017). Selenium and vitamin E in pre-partum diets reduced the incidence of retained placenta, improved fertility, and decreased cases of clinical mastitis (Brzezinska-Slebodzinska et al., 1994, Weiss et al., 1997, Hemingway, 2003). Also, Vitamin E and selenium improved immune responses through enhanced neutrophil function and increased antioxidant capacity (Hogan et al., 1990, Sordillo and Raphael, 2013). More recently, research focused on the impact of vitamin E and selenium to reduce in-vitro models of oxidative stress in bovine aortic and mammary endothelial cells (Sordillo et al., 2008, Kuhn and Sordillo, 2021). Collectively, the existing literature supports the widespread use of vitamin E and selenium supplementation in transition cows to decrease risk for disease (NRC, 2001). Despite the body of literature on enhancing immune function and improving redox balance with supplemental vitamin E and selenium, dairy cows still experience oxidative stress in the periparturient period which further increases the risk for disease (Kuhn et al., 2018). Indeed, approximately 45-75% of disease incidence occur during the first 30 d in milk

when cows are at highest risk for oxidative stress (LeBlanc et al., 2006, Lopes et al., 2020). Thus, alternative methods for reducing oxidative stress and disease risk in the periparturient period are needed.

In addition to vitamin E and selenium, supplemented or naturally occurring vitamins such as -A, -D, and β-carotene in the diets of dairy cows demonstrated the ability to optimize immunity and antioxidant defenses (Jin et al., 2014, Oliveira et al., 2015, Vieira-Neto et al., 2017). Indeed in human studies, vitamins A, D, and β-carotene were found to have critical roles in reduced oxidative stress and improved disease risk and progression (Sowers and Lachance, 1999). However, during the time-period for increased risk for oxidative stress for dairy cattle in the periparturient period, vitamins A, D, and β-carotene reached their lowest serum concentrations and tended to remain low through the critical transition period (Goff et al., 2002, Holcombe et al., 2018). Moreover, decreased serum vitamin concentrations in the periparturient period are related to increased risk for disease morbidity (LeBlanc et al., 2004a). For example, decreased serum vitamin A concentrations in the periparturient period were associated with increased risk of mastitis, uterine diseases, and hyperketonuria (LeBlanc et al., 2004a, Strickland, 2021). Supplemental β-carotene reduced the incidence of retained placenta (Michal et al., 1994). Decreased concentrations of serum vitamin D were associated with increased risk for uterine disease, but conversely presence of urine ketones were associated with elevated concentrations of vitamin D (Wisnieski et al., 2020). However, the association between serum concentrations of vitamins A, D, and β-carotene with biomarkers of oxidative stress in the periparturient period remained unknown. Therefore, the objective of this research was to determine the associations between vitamins A, D, E, and β-carotene with biomarkers of oxidative stress and antioxidant

potential. We hypothesized that serum concentrations of vitamins A, D, E, and β-carotene would be negatively associated with biomarkers of oxidative stress in periparturient cows.

MATERIALS AND METHODS

Animals

A total of 353 cows from 5 commercial Michigan dairy herds were enrolled from September 2014 to August 2018. Cows were enrolled in cohorts of up to 15 animals/cohort evenly divided between heifers <25 mo old, second-parity cows, and third or greater parity cows. Cows and heifers were randomly selected at enrollment when cows and heifers were approximately 200 to 230 d pregnant, and cows had fewer than 380 days in lactation. Exclusion criteria for the current study included diagnosis of any disease from the time of enrollment to 30 days post-parturition. These diseases included metritis, retained placenta, mastitis, milk fever, lameness, displaced abomasum, ketosis, pneumonia, abortion, and death. This resulted in 240 healthy cows that were included in our investigation into the association of serum fat soluble vitamins and oxidative stress.

Sample collection

Blood samples were collected from the coccygeal vein at dry off (DO) (-48±12d pre-calving), close-up (CU) (-17±7d pre-calving), and fresh (DIM2-10) (7±3d post-calving) in serum or plasma EDTA tubes (BD Vacutainer, Franklin Lakes, NJ). Blood tubes were transported to the laboratory on ice but were then allowed to clot at room temperature for approximately 1 hour. Serum was separated by centrifugation at 2,000 x g for 20 min at 20° C. Aliquots of serum were placed into 1.5 mL microcentrifuge tubes and stored at -20°C until analysis. In order to prevent ex-vivo lipid peroxidation in samples for oxylipid analysis, an antioxidant reducing agent was mixed with plasma samples both prior to freezing and post-thawing as previously described

(Mavangira et al., 2015). These samples were flash frozen in liquid nitrogen and stored at -80 °C until analysis.

Vitamin analysis

All samples were analyzed within 1 to 6 mo of collection to prevent vitamin degradation (Comstock et al., 1993). The concentrations for vitamin A, vitamin E, and β-carotene were analyzed at the Michigan State University Veterinary Diagnostic Laboratory (Lansing, MI 48910) by liquid chromatography mass spectrometry (LC/MS) utilizing a method by Arnaud et al. (1991) and were well described in Strickland et al. (2021) (Arnaud et al., 1991, Strickland et al., 2021). Briefly, five-point calibration curves were constructed using stock alpha-tocopherol (Sigma-Aldrich, St. Louis, MO, USA) solution (absorbance of 0.09 to 0.11 at 292 nm), retinol standard (Sigma-Aldrich, St. Louis, MO, USA) (absorbance of 0.085 to 0.095 at 325 nm), and beta-carotene standard (Sigma-Aldrich, St. Louis, MO, USA) (absorbance of 0.18 to 0.22 at 450 nm). Apocarotenal (Trans-β-APO-8”-carotenal, Fluka, St. Louis, MO, USA) was dissolved in methylene chloride to an absorbance of 0.10 to 0.15 at 450 nm to make the internal standard solution. Samples were analyzed by LC/MS using a Waters Acquity system and Waters Empower Pro Chromatography Manager software (Waters Corporation, Milford, MA, USA). Peak integration was by the ApexTrack method of Empower Pro with manual review. Serum vitamin D was analyzed by radioimmunoassay by Heartland Assays (Iowa State University Research Park, Ames, IA, USA) in the same manner as Holcombe et al. (2018) (Farrell et al., 2012, Holcombe et al., 2018). Briefly, the detection range was 2.5-100 ng/mL, the interassay and intraassay coefficients of variation were 13.3 and 6.5% respectively, and assay was validated by serial dilution and test for linearity (Holcombe et al., 2018).

Antioxidant potential analysis

The AOP was quantified in serum samples as described previously (Re et al., 1999). The monocation, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfric acid) (ABTS) (SigmaAldrich, St. Louis, MO, USA) was oxidized by potassium persulfate to create a stable radical in a glass container that was sealed and covered with foil to protect it from light exposure (Re et al., 1999). The ABTS radical solution was used between 12 and 24 hours of production and was then discarded after each use. The AOP of a sample was standardized to the reduction capacity of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or synthetic vitamin E analog) (SigmaAldrich, St. Louis, MO, USA) which was diluted to create a 6-point standard curve at concentrations of 25 µM, 12.5 µM, 6.25 µM, 3.125 µM, and 1.56 µM. The known reducing capacity of Trolox was used to determine the reducing capacity of each sample. The dilution of ABTS in phosphate buffered solution (PBS) was determined by analyzing serial dilutions (1:70, 1:75, 1:80, 1:85, 1:90, 1:95) in triplicate in colorimetric plate reader (Tecan, Mannedorf, Switzerland) at 730 nM for concentration of 0.7 ± 0.02. Samples were diluted 1:20 in PBS prior to being plated in triplicate with the standards. Once ABTS was added to wells, plates were covered with aluminum foil and incubated for 6 min and read at 730 nM. For the present study, the interassay and intraassay coefficient of variance was 4.2% and 0.92%, respectively.

Reactive oxygen species analysis

The pro-oxidant concentration of serum samples, or ROS was determined by the d-ROMS assay (Diacron International, Grosseto, Italy). The d-ROMS assay is an indirect measurement of hydrogen peroxide by addition of N,N-diethylparaphenylinediamine. This substrate forms a stable cation with hydrogen peroxide derivatives (Alberti et al., 2000). The results are expressed

as “Carratelli units” (CarrU) with 1 CarrU having the equivalent oxidizing capacity as 0.08 mg/dL of hydrogen peroxide.

Liquid chromatography tandem mass spectrometry

For this analysis, 7 cows were selected from the group of 220 healthy cows. Because ex-vivo alterations in oxylipids occurred over time in storage and this study was carried out over the course of 3 years, only cows sampled within the final 6 months were used (Koch et al., 2020). Of the 25 cows in this cohort, 9 were excluded for having 1 or more disease, and of the remaining 16 cows, only 7 had all 3-sample time points available for analysis. Targeted oxylipids were analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS). Similarly, the mixture of deuterated internal standards including 5(S)-hydroxyeicosatetraenoic-d8, 15(S)-hydroxyeicosatetrsenoic-d8 acid, 8(9)-epoxyeicosatrienoic-d11 acid, prostaglandin E2-d9, arachidonic acid-d8, 8(9)-epoxyeicosatrienoic-d11 acid, 2-arachidonoyl glycerol-d8, and arachidonoyl ethanolamide-d8 was prepared in the same manner as Vengai et al. with final concentrations of 0.25, 0.25, 0.5, 0.5, 50, 2, and 0.25 µM and added to every sample (Mavangira et al., 2015). This mixture was then used to create a 6-point standard curve ranging from 0.001 to 500 µM. First, frozen plasma samples were thawed on ice and then 2 mL of plasma from each sample was combined with 5 mL, methanol, 2 µl of formic acid, and 15 µl of internal standard mixture. Samples were vortexed for 2 min, allowed to incubate for 15 min at room temperature, and then were centrifuged for 20 min at 4 °C and 4,816 x g. The supernatant was diluted with 190 µl of 50:50 HPLC-grade water and formic acid. Solid-phase extraction columns (waters, Milford, MA). Prior to extraction columns were conditioned with 6 mL of methanol and then 6 mL of high-performance liquid chromatography (HPLC) water. Samples were added to columns and washed with 20% methanol and then 4 min of vacuum was used to fully dry the columns. A

50:50 mixture of methanol:acetonitrile was used to elute the samples which were then dried under vacuum using a Savant SpeedVac at 45 °C for at least 3 h run-time or until dried (ThermoQuest, Holbrook, NY). The dried residues were reconstituted using 150 ul of 2:1 methanol:water and transferred to chromatography vials with inserts and stored at -20 °C until analysis. The quantification of analytes was performed on a Waters Acquity HPLC with a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Analyte separation was accomplished using an Ascentis Express C18 HPLC column, 10 cm x 2.1 mm, 2.7 µm (Supelco, Bellefonte, PA, USA). Samples were held at 50 °C, mobile phase A was water with 0.1% formic acid, mobile phase B was acetonitrile and a flow rate of 0.3 mL/min was maintained. Ratios of mobile phase A:B were as follows: 99:1 for time 0 to 0.5 min, 60:40 at 2.0 min, 20:80 at 8.0 min, 1:99 at 9 min until 13.0 min, and then 99:1 from 13.1 min until 15 min at the end of liquid chromatography separation for each sample. Oxylipids were detected in negative ion mode using electrospray ionization. Each oxylipid was analyzed with QuanOptimize software (4.0, Waters, Milford, MA, USA).

Cell culture and treatments

Cell culture experiments using bovine aortic endothelial cells (BAEC) were perfomed using established protocols for inducing oxidative stress in-vitro (Mavangira et al., 2020). The BAEC were chosen because of their central role in imune responses and their location at the vascular and tissue interface were they are targets of prooxidant damage during oxidative stress. The BAEC were harvested previously from sections of aortas collected immediately after slaughter using methods previously described by our group (Aherne et al., 1995). Pro-oxidant challenge of BAECs was induced using 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH, Cayman Chemical, Ann Arbor, MI). Treatments with calcitriol (1α,25-

Dihydroxycholecalciferol/1α,25-DihydroxyvitaminD3/25-$(OH)_2$-D_3, Biovision, Milpitas, CA, USA) were performed to assess the effect of vitamin D on the AAPH-induced pro-oxidant challenge. The working doses of AAPH and vitamin D were determined by assessing cellular viability based on quantifying ATP production using the Cell Titer-Glo assay (Promega Corp., Madison, WI, USA) as previously described (Mavangira et al., 2020).

mRNA quantification in BAEC

The quantification of gene expression of target genes was performed as previously described by our group (Aitken et al., 2011) (Table 3.1). The RNA from BAECs was extracted using the (Promega Maxwell RSC instrument, Madison, WI, USA) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using predesigned and custom-designed TaqMan primers from Applied Biosystems (St. Louis, MO, USA) quantitative real-time (qRT-PCR) was performed. The qRT-PCR was performed in triplicate for each sample using reaction mixtures containing actin beta (ACTB); Glucuronidase beta (GUSB), and ribosomal protein S9 (RPS9) as endogenous controls. The cytochrome P (CYP) 24A1, nuclear erythroid factor 2 like 2 (NEF2L2), and vitamin D receptor (VDR) were the target genes. The thermal cycling conditions for fast 2-step PCR were used. Stage 1, enzyme activation at 95ºC for 20 seconds; stage 2, 95ºC for 3 seconds; stage 3, 60ºC for 30 seconds; with 40 replications through stages 2 and 3. The abundance of target genes was normalized to endogenous control genes and calculated using the ΔCt method for statistical analyses. The $2^{-\Delta\Delta Ct}$ method for relative expression was used to display the data (Aitken et al., 2011).

Table 3.1: ***Proprietary TaqMan primer reference information***

Gene	NCBI Reference Sequence[1]	TaqMan Assay ID
CYP24A1	NM_001191417.1	Bt04306544_m1
NFE2L2	NM_001011678.2	Bt03251878_m1
VDR	NM_001167932.2	Bt04301663_m1
ACTB	NM_173979.3	Bt03279174_g1
GUSB	NM_001083436.1	Bt03256165_m1
RPS9	NM_001101152.2	Bt03272017_m1

[1]National Center for Biotechnology Information reference sequence found in the nucleotide da-tabase (https://www.ncbi.nlm.nih.gov/nuccore/, accessed on 4 January 2021). CYP24A1, Cytochrome P24A1; NFE2L2, Nuclear erythroid factor 2 like 2; VDR, Vitamin D receptor; ACTB, _-actin; GUSB, Glucuronidase beta; RPS9, Ribosomal protein S9.

Endothelial Cell-substrate Impedance Sensing (ECIS)

Barrier integrity of BAEC was performed using a protocol previously described by our group (Ryman et al., 2016) with minor modifications. Briefly, BAEC were cultured in 96-well arrays with a 10+ electrode system until approximately 80% confluency in the 5% serum-containing media. Cells were preincubated with 10 nM of vitamin D for 12 hours before adding AAPH at 3 mM. Resistance to electrical passage across the confluent monolayer was monitored using the electric cell-substrate impedance sensing system (ECIS, Applied Biophysics, Inc., Troy, NY). Resistance measurements immediately before treatment additions were used to normalize all subsequent values.

Data analyses

Cows that had one or more of the following health events during the study period were excluded: metritis, mastitis, ketosis, lameness, displaced abomasum, retained placenta, pneumonia, milk fever, abortion, death, and/or death of calf. Stata version 14.2 (College Station, TX, USA) was used for all analyses.

Mixed linear regression models for vitamins and AOP. Mixed linear regression models were built to assess factors associated with AOP. Three models were built: one for DO, one for CU, and one for DIM2-10. Candidate variables for the models included the four vitamin biomarkers

(B-carotene, vitamin A, vitamin D, and vitamin E), parity, year (to adjust for differences between the two machines used), and all possible two-way interactions. A backwards selection was performed to select the variables in the final model (alpha=0.05). Multicollinearity was carefully monitored throughout the model building process by assessing standard errors for inflation and by calculating the variance inflation factors (VIFs). Normality of residuals was visually checked using histograms and Quartile-Quartile (Q-Q) plots. If normality of residuals was violated, then the outcome variable was transformed (e.g. log base 10 function). A random intercept for farm and/or cohort was included unless the estimates were very small and did not affect the other model coefficients when they were excluded.

Spearman correlation statistics for association of vitamins with oxylipids. 20-HETE, and ROS. Spearman correlation statistics were calculated to assess the association of each vitamin (B-carotene, vitamin A, vitamin D, and vitamin E) with multiple oxylipids (5-iPF2alphaVI, 8,12-isoprostane, 8-isoprostane PGA2, 8-isoprostane PGF2alpha), 20-HETE, and ROS concentrations at DO, CU, DIM2-10.

Data Analysis for cell culture. One- or two-way ANOVA was performed using the proc mixed procedure with or without repeated measures where appropriate using the SAS software (SAS 9.4, Cary Inc., NC, USA) to analyze the cell culture treatment outcomes. One-way ANOVA with Dunnett's post hoc correction for multiple comparisons was used to analyze cell viability and mRNA expression data. A two-way ANOVA with a Tukey adjustment for multiple comparisons was used to compare the barrier integrity data for treatment and time factors and their interactions. Significance was declared when $P \leq 0.05$.

RESULTS

Summary statistics for the oxidative stress biomarkers and vitamins are shown in Table 3.2

Table 3.2: ***Summary statistics of oxidative stress biomarkers and vitamins by time point***

Biomarker	Time point	N	Mean	SEM	Min	25th percentile	Median	75th percentile	Max
Vitamin E	DO	239	2.66	0.06	0.71	1.99	2.60	3.29	5.28
	CU	214	3.12	0.10	0.61	2.02	2.78	4.06	9.44
	DIM 2-10	198	1.79	0.06	0.001	1.15	1.81	2.37	4.57
AOP	DO	240	5.08	0.13	1.31	3.57	4.69	6.43	10.80
	CU	222	5.05	0.13	2.38	3.59	4.57	5.83	10.80
	DIM 2-10	207	4.36	0.10	1.05	3.38	4.27	5.20	8.63
B- carotene	DO	238	5.00	0.25	0.21	2.62	4.07	6.00	23.62
	CU	221	3.35	0.16	0.10	1.77	2.62	4.53	13.87
	DIM 2-10	206	1.63	0.09	0.10	0.74	1.15	2.02	6.39
ROS	DO	35	169.40	10.44	16.3	130.7	155.2	212.3	310.3
	CU	12	132.71	11.43	49.0	110.3	138.8	155.2	204.2
	DIM 2-10	22	181.52	8.80	122.5	155.2	175.6	212.3	277.7
Vitamin A	DO	239	302.63	5.41	102	238	296	358	528
	CU	221	277.15	5.88	112	213	278	331	561
	DIM 2-10	205	256.81	8.33	53	177	241	316	764
Vitamin D	DO	186	99.12	2.02	25.40	81.0	97.60	112.50	198.60
	CU	173	93.77	2.19	20.10	77.20	94.80	110.00	210.40
	DIM 2-10	184	82.06	1.71	9.80	63.95	82.30	96.30	166.40
5- iPF2alphaVI	DO	7	0.07	0.01	0.05	0.05	0.05	0.10	0.10
	CU	7	0.06	0.01	0.05	0.05	0.05	0.05	0.10
	DIM 2-10	7	0.06	0.01	0..05	0.05	0.05	0.10	0.10
8,12-isoprostane	DO	7	0.26	0.05	0.1	0.10	0.30	0.40	0.40
	CU	7	0.23	0.07	0	0.10	0.20	0.30	0.60
	DIM 2-10	7	0.21	0.04	0.1	0.10	0.20	0.30	0.40
8-isoprostane PGA2	DO	7	0.29	0.05	0.10	0.20	0.30	0.40	0.50
	CU	7	0.73	0.13	0.40	0.50	0.60	0.90	1.4
	DIM 2-10	7	0.16	0.04	0.10	0.10	0.10	0.20	0.40
8-isoprostane PGF2alpha	DO	7	0.26	0.15	0	0	0.10	0.30	1.1
	CU	7	0	0	0	0	0	0	0
	DIM 2-10	7	0.73	0.17	0	0.50	0.70	1.0	1.5
20-HETE	DO	27	2.22	0.27	0.01	1.20	1.90	3.19	6.54
	CU	24	5.28	1.66	0.28	1.71	3.60	5.75	41.41
	DIM 2-10	27	9.31	2.31	0.50	2.78	4.80	8.50	51.95

AOP = antioxidant potential, ROS = reactive oxygen species, DO = dry off, CU = close up, DIM2-10 = 2-10 days in milk

Correlations between vitamins and oxylipids

The correlation analyses shown in Table 2 were interpreted based on ranges determined by Mukaka, 2012 (Mukaka, 2012). Briefly, correlations between 0-0.3 have a negligible association, 0.3-0.5 have a low association, 0.5-0.7 have a moderate association, 0.7-0.9 have a high association, and greater than 0.9 have a very high association (Mukaka, 2012). At dry off,

vitamin E had a low positive correlation with ROS (r = 0.36, p<0.01). Similarly, β-carotene also had a low positive correlation with serum ROS (r=0.35, p<0.05) as well as with vitamin A concentrations (r=0.46, p<0.01). β-carotene also had a high negative correlation with 8,12-isoprostane (r=-0.77, p<0.05) and 8-isoprostane PGA2 (r=-0.80, p<0.05). Serum vitamin A concentrations had a negligible positive correlation with serum vitamin D concentrations (r=0.20, p<0.01), a low positive correlation with serum 20-HETE (r=0.42, p<0.05), and a high positive correlation with 8-isoprostane PGA2 (r=0.76, p<0.05).

Table 3.3: ***Mixed linear regression results for the association of vitamin D with AOP at CU and DIM2-10***

Variable	Coefficient	L95% CI	U95% CI	SE	z	P-value
CU[1] (N=173)						
Vitamin D	0.006	0.0001	0.01	0.003	2.01	0.04
Intercept	3.79	3.13	4.46	0.34	11.18	<0.01
DIM2-10[2] (N=184)						
Vitamin D	0.008	0.003	0.01	0.003	2.96	0.01
Intercept	3.43	2.82	4.03	0.31	11.15	<0.01

[1]Random intercept estimate for Cohort: 0.84, 95% CI (0.59, 1.19).
[21]Random intercept estimate for Cohort: 0.83, 95% CI (0.57, 1.20).
CU = close up, DIM2-10 = 2-10 days in milk, AOP = antioxidant potential

At the close-up, vitamin A concentrations had a positive negligible correlation with serum β-carotene (r=0.19, p<0.01), vitamin E (r=0.30, P<0.01), and vitamin D concentrations (r=0.16, p<0.05). Serum concentrations of β-carotene had a low positive correlation with serum vitamin E concentrations (r=0.32, p<0.01). At DIM 2-10, serum vitamin A concentrations had a negligible positive correlation with serum vitamin E concentrations (r=0.18, P<0.05) as well as with serum vitamin D concentrations (r=0.29, p<0.01). Serum vitamin A concentrations were moderately positively associated with β-carotene concentrations (r=0.61, p<0.01) and moderately negatively correlated with serum 20-HETE (r=-0.57, p<0.01). β-carotene concentrations had a negligible positive correlation with serum vitamin E concentrations (r=0.23, p<0.01) and a low negative correlation with 20-HETE (r=-0.42, p<0.05). When all sample time points were combined, serum

concentrations of vitamins E had a low positive correlation with β-carotene concentrations ($r=0.35$, $p<0.01$), and a positive negligible correlation with vitamin A concentrations ($r=0.26$, $P<0.01$), as well as with vitamin D concentrations ($r=0.16$, $p<0.01$). Vitamin E also had a negative negligible correlation with serum 20-HETE concentrations ($r=-0.23$, $p<0.05$). β-carotene concentrations had a positive low correlation with serum vitamin A concentrations and a positive but negligible correlation with vitamin D concentrations ($r=0.16$, $p<0.01$). Serum β-carotene concentration had a low negative correlation with serum 20-HETE concentrations ($r=-0.42$, $p<0.01$). Serum vitamin A had a positive negligible correlation with serum vitamin D concentrations ($r=0.26$, $p<0.01$) and a negative negligible correlation with 20-HETE concentrations in the post-partum period ($r=-0.25$, $p<0.05$).

Table 3.4: ***Correlation analysis results for vitamins, oxylipids. 20-HETE, and ROS***

	Vitamin E	B-carotene	ROS	Vitamin A	Vitamin D	5-iPF2alphaVI	8,12-isoprostane	8-isoprostane PGA2	8-isoprostane PGF2alpha	20-HETE
Dry off										
Vitamin E	1									
B- carotene	0.04	1								
ROS	0.36**	0.35*	1							
Vitamin A	0.08	0.46**	0.3	1						
Vitamin D	0.08	-0.002	.	0.20**	1					
5- iPF2alphaVI	0.43	-0.43	0.80*	0.43	.	1				
8,12-isoprostane	-0.13	-0.77*	0	0.4	.	0.07	1			
8-isoprostane PGA2	-0.13	-0.80*	-0.19	0.76*	.	0.15	0.74	1		
8-isoprostane PGF2alpha	-0.07	0.15	0.2	-0.3	.	0.15	-0.56	-0.28	1	
20-HETE	-0.32	0.12	0.2	0.42*	-0.04	0.29	-0.78*	-0.35	0.27	1
Close up										
Vitamin E	1									
B- carotene	0.32**	1								
ROS	0.16	-0.03	1							
Vitamin A	0.30**	0.19**	0.07	1						
Vitamin D	0.19*	-0.04	.	0.16*	1					
5- iPF2alphaVI	0.39	-0.61	0.77	0	.	1				
8,12-isoprostane	0.35	-0.11	0.2	-0.38	.	0.62	1			
8-isoprostane PGA2	-0.17	-0.67	0.95	-0.04	.	0.62	0.33	1		
8-isoprostane PGF2alpha	.	.	.	.	.	.	.	0.37*	1	
20-HETE	-0.38	0.15	0.4	-0.12	0.19	-0.41	-0.33	0.14	.	1
DIM2-10										
Vitamin E	1									
B- carotene	0.23**	1								
ROS	0.16	-0.13	1							
Vitamin A	0.18*	0.61**	0.01	1						
Vitamin D	0	0.07	.	0.29**	1					
5- iPF2alphaVI	-0.39	-0.32	0.48	0.39	.	1				
8,12-isoprostane	-0.03	-0.62	0.05	0.76	.	0.08	1			
8-isoprostane PGA2	0.39	0.18	0.23	0.65	.	0.39	-0.19	1		
8-isoprostane PGF2alpha	-0.26	-0.54	0.73	0.66	.	0.79*	0.24	0.37*	1	
20-HETE	0.04	-0.42*	-0.02	-0.57**	-0.4	0.79*	-0.13	0.04	0.46	1
All time points										
Vitamin E	1									
B- carotene	0.35**	1								
ROS	0.03	0.14	1							
Vitamin A	0.26**	0.45**	0.24	1						
Vitamin D	0.16**	0.16**	.	0.26**	1					
5- iPF2alphaVI	0.26	-0.31	0.65**	0.25	.	1				
8,12-isoprostane	0.05	-0.3	0.08	0.23	.	0.3	1			
8-isoprostane PGA2	0.42	0.09	-0.23	-0.05	.	0.05	0.13	1		
8-isoprostane PGF2alpha	-0.45	-0.38	0.42	0.21	.	0.24	-0.1	0.37*	1	
20-HETE	-0.23*	-0.42**	0.13	-0.25*	-0.19	-0.02	-0.4	-0.07	0.23	1

*P<0.05, **P<0.01

Missing values: not enough data points to calculate correlation coefficient

Vitamins and antioxidant potential

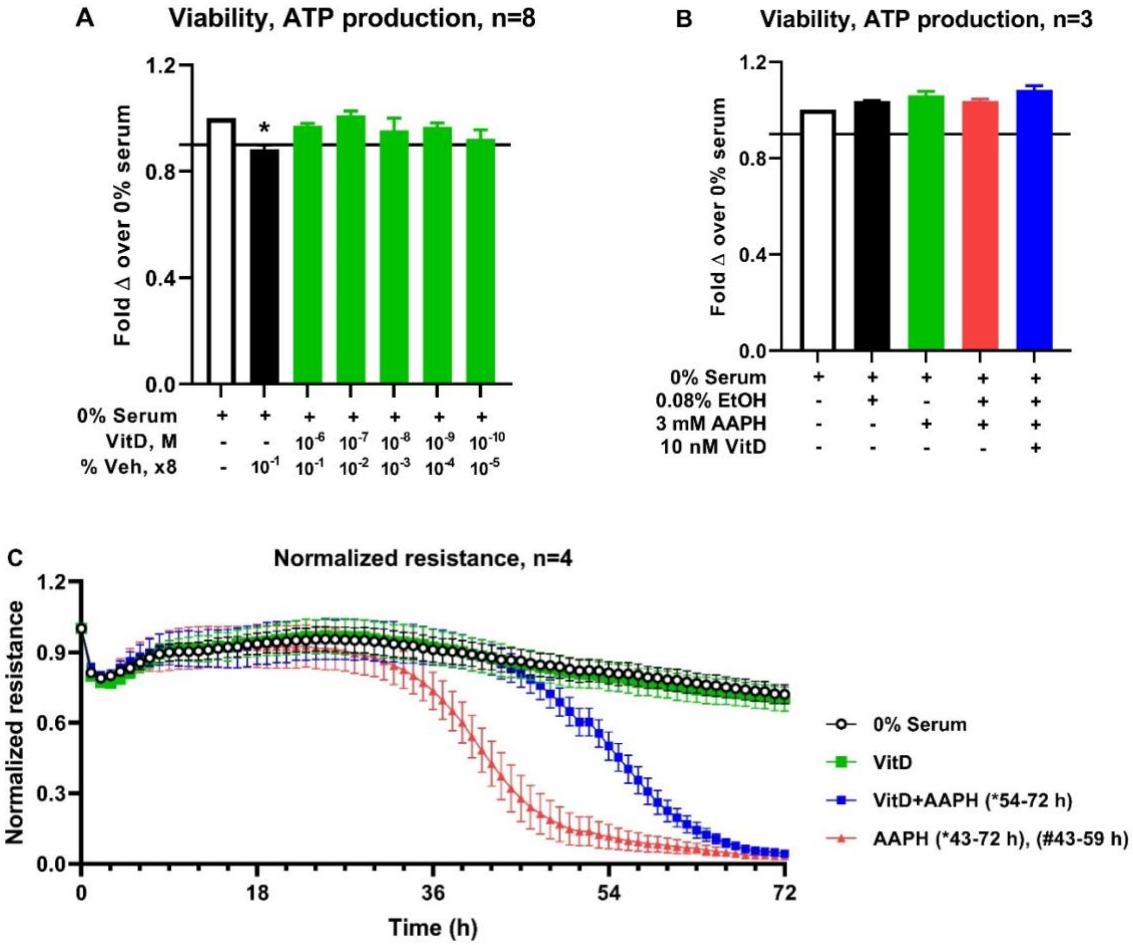
There were no variables that were significantly associated with AOP at DO, therefore these results were omitted. Vitamin D concentrations were associated with AOP at CU and DIM-2-10 (Table 2). Higher vitamin D concentrations correlated to a higher concentration of AOP at both CU and DIM2-10 ($P=0.04$ and $P<0.01$, respectively).

Cell Culture

Based on the positive correlation between vitamin D and AOP, the potential protective effects of vitamin D against oxidative stress were explored using an in-vitro endothelial cell model of oxidative stress. A dose titration was performed to identify a vitamin D concentration within physiologic range for dairy cattle to assess potential effects on barrier integrity of BAEC (Figure 1). Exposure of BAEC to vitamin D doses ranging from 1000 nM to 0.1 nM for 24 h maintained cell viability above 90% (Figure 1A). Treatment with vehicle alone (ethanol 0.8% by volume) which as equivalent to vehicle content in the highest vitamin D concentration tested (1000 nM) induced significant decrease ($P<0.05$) in cell viability relative to untreated control (Figure 1A). Consequently, the highest dose of 10 nM of vitamin D (which contained 0.08% ethanol) was selected for assessing effects of vitamin D on barrier integrity in BAEC exposed to 3 mM AAPH. The 10 nM of vitamin D maintained BAEC viability at levels similar to untreated controls for the duration closer to that of the barrier integrity assessment (Figure 1B). The presence of VDR in BAEC was confirmed, however, mRNA expression for VDR was not affected by treatment (Figure 2). Similarly, neither CYP24A1 nor NrF2 differed among treatments (Figure 2). The treatment of BAEC with vitamin D alone did not alter barrier integrity relative to untreated controls. Barrier integrity was decreased by treatment with AAPH.

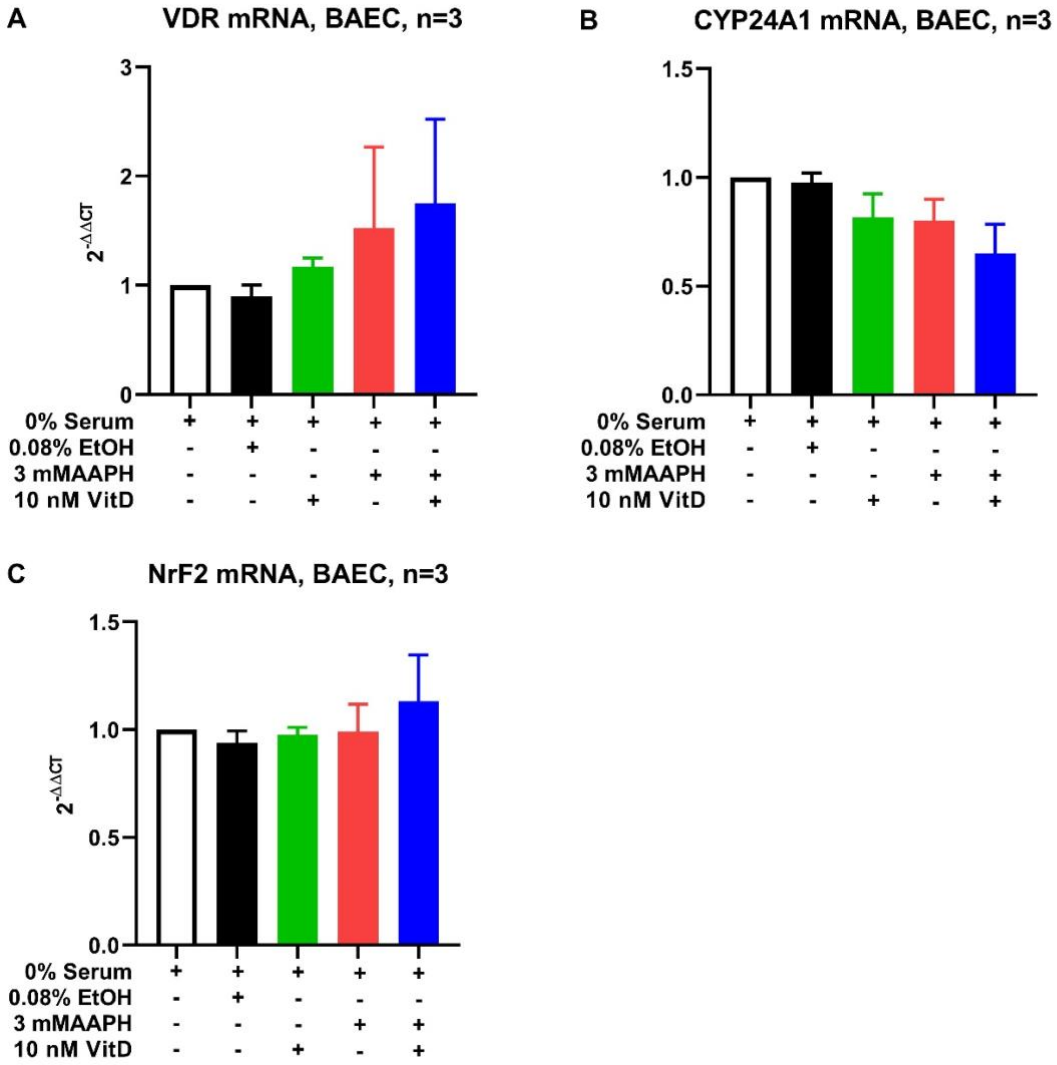
Pretreatment of BAEC with vitamin D significantly ($P<0.05$) prevented the loss of barrier resistance induced by AAPH by 16 hours (Figure 1C).

Figure 3.1: ***Bovine arterial endothelial viability and barrier integrity with treatment of Vitamin D***



Effect of Calcitriol (vitamin D) on barrier integrity of BAEC treated with AAPH. A dose-response curve was performed to test physiological vitamin D concentrations (10^{-6} M – 10^{-10} M) on cell viability for 24 h (A). The highest vitamin D concentration (10^{-7} M or 10 nM) maintaining at least 90% viability relative to untreated control was assessed for effect on barrier integrity in cells exposed to 3 mM AAPH (C). The viability of BAEC in cells exposed to treatments for the duration of barrier integrity assessment was determined (B). Viability data were each compared to untreated control and analyzed by the one-way ANOVA with Dunnett's post hoc adjustment for multiple comparisons. Two-way repeated-measures ANOVA analyzed barrier integrity data with Tukey adjustment for multiple comparisons. Asterisk (*) indicates a significant difference from untreated control for viability (1A) and barrier integrity (1C). Duration of differences in barrier integrity is indicated in parentheses in C. Pound (#) shows the comparison between treatment with 3 mM AAPH with 10 nM Vit D vs. 3 mM AAPH alone. For all comparisons, $\alpha=0.05$.

Figure 3.2: ***Gene expression in BAEC treated with AAPH with or without vitamin D***



Gene expression in BAEC treated with AAPH with or without vitamin D. The mRNA expression was quantified for vitamin D receptor (VDR, A), cytochrome P 450 24A1 (CYP24A1, B), and nuclear erythroid factor 2 like (NrF2, C) after incubation with treatments for 60 h. The mRNA expression data are presented as fold change relative to untreated controls (open bars). The mRNA expression data for each treatment was compared to untreated control and analyzed by the one-way ANOVA with Dunnett's post hoc adjustment for multiple comparisons. For all comparisons, α=0.05.

DISCUSSION

Dietary supplementation of vitamins and minerals are commonly delivered to cattle at concentrations greater than required for normal physiological processes in order to enhance immune function and decrease the risk for oxidative stress (Abuelo et al., 2015). Indeed, vitamin

E, β-carotene, and selenium are expressly formulated into rations to promote antioxidant defenses, particularly in peri-parturient period (NRC, 2001). Surprisingly, the current study found that only vitamin D, but not vitamin E or β-carotene, was positively associated with AOP at both CU and DIM2-10. These results were surprising because vitamin E and β-carotene have antioxidant function (Sies and Krinsky, 1995). Vitamin D, conversely, is well known for its role in promotion of gene transcription and regulation of calcium, however current literature on the effects of vitamin D on oxidative stress in cattle is still limited (Bikle, 2014, Tagliaferri et al., 2019). These results indicate that vitamin D may have a role in regulating the oxidant status in cows and it should be more fully investigated.

The increased risk for oxidative stress in periparturient period is a widespread challenge in the dairy industry negatively affecting milk production and cow welfare through increased risk of disease (Abuelo et al., 2019). During the periparturient period the availability circulating serum vitamins E and β-carotene available to quench free radicals greatly decreases (Goff et al., 2002, LeBlanc et al., 2004a). For this reason, increased dietary supplementation of antioxidants, such as vitamin E, is employed to decrease risk of oxidative stress in the periparturient period (Abuelo et al., 2015). Merely increasing antioxidant supplementation may not be effective. For example, Bouwstra et al. demonstrated that dietary supplementation of vitamin E at 3 times recommended concentrations actually increased oxidative stress and mastitis incidence in dairy cows (Bouwstra et al., 2010). This paradox is demonstrated at the dry-off time point where both vitamins E and β-carotene concentrations are positively correlated with serum ROS ($r = 0.35, 0.35$, $p < 0.5$ respectively). Indeed, vitamin E has no other significant associations with any other biomarker of oxidative stress at the individual time points in this study, including the periparturient period ($P > 0.05$).

Vitamins A and D function more like hormones, affecting transcription of a wide variety of genes and are therefore not antioxidants by definition, but both demonstrate antioxidant effects in cattle in response to supplementation (Herdt and Stowe, 1991, Jin et al., 2014, Kweh et al., 2021). For example, vitamin D treatment can alter antioxidant potential indirectly by increasing glutathione peroxidase concentrations in humans (Tagliaferri et al., 2019). This is important because total AOP is composed of 3 elements: enzymes such as glutathione peroxidase, non-enzymatic protein antioxidants primarily with sulfhydryl groups, and low molecular weight molecules such as glutathione, vitamin E, and β-carotene (Celi, 2011). The association between serum vitamin D concentrations and AOP at CU and DIM 2-10 may be a result of increases in AOP components from vitamin D gene transcription (Kim et al., 2020).

The Pearson correlation analysis reveals interesting insights into the relationships of serum vitamins and biomarkers of oxidative stress and the most significant finding is the negative correlation between 20-HETE concentrations with serum vitamin A and β-carotene concentrations at DIM2-10 and in the combined time point analysis. The oxylipid 20-HETE is derived from cytochrome P-450 metabolism of arachidonic acid (Kuhn et al., 2018). 20-HETE is not only increased in serum during the periparturient period but is also elevated in serum and milk during severe coliform mastitis (Mavangira et al., 2015, Kuhn et al., 2017). Indeed, 20-HETE induces oxidative stress, while vitamin A and β-carotene improve antioxidant status (Han et al., 2013, Fiedor and Burda, 2014, Jin et al., 2014). Elevated serum 20-HETE concentrations during the periparturient period concentrations may occur in part due to a depletion vitamin A and β-carotene serum concentrations and thus a decrease in free radical quenching capacity. Serum vitamin E concentrations were negatively correlated with serum 20-HETE concentrations when all time points were combined as well. Kuhn et al. found that vitamin E could reduce 20-

HETE production through competitive cytochrome metabolism with arachidonic acid (Kuhn and Sordillo, 2021). These findings indicate that redox balance is more complicated than quenching of free radicals and the relationships between serum vitamins and 20-HETE concentrations merits further investigation.

The association between serum concentrations of vitamin D at CU and DIM2-10 is particularly interesting because vitamin D is not a direct antioxidant and thus the mechanism behind the association is not immediately evident. The functional significance of the relationship between vitamin D and AOP was investigated using an in-vitro model of oxidative stress using bovine aortic endothelial cells. Vascular endothelial cells are essential in regulating inflammatory responses and orchestrating the barrier integrity between the circulating blood and local tissues (Ryman et al., 2015). Damage to endothelial cells through oxidative stress may lead to aberrant inflammation and a dysfunctional endothelial cell barrier (Ryman et al., 2016). The model of oxidative stress using a free radical generator, AAPH, was previously reported by our group as inducing loss of barrier integrity in BAECs (Mavangira et al., 2020). Thus, the effect of vitamin D on the loss of barrier integrity in this model was assessed. We demonstrated that vitamin D could prevent the functional disruption of endothelial cell barrier resistance with an in-vitro model of prooxidant challenge. Our findings agree with previous studies that showed that vitamin D improved barrier integrity in endothelial progenitor cells during tumor necrosis factor α-induced inflammatory conditions (Oh, 2019). Although the mechanism was not investigated in our studies, inhibition of pro-oxidant generation pathways may be responsible for the protective effects on barrier integrity (Uberti et al., 2014, Won et al., 2015). It is also unclear if the protective effects on barrier integrity of the BAECs are meditated via the vitamin D receptor (VDR) because of a lack of differences in mRNA expression on exposure to vitamin D. Other

studies reported presence of VDR in BAECs and that mRNA expression increases upon exposure to vitamin D (Kim et al., 2020).

The current study also did not find mRNA expression changes of CYP24A1, the enzyme responsible that metabolizes vitamin D. The CYP24A1 gene expression generally increases upon exposure to vitamin D in BAECs or other cells (Jones et al., 2012). These findings suggest that vitamin D may protect endothelial barrier integrity through a mechanism independent of transcriptional regulation. The fact that vitamin D was protective in a prooxidant challenge suggests a direct or indirect effect on antioxidant protective mechanisms against oxidative stress and changes in redox balance. Other oxidative stress models in cell culture systems report augmentation of antioxidant response systems, increased reducing capacity and decreased formation of isoprostanes following treatment with vitamin D (Pfeffer et al., 2018). Follow-up studies will investigate the specific nature of the correlation between vitamin D and AOP and the mechanisms responsible for maintaining barrier integrity of BAEC in vitro.

Our in-vivo study showed a significant positive correlation between serum vitamin D concentrations and serum AOP in periparturient cattle at high risk for oxidative stress. Dairy cattle are at an increased risk of developing inflammatory disorders after calving (Lopes et al., 2020). Our in-vitro prooxidant challenge did not evaluate changes in antioxidant potential but assessed for changes in the gene expression for the master regulator of antioxidant responses, NrF2 (Kim et al., 2020). The lack of effects in the expression of NrF2 likely further supports that AAPH treatments did not induce further prooxidant challenge commonly affecting cell culture systems (Halliwell, 2003). Since the assessment of Nrf2 was assessed at 60 h, this precluded the identification of potential early changes in gene expression of NrF2. Future studies should assess

antioxidant potential, temporal changes in NrF2 and other specific antioxidant response genes to understand the mechanism by which vitamin D exerts its endothelial stabilizing effects.

CONCLUSION

Serum concentrations of vitamin D are positively associated with AOP in periparturient cattle, but the common antioxidant vitamins including vitamins A, E, and β-carotene are not associated with AOP in the current study. The results of this study indicate that the relationship between vitamins A, D, E, and β-carotene and oxidative stress must be re-evaluated. As the understanding of oxidative stress in dairy cattle continues to grow, re-examination of the effectiveness of common oxidative stress-mitigation strategies such as dietary vitamin supplementation is paramount. The re-examination of oxidative stress-mitigation strategies is particularly salient because despite increased supplementation of vitamins such as vitamin E in the periparturient period, oxidative stress and periparturient diseases remain a consistent problem in the dairy industry. The potential benefits of supplementary vitamin D on serum AOP could have a major impact on prevention of oxidative stress and disease periparturient cows and therefore further research is needed.

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CHAPTER 4: OXYLIPID PROFILES IN MILK SECRETIONS VARY DURING EARLY MAMMARY GLAND INVOLUTION IN HEALTHY DAIRY COWS

ABSTRACT

The early period of mammary gland involution is a critical juncture in the lactation cycle that can have significant effects on milk production and mammary gland health. Methods to optimize early mammary gland involution have been studied for several decades. Yet, there are still many processes during this period that are poorly understood, including the role of oxylipids. Oxylipids are potent metabolites of polyunsaturated fatty acids (PUFA) and are important mediators of inflammation. The objective of this study was to evaluate effects of Pegbovigrastim (PEG) given 1 wk before and at the day of dry-off (D0) on concentrations of oxylipids in plasma and milk from 7 d before D0 to 14 after, as well as the effects during the first 14 d of the subsequent lactation. We hypothesized that both pro- and anti-inflammatory oxylipids would vary as a result of initiation of mammary gland involution and that pegbovigrastim would have an affect on oxylipids, particularly those related to leukocytes. A randomized, blocked method was used to enroll cows into either the PEG treatment group (n = 10) or control group (n = 10; CON). Blood samples were collected -7, -2 , -1 , 0 , 1 , 2, 4, 7, 14 d relative to dry-off and 5, 10, and 14 d post-calving. Cows were treated with PEG or a sham injection of saline on 7 d before and at dry off. Samples were analyzed for PUFA and oxylipids in milk and plasma by ultra-performance mass-spectrometry and liquid chromatography tandem quadrupole mass spectrometry, respectively. Repeated measures analyses revealed that, with the exception of prostaglandin F2α and 8-iso-keto-prostaglandin E2 in milk, PEG had no effect on milk oxylipids. For oxylipids extracted from plasma, there was a significant treatment by time interaction for plasma 8,12-iso-prostaglandin F2α VI. Nearly all other PUFA and oxylipids varied over time during the study period with most increasing or decreasing on D+1 in plasma and D+7 and +14

in milk. This study demonstrated changes in oxylipids in milk secretions during early involution and further investigation may illuminate multiple complex processes and reveal targets for optimization of mammary gland involution.

INTRODUCTION

The mammary gland is a dynamic and complex organ that undergoes major transitions throughout the lactation cycle requiring significant adaptations in immunological, structural, and physiological processes (Capuco et al., 1997). Important mammary adaptation periods include transitions from lactation to involution, from involution to colostrogenesis, and from colostrogenesis to lactation (Schanbacher and Smith, 1975, Oliver and Sordillo, 1989). The transition from lactation to mammary involution, , can determine the potential future milk production and the mammary gland's resistance to infection (Nickerson, 1989, Capuco and Choudhary, 2020). Dry off is the abrupt cessation of milking and serves as the trigger for the onset of involution. During the early dry period the mammary gland is particularly susceptible to new intramammary infections (IMI), in part due to the increased intramammary pressure causing teat canal widening which impairs formation of keratin plug (Nickerson, 1989). Indeed, the highest rate of new IMI often occurs during the initial dry period (Dingwell et al., 2003). The occurrence of IMI during the dry period negatively affects mammary gland involution and can result in decreased milk production in the subsequent lactation (Smith et al., 1968, Sordillo and Nickerson, 1988). Additionally, the early involution period is characterized by a rapid turnover of senescent milk producing epithelial cells, remodeling of the extracellular matrix, and an impairment of the blood-milk barrier (Sordillo and Nickerson, 1988, Capuco et al., 1997, Wellnitz and Bruckmaier, 2021). An appropriate immune response facilitates morphological changes in early involution including recruitment of inflammatory cells to the mammary gland to

aid in clearing apoptotic cells and milk components (Atabai et al., 2007, Chou et al., 2009). The most abundant inflammatory cells involved in mammary gland involution include macrophages, lymphocytes, and polymorphonuclear (PMN) cells including neutrophils (Sordillo and Nickerson, 1988). Sufficient inflammatory cell recruitment and activity are essential to enhance the health and function of the involuting mammary gland (Aitken et al., 2011).

The innate immune system is the first line of defense against IMI and has direct effects on the mammary involution process. Inflammation is an essential component of the innate immune system. A normal inflammatory response is transient in nature and neutralizes pathogens without clinical signs, but aberrant magnitudes or duration of the inflammatory process can lead to tissue damage (Sordillo, 2018b). Due to potential of harm to the host, the inflammatory process is tightly regulated. Oxylipids, (also known as oxylipins), are derived from polyunsaturated fatty acids (PUFA) and serve as important mediators for both magnitude and resolution of inflammation (Mavangira and Sordillo, 2018). Oxylipid substrates include omega-6 fatty acids such as arachidonic acid (C20:4n-6, AA) and linoleic acid (C18:2n-6, LA) as well as omega-3 fatty acids alpha-linolenic acid (C:18:3n-3, ALA), eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA) (Gabbs et al., 2015). Traditionally, omega-6 fatty acid metabolites are considered pro-inflammatory while metabolites of omega-3 fatty acids are considered as anti-inflammatory, although recent advances reject that overly simplistic understanding (Sordillo, 2018b). The PUFA are bound to phospholipids in cell membranes and their release depends on signaling from specific immune mediators (Gabbs et al., 2015). Free PUFA are metabolized to oxylipids through enzymatic pathways such as cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes or by non-enzymatic pathways like free radical induced oxidation (Buczynski et al., 2009). Once formed, oxylipids have potent

biological function that determines both the magnitude and the duration of an inflammatory response (Sordillo, 2018b). Among their innumerable biological functions, oxylipids can regulate other components of the innate immune system such as polymorphonuclear (PMN) cells. For example, 15-HETE can prevent degranulation of PMN and thromboxane can serve as a chemoattractant to PMN in humans (Gabbs et al., 2015). Previous work from our laboratory investigated oxylipid concentrations during the transition from lactation to involution with blood samples taken from 6 days before to 12 days after dry-off (Putman et al., 2019). Putman et al. found that the majority of PUFA substrates and oxylipids varied during the sample period and many plasma PUFA and oxylipids increased 1 or 2 days after dry-off which suggests that oxylipids are an important component of involution.

Bovine granulocyte colony stimulating factor, also known as Pegbovigrastim (PEG), is a protein that stimulates neutrophil production in the bone marrow and enhances the immune function of neutrophils in circulation (Canning et al., 2017). Neutrophils are the first innate immune cell recruited to the infected mammary gland during the early stages of inflammation (Aitken et al., 2011). Impaired neutrophil chemotaxis is positively associated with increased incidence of mastitis in periparturient cows (Cai et al., 1994). During involution, neutrophils are also recruited to the mammary gland and can increase 14-fold in the epithelial lining of the alveoli (Sordillo and Nickerson, 1988). Additionally, there is evidence that increased neutrophil infiltration in reaction to intramammary infusion of LPS or interleukin-2 at the time of dry off accelerate mammary gland involution (Nickerson et al., 1992, Dallard et al., 2010). Furthermore, PEG can alter the expression of lipoxygenase (LOX) genes in neutrophils when cows are treated during the periparturient period which suggests that treatment with PEG may alter oxylipid profiles (Lopreiato et al., 2020).

The early involution period is a critical time for mammary gland health because of the complex physiological changes coupled with the increased susceptibility to infection. Optimizing immune response during dry off may help prevent new IMI and enhance the essential remodeling processes required for milk production. Treatment with PEG, 7 days prior to and on the day of dry-off may improve the involution process by increasing circulating neutrophils and by improving neutrophil function required for mammary involution. Treatment with PEG may affect oxylipid profiles which in turn could alter the magnitude and duration of the inflammatory response. The objective of this study was to determine the effects of treatment using PEG on oxylipid profiles in plasma and milk during the transition from lactation to mammary gland involution state. We hypothesized that administration of PEG before and at dry off, would alter oxylipid profiles compared to controls during dry-off which may indicate enhancement of mammary gland involution.

MATERIALS AND METHODS

Holstein cows (n=20) were enrolled from the Michigan State University Dairy Cattle Teaching and Research Center. Eligibility criteria included clinically healthy, lactating, pregnant cows in 1^{st}-5^{th} lactations. Cows were ineligible if they exhibited any clinical signs, had not completed a withdrawal period from any medication, had undergone any surgical procedures in the prior 30 days, had SCC > 200,000 cells/mL, or had been treated for mastitis during the previous 90 days. Any cows with lymphocytes >10 x 10^9/L were considered to have lymphocytosis and were not eligible for the study (Ruggiero et al., 2019). Bovine leukosis virus (BLV) can alter immune function such as altered lymphocyte function and is associated with increased risk of mastitis (Erskine et al., 2011, Bartlett et al., 2020). To reduce the potential effects of BLV in the current study, all cows were screened by blood leukocyte differential (QScoutBLD, Advanced Animal

Diagnostics, Morrisville, NC) 1 wk prior to study onset. Cows were milked three times daily prior to dry-off and were housed in tie stalls with foam mattresses which were cleaned daily. They were fed a total mixed ration formulated to meet their requirements both prior to and after dry off (Table 1). After enrollment, one cow was found to have a SCC of 400,000 cells/mL at the at the last herd test prior to study enrollment, that cow was allowed to remain in the study. No cows enrolled in this study were treated with intramammary antibiotics at dry off. After collection of the final dry cow sample (14 d post dry off), an external teat sealant was applied to all quarters. This study was approved by the Michigan State University Institutional Animal Care and Use Committee (protocol #PROTO201900347).

Table 4.1: ***Diet ingredients and nutrient analysis of late lactation, far-off dry, and fresh cows during the study period on a dry matter basis***

Item	Late lactation	Far-off dry	Fresh cow
Ingredient[1]	Corn silage Grass silage Ground corn Soybean hulls Cotton seed Soybean meal Corn gluten Mineral supplement	Grass silage Corn silage Soybean meal Straw Mineral supplement	Corn silage Grass silage Ground corn Soybean meal Alfalfa hay Dry corn gluten Mineral supplement
Nutrient analysis			
NE_L (Mcal/kg)	1.6	1.3	1.6
CP (%)	16.5	15.6	17.9
Fat (%)	4.7	2.7	2.9
NFC (%)	38	26	40
Calcium (%)	0.99	1.2	1.1
Phosphorus (%)	0.44	0.35	0.46
Magnesium (%)	0.33	0.34	0.28
Potassium ((%)	1.2	1.7	1.4
Sodium (%)	0.38	0.14	0.47
Chloride (%)	0.27	0.36	0.38
Sulfur (%)	0.21	0.19	0.21
Added selenium (mg/kg)	0.23	0.26	0.28

[1] Ingredients listed in order of decreasing dry matter weight

Abbreviations: NE_L = net energy for lactation, CP = crude protein, NFC = non-fiber carbohydrate

Treatment

Cows were blocked by parity, milk production (305 d yield), and BLV status before random assignment to treatment groups. Pegbovigrastim (PEG), (Imrestor, Elanco Animal Health, Greenfield, Indiana), is a synthetic compound similar to naturally occurring bovine granulocyte colony-stimulating factor and the FDA approved product is labelled for administration of a first dose 2 weeks prior to calving and a second dose at calving to prevent mastitis in the first 30 DIM (Canning et al., 2017). For the current study, 15 mg of PEG was administered subcutaneously 7 days prior to dry off and on the day of dry off. Control cows were given an equal volume (2.7 mL) of sterile saline subcutaneously. All injections were given after samples were collected by a researcher who was not involved with data collection or analysis while other researchers were blinded to treatment. All subcutaneous injections were given in the neck.

Samples

Samples were collected prior to treatment on 7 d (D-7) and then 2 d (D-2), 1 d (D-1) prior to dry off, on the day of dry off (D0), and on 1 d (D+1), 2 d (D+2), 4 d (D+4), 7 d (D+7), and 14 d (D+14) after dry off. Post-calving samples were collected 5 d (C+5), 10 d (C+10), and 14 d (C+14) after parturition. Blood samples were collected from the coccygeal vein in EDTA plasma blood tubes (BD Vacutainer, Pittsburgh, PA). Blood samples were collected at 6:00 AM every morning around dry off and were collected at approximately 1:00 PM in the post-partum period. Milk samples were collected from each quarter using aseptic technique. Milk quarter samples were pooled prior to sample storage. Prior to processing, 4 µl/mL of an antioxidant reducing agent (ARA) consisting of 0.9 mM butylated hydroxytoluene (BHT), 0.54 mM EDTA, 3.2 mM triohenylphosphine (TPP), and 5.6 mM indomethacin diluted in a mixture of 50% methanol, 25% ethanol, and 25% HPLC-grade water was added to plasma and milk samples. Blood samples were centrifuged at 1449 x g at 4°C for 15 min with the centrifuge brake turned off. Plasma and

whole milk were aliquoted into 2 mL tubes and flash frozen in liquid nitrogen. Samples were stored at -80 Celsius until analyzed.

Reagents

Methanol, acetonitrile, and formic acid of LC–MS grade were from SigmaAldrich (St. Louis, MO). Potassium hydroxide made by Sigma Aldrich. Deuterated and non-deuterated PUFA, oxylipid, and isoprostane standards were purchased from Cayman Chemical (Ann Arbor, MI, USA). Butylated hydroxy toluene (BHT) was purchased from Acros (Waltham, MA, USA), 156 EDTA and triphenylphosphine were purchased from SigmaAldrich, and indomethacin was purchased from Cayman Chemical.

Targeted Lipidomics

Milk and secretions collected during the dry period were analyzed approximately 3 wk after collection was completed to minimize sample degradation. However, research restrictions caused by SARs CoV 2 pandemic, delayed analysis of all plasma and post calving milk samples by 4-5 months. A detailed description of the lipid extraction and quantification by ultra-performance liquid chromatography (UPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) used in the present study can be found in Ryman et al., 2017 and, Mavangira et al., 2015. Frozen plasma samples were thawed on ice and mixed with 1 mL 4% formic acid and 4 µl ARA to prevent ex vivo peroxidation and degradation (O'Donnell et al., 2009). Fifteen µl of internal standard mix made of 0.25 µM 5(S)-HETE, 0.25 µM 8,9-DHET-d_{11}, 0.5 µM 8(9)-EET-d_{11}, 0.25 µM 15(S)-HETE-d_8, and 0.5 µM PGE_2-d_9 (Cayman Chemical, Ann Arbor, MI, USA) was added to samples, vortexed for 2 min, and centrifuged at 4 °C for 20 min at 4,816 x g. The supernatant was separated and diluted with 95 µl of formic acid. Solid phase extraction was performed using Oasis Prime HLB 3cc columns (Waters Corporation, Milford, MA, USA) on an

Biotage Extrahera Classic robot (Biotage, Charlotte, NC, USA). Samples were eluted with 2.5 mL of a 9:1 acetonitrile:methanol mixture and then placed in a Savant Speedvac (Thermo Fischer Scientific, Waltham, MA, USA) until volatile solvents were fully evaporated. Samples were reconstituted with 150 µl of a 2:1 methanol:water mixture and stored in chromatography vials at -20 °C until analysis. Extraction of milk oxylipids followed a similar process with the following exceptions. Twenty µl of ARA was added to 0.5 mL of thawed whole milk. Hydrolysis was performed by mixing samples with 355 µl of 6M potassium hydroxide and then incubated for 45 min at 45 °C. Samples were mixed with 1.5 mL of acetonitrile with 1% formic acid. Samples were centrifuged and the supernatant was added to 20 mL of water. Samples were passed through a 12 cc Oasis HLB column (Waters Corporation, Milford, MA, USA) using a Biotage Extrahera Classic robot (Biotage, Charlotte, NC, USA), washed with 10 mL of 5% methanol, and then eluted into vials containing 10 µl of 20% glycerol in methanol. Next, volatile components were removed in the Savant Speedvac (Thermo Fischer Scientific, Waltham, MA, USA) and reconstituted with 150 µl of 2:1 methanol:water. To remove impurities, samples were centrifuged at 16 x g for 10 min at 4 °C. The supernatant was pipetted into chromatography vials and stored at -20 °C until analysis.

Mass spectrometry analysis

Fatty acid analysis was performed using reverse-phase liquid chromatography on a Waters Acquity UPLC (Waters Corporation, Milford, MA, USA) using an Ascentis Express C18 10 cm x 2.1 mm, 2.7 µm column (Supelco, State College, PA, USA). Samples were processed in electrospray negative ionization mode with a flow rate of 0.37 mL/min at 50 °C and were quantified using Empower 3 Software (Waters Corporation, Milford, MA, USA) calibrated to a linear 7-point curve. Oxylipids and isoprostanes were quantified using a Waters Xevo-TQ-S LC-

MS/MS as described previously (Mavangira et al., 2015). Briefly, PUFA metabolites were chromatographically separated using a C18 column (10 cm x 2.1 mm; 2.7 μM particles) (Ascentis Express, Sigma Aldrich). Sample analysis was 15 min per sample with a flow rate of 0.3 mL/min. Metabolites were detected using negative ion electrospray ionization with optimization of cone and collision voltages performed by Waters QuanOptimize Software. Finally, determination of chromatography peak area for each metabolite was accomplished using Waters MassLynx software.

Statistical analysis

All statistical analyses were carried out using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Graphs and heat maps were created using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Prior to analysis, the normality of distribution of each variable was visually assessed using histograms and quantile-quantile plots using PROC UNIVARIATE. Variables that violated assumptions of normality were $\log_{10}$ transformed prior to further analysis. To assess effectiveness of our randomized, blocked enrollment protocol, differences in milk production, parity, and BLV status between treatment groups were tested with t-tests performed using PROC ANOVA. Potential differences between treatment groups were assessed for each variable using PROC ANOVA. When variables differed at D-7 (before treatment was administered), D-7 values were included in model as covariates. If an oxylipid was present during mammary gland involution, but below the limit of detection after calving, values were imputed as 50% of the limit of detection for repeated measures analysis. To assess effects of time and treatment on oxylipid concentrations, repeated measures models were constructed using PROC MIXED. Each oxylipid served as a dependent variable while treatment and sampling period were included as independent variables. When neither treatment, nor treatment by time effects were significant,

treatment groups were combined and PROC MIXED was used to assess the effects of time on oxylipid concentrations during early mammary gland involution (irrespective of treatment). Differences in concentrations of PUFA and oxylipids between sample points were assessed using multiple pairwise comparison with a Bonferroni correction. Estimates of least square means were back-transformed for ease of interpretation. Statistical significance was considered at $P < 0.05$.

RESULTS

Cows were 323 DIM on average (range: 302-439 DIM) and had an average parity of 1.8 (range: 1-4). The average milk production at D-7 was 25.5 kg/d (range: 5.4-46.1 kg/d). The average SCC at study enrollment was 92,750 cells/mL (range: 13,000-400,000 cells/mL). There were no differences between study groups for SCC, BLV status, previous lactation milk yield, or parity (data not shown, $P > 0.33$).

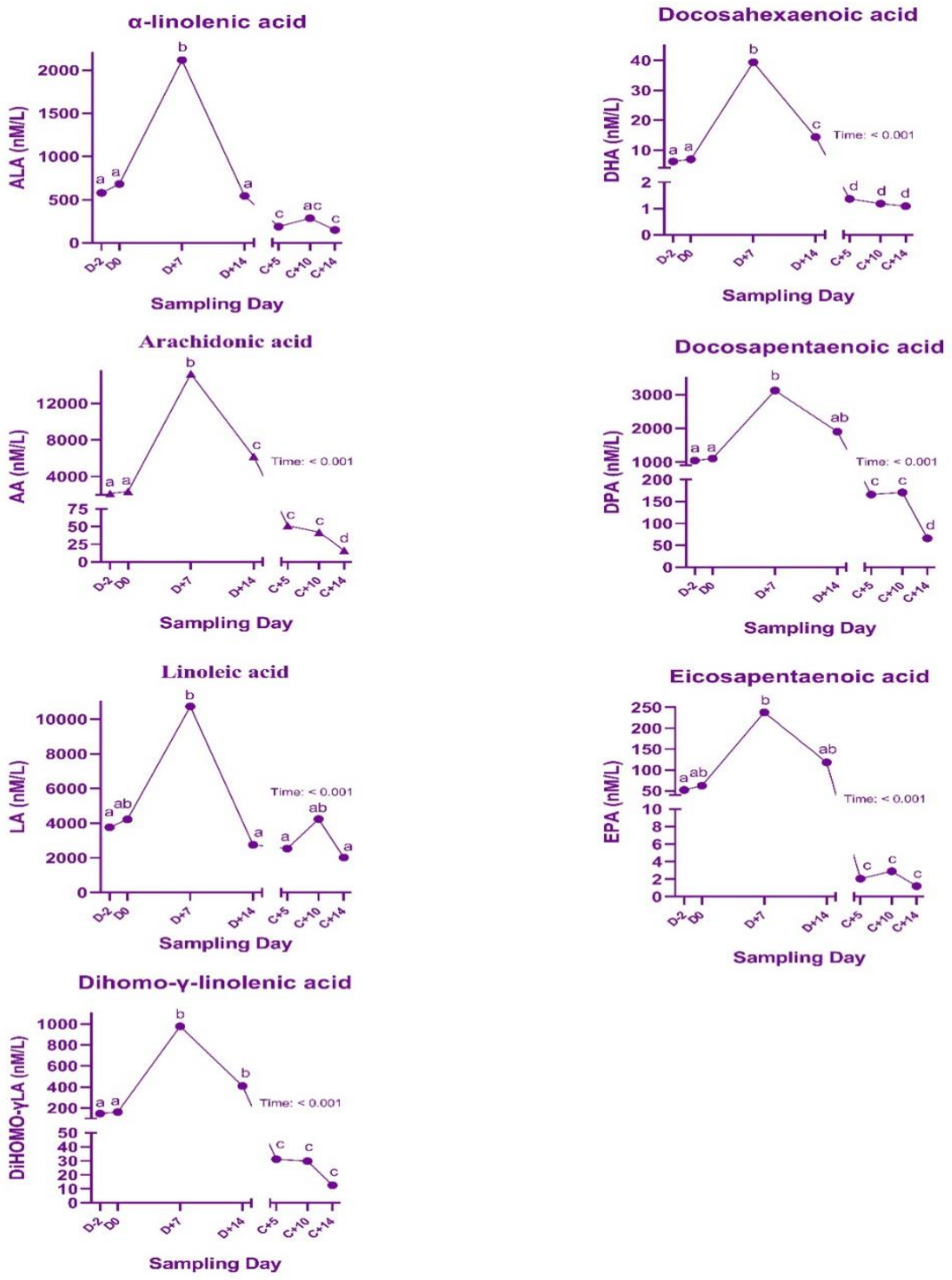
Of the 64 lipid standards used for UPLC and LC-MS/MS analysis, 28 milk and 21 plasma lipid mediators and their metabolites were detected (acronyms defined in Table 2). In milk samples, 7 oxylipids and isoprostanes were only present in the D-7 to D+14 sampling period, but were below the limit of detection (<0.1 nM) after calving, including prostaglandin F2α (PGF2α) (Figure 3), 9-hydroxyeicosatetraenoic acid (HETE), 11,12-dihydroxyeicosatrienoic acid (DHET), 14,15-DHET, 20-HETE (Table 3), 8-iso-15-keto-PGE2 (Figure 3), and 8-iso-15-PGF2α (Table 4). Milk PUFA concentrations did not vary between PEG and control cows at D-7 ($P > 0.28$; data not shown). Baseline concentrations (D-7) of 9,10-dihydroxyoctadecenoic acid (DiHOME) in milk were greater in PEG cows than control cows ($P = 0.04$; data not shown). There were no differences between treatment groups in any baseline concentrations plasma PUFA, oxylipids, or isoprostanes ($P > 0.13$; data not shown).

Table 4.2: ***Abbreviations and classes of lipids used in this manuscript***

Abbreviation	Lipid	Class
AA	Arachidonic acid	PUFA
ALA	α-Linolenic acid	PUFA
DHA	Docosahexaenoic acid	PUFA
DPA	Docosapentaenoic acid	PUFA
EPA	Eicosapentaenoic acid	PUFA
Dihomo-γLA	Dihomo-γ-linolenic acid	PUFA
LA	Linoleic acid	PUFA
PGF2α	Prostaglandin F2α	Oxylipid
PUFA	Poly-unsaturated fatty acid	
5-HETE	5-Hydroxyeicosatetraenoic acid	Oxylipid
5-IPFαVI	5-iso-prostaglandin FαVI	Isoprostane
5-oxoETE	5-Oxoeicosatetraenoic acid	Oxylipid
8,9-DHET	8,9-Dihydroxyeicosatrienoic acid	Oxylipid
8-iso-keto-PGE2	8-iso-keto-15-prostaglandin E2	Isoprostane
8-iso-15R-PGF2α	8-iso-15R-prostaglandin F2α	Isoprostane
8-iso-PGA2	8-iso-prostaglandin A2	Isoprostane
8,12-iso-PGF2α VI	8,12-iso-Prostaglandin-F2α VI	Isoprostane
9,10-EpOME	9,10-Epoxyoctadecenoic acid	Oxylipid
9,10-DiHOME	9,10-Dihydroxyoctadecenoic acid	Oxylipid
9-HETE	9-Hydroxyeicosatetraenoic acid	Oxylipid
9-HODE	9-Hydroxyoctadecadienoic acid	Oxylipid
9-oxoODE	9-Oxooctadecadienoic acid	Oxylipid
11-HETE	11-Hydroxyeicosatetraenoic acid	Oxylipid
11,12-DHET	11,12-Dihydroxyeicosatrienoic acid	Oxylipid
12,13-DiHOME	12,13-Dihydroxyoctadecenoic acid	Oxylipid
13-HODE	13-Hydroxyoctadecadienoic acid	Oxylipid
13-oxoODE	13-Oxooctadecadienoic acid	Oxylipid
14,15-DHET	14,15-Dihydroxyeicosatrienoic acid	Oxylipid
14,15-DiHETE	14,15-Dihydroxyeicosatetraenoic acid	Oxylipid
15-HETE	15-Hydroxyeicosatetraenoic acid	Oxylipid
15-oxoETE	15-oxoeicosatetraenoic acid	Oxylipid
17,18-DiHETE	17,18-Dihydroxyeicosatetraenoic acid	Oxylipid
20-HETE	20-Hydroxyeicosatetraenoic acid	Oxylipid

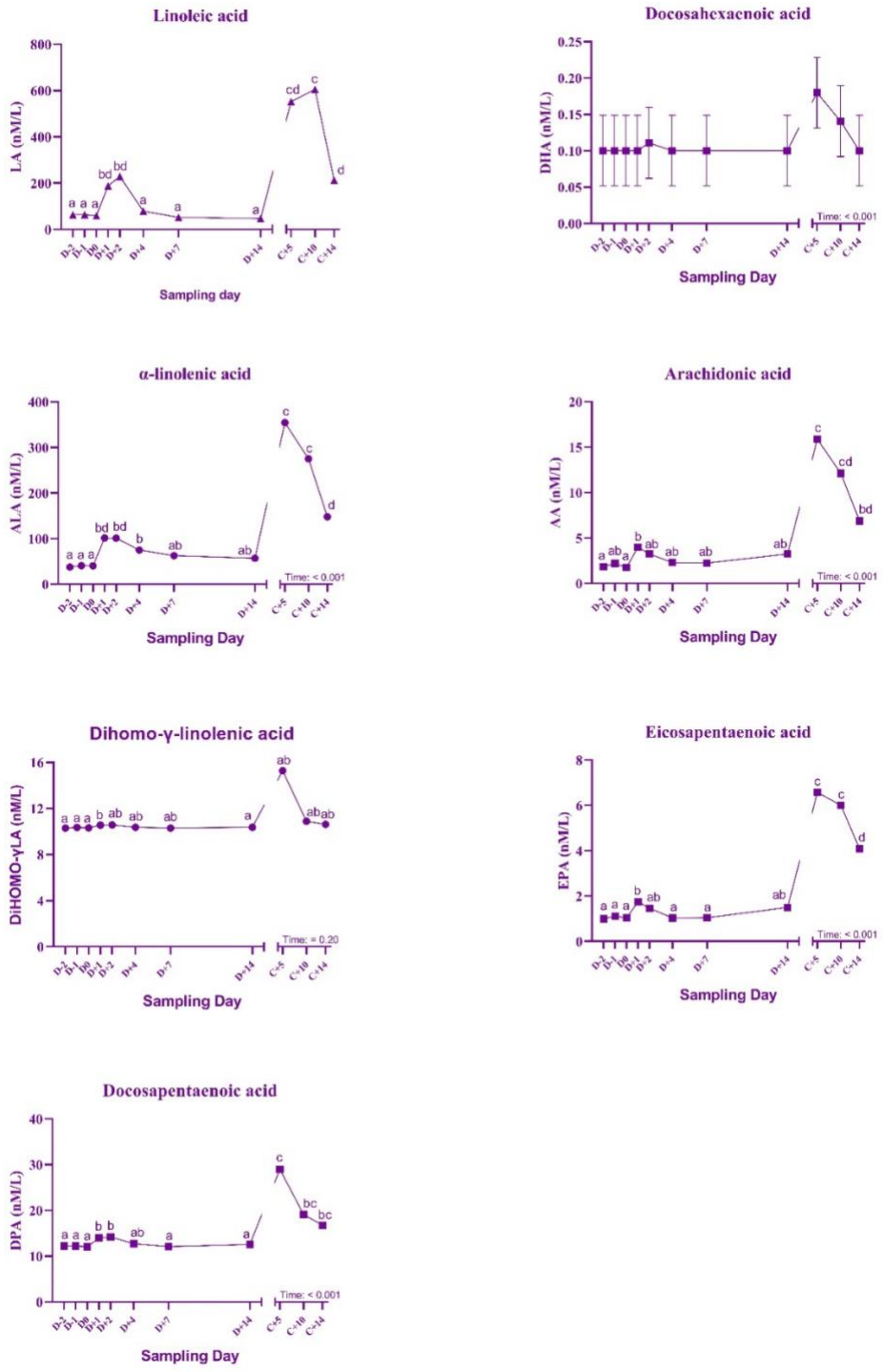
No PUFA concentrations in milk (Figure 1) or plasma (Figure 2) were affected by treatment ($P > 0.12$) nor time by treatment interactions ($P > 0.14$, Table 5). Concentrations of all lipid mediators in milk were affected by time ($P < 0.001$). In plasma, there was no effect of time on dihomo-γ-linolenic acid (Dihomo γ LA)(Figure 2), 5-HETE, 8,9-DHET, 11-HETE, 11,12-DHET, 14,15-DHET (Table 6), or 5-iso-prostaglandin FαVI (5-IPFαVI) ($P > 0.10$; Table 4). In milk, there was an effect of time ($P < 0.001$), treatment ($P = 0.03$), and a treatment by time interaction ($P = 0.002$) on milk prostaglandin F2α (PGF2 α) concentrations (Figure 3). In addition, milk PGF2 α concentrations were higher in PEG-treated cows compared to controls ($P = 0.005$, Figure 3). We observed a treatment by time interaction for 8-iso-15-keto-PGE2 ($P = 0.02$; Figure 3). Cows that were treated with PEG had increased milk 8-iso-15-keto-PGE2 concentrations on D+7 ($P = 0.037$). In plasma, no effects of treatment were observed for any variables ($P > 0.09$). A treatment by time interaction was observed for 11-HETE ($P = 0.045$, Figure 4). An interaction of treatment by time was also observed for 8,12-iso-PGF2α plasma concentrations ($P = 0.03$, Figure 4).

Figure 4.1: ***Concentrations of milk polyunsaturated fatty acids during early mammary gland involution and post-calving***



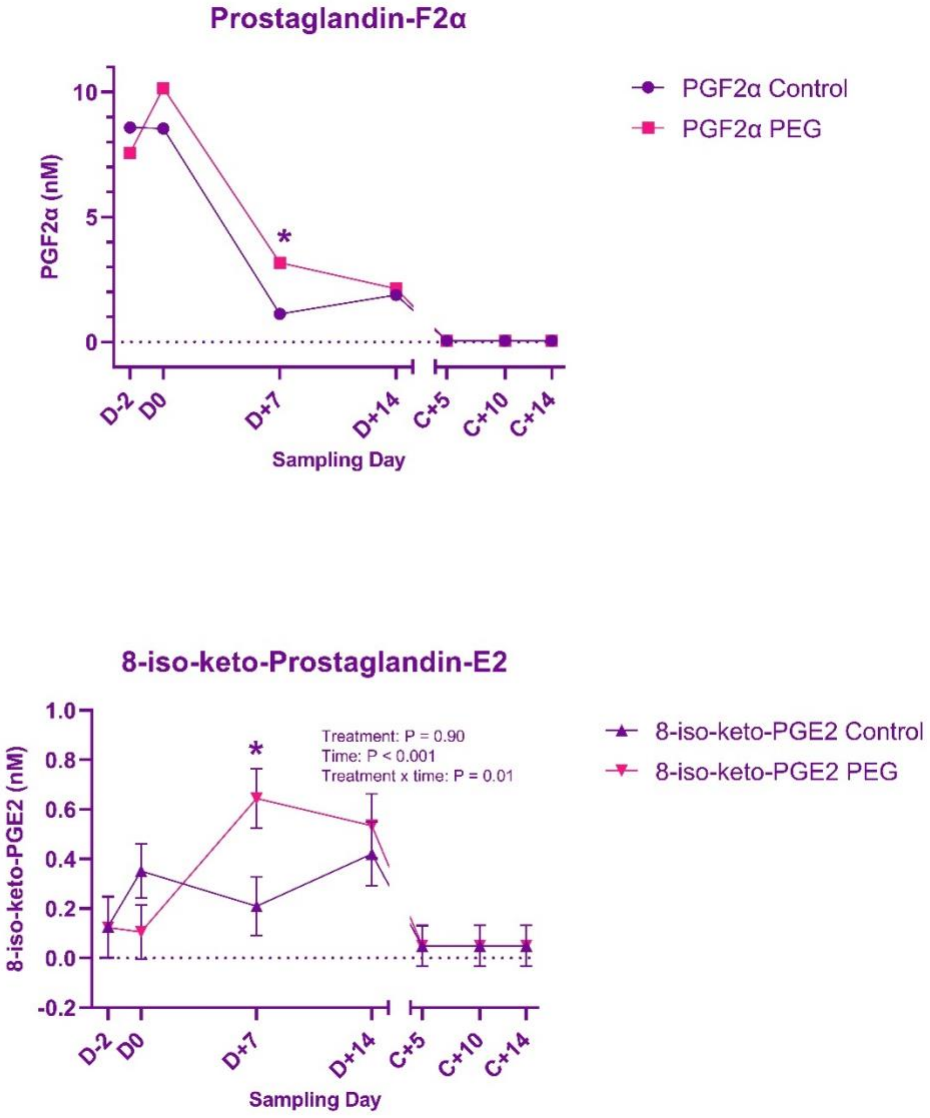
Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
[abcd] differences in superscripts within rows signifies significance between sampling time points ($P < 0.05$).

Figure 4.2: ***Concentrations of plasma polyunsaturated fatty acids during early mammary gland involution and post-calving***



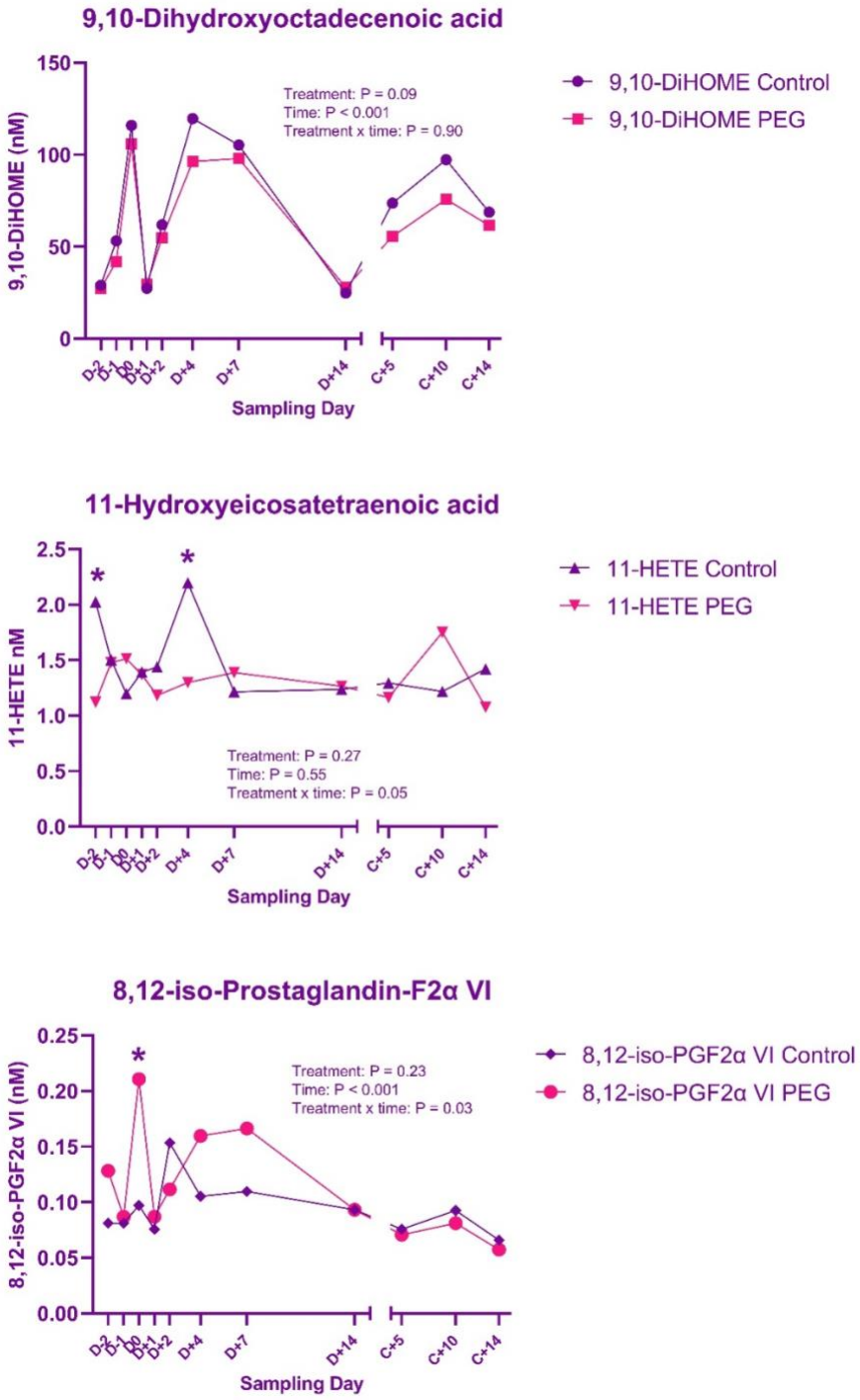
Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
[abcd] differences in superscripts within rows signifies significance between sampling time points ($P < 0.05$).

Figure 4.3: ***Concentrations of milk prostaglandin F2α and 8-iso-keto-prostaglandin E2 during early mammary gland involution and post-calving***



Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
* signifies significance between treatments at a given sampling time point ($P < 0.05$).

Figure 4.4: ***Concentrations of plasma 9,10-dihydroxyoctadecenoic acid, 11-hydroxyeicosatetraenoic acid, and 8,12-iso-prostaglandin-F2α VI during early mammary gland involution and post-calving***



Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
* signifies significance between treatments at a given sampling time point ($P < 0.05$).

Variations in milk PUFA, oxylipid, and isoprostane concentrations during the transition from lactating to active mammary gland involution can be visualized as heat maps (Figure 5). This heat map demonstrates the changes in lipid mediator populations in relation to the available substrate. By way of illustration, as milk PUFA increase in concentration on D+7 from D-2 (P = 0.03), concentrations of most milk oxylipids increase on D+7 as well (Table 3). In contrast, milk isoprostanes 8,12-iso-prostaglandin-F2α VI (8,12iPGF2 α VI) and 5-iso-prostaglandin FαVI (5-IPFαVI) decreased in concentration after dry off (Table 4). In contrast, milk 8-iso-prostaglandin A2 (8iPGA2) increased on D+14 (Table 4). Similarly, to milk PUFA, concentrations of oxylipids in milk varied throughout the study period (P < 0.0001; Table 3). Except for 5-HETE, concentrations of oxylipids increased in milk from D-2 to D+7 (P = 0.003; Table 3).

Figure 4.5: ***Heat map of PUFA and oxylipid milk concentrations during early mammary gland involution***

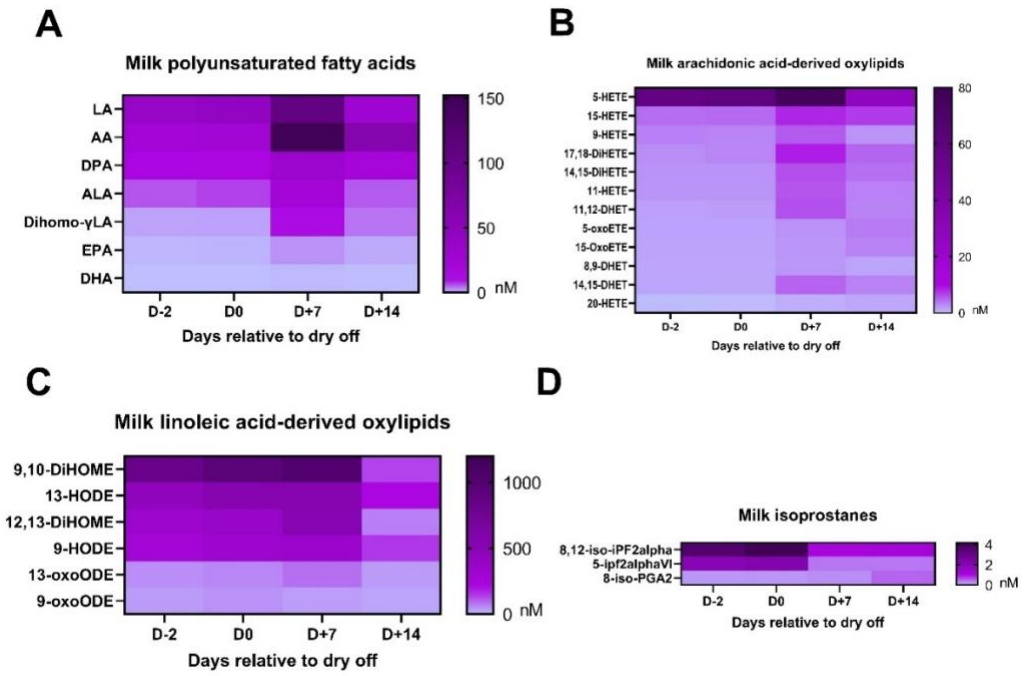


Figure illustrates the relative concentrations of lipid mediators during the transition from lactation to early mammary gland involution. The darker shades of purple represent increased lipid concentrations in milk. A.) Polyunsaturated fatty acids serve as substrate for both oxylipids and isoprostanes. B.) Oxylipids derived from arachidonic acid generally increase at the same time points as arachidonic acid. C.) Milk oxylipids derived from linoleic acid also tend to increase when milk linoleic concentrations increase after dry off. D.)

Concentrations of 5-oxoETE and 15-oxoETE in milk increased further from D+7 to D+14 ($P = 0.01$; Table 3). In contrast, concentrations of 5-HETE in milk remained stable and then decreased at D+14 ($P = 0.003$; Table 3). Alternatively, of 6 LA-derived milk oxylipids, 4 (9-HODE, 13-HODE, 9,10-DiHOME, and 11,12-DiHOME) remained stable and then decreased at D+14 (Table 3; $P < 0.0001$). Milk concentrations of 13-oxoODE increased from D-2 to D+7 (Table 3; $P = 0.046$) and 9-oxoODE remained stable through the dry period (Table 3; $P = 0.55$). Concentrations in milk of all LA-derived oxylipids decreased in the early post-calving period (C+5-C+14) compared to dry period samples (Table 3; $P = 0.03$). Milk AA-derived 11-HETE and 15-HETE concentrations decreased at C+14 compared to pre-dry samples (Table 3; $P = 0.02$) while 14,15-DiHETE concentrations were increased at C+10 compared to pre-dry samples (Table 3; $P = 0.046$). The remaining 5 AA-derived milk oxylipids post-calving concentrations were similar to pre-dry concentrations (Table 3; $P = 0.15$).

Table 4.3: ***Milk oxylipid concentrations throughout the study period***

		Day Relative to Dry Off or Calving[1]								
PUFA [2]	Oxylipid (nM)	D-2	D0	D+7	D+14	C+5	C+10	C+14	SE	P-value
AA	5-HETE	57.06^{a}	59.42^{a}	76.70^{a}	26.85^{b}	69.15^{a}	75.61^{a}	55.16^{a}	0.06	<0.01
AA	5-oxoETE	1.61^{a}	1.61^{a}	2.44^{b}	3.70^{c}	2.14^{ab}	1.98^{abc}	1.81^{abc}	0.03	<0.01
AA	8,9-DHET	1.43^{a}	1.43^{a}	2.17^{b}	1.42^{a}	1.32^{a}	1.29^{a}	1.21^{a}	0.02	<0.01
AA	9-HETE	3.37^{a}	3.17^{a}	5.46^{b}	2.35^{a}	<0.10	<0.10	<0.10	0.05	<0.01
LA	9-HODE	283.86^{a}	337.13^{a}	363.41^{a}	147.74^{b}	84.47^{c}	104.02^{b}^{c}	77.59^{c}	0.05	<0.01
LA	9-oxoODE	37.91^{a}	48.60^{a}	35.89^{a}	28.38^{a}	6.29^{b}	9.23^{b}	9.32^{b}	0.07	<0.01
LA	9,10-DiHOME	822.24^{a}	958.08^{a}	1038.96^{a}	134.40^{b}	127.85^{b}	156.14^{b}	134.87^{b}	0.07	<0.01
AA	11-HETE	2.38^{ac}	2.33^{ac}	5.76^{b}	3.38^{a}	1.90^{acd}	2.09^{c}	1.39^{d}	0.04	<0.01
AA	11,12-DHET	1.68^{a}	1.81^{a}	6.02^{b}	3.32^{c}	<0.10	<0.10	<0.10	0.05	<0.01
LA	12,13-DiHOME	353.43^{a}	391.74^{a}	538.77^{b}	68.39^{c}	68.77^{c}	71.71^{c}	67.38^{c}	0.07	<0.01
LA	13-HODE	455.83^{ab}^{c}	556.03^{a}	552.59^{a}	200.77^{d}	251.83^{d}	300.75^{c}^{d}	216.17^{d}	0.06	<0.01
LA	13-oxoODE	52.35^{a}	61.01^{ab}	86.80^{b}	38.99^{a}	19.53^{c}	23.25^{c}	20.51^{c}	0.05	<0.01
AA	14,15-DHET	1.43^{a}	1.43^{a}	4.49^{b}	3.26^{c}	<0.10	<0.10	<0.10	0.04	<0.01
AA	14,15-DiHETE	2.48^{a}	2.48^{a}	6.07^{b}	4.31^{bc}	3.85^{ac}	3.88^{c}	3.21^{ac}	0.04	<0.01
AA	15-HETE	4.48^{a}	4.66^{a}	8.29^{b}	7.14^{b}	4.18^{ac}	4.30^{ac}	2.98^{c}	0.04	<0.01
AA	15-oxoETE	1.47^{a}	1.48^{a}	2.31^{b}	3.24^{c}	1.50^{a}	1.38^{a}	1.39^{a}	0.03	<0.01
AA	17,18-DiHETE	2.74^{a}	3.13^{ac}	8.73^{b}	4.90^{c}	2.56^{a}	2.29^{a}	1.94^{a}	0.05	<0.01
AA	20-HETE	0.20^{a}	0.22^{a}	0.95^{b}	1.28^{b}	<0.10	<0.10	<0.10	0.03	<0.01

[1]Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
[2] PUFA refers to polyunsaturated fatty acids that serve as substrate for oxylipids. Each oxylipid is paired with its substrate PUFA which is either AA (arachidonic acid) or LA (linoleic acid).
^{abcd} differences in superscripts within rows signifies significance between sampling time points ($P < 0.05$).
The limit of detection for oxylipids was 0.1 nM so concentrations <0.1 were not detected at that sampling point.
Abbreviations for HETE = hydroxyeicosatetraenoic acid, oxoETE = oxoeicosatetraenoic acid, DHET = dihydroxyeicosatrienoic acid, HODE = hydroxyeicosatetraenoic acid, oxoODE = hydroxyoctadecadienoic acid, DiHOME = dihydroxyoctadecenoic acid, DiHETE = Dihydroxyeicosatetraenoic acid

Plasma PUFA concentrations also increased after dry off with concentrations of AA, LA, ALA, Dihomo-γLA, DPA, and EPA increased in plasma on D+1 as compared to D0 (Figure 2; P =

0.02). After D+2, concentrations of plasma AA did not vary in concentrations collected during the dry period (Figure 2; P > 0.48). Conversely, plasma concentrations of Dihomo-γLA did not change during the dry period (Figure 2; P = 0.2). Unlike milk, as compared to concentrations during the pre-dry period, plasma concentrations of LA, ALA, DPA, AA, and EPA increased post calving (C+5) (Figure 2; P < 0.001). Post-calving concentrations of EPA remained elevated above pre-dry concentrations through C+14 (Figure 2; P = 0.005).

Table 4.4: ***Milk and Plasma isoprostanes***

	Day Relative to Dry Off or Calving[1]								
Milk Isoprostanes (nM)	D-2	D0	D+7	D+14	C+5	C+10	C+14	SE	P-value
5-ipf2alphaVI	2.03[a]	2.21[a]	0.40[b]	0.39[b]	0.72[c]	0.90[c]	0.74[c]	0.044	<0.01
8,12-iso-iPF2alpha-VI	3.60[a]	4.16[a]	1.14[b]	1.19[b]	1.40[b]	1.54[b]	1.26[b]	0.042	<0.01
8isoPGA2	0.21[a]	0.23[a]	0.27[a]	0.49[b]	<0.10	<0.10	<0.1	0.02	<0.01

Plasma Isoprostanes (nM)	D-2	D-1	D0	D+1	D+2	D+4	D+7	D+14	C+5	C+10	C+14	SE	P-value
5-ipf2alphaVI	0.10	0.09	0.11	0.08	0.08	0.07	0.07	0.08	0.09	0.09	0.09	0.005	0.1
8,12-isoiPF2alpha-VI	0.14[a]	0.13[a]	0.13[a]	0.09[ab]	0.08[ac]	0.08[ac]	0.11[ad]	0.13[a]	0.07[bcd]	0.06[c]	0.07[bc]	0.005	<0.01

[1]Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
[abcd] differences in superscripts within rows signifies significance between sampling time points (P < 0.05).
5-ipf2alphaVI = 5-iso-prostaglandin FαVI, 8,12-iso-Prostaglandin-F2α VI = 8,12-iso-iPF2alpha-VI , 8-iso-prostaglandin A2 = 8isoPGA2

Plasma oxylipids also varied during the transition from lactation to mammary gland involution, but to a lesser degree and at different time points than milk oxylipids (Table 6). For instance, plasma 13-HODE, like milk 13-HODE, decreased after dry off, but concentrations decreased earlier in plasma than in milk (D+1) (Table 6; P = 0.008). Other LA-derived plasma oxylipids such as 9-oxoODE also decreased on D+1 (Table 6; P = 0.02) while 13-oxoODE did not vary during the early involution period (Table 6; P = 0.12). Unlike in milk, AA-derived 17,18-DiHETE plasma concentrations decreased on D+2 as compared to D-1 (Table 6; P = 0.04). No other plasma AA-derived oxylipids varied over the early mammary involution period (Table 6; P

= 0.50). After calving, plasma concentrations of half of the AA-derived oxylipids increased in plasma compared to the dry period which was similar to most of those in milk (Table 6; P = 0.009).

Table 4.5: ***Effects of Pegbovigrastim treatment and time by treatment interactions on milk and plasma oxylipids***

	Milk			Plasma		
Variable	Treatment	Time	Treatment x Time	Treatment	Time	Treatment x Time
AA	0.32	<.001	0.27	0.36	<.0001	0.70
ALA	0.19	<.001	0.44	0.43	<.0001	0.50
Dihomo-γLA	0.50	<.001	0.19	0.24	0.29	0.53
DHA	0.29	<.001	0.84	0.36	0.10	0.96
DPA	0.23	<.001	0.14	0.15	<.0001	0.34
EPA	0.40	<.001	0.31	0.12	<.0001	0.61
LA	0.58	<.001	0.33	0.74	<.0001	0.51
PGF2α	0.03	<.001	0.00			
5-HETE	0.25	<.001	0.65	0.89	0.41	0.68
5-oxoETE	0.93	<.001	0.99			
8,9-DHET	0.97	<.001	0.53	0.89	0.19	0.51
8-iso-keto-PGE2	0.90	<.001	0.01			
8-iso-PGA2	0.49	<0.001	0.98			
9,10-DiHOME	0.50	<.001	0.26	0.09	<.001	0.90
9-HETE	0.64	<.001	0.38			
9-HODE	0.99	<.001	0.63	0.74	<.001	0.36
9-oxoODE	0.30	<.001	0.87	0.97	<.001	0.39
11-HETE	0.85	<.001	0.19	0.27	0.55	0.05
11,12-DHET	0.77	<.001	0.76	0.40	0.12	0.37
12,13-DiHOME	0.65	<.001	0.67			
13-HODE	0.31	<.001	0.47	0.86	<.001	0.32
13-oxoODE	0.47	<.001	0.84	0.15	0.00	0.77
14,15-DHET	0.78	<.001	0.81	0.92	0.20	0.76
14,15-DiHETE	0.98	<.001	0.67	0.36	0.04	0.31
15-HETE	0.90	<.001	0.55	0.62	0.03	0.39
15-oxoETE	0.48	<.001	0.98			
17,18-DiHETE	0.84	<.001	0.91	0.27	0.01	0.33
20-HETE	0.15	<0.001	0.54	0.66	0.01	0.40
5-IPFαVI	0.46	<.001	0.37	0.50	0.15	0.91
8,12-iso-PGF2αVI	0.58	<.001	0.55	0.23	<.001	0.03

Table 4.6: ***Plasma oxylipids throughout the study period***

		Day relative to dry off or calving[1]												
PUFA[2]	Oxylipid (μM)	D-2	D-1	D0	D+1	D+2	D+4	D+7	D+14	C+5	C+10	C+14	SE	P-value
AA	5-HETE	4.15^{ab}	3.68^{ab}	4.79^{b}	3.85^{b}	3.83^{b}	3.51^{ab}	2.82^{a}	3.11^{a}	5.83^{b}	4.54^{ab}	2.98^{a}	0.05	<0.01
AA	8,9-DHET	0.50^{b}	0.45^{ab}	0.36^{a}	0.44^{b}	0.45^{b}	0.36^{a}	0.40^{ab}	0.35^{a}	0.61^{b}	0.53^{ab}	0.45^{ab}	0.04	<0.01
LA	9-oxoODE	9.46^{bd}	11.06^{ab}	13.00^{a}	5.67^{c}	7.30^{cf}	6.71^{bce}	6.44^{cde}	5.91^{d}	10.10^{abe}	12.73^{a}	11.78^{af}	0.05	<0.01
AA	11-HETE	1.27^{b}	1.67^{a}	1.33^{b}	1.53^{a}	1.52^{a}	1.31^{ab}	1.31^{ab}	1.45^{b}	1.59^{a}	1.51^{a}	0.93^{b}	0.05	0.01
LA	13-HODE	166.92^{a}	174.18^{a}	172.31^{a}	109.30^{bc}	116.47^{bc}	94.86^{b}	94.00^{b}	85.20^{b}	141.25^{ac}	136.18^{a}	145.51^{a}	0.03	<0.01
LA	13-oxoODE	1.01^{c}	1.19^{ab}	1.23^{b}	1.41^{b}	0.94^{a}	0.98^{a}	1.05^{ac}	0.79^{a}	1.40^{b}	2.04^{b}	1.88^{bc}	0.06	<0.01
AA	14,15-DiHETE	5.58	5.85	6.85	4.75	5.09	5.55	6.99	6.15	7.52	5.80	7.27	0.04	0.02
AA	15-HETE	2.97	3.03	2.75	2.60	2.67	2.26	2.69	2.73	2.36	1.93	1.90	0.05	0.01
AA	17,18-DiHETE	56.25^{abc}	59.08^{ac}	58.51^{abc}	50.35^{ab}	46.51^{b}	50.46^{ab}	56.65^{abc}	54.87^{abc}	66.34^{c}	62.97^{a}	61.53^{ac}	0.03	<0.01

[1]Sampling time periods around the time of dry off are labeled D±number of days either before (-) or after (+) dry off (D0). Samples acquired post-calving are denoted with a C+number of days after calving.
[2] PUFA refers to polyunsaturated fatty acids that serve as substrate for oxylipids. Each oxylipid is paired with its substrate PUFA which is either AA (arachidonic acid) or LA (linoleic acid).
[abcd] differences in superscripts within rows signifies significance between sampling time points ($P < 0.05$)
The limit of detection for oxylipids was 0.1 nM so concentrations <0.1 were not detected at that sampling point.
Abbreviations for HETE = hydroxyeicosatetraenoic acid, oxoETE = oxoeicosatetraenoic acid, DHET = dihydroxyeicosatrienoic acid, HODE = hydroxyeicosatetraenoic acid, oxoODE = hydroxyoctadecadienoic acid, DiHOME = dihydroxyoctadecenoic acid, DiHETE = Dihydroxyeicosatetraenoic acid

DISCUSSION

Administration of Pegbovigrastim 7 d before dry off and on the day of dry off had no effect on most milk and plasma oxylipids analyzed in the current study. Indeed, only 1 oxylipid (PGF2α) and 1 isoprostane, (8-iso-keto-PGE2) in milk were affected by PEG treatment at dry off. This finding was surprising because PEG stimulates the bovine immune system and the involvement of lipid mediators are ubiquitous across both innate and adaptive immune functions (Kimura et al., 2014, Gabbs et al., 2015). The lack of treatment effects in our study indicates that the variation in oxylipid concentrations in milk and plasma between the D-2 and D+14 sampling points are likely a result of the physiological alterations associated with mammary gland involution.

The abrupt cessation of lactation at dry off triggers a series of immunological, physiological, and morphological changes as the mammary gland transitions from lactating to nonlactating (Hurley, 1989). This study demonstrated dynamic shifts in milk and plasma concentrations of oxylipids during the early mammary involution period that likely reflect immune processes occurring in the mammary gland and systemic circulation. Oxylipid concentrations are dependent upon substrate availability which can be influenced by dietary PUFA supplementation (Ryman et al., 2017). Both the PUFA substrate composition and the type of stimulus can affect the nature of oxylipid biosynthesis and inflammatory response (Calder, 2008, Ryman et al., 2017). Milk concentrations of PUFA peaked 7 d after dry off which could indicate increased release of PUFA from membrane phospholipids via increased phospholipase activity (Burke and Dennis, 2009). In humans, phospholipase activity is increased in coordination with systemic inflammatory events and in response to cytokines such as interleukin-1 and tumor necrosis factor-α (TNFα) (Crowl et al., 1991). During the first 2 wk of mammary involution, gene expression of factors known to catalyze phospholipase activity include genes related to cytokine activity, leukocyte chemotaxis, and redox homeostasis is increased in bovine mammary tissue (Piantoni et al., 2010). In support of this, the most dramatic alterations in milk PUFA and oxylipids occurred between D0 and D+14 in the current study. Consistent with other studies (Średnicka-Tober et al., 2016, Kuhn et al., 2017), prior to dry off, LA was the dominant PUFA in milk which may be related to the LA-rich content of corn and soy commonly included in dairy cow rations. Cows in our study transitioned from a diet primarily composed of corn silage to one primarily composed of grass silage. Rations with relatively increased corn silage and less grass were associated with increased milk AA and decreased ALA (Kusche et al., 2015). The change of ration after dry off may not have had an effect on milk PUFA in our study, , because milk AA concentrations

increased more than 6-fold after dry off and became the most abundant milk PUFA. The shift in the PUFA profile of milk secretions after dry off is likely affected by the rapid decline in de novo fat synthesis and secretion in the first 3 d mammary gland involution (Hurley et al., 1987). Moreover, organelles essential for secretion of lipid droplets from the mammary epithelial cell decline resulting in large fat vacuole formation in the cell (Sordillo and Nickerson, 1988). In addition to intracellular lipid droplets, milk fat globules in the mammary gland do not decline in concentration for approximately 3-7 d post dry off and are potentially susceptible to oxidation or hydrolyzation (Hurley, 1989, Vanderghem et al., 2011). It is presumed that lipids are removed by macrophage and neutrophil phagocytosis during mammary gland involution, however, increased concentrations of lipases have been documented during involution in goats and could affectavailable PUFA substrate (Thompson, 1988, Zhao et al., 2019). The inversion of concentrations of AA and LA that we observed may have been related to the transition from milk fat synthesis to milk fat clearance from the mammary gland.

The immune response during mammary gland involution is similar to responses to an invading pathogen and include infiltration of leukocytes into the mammary gland, impairment of the blood milk barrier, and increases in endogenous soluble defenses such as lactoferrin and cytokines (Aitken et al., 2011, Hughes and Watson, 2018, Wellnitz and Bruckmaier, 2021). So it was not surprising that some shifts of PUFA and oxylipid profiles in milk secretions during mammary gland involution were similar to those occurring during an intramammary infection (Mavangira et al., 2015). These findings likely reflect the localization and magnitude of inflammatory processes occurring during mammary gland involution. The relative specificity of oxylipids to sample type and physiological process of interest should be considered when interpreting oxylipids as biomarkers. For example, prior researchers investigated plasma oxylipids as

biomarkers for mammary gland involution and hypothesized that peak inflammation occurs on days 1 and 12 of mammary gland involution (based on abundance of serum inflammatory markers) however in our study milk oxylipids peaked 7 or 14 d after dry off (Putman et al., 2018, Putman et al., 2019). Certain oxylipids such as 9-oxoODE increased 1 d after dry off in both studies, but 13-HODE decreased 11 d earlier in the current study (Putman et al., 2019). It is possible that there were significant variations in lipid mediators between D0 and D+7 in milk, similar to plasma lipids. Samples were not collected between D0 and D+7 in order to avoid stimulating milk production. However, many important functions occur during the first week of mammary gland involution and analysis milk oxylipid concentrations during that period may provide new insight about mammary gland involution.

Administration of PEG 7 d before dry off and on the day of dry off affected concentrations of milk PGF2α and 8-iso-keto-PGE2 during early mammary gland involution. Treatment of dairy cows with PEG was developed to increase circulating leukocyte concentrations and to improve leukocyte function during the early lactation when immune function is suppressed (Kimura et al., 2014). Neutrophils collected from periparturient cows treated with PEG had enhanced myeloperoxidase activity compared to neutrophils collected from control cows during ex vivo stimulation (McDougall et al., 2017). Myeloperoxidase catalyzes the formation of free radicals to kill pathogens, but can also alter oxylipid profiles, particularly through nonenzymatic oxidation of PUFA (Capuco et al., 1986, Sordillo, 2018a). Indeed, myeloperoxidase activity affected oxylipid profiles in mice in response to treatment with endotoxin (Kubala et al., 2010). Conversely, cows enrolled in our study were healthy with only 1 cow diagnosed with clinical mastitis during the early dry period (data not shown). Thus, it is possible that leukocytes were not activated in a way that would substantially alter oxylipid profiles. For example, when healthy

periparturient cows were treated with PEG, serum myeloperoxidase concentrations were not affected (Lopreiato et al., 2019). Additionally, neutrophils from healthy humans treated with a medication similar to PEG, (granulocyte colony-stimulating factor) , did not have increased activity of enzymes involved with the oxidative burst (Höglund et al., 1997). In contrast, neutrophilia in severely ill humans was associated with increased oxidative stress and altered PUFA concentrations (Das, 2021).

Another potential explanation for the lack of treatment effects we observed is that leukocytes from PEG treated cows do not seem to infiltrate the mammary gland at a greater rate than those of untreated cows. For instance, administration of PEG in healthy periparturient cows did not affect SCC of milk, despite leukocytosis (Canning et al., 2017). Additionally, PEG-treated cows with experimental acute and chronic mastitis did not differ in SCC compared to control cows (Powell et al., 2018, Putz et al., 2019). This implies that PEG may not affect leukocyte infiltration into the mammary gland, regardless of infection status. Interestingly, PEG-treated cows with acute mastitis had increased neutrophil extracellular traps (NET) formation in milk fat globules compared to untreated cows 24 hr post-infection (Powell et al., 2018). Production of free radicals occurs during NET formation which could result in oxidation of lipids within milk fat globules (Bassel and Caswell, 2018). Enzymes associated with NET formation increased production of PGF2α in an in vitro model of equine endometriosis (Rebordão et al., 2021). Thus, the increase in PGF2α in PEG treated cows in our study may have been related to NET formation in milk fat globules during early mammary gland involution. However, we would have expected changes in other oxylipids associated with NET-formation such as leukotriene and 5-oxo-ETE (Surmiak et al., 2020).

Certain oxylipid profiles may indicate whether current redox balance and inflammatory status will be conducive to an orchestrated or aberrant immune response to stimuli (Sordillo and Mavangira, 2014), which could include the response to PEG. For example, altered oxylipid profiles during early mammary gland involution, (approximately 40-60 d before calving), were associated with increased disease risk after calving (Putman et al., 2022). Most relevant to our study was the finding that plasma PGF2α was increased on the day of dry off in cows that were later diagnosed with disease compared to cows that remained healthy through 40 DIM(Putman et al., 2022). Plasma PGF2α was not detected in the current study, but alterations in PGF2α concentrations might have been indicative of aberrations in inflammatory processes occurring in their respective anatomical compartments. The degree of increase of PGF2α concentrations was less in our study compared to Putman et al. (2022) (~2.8 vs 4 fold change, respectively). The lower magnitude of PGF2α concentration change may be reflective the differences in disease incidences between the 2 studies which included 1 out of 20 cows in our study and 9 out of 16 cows in Putman et al. (2022). Thus, the potential implications of the PEG-related effects on PGF2α milk concentrations in the current study are not understood and may have been an effect of random variation between individual cows and our small sample size.

In our study we identified significant treatment (PEG) by time interactions for plasma 11-HETE and 8,12-iso-PGF2α VI. Isoprostanes, (such as 8-iso-keto-PGE2 and 8,12-iso-PGF2α VI), are formed when PUFA in cellular membranes are damaged as a result of peroxidation from excess free radicals (Milne et al., 2011). Since isoprostanes are a direct measure of pro-oxidant damage, they serve as excellent biomarkers of oxidative stress and are associated with disease in dairy cows (Putman et al., 2021). Plasma isoprostanes did not vary during the dry period, but milk 5-iPF2αV and 8,12-iPF2α decreased after dry off. The decrease in milk isoprostanes may have

been a result of the cessation of milk production resulting in reduced mitochondrial production of free radicals. In support of this, shifts in redox balance towards a pro-oxidant state and increased milk isoprostanes are associated with increased milk production in healthy cows (Kuhn et al., 2018).

Regardless of biosynthetic pathway, the changes of substrate concentrations were fairly similar to their metabolites. Indeed, 7 of the 8 AA-derived oxylipids in milk increased on D+7 when milk AA concentrations peaked. Conversely, a majority of the LA derived oxylipids decreased on D+14 similar to LA. These findings were consistent with previous literature investigating oxylipid profiles (Sordillo, 2018a). The LA-derived DiHOMEs are related to lipid metabolism, mitochondrial dysfunction, and cytotoxicity in humans (Hildreth et al., 2020). In cows, both 9,10- and 12,13-DiHOME were significantly elevated in milk in cows with coliform mastitis compared to healthy controls (Mavangira et al., 2015). In our study, both milk 9,10- and 12,13-DiHOME increased on D+7. These findings could indicate that these DiHOME oxylipids are associated with certain immune processes in the mammary gland.

Finally, our study also investigated the effects of PEG treatment 7 d before and on the day of dry off on oxylipid concentrations in the first 2 wk of lactation. Interestingly, milk AA concentrations were markedly reduced while milk LA concentrations were not different between the pre-dry and post-parturient samples. Some difference in concentration may have occurred because the dry period samples were analyzed at a different time point than the post-parturient ones so we could avoid storage degradation in the former. Although the relative stability between specific PUFA is unknown, total PUFA have been found to be stable for more than 1 yr when stored at -80 °C (Metherel and Stark, 2016). One study found that milk PUFA concentrations were negatively correlated with milk production and when PUFA concentrations were corrected

for milk production they followed the same pattern as plasma PUFA over the lactation cycle (Kuhn et al., 2017). This does not account for the lack of change in milk LA concentrations, however. Sample handling and extraction method can have significant effects on oxylipid results (Teixeira et al., 2021). Strategies for enhancing sampling and analysis methods to ensure accurate oxylipid analysis could result in improved use of oxylipids as biomarkers.

CONCLUSION

For the first time, this study reveals the changes in milk oxylipid concentrations in dairy cows as the mammary gland transitions from lactation to the non-lactating state. Moreover, PEG treatment 1 wk prior and on the day of dry-off had no effect on concentrations of most milk or plasma oxylipids. Both milk and plasma oxylipids varied during early mammary gland involution, but changes occurred at different sampling points and with greater magnitude in milk compared to in plasma. The differences between plasma and milk oxylipids may reflect the inflammatory processes occurring systemically or in the mammary gland during early mammary gland involution. Future studies should focus on the biological effects of oxylipids on mammary gland involution and subsequent milk production and mastitis incidence.

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CHAPTER 5: PLASMA CONCENTRATIONS OF RETINOIC ACID ISOMERS DECREASE DURING EARLY MAMMARY GLAND INVOLUTION IN DAIRY COWS

ABSTRACT

Retinoic acid, the active form of vitamin A, is known to have pleotropic effects on the immune system and may play a role in mammary gland involution during the early dry period . The objective of our study was to investigate plasma concentrations of several retinoic isomers: all-trans retinoic acid (ATRA), 9-cis retinoic acid (9CisRA), and 13-cis retinoic acid (13CisRA) during the transition from lactation to mammary gland involution in cows treated with pegbovigrastim or non-treated controls. Additionally, we investigated associations of retinoic acid isomers with redox balance, inflammation, and biomarkers of mammary gland involution. Cows were randomly assigned to treatment of pegbovigrastim (n = 10) or saline -injected controls (n = 10). Cows were treated with PEG -7 d before and on the day of dry off. Plasma was collected using foil-wrapped EDTA tubes -7, -2, -1, 0, 1, 2, 4, 7, and 14 d relative to dry off. Quantification of ATRA, 9CisRA, and 13CisRA was performed with liquid chromatography tandem mass spectrometry. Linear mixed models were used to investigate the effect of treatment and time on retinoic acid isomers. Polynomial regression models were created to investigate the association of retinoic acid isomers with biomarkers of involution. Treatment had no effect on concentrations of any retinoic isomer. Plasma concentrations decreased after dry off and reached their nadir on days 2, 4, and 1 d post dry off for ATRA, 9CisRA, and 13CisRA, respectively. Concentrations of ATRA were associated with milk yield prior to dry off, systemic redox balance, serum haptoglobin, and milk citrate. This study identified that ATRA, 9cisRA, and 13cisRA vary during dry off and ATRA may affect mammary gland involution.

INTRODUCTION

Mammary gland involution, which occurs after the abrupt cessation of lactation in dairy cows, is a pivotal point of the lactation cycle. As the mammary gland transitions from lactating to non-lactating it goes through morphological, physiological, and immunological changes increasing the risk for mastitis (Dingwell et al., 2003). The increased risk for mastitis in the early dry period is one reason why intramammary antimicrobial therapy in all quarters of cows at dry off is used on the majority of U.S. dairy farms (NAHMS, 2016). Thus, intramammary antimicrobial therapy at dry off accounts for majority of antimicrobial doses given on dairy farms (Pol and Ruegg, 2007, Schrag et al., 2020, de Campos et al., 2021). Reducing antimicrobial use at dry off by selectively treating only at risk quarters can be implemented without compromising milk quality (Weber et al., 2021). Alternative methods (other than antimicrobials) that help to optimize mammary glandimmune responses during involution are also of great interest (Zhao et al., 2019).

The mammary gland requires an appropriate immune response to support the complex physiological changes occurring during involution (Nickerson, 1989). Mammary involution can be divided into 3 phases: 1.active involution, during which the mammary gland switches from milk synthesis to epithelial cell turnover, 2. static involution, when the mammary gland is most resistant to infection, and 3. lactogenesis, as the mammary gland prepares for the next lactation (Sordillo and Nickerson, 1988). Active involution is characterized by a massive turnover of mammary epithelial cells (MEC), infiltration of leukocytes into the mammary gland, and the disruption of the milk blood barrier (Sordillo and Nickerson, 1988, Capuco and Choudhary, 2020, Wellnitz and Bruckmaier, 2021). The immune response initiated by active involution causes alterations in metabolism, oxidative stress, and inflammatory markers in circulation

(Sordillo, 2018). For example, serum concentrations of inflammatory biomarkers, such as haptoglobin and oxylipids, vary in concentrations during early mammary gland involution (Putman et al., 2018, Putman et al., 2019). Alterations in mammary gland function during involution can be more specifically investigated by measuring biomarkers in milk secretions collected after dry off. For example, citrate is secreted into milk in association with MEC de novo fat production and citrate concentrations decrease in milk secretions as MEC activity declines in early involution (Sordillo et al., 1987, Garnsworthy et al., 2006). Alternatively, as compared to during lactation, antimicrobial factors such as lactoferrin are secreted at a greater rate by MEC during mammary gland involution (Hurley, 1989, Schanbacher et al., 1993). Additionally, lactoferrin was decreased nearly 60-fold during early mammary gland involution in cows with chronic *Staphylococcus aureus* mastitis as compared to healthy control cows (Renna et al., 2019). This indicates that lactoferrin and other biomarkers can provide valuable insight for methods to optimize mammary gland function during early involution.

During the dry period, increased concentrations of serum retinol (the predominant form of vitamin A in circulation) are associated with decreased risk of disease after calving (LeBlanc et al., 2004b, Strickland et al., 2021). Vitamin A is an essential nutrient for dairy cows and increased dietary supplementation can improve immune function and mammary gland health (Chew and Johnston, 1985, Jin et al., 2014). Vitamin A is a blanket term for a family of molecules that include retinol, retinyl esters, and isomers of retinoic acid (RA) (Blomhoff and Blomhoff, 2006). The most commonly studied RA isomers in dairy cows include all-trans retinoic acid (ATRA), 9-cis retinoic acid (9cisRA), and 13-cis retinoic acid (13cisRA) (Horst et al., 1995, Puvogel et al., 2005). Both ATRA and 9cisRA bind to specific nuclear receptors which mediate expression of a multitude of genes affecting processes relevant to mammary gland

involution (such as mammary gland remodeling) and promote granulocytosis (Meyer et al., 2005). The nuclear receptor for 13cisRA has not been identified but 13cisRA is thought to have some biological activity either directly or by serving as a precursor for ATRA (Blaner, 2001). For example, treatment using 13cisRA improved leukocyte function in vitro and increased endogenous 13cisRA was associated with increased milk production during recovery from coliform mastitis in cows (Van Merris et al., 2004, Meyer et al., 2005). In a murine model, treatment with RA during weaning resulted in accelerated mammary gland involution (Zaragoza et al., 2007). In dairy cows, serum ATRA concentrations increased while 13cisRA decreased in response to experimental coliform mastitis (Van Merris et al., 2004). This indicates that RA isomers may have important functions related to bovine mammary gland involution and could serve as a valuable involution biomarker.

Isomers of RA are known to be associated with immune functions, but nothing is known about endogenous RA concentrations in healthy dairy cattle during the transition from lactating to active involution. Further, RA isomers can improve mammary gland involution in other species, but little is known about the associations between RA isomers and physiological processes occurring during involution. We hypothesized that concentrations of RA isomers would change after dry off and that they would be associated with biomarkers of mammary gland involution, inflammation, and oxidative stress in dairy cows. The objective of this study was to describe concentrations of ATRA, 9CisRA, and 13CisRA in dairy cows and to investigate associations among RA isomers and biomarkers for mammary gland involution, inflammation, and oxidative stress during the transition from lactation to active involution.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Michigan State University Animal Use and Care Committee (PROTO201900347). Pregnant late lactation, primiparous (n = 11) and multiparous (n = 9) cows were enrolled in a blinded, randomized clinical trial from November 2019 through February 2020. Cows were blocked by parity, milk production, and bovine leukosis virus (BLV) status. Exclusion criteria included having had a surgical procedure within the previous 30 d, any treatment for mastitis within the previous 90 d, and somatic cell count (SCC) >200,000 cells/mL at the most recent DHIA test. All cows were examined by a veterinarian a week prior to enrollment to ensure they were clinically healthy, had a BCS between 3.0-3.75, and were aleukemic (or if BLV positive; <10,000 lymphocytes/mL). Lymphocytic cows were excluded from the study. Cows were housed in a tie-stalls topped with saw dust and were fed rations individually to meet their nutrient requirements according to the NRC guidelines (Table 1). The individuals collecting and processing samples were blinded to treatment for the duration of the study. Cows were randomly assigned to received either 15 mg of Pegbovigrastim (Elanco, Greenfield, IN, USA) or an equivalent volume of normal saline that was injected in the subcutaneous space of the periscapular region by a trained technician after sample collection was complete. Injections were given 7 d before and again on the day of dry off.

Table 5.1: ***Study farm ration ingredients and nutrient analysis***

Item	Late lactation	Far-off dry
Ingredient	Corn silage	Grass silage
	Grass silage	Corn silage
	Ground corn	Soybean meal
	Soybean hulls	Straw
	Cotton seed	Mineral supplement
	Soybean meal	
	Corn gluten	
	Mineral supplement	
Nutrient analysis		
NEL2 Mcal/kg	1.6	1.3
CP (%)	16.5	15.6
Fat (%)	4.7	2.7
NFC (%)	38	26
Calcium (%)	0.99	1.2
Phosphorus (%)	0.44	0.35
Magnesium (%)	0.33	0.34
Potassium ((%)	1.2	1.7
Sodium (%)	0.38	0.14
Chloride (%)	0.27	0.36
Sulfur (%)	0.21	0.19
Added selenium (mg/kg)	0.23	0.26
Vitamin A (KIU/d)	137.4	82.5
Vitamin D (KIU/d)	25.6	15.3

Sample collection and processing

Blood samples were collected by venipuncture of the coccygeal vein -7 d (D-7; samples were collected before treatment), -2 d (D-2), -1 d (D-1), day 0 (D0), 1 d (D+1), 2 d (D+2), 4 d (D+4), 7 d (D+7), and 14 d (D+14) relative to day of dry off. Milk and milk secretions were collected D-7, D-2, D0, D+7, and D+14. Plasma was collected in foil-wrapped glass EDTA tubes and serum was collected in serum separator tubes (Thermo Fischer Scientific, Waltham, MA). Quarter milk samples were collected aseptically from each cow in the parlor for samples D-7 to D0, and in tie-

stalls for D+7 and D+14. Equal volumes of milk from each quarter were combined in the laboratory prior to processing.

Blood tubes were stored in closed containers on ice before being transferred to the laboratory. Serum separator tubes were allowed to clot for approximately 1 hour prior to centrifugation. In the laboratory, plasma samples were handled exclusively under amber light as quickly as possible to minimize potential UV light exposure. Blood samples were centrifuged at 1449 x *g* for 15 min at 4 °C to separate plasma and serum from cells. After separation , plasma was placed in 1 mL aliquots into glass amber chromatography vials. Samples were then gently lowered into liquid nitrogen to prevent vial rupture during flash freezing. Serum samples were aliquoted into minicentrifuge tubes and flash frozen in liquid nitrogen. Skim milk was produced by centrifuging milk at 10,000 x*g* for 20 min at 4 °C. Milk fat was scraped away so that skim milk could be aliquoted into minicentrifuge tubes and flash frozen. Samples were stored at -80 °C until analysis. Serum biomarkers of inflammation included non-esterified fatty acids (NEFA), haptoglobin, and albumin and were analyzed using commercially available kits for CataChem Well-T biochemistry analyzer (Catachem Inc., Oxford, CT). Lactoferrin and bovine serum albumin (BSA) in skim milk were analyzed using commercially available ELISA kits containing bovine-specific antibodies (Bethyl Laboratories Inc., Waltham, MA). Milk and milk secretion samples were diluted with deionized water as needed to fit the standard curve. Milk ELISA samples were analyzed in triplicate by colorimetric analysis using the Biotek H1 plate reader (Biotek, Winooski, VT).

Reactive oxygen species and antioxidant potential

Reactive oxygen and nitrogen species (ROS) were analyzed by fluorescence using a commercially available kit (OxiSelect, Cell BioLabs Inc., San Diego, CA) on the Biotek H1 plate reader. Whole milk and serum ROS concentrations were determined at 480 nm of excitation and 530 nm of emission. Background fluorescence was accounted for by subtracting blank well values from sample well values. Each sample was analyzed in duplicate and results are presented as relative fluorescence unit (RFU) per microliter sample. Total antioxidant potential (AOP) concentrations were determined by measuring the reduction of the radical 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich, St. Louis, MO) of each milk and serum sample as previously described (Re et al., 1999). The synthetic vitamin E analog, trolox, was used to create the standard curve and results are presented in trolox equivalents (TE).

Citrate

Solid phase extraction of citrate was conducted by combining 500 μL of thawed whole milk with 1.5 mL of acetonitrile 1% Formic acid and then vortexed for 30 sec. The sample was then centrifuged 5 min at 10,000 *xg*. The supernatant was removed from each sample and added to 20 mL of water. Then 15 uL of internal standard which included 65 μM of citric acid (2,2,4,4,-D4) (Cambridge Isotope Laboratories, Inc., Andover, MA) and 65 μM of citrate (Sigma-Aldrich Corp., St. Louis, MO). The solid phase extraction columns (12 cc, Waters Oasis, Milford, MA) were conditioned with 12 mL of methanol followed by 12 mL of water. After conditioning, the sample was slowly passed through the columns which were then washed with 12 mL of 5% methanol. The samples were eluted from the columns and captured by slowly adding 6 mL of acetonitrile:methanol (90:10). The samples were dried with heated vacuum using a Savant SpeedVac (ThermoFischer Scientific, Waltham, MA) and resuspended in 150 μL of

methanol:water (2:1) and centrifuged at 10,000 *xg* for 5 min before they were transferred to chromatography vials. Liquid chromatography Mass Spectrometry was accomplished with by Waters Acquity (Waters, Waltham, MA) connected to a signal quad detector mass spectrometer. A 10CM x 2.1mm, 2.7µm C18 high performance liquid chromatography (HPLC) column (Supelco, Ascentis Express 90, Sigma Aldrich) was used for sample separation. Column temp was set at 50^{o}C, and the flow rate was 0.3 mL per minute. The gradient began with 25% A, 5% B, 70% D. and flowed for 4 minutes, 40% A, 10% B, 50% D for 1 minute, then returned to 25% A, 5% B, 70% D for 2 minutes (A = acetonitrile, B = methanol, D = 0.1% formic acid water). The mass spectrometer was set to electro spray ionization negative; the dwell seconds were 0.03, and the cone voltage was 30. The masses monitored were SIR mass 191 for citrate and 199.3 for labeled citrate.

Retinoic Acid Extraction

Liquid chromatography grade formic acid, methanol, and acetonitrile used for extraction and analysis as well as potassium phosphate buffer components dibasic potassium phosphate and monobasic potassium phosphate were purchased from Sigma Aldrich (Waltham, MA, USA).During extractions all plasma samples and standards were handled under amber light. Plasma samples were thawed on ice. Potassium phosphate buffer was made by mixing 1.017 g dibasic potassium phosphate (K_2HPO_4) and 12.814 g of monobasic potassium phosphate (KH_2PO_4) and dissolved in 800 mL distilled water. Once dissolved, potassium phosphate buffer volume was brought to one L with distilled water to achieve a 0.1 M solution and the pH was adjusted to 5.4. The deuterated internal standards all-trans retinoic acid-d_5 and 13-cis-retinoic acid-d_5 were dissolved in DMSO at 6.55 mM and 3.27 mM, respectively. The internal standards included all-trans retinoic acid, 9-cis retinoic acid, isotretinoin (13-cis retinoic acid), all-trans

retinoic acid-d_5, and 13-cis-retinoic acid-d_5 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Serial dilutions were performed to dilute internal standards to 100 nM. A standard curve was created by dissolving all-trans retinoic acid, 9-cis retinoic acid, and 13-cis retinoic acid in DMSO to 66.6 mM, 3.33 nM, and 3.33 mM, respectively. The standard curve was created by diluting standards to 75 μM, 15 μM, 3 μM, 0.6 μM, 0.12 μM, and 0.024 μM which were placed into amber chromatography vials and stored at -20 °C until analysis. Next, 2 mL of plasma was combined with 4 mL 100% ethanol, 8 mL of 0.1 M potassium buffer, and 20 μl of internal standards and mixed by vortex for 1 min. Then, 1 mL of hexane was added and samples were again mixed by vortex for 5 min. Samples were centrifuged at 1,449 × *g* for 10 min at 4 °C. After centrifugation, the supernatant was removed and put into a clean vial. The steps of adding hexane, mixing, and centrifugation were repeated twice more with the supernatants of each sample combined into a single vial each time resulting in approximately 3 mL of hexane supernatant per sample. Samples were dried under vacuum using a Savant SpeedVac (Thermo Quest, Hollbrook, NY, USA) for approximately 20 min at 45 °C and resuspended in 150 μl of 2:1 methanol:water with 0.1% formic acid and placed into chromatography vials. Samples were stored at -20 °C until analysis.

Liquid chromatography tandem mass spectrometry analysis

Samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using Waters acquity UPLC with Waters Xevo-TQ-S (Waters Corporation, Milford, MA, USA). The chromatography separation was performed on a C18 1.7 uM 2.1 x 150 mm column at 50 °C (Waters Corporation). The autosampler was maintained at 10 °C. Mobile phase bottle A was 0.1 % formic and mobile phase bottle D was acetonitrile. The flow rate was 0.3 mL/min and each sample ran for approximately 10 min. The linear gradient steps were (using A:D ratio): 60:40 for

0.3 min with a slow change to 20:80 at 2 min, at 6.5 min 1:99 held until 8 min, at 8:01 min the ratio reversed back to 60:40 and held until 10 min. All retinoic acid isomers were detected using electrospray ionization in negative mode. QuantOptimize software (Waters) was used to optimize cone voltages and collision voltages for each isomer. Target MassLynx software was used to analyze peaks for retinoic acid isomer concentrations.

Statistical analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc, Cary, NC, USA) and graphs were created using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The experimental unit was the cow. Variables were analyzed for normality using Q-Q plots and histograms. Non-normally distributed variables included SCC, milk yield the day before dry off ATRA, 9CisRA, and 13CisRA which were transformed prior to analysis. Variables used to block cows upon enrollment included previous lactation milk yield, SCC, parity, and BLV status. Differences between treatment groups in previous lactation milk yield, SCC, parity, and BLV status were assessed by T-test using PROC ANOVA. The D-7 samples were collected prior to treatment and were used to test for innate differences in variables between treatment groups with T-tests using PROC ANOVA. The effects of sampling period, treatment, and treatment and sampling period interactions on each plasma retinoid was tested by creating a mixed model with repeated measures using an auto regressive covariance structure with PROC MIXED. Within the repeated measures mixed model, cow was the random effect, treatment and sampling period were dependent variables, and retinoid concentrations were the independent variable. Bonferroni correction was used to control type-1 errors in pairwise differences and adjust for multiple comparisons. When treatment did not affect retinoic isomer concentrations, treatment was removed from the model to better assess the effects of sampling period over the dry period on

retinoic acid isomers. The effects of sampling period on retinoid isomers over dry-off were assessed using PROC MIXED with cow as the random effect, sampling period as independent variable, and retinoid concentrations were dependent variables. Bonferroni correction was used to correct of multiple comparisons and results were considered significant at $p < 0.05$. Results were back-transformed and displayed at geometric means (Figure 1).

Regression models were created in SAS 9.4 to test the association between retinoic acid isomer concentrations and biomarkers of redox balance, inflammation, and mammary gland involution during the transition from lactation to non-lactation. Using the PROC MIXED command, models were built for each physiological category (redox balance, inflammation, and mammary gland involution). Correlation analysis was performed with PROC CORR to assess correlations between retinoic acid isomers and correlation between biomarkers within each physiological category. Spaghetti plots were created using R (R Studio, Boston, MA) for each variable to assess whether the variable relationship with time was linear, quadratic, or polynomial. Further analysis was performed using PROC MIXED to identify the most appropriate type of regression for each variable. Analysis for linear, quadratic, or polynomial regression was completed for each variable and the best fit between the independent and dependent variables was decided based on the lowest Akaike information criterion (AIC) and level of significance ($P < 0.05$). The explanatory candidate variables for each model included treatment (pegbovigrastim vs. control) and parity group (1, 2, 3+). Backward selection was used to select variables for the final model with an α of 0.05 and variables that were not significant were systematically removed from the model. Although prior analyses demonstrated no effect of treatment, treatment was included in the models to investigate any potential treatment effects which may be associated in combination with multiple biomarkers. The Tukey adjustment was used to account for multiple comparisons

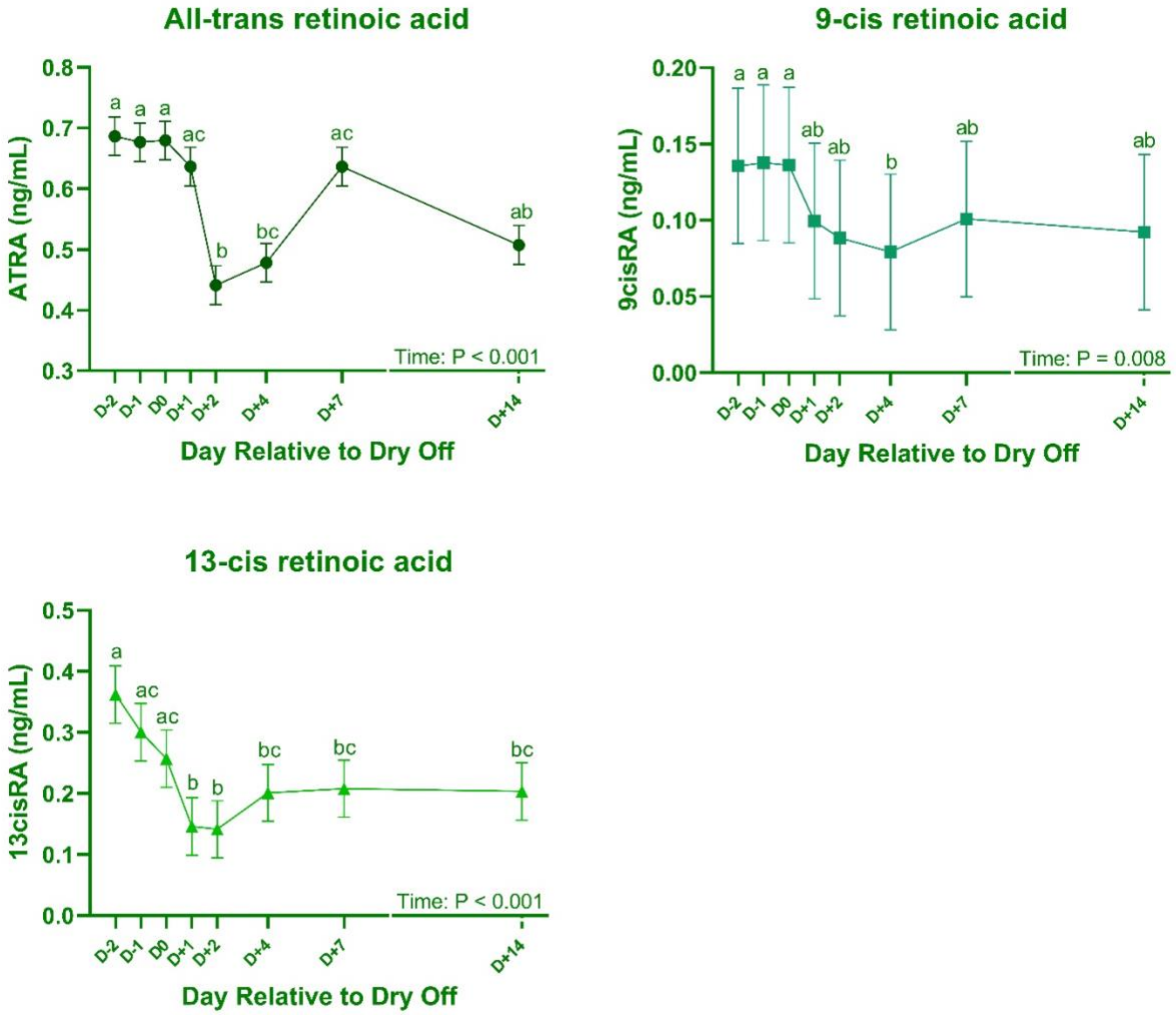
between levels of categorical variables. The redox balance model was built to explore the association between redox balance in serum and milk with retinoic acid isomer concentrations during early mammary gland involution. Separate redox models were built for redox balance in serum and in milk as opposed to a single model to improve model fit and decreases in error. Candidate variables for the systemic redox balance model included serum ROS, AOP, oxidative stress index (OSi), interactions between redox biomarkers, daily milk yield on D-1 (MY), and sampling time point. Candidate variables for the milk redox balance model included milk ROS, AOP, OSi, interactions between milk redox biomarkers, MY, and sampling time point. The inflammation model contained systemic inflammatory biomarkers such as serum albumin, haptoglobin, non-esterified fatty acids (NEFA), MY, and sampling time point. The involution model included biomarkers for mammary gland involution in milk including milk lactoferrin, citrate, serum albumin, MY, and sampling time point. Final models were determined by the significance of continuous variables, goodness of fit, and monitoring coefficient estimates for evidence of multicollinearity.

RESULTS

No differences were found between treatment groups for SCC, BLV status, previous lactation milk yield, or parity (data not shown, $P > 0.33$). Furthermore, none of the retinoic acid isomers were different between treatment groups on D-7 and therefore the D-7 sampling point was not included in further models as covariate ($P > 0.79$). The cows in this study ($n = 20$) had a mean parity of 1.8 (SE= 0.2,), 323 DIM (SE: 8.3), 57.5 days dry (SE: 1.2), 92,750 SCC (SE: 21,830, milk production at dry-off 25.5 kg/d (SE:2.2,), and a mean previous lactation milk yield of 13,078 kg (SE: 634).

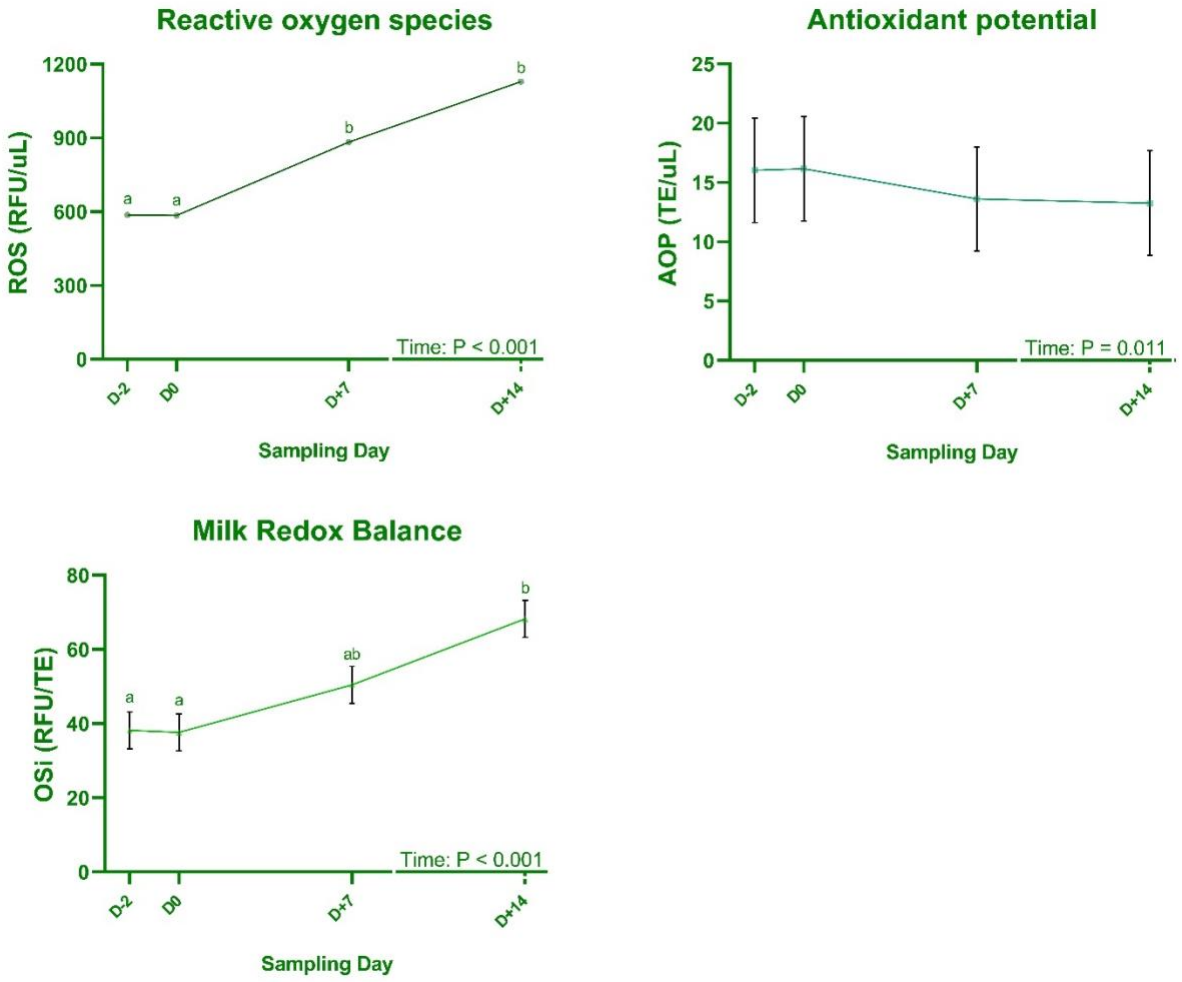
Concentrations of ATRA, 9CisRA, and 13CisRA were associated with time (Figure 1; $P < 0.008$). Plasma ATRA decreased after dry off to 0.44 ng/mL and was less than pre-dry concentrations on D+2 and D+4 ($P = 0.021$) and then did not vary through D+14 ($P > 0.06$: Figure 1). Similarly, plasma concentrations of 9CisRA decreased after dry off and reached the least concentration on D+4 (0.079 ng/mL; $P = 0.038$, Figure 1). Plasma concentrations of 13CisRA also decreased after dry off and were least on D+1 and D+2 ((0.14 ng/mL; $p < 0.001$, Figure 1) as compared to the lactating period. Concentrations of 13CisRA did not recover to pre-dry concentrations through D+14 ($P = 0.0.06$; Figure 1). There were no effects of PEG treatment on plasma concentrations of ATRA, 9CisRA, or 13CisRA during the transition from lactation to mammary gland involution (data not shown; $P > 0.22$). There were no effects of PEG on milk redox biomarkers milk ROS, AOP, or OSi (data not shown; $P > 0.27$). Milk ROS, AOP, and OSi varied during the transition from lactating to nonlactating ($P = 0.011$; Figure 2). No biomarkers included in the systemic redox balance model were affected by PEG (data not shown; $P = 0.10$), but serum ROS, AOP, and OSi varied over time ($P = 0.014$; Figure 3). Additionally, there were no effects of PEG treatment on serum inflammatory biomarkers including serum albumin, haptoglobin, and NEFA (data not shown, $P = 0.16$), but all variables changed significantly over time ($P < 0.001$; Figure 4). Finally, no mammary involution biomarkers were affected by PEG (data not shown; $P = 0.28$). Milk concentrations of serum albumin and lactoferrin increased after dry off ($P < 0.001$; Figure 5). Milk concentrations of citrate, however, did not change over the study period ($P = 0.36$; Figure 5).

Figure 5.1: ***Concentrations of retinoic acid isomers during the transition from the lactating period to the nonlactating period***



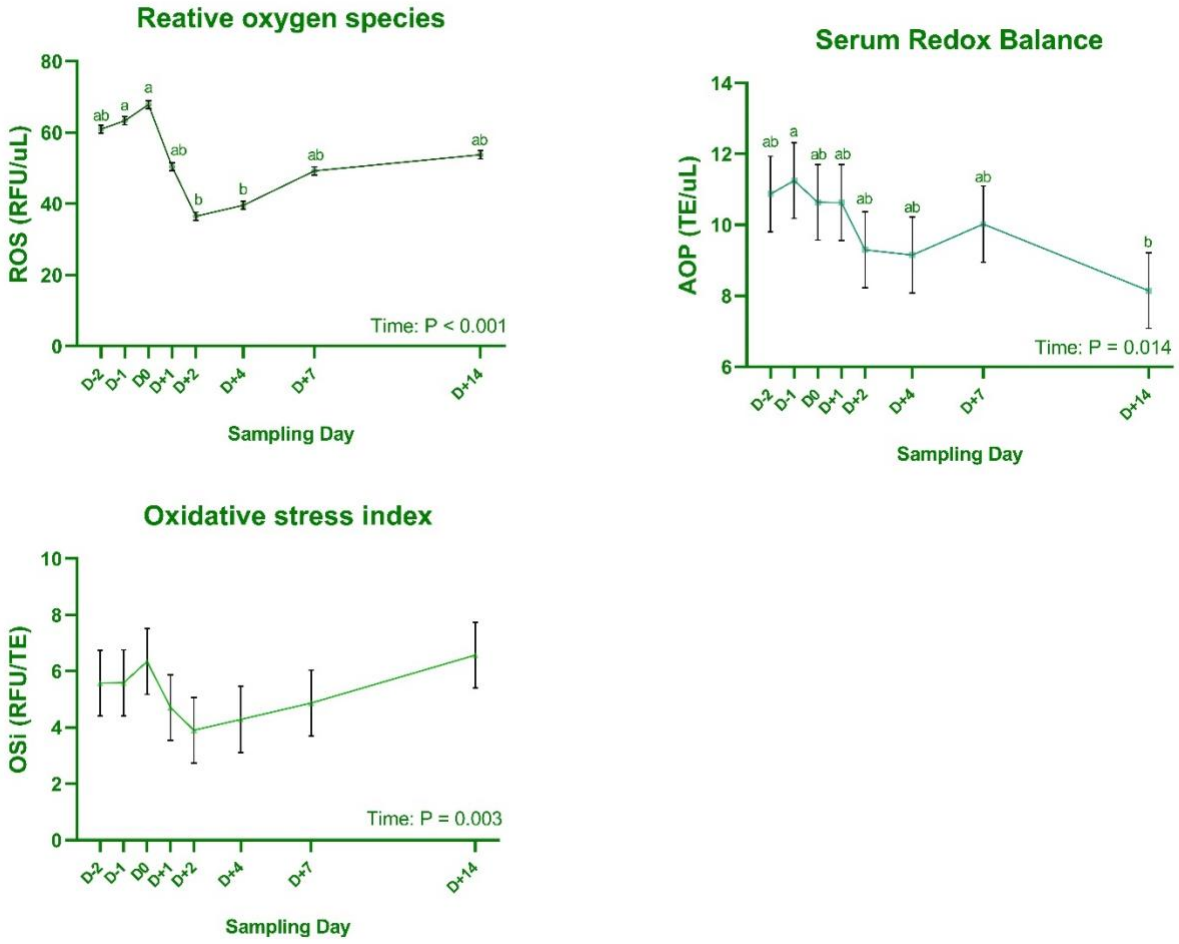
Concentrations of all-trans retinoic acid, 13-cis retinoic acid, and 9-cis retinoic acid as geometric means +/- standard error during the transition from lactation to mammary gland involution.
Differing superscripts (a, b, c) note means at different time points differ (P = 0.04)

Figure 5.2: ***Milk redox balance biomarkers during the transition from the lactating period to the nonlactating period***



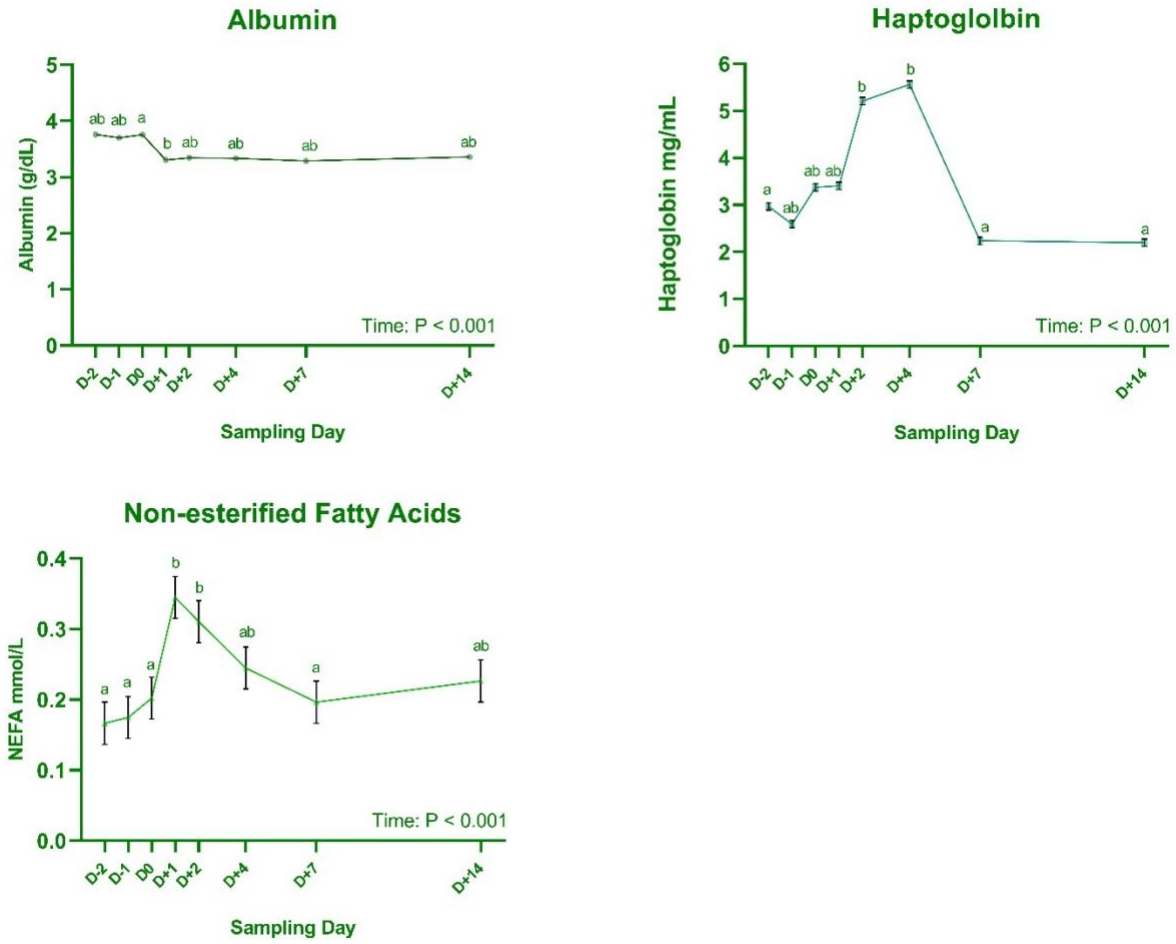
Concentrations of milk reactive oxygen species (ROS), antioxidant potential (AOP), and the oxidative stress index (OSi) as geometric means +/- standard error during the transition from lactation to mammary gland involution. Differing superscripts (a, b) note means at different time points differ (P = 0.01)

Figure 5.3: ***Systemic redox balance biomarkers during the transition from the lactating period to the nonlactating period***



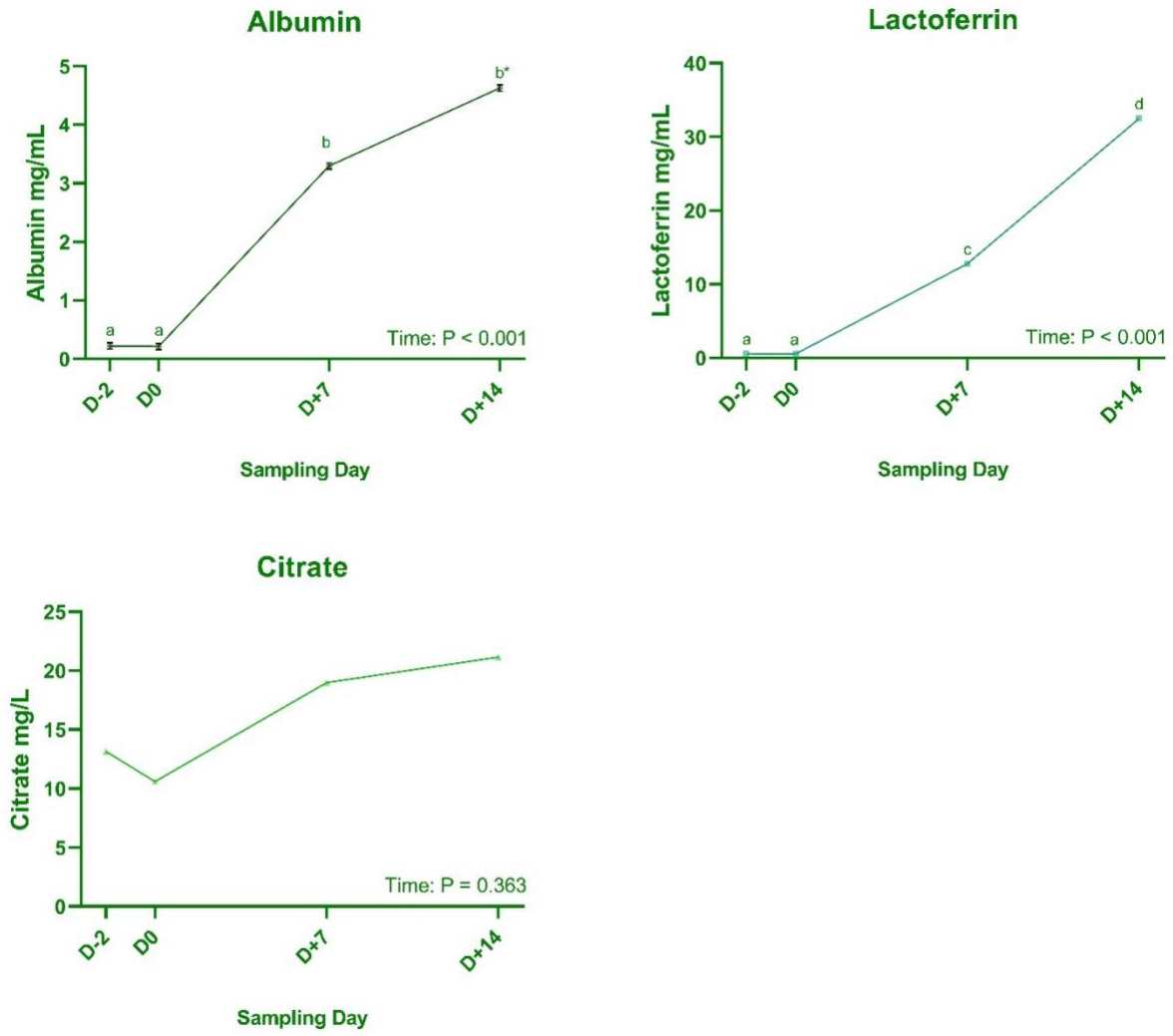
Concentrations of serum reactive oxygen species (ROS), antioxidant potential (AOP), and the oxidative stress index (OSi) as geometric means +/- standard error during the transition from lactation to mammary gland involution. Differing superscripts (a, b) note means at different time points differ (P = 0.04)

Figure 5.4: ***Inflammation biomarkers during the transition from the lactating period to the nonlactating period***



Concentrations of serum inflammatory biomarkers including albumin, haptoglobin, and non-esterified fatty acids as geometric means +/- standard error during the transition from lactation to mammary gland involution.
Differing superscripts (a, b) note means at different time points differ (P = 0.047)

Figure 5.5: ***Mammary gland involution biomarkers during the transition from the lactating period to the nonlactating period***



Concentrations of mammary gland involution biomarkers in milk secretions including serum albumin, lactoferrin, and ctrate as geometric means +/- standard error during the transition from lactation to mammary gland involution. Differing superscripts (a, b, c, d) note means at different time points differ (P = 0.003)

Concentrations of ATRA, 9cisRA, and 13cisRA were all significantly correlated with each other (P< 0.001; Table 2). Therefore, to avoid collinearity only ATRA was used in the involution models. The final model for milk redox balance included time (P =0.001), MY (P = 0.011), parity (P = 0.046), and treatment (P = 0.34; Table 3). Parity 3+ cows had lower serum ATRA than parity 2 cows (P = 0.03; Table 3). The final systemic redox model included MY (P = 0.013), serum ROS (P = 0.001), serum ROS x time interaction (P = 0.005), serum OSi (P = 0.002),

parity (P = 0.08), and treatment (P = 0.65; Table 4). The final inflammation model contained haptoglobin (P = 0.03), time (P = 0.002), MY (P = 0.013), parity (P = 0.07), and treatment (forced; P = 0.51; Table 5). The final mammary gland involution model contained MY (P = 0.009), milk citrate concentrations (P = 0.02), parity (P = 0.15), and treatment (forced; P = 0.26; Table 6). There were no differences between treatment or parity in the mammary gland involution model (P = 0.16).

Table 5.2: ***Correlation analysis of retinoic acid isomers***

	ATRA	9cisRA	13cisRA
ATRA[1]	1	0.65738	0.25907
		<.0001	0.0005
9cisRA		1	0.43222
			<.0001
13cisRA			1

[1] ATRA: all-trans retinoic acid, 9cisRA: 9-cis retinoic acid, 13cisRA: 13-cis retinoic acid

Table 5.3: ***Polynomial mixed model results for milk redox balance model***

Variable	Coefficient	SEM	LSM	SE for LSM	T value	P-value
Intercept	-0.458	0.091			-5.03	0.0001
Milk Yield	0.048	0.019			2.55	0.022
Time	-0.00015	0.000047			-3.28	0.0013
Treatment						
Pegbovigrastim	Referent	0.02099				
Control	-0.0284	0.0295	0.9368	0.029	-0.96	0.3521
Parity group						
1	0.0496	0.091	0.8568	0.0458	1.47	0.16
2	0.117	0.048	1.1209	0.0338	2.45	0.027
3+	Referent					

Table 5.4: ***Polynomial mixed model results for systemic redox balance model***

Variable	Coefficient	SEM	LSM	SE for LSM	T value	P-value
Intercept	-0.86	0.17			-5.02	<0.001
Milk Yield	0.054	0.022			2.52	0.029
ROS	0.265	0.083			3.19	0.002
Osi	-0.014	-0.014			-3.11	0.002
TreatmentROS*time	-0.000008	0.000027			-2.87	0.006
Control	-0.015	0.034	0.59	0.024	-0.46	0.66
Pegbovigrastim	Referent	0.03	0.61	0.025		
Parity group						0.08
1	0.05	0.0538	0.59	0.02	1.33	0.38
2	0.118	0.054	0.69	0.04		0.05
3+	Referent	0.05	0.520	0.03		0.07

Table 5.5: ***Polynomial mixed model results for inflammation model***

Variable	Coefficient	SEM	LSM	SE for LSM	T value	P-value
Intercept	-0.509	0.096			-5.3	<0.001
Milk Yield	0.049	0.019			2.51	0.024
Time	-0.0002	0.00005			-3.2	0.002
Haptoglobin	-0.093	0.043			-2.15	0.03
Control	-0.02	0.03	0.58	0.02	-0.67	0.51
Pegbovigrastim	Referent	0.03	0.61	0.02		
Parity group						
1	0.04	0.035	0.58	0.02	1.18	0.29
2	0.11	0.049	0.69	0.04	2.3	0.47
3+	Referent	0.04	0.53	0.03		0.06

Table 5.6: ***Polynomial mixed model results for mammary gland involution model***

Variable	Coefficient	SEM	LSM	SE for LSM	T value	P-value
Intercept	-0.4	0.13			-3.04	0.007
Milk Yield	0.07	0.03			2.67	0.02
Citrate	-0.06	0.03			-2.25	0.03
Treatment						
Conrtol	-0.04	0.04	0.62	0.028	-1.14	0.27
Pegbovigrastim	Referent		0.68	0.028		
Parity group						
1	0.001	0.05	0.60	0.028	0.03	0.98
2	0.113	0.06	0.77	0.028	1.79	0.09
3+	Referent		0.59	0.028		

DISCUSSION

For the first time, this study demonstrated that the active forms of vitamin A (ATRA, 9cisRA, and 13CisRA) decrease in plasma concentration during the early active involution period in dairy cows. These findings indicate that RA isomers may affect physiological aspects of mammary gland involution. It is well known that the transition from lactating to non-lactating state is a critical juncture for mammary gland health and function (Dingwell et al., 2003). Concentrations of serum retinol and its active metabolites (such as ATRA) have been associated with decreased disease risk including mastitis (LeBlanc et al., 2004a, Van Merris et al., 2004). Moreover, ATRA enhances functions of entities essential to mammary gland involution such as immune cell function and protease function (Chew, 1987, Zaragoza et al., 2007). Therefore, investigating associations between RA isomers and processes involved in mammary gland involution could identify targets for optimization of mammary gland involution, mammary gland health, and milk production. The decrease in plasma concentrations of ATRA after dry off that we observed was surprising because plasma ATRA concentrations increased in response to experimental coliform mastitis in dairy cows (Van Merris et al., 2004). The immune response during mammary gland involution we observed was not as severe as inflammation induced by clinical mastitis in the aforementioned study which (Van Merris et al., 2004). The cows in our study were healthy throughout the duration of the study. However, early mammary gland involution involves the inflammatory response as evidenced by upregulation of mammary tissue gene transcription and inflammatory mediators in milk related to the acute inflammatory response during the first 2-3 weeks after dry off (Boggs et al., 2015, Dado-Senn et al., 2018). Although coliform mastitis and mammary involution do not elicit equivalent immune responses, Van Merris et al. (2004) also reported a repression of plasma 13CisRA concentrations after the onset of inflammation, similar to our study (Van Merris et al., 2004). Additionally, increased plasma 13cisRA was associated

with elevated levels of mammary epithelial cell apoptosis the day after calving (Puvogel et al., 2005). These findings indicate that 13cisRA may have a role in factors essential to mammary gland involution (inflammation and mammary epithelial cell apoptosis), and therefore requires further investigation.

Milk yield prior to dry off was associated with ATRA concentrations in mammary gland involution, inflammatory biomarkers, and systemic and mammary gland redox balance. High levels of milk production at dry off is a growing concern as advances in nutrition, genetics, and fertility result in ever greater milk yields in later lactation. Indeed, a recent large study reported that half of the study population were producing >28 kg/d milk at dry off (Olagaray et al., 2020). This result was significant because as compared to lower producing cows, herd mates producing >20 kg/d milk at dry off had increased mammary gland pressure and fecal glucocorticoids during the first week of the dry period (Bertulat et al., 2013). Moreover, every 5 kg above 12.5 kg/d milk at dry off increased the odds of mastitis at parturition 1.8 fold (Rajala-Schultz et al., 2005). The average milk yield at dry off in our study exceeded 20 kg/d indicating they were potentially at increased risk for negative effects on mammary gland health. Thus, association with milk yield prior to dry off and ATRA indicates that ATRA may be associated with mammary gland health. Greater retinoic acid activity during early mammary gland involution in rats resulted in decreased milk production and increased proteases important for mammary tissue remodeling (Zaragoza et al., 2007). Cows supplemented with 8-fold the recommended vitamin A concentrations during the dry period had increased milk ATRA prior to calving, increased apoptosis in milk secreting epithelial cells, and moderately decreased milk production during the subsequent lactation as compared to supplemented controls (Puvogel et al., 2005). Although the milk ATRA analytical methods were not ideal in the Puvogel et al. (2005) study, it may indicate

that ATRA may decrease milk yield in cows. Decreasing milk yield prior to dry off with ATRA prior to dry off could be beneficial because it is associated with improved cow welfare and decreased mastitis risk during the dry and early lactation period (Gott et al., 2016, Vilar and Rajala-Schultz, 2020). However, future investigations must protect samples for UV exposure and identify RA isomers by LC-MS/MS to minimize post sampling degradation and misidentification of isomers.

Concentrations of ATRA were significantly associated with a pro-oxidant balance in serum, but not with any redox parameters milk during mammary gland involution. Contradictions in biomarkers of oxidative stress can occur among different body compartments may reflect the respective homeorhetic responses to different stimuli. For example, isoprostanes are products of oxidative damage to lipids and are considered to be direct measures of oxidative stress (Celi, 2011). In healthy dairy cows, 15-F_{2t}-isoprostane decreased in plasma, but increased in milk, when comparing periparturient cows with mid-lactation cows (Kuhn et al., 2018). The increased oxidative stress in the periparturient period may have been related to the increased ROS production that can occur as a result of negative energy balance and NEFA mobilization (Sordillo and Mavangira, 2014). In contrast, peak lactation cows may have increased oxidative stress in milk as a result of increased ROS production as a by-product of increased mitochondrial function for milk production (Celi, 2011). The differences between the systemic and mammary redox models may also be a consequence of the difference between blood and milk components. In cows with coliform mastitis, free plasma 15-F_{2t}-isoprostane was highly correlated with plasma ROS ($r = 0.83$), but free milk 15-F_{2t}-isoprostane was negatively correlated with milk ROS ($r = -0.86$) (Mavangira et al., 2016). Mavangira et al. (2016) hypothesized that lipids, including isoprostanes, are primarily esterified and contained within fat globules. Given these findings, it

may be difficult to use a serum biomarker, such as ATRA, to determine the magnitude of oxidative stress in the involuting mammary gland. Milk ATRA was not analyzed in this study, but it would be interesting to investigate the potential association between milk ATRA and milk oxidative stress.

Haptoglobin is an positive acute phase protein because liver secretion increases in response to inflammation resulting in a significant increase in serum concentrations (Eckersall and Bell, 2010). During mammary gland involution circulating concentrations of haptoglobin increase and the magnitude change is positively associated with milk production prior to dry off (Putman et al., 2018, Mezzetti et al., 2020). This agrees with the findings of the current study because both MY and haptoglobin were significantly associated with ATRA in the inflammation model. This indicates that milk yield prior to dry off may be associated with increased inflammation along with ATRA. Lactoferrin is an iron-binding protein that increases in milk secretions during early involution and is thought to slow bacterial growth by binding free iron (Shimazaki and Kawai, 2017). In-vitro analysis of bovine epithelial cells demonstrated that ATRA was essential for translocation of lactoferrin in the cell and thus may prove critical for lactoferrin secretion during involution (Baumrucker et al., 2006). In mice the RA signaling pathway is fully activated demonstrating the increased uptake of RA, transfer of RA to the nucleus, and metabolism of RA (Cabezuelo et al., 2019). Lactoferrin was not associated with systemic ATRA in the current study, but future research should investigate the association of lactoferrin and RA isomers in mammary tissue.

Citrate is produced and secreted by mammary epithelial cells converting organic molecules into energy as part of the tricarboxylic acid cycle (Faulkner and Peaker, 1982). Citrate does not effectively permeate the mammary gland from blood and is highly associated with de novo fat

synthesis making it an excellent biomarker for mammary epithelial cell function (Faulkner and Peaker, 1982). Prior studies investigated milk citrate as a biomarker for mammary gland involution because it decreased in milk secretions after dry off {Zhao, 2019 #2024, Garnsworthy et al., 2006). In the present study, milk citrate concentrations did not vary during early mammary gland involution. Increased milk production prior to dry off did prevent the decrease in milk citrate that occurred in low producing cows, but samples were only collected for 3 d after dry off (Silanikove et al., 2013). The reason for differing results may have been due to the differences in previous studies which included enzymatic methods which were sensitive but could not specify between citrate and its isomer isocitrate (Wong, 2012) or citric acid (Boutinaud et al., 2016). A comparison of urine citrate analysis between mass spectrometry and enzymatic methods did not find robust agreement between methods (Keevil et al., 2005). Milk citrate concentrations can also vary as a result of season, diet, mastitis, and vary between cows (Garnsworthy et al., 2006, Hyvönen et al., 2010). This is the first study to our knowledge to analyze milk citrate by LC-MS during early mammary gland involution. More research is needed to investigate how the agreement between different citrate assay methods in milk and milk secretions is needed. Retinoic acid concentrations may be determined in part by availability of precursors which include retinol, retinal, and retinyl esters (Blomhoff and Blomhoff, 2006). Serum retinol concentrations were not evaluated as part of this study. It is possible that decreased serum retinol concentrations may have affected substrate availability and resulted in decreased RA isomers. However, in cows that are supplemented with vitamin A during the dry period, serum retinol does not decrease during the dry period (Puvogel et al., 2005, Strickland et al., 2021). Puvogel et al. (2005) did find a moderate increase in milk RA isomers, but only after 60 d of 8-fold the recommended dietary vitamin A supplementation compared to supplemented cows. Regardless

of dietary supplementation, circulating concentrations of retinol do not tend to deviate until hepatic reserves reach 20 μg/g (Kohlmeier and Burroughs, 1970). Therefore, it is not very likely that dietary vitamin A concentrations would have a great affect.

Differences in sample handling and analysis of RA isomers may account for differing results from prior research. The methods for sample handling, extraction, and quantification can greatly affect the accuracy of retinoic acid analysis. High performance liquid chromatography (HPLC) was used in previous studies to identify concentrations of RA isomers in cattle (Van Merris et al., 2004, Puvogel et al., 2005, Agustinho et al., 2021). The RA isomers have overlapping ultraviolet spectral profiles and so individual isomers cannot be identified without chromatographic separation and results will have inflated concentrations (Kane et al., 2008). HPLC lacks the sensitivity required for analysis of RA isomers (Kane and Napoli, 2010). In particular, 9-cisRA commonly coelutes with any of the other RA-isomers and therefore is rarely reported (Kane, 2012). We were able to successfully identify 9cisRA and are the first to report endogenous 9cisRA concentrations in cattle to our knowledge. The accurate identification of 9cisRA is important because it binds to retinoid X receptors and has many effects on immune function, development, and metabolism which are unique from ATRA (Kane, 2012). Retinoids can stick to plastic and results could be compromised which is why we collected blood samples in glass tubes and stored plasma in glass vials in this study (Kane and Napoli, 2010). Additionally, samples must be protected from UV light from the time of collection on farm to analysis in the laboratory to prevent degradation and isomerization (Kane et al., 2008). Our samples were handled and analyzed according to these strict parameters and may provide the most accurate insight of RA isomer dynamics in the dairy cow to date.

CONCLUSION

This study demonstrated for the first time that concentrations of ATRA, 9CisRA, and 13CisRA decrease in plasma during the transition from lactation to mammary gland involution in healthy dairy cows. We also found that ATRA is associated with biomarkers of redox balance, inflammation, and MEC activity during mammary gland involution. There was no effect of pegbovigrastim on any of the biomarkers investigated in this study. The current study was limited in sample size, but cows were healthy and sample handling and analysis were performed at the highest standard. Therefore, the changes in biomarker concentrations likely reflected the physiological processes of mammary gland involution. Further research should investigate the associations between RA isomers in plasma and milk to better identify the effects of RA isomers in the mammary gland.

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CHAPTER 6: SUMMARY

Mammary gland involution which occurs after the abrupt cessation of lactation is a vulnerable period for dairy cows in the lactation cycle with implications for mammary gland health and milk production. A better understanding of the processes involved in mammary gland involution and how they relate to future health and production could reduce disease in dairy cattle and improve dairy cow welfare. Biomarkers associated with mammary gland involution (including vitamins and lipid mediators) found in blood and milk can provide valuable insight into physiological processes that occur during the transition from lactation to the dry period. Serum vitamins including retinol, β-carotene, vitamin D, and α-tocopherol are commonly analyzed in dairy cows to assess the dietary supplementation sufficiency. However, we found that serum retinol was negatively associated with fresh cow disease and vitamin D was positively associated with antioxidant potential during the periparturient period. These findings indicated that serum vitamins may provide valuable insight into inflammatory, metabolic, and redox processes occurring between dry off and early calving. During early mammary gland involution, there were variations in lipid mediators in milk secretions after dry off. Indeed, lipid mediators associated with both pro- and anti-inflammatory processes changed in response to dry off which indicated that mammary gland involution in healthy cows was associated with inflammation and oxidative stress. Finally, we found that plasma concentrations of all-trans retinoic acid, 9-cis retinoic acid, and 13-cis retinoic acid decreased in concentration after dry off. Furthermore, we used regression models to investigate the association of plasma all-trans retinoic acid with other biomarkers of mammary gland involution during the transition from lactation to involution. All-trans retinoic acid was associated with serum haptoglobin, a systemic pro-oxidant balance, and citrate concentrations in milk secretions. These findings indicated that retinoic acid may have

had some effects on inflammation, oxidative stress, and mammary epithelial cell function during involution. Overall, this body of work demonstrates that fat-soluble vitamins and lipid mediators likely represent complex physiological processes and could be useful biomarkers for mammary function in dairy cows.

Many unanswered questions arose as a result to our studies. Future studies should further investigate the validity of fat-soluble vitamins and lipid mediators as biomarkers for mammary gland involution. Both fat-soluble vitamins and lipid mediators exist at the nexus of inflammation, oxidative stress, and metabolism and thus represent many important characteristics of mammary gland involution. In addition to serving as biomarkers, vitamins and lipid mediators can be altered through dietary supplementation. If these alterations could be utilized to optimize mammary gland involution, mammary gland involution and production could be improved without the use of antimicrobials.