UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

CYTOLOGICAL ENDOMETRITIS IN DAIRY CATTLE: NEW INSIGHTS INTO PATHOGENESIS, DIAGNOSIS AND THERAPY

GONÇALO DA SILVA PEREIRA

Orientador(es): Doutora Maria Elisabete Tomé Sousa Silva

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade Clínica

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“Imperfect understanding is often more dangerous than ignorance”, Newt Scamander

J. K. Rowling

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“It takes a village to raise a child” is a popular saying that some believe is an ancient African proverb. The overall interpretation is that an entire community of people must care for children in order for them to grow in a safe and healthy environment. In many ways, a PhD student is like a child, and in the following lines, I will acknowledge the members of my “village”.

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Endometrite citológica em vacas leiteiras: Novos desenvolvimentos em patogénese, diagnóstico e tratamento

RESUMO

Em vacas leiteiras pós-parto, a endometrite subclínica (ESC) é caracterizada por inflamação persistente do endométrio, com profundos efeitos prejudiciais na fertilidade subsequente. Apesar do conhecido papel das adipocinas na regulação do metabolismo e da inflamação, a regulação destas moléculas em vacas com ESC é ainda pouco compreendida. Além disso, o efeito da ESC na transcrição de células do endométrio tem sido explorado a partir de biópsias endometriais totais, mascarando assim os efeitos na transcrição específica de cada tipo celular. Além disso, apesar da reconhecida modulação da função imune após a suplementação com ácidos gordos polinsaturados (PUFA) ómega-3, o potencial terapêutico dos mesmos para o controlo da ESC ainda não foi determinado. Assim, os principais objetivos deste trabalho foram avaliar a relação entre adipocinas e a ESC (Capítulo III), elucidar os efeitos da progesterona e ESC nos perfis de transcrição de células endometriais do estroma, epiteliais glandulares e epiteliais luminais (Capítulos IV, V), e os efeitos da suplementação com PUFA ómega-3 com protecção ruminal na homeostase endometrial e fertilidade em vacas leiteiras pós-parto (Capítulo VI).

Os resultados mostraram que a adiponectina no plasma e no lavado uterino, além da quemerina no líquido uterino, possuem alto poder discriminatório para o diagnóstico de ESC. Este trabalho evidenciou também que a progesterona e a ESC afetam os perfis de transcrição endometriais das diferentes populações celulares de uma forma específica, que a presença prévia de leucócitos ainda afeta o perfil de transcrição das células endometriais no final do período voluntário de espera e que a recuperação ou persistência da inflamação está associada a padrões de transcrição envolvidos não apenas na função imunológica, mas também na remodelação tecidular, adesão celular e receptividade uterina. Além disso, apesar de aparentemente não produzir efeito sobre o estado inflamatório endometrial no final do período voluntário de espera, a suplementação com PUFA ómega-3 diminuiu o intervalo parto-concepção.

No geral, esta tese estabelece a adiponectina como um biomarcador adequado de ESC, capaz de prever o risco de persistência de inflamação em vacas leiteiras no pós-parto, fornece novas perspectivas sobre a persistência e recuperação da ESC, apresentando assim novas hipotéticas estratégias terapêuticas. Além disso, este trabalho substancia os PUFA ómega-3 como nutracêuticos válidos para melhorar os parâmetros reprodutivos em vacas leiteiras no pós-parto.

Palavras-Chave: Vaca leiteira, Adipocinas, Ácidos gordos polinsaturados n-3, Transcriptoma, Endometrite subclínica.

Cytological endometritis in dairy cattle: New insights into pathogenesis, diagnosis and treatment

ABSTRACT

In postpartum dairy cows, subclinical endometritis (SCE) is characterized by persistent endometrial inflammation, with profound detrimental effects on subsequent fertility. Despite the known role of adipokines regulating metabolism and inflammation, the association of adipokine signaling with SCE is still poorly understood. Moreover, the effect of SCE on endometrial transcription was mainly determined from biopsy-derived whole tissue, thus masking effects on cell type-specific gene transcription. In addition, despite the recognized improvement in immune function following n-3PUFA supplementation, the therapeutic potential of these fatty acids for SCE control is still to be determined. Therefore, the main objectives of this work were to assess the relationship between adipokines and SCE (Chapter III), to elucidate the effects of progesterone and SCE in the transcription profiles of endometrial glandular, luminal and stromal cells (Chapters IV, V), and the effects of feeding rumen-protected n-3PUFA on endometrial homeostasis and fertility in postpartum dairy cows (Chapter VI).

The results showed that adiponectin in plasma and uterine fluid in addition to chemerin uterine fluid have high discriminatory power for the diagnosis of SCE. This work additionally evidenced that progesterone and SCE affect endometrial transcriptomic profiles in a cell type-specific manner. This work also evidenced that the previous presence of immune cells is still impacting the transcriptome of endometrial cells at the end of the voluntary waiting period and that recovery or persistence of inflammation is associated with transcription patterns involved not only in immune function but also in tissue remodeling, cell adhesion, and uterine receptivity. Furthermore, despite having no apparent effect on the endometrial inflammatory status at the end of the voluntary waiting period, dietary supplementation with n-3PUFA decreased calving to conception interval.

Overall, this thesis establishes adiponectin as a suitable biomarker of SCE, able to predict the risk of persistence of inflammation in postpartum dairy cows, provides new insights into the persistence and recovery of SCE, thus presenting putative alternative therapeutic strategies. Moreover, this work substantiates dietary n-3PUFA as valid nutraceuticals to improve reproductive parameters in postpartum dairy cows.

Keywords: Dairy cow, Adipokines, n-3 polyunsaturated fatty acids, Transcriptome, Subclinical Endometritis.

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LIST OF ABREVIATIONS

ADF	Acid detergent fiber
ADL	Acid detergent lignin
AGT	Angiotensinogen
AI	Artificial insemination
ALA	Alpha-linolenic acid
ANXA1	Annexin A1
ARA	Arachidonic acid
AUC	Area under the curve
BCS	Body condition score
BHB	β-hydroxybutyric acid
CCI	Calving-conception interval
CCRL2	C-C chemokine receptor-like 2
CL	Corpus luteum
CMKLR1	Chemokine like receptor 1
Cq	Quantification cycle
CYTO	Cytological endometritis
d	Days
DEG	Differentially expressed genes
DES	Desmin
DHA	Docosahexaenoic acid
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DPA	Docosapentaenoic acid
DPP	Days postpartum
E	Efficiency
E2	Estradiol
EMT	Epithelial-mesenchymal transition
EPA	Eicosapentaenoic acid
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FA	Fatty acid
FAME	Fatty acid methyl esters
FGF	Fibroblast Growth Factor
FOXA2	Forkhead Box A2

FPCM	Fat (4%) and protein (3.3%) corrected milk
G-CSF	Granulocyte colony-stimulating factor
GE	Glandular epithelial
GO	Gene ontology
GPR1	G Protein-Coupled Receptor 1
H	Healthy
INF	Interferon
INFT	Interferon tau
KDF1	Keratinocyte differentiation factor
Kg	Kilogram
LA	Linoleic acid
LCM	Laser capture microdissection
LE	Luminal epithelial
LPS	Lipopolysaccharide
MSX1	Msh Homeobox 1
n-3PUFA	Omega-3 Polyunsaturated Fatty Acids
NAMPT	Nicotinamide phosphoribosyl-transferase
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NSAID	Nonsteroidal anti-inflammatory drug
OR	Odds ratio
OXTR	Oxytocin receptor
P4	Progesterone
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCA	Principal component analysis
PE	Persistent subclinical endometritis
PFA	Paraformaldehyde
PGF2α	Prostaglandin F2α
PMN	Polymorphonuclear neutrophils
PPI	Protein-protein interaction
PUFA	Polyunsaturated fatty acids
QC	Quality control
RARRES2	Retinoic acid receptor responder protein 2
R	Recovery
RIN	RNA integrity number

ROC	Receiver operating characteristic
RT	Reverse transcription
SCE	Subclinical endometritis
SEM	Standard error of the mean
SOX17	SRY-Box transcription factor 17
SPM	Specialised pro-resolving mediators
ST	Stromal
TAC3	Tachykinin precursor 3
TGF-β	Transforming growth factor beta
TLR4	Toll-like receptor 4
TMR	Total Mixed Ration
TNF	Tumor necrosis factor
TPM	Transcripts per million
WPP	Weeks postpartum
WT1	Wilms' tumor suppressor gene

CHAPTER 1 – GENERAL INTRODUCTION

The dairy industry will face numerous challenges in the upcoming decades as production systems are expected to shift to more modernised dairy farms with higher yields per cow (Britt et al. 2018). The sustainability of dairy cattle farming systems relies largely on the ability of cows to maintain reproductive performance while coping with the negative energy balance imposed by increased milk production (Diskin and Lonergan 2014).In this context, dairy cows are expected to conceive in an optimal time interval following the previous calving to maintain economic viability. To do so, a physiological inflammation process termed uterine involution must occur in a timely manner. However, due to hampered host defence mechanisms and uterine bacterial contamination, this process is sometimes compromised, resulting in reproductive disorders characterised by persistent uterine inflammation and tissue damage (Sheldon et al. 2009a).

Unlike clinical presentations, cytological endometritis (CYTO), also referred to as subclinical endometritis (SCE), is often overlooked by practitioners and farmers due to the absence of clinical signs and straightforward diagnostic methods. Moreover, due to imperfect understanding of the mechanisms of endometrial inflammation persistence (Leblanc 2012) and inconsistent evidence of how successful the current therapeutic approaches are (Dubuc et al. 2011a; Haimerl et al. 2013; 2018), endometritis continues to have a significant economic impact in dairy cattle farming systems.

Adipokines, cytokines with both pro- and anti-inflammatory effects that are secreted mainly by adipose tissue (Mancuso 2016), have emerged as important regulators of metabolism and inflammation in several scenarios including inflammation and reproductive function (Reverchon et al. 2014a). Owing to the regulation of metabolism and inflammation by adipokines, these molecules exhibit promising potential for the elucidation of the link between negative energy balance and immune function in postpartum dairy cows. Therefore, it is hypothesized that these molecules can be useful biomarkers for the identification of cows affected by SCE.

Also, SCE is characterised by altered transcriptomic profiles of the endometrial cells. However, as in other heterogeneous tissues, quantification of gene expression from the whole endometrium may not reflect the specific transcription of the different cell types. Laser capture microdissection (LCM) emerged as a research tool to isolate cell populations for further molecular analyses (Bevilacqua and Ducos, 2018). This method shows exciting potential to elucidate how SCE might differentially regulate the transcription of the different endometrial cell types and how this regulation can be key to understand the mechanisms of endometrial inflammation persistence.

Moreover, diets enriched or supplemented with n-3 polyunsaturated fatty acids (PUFA) are known to benefit the health status in several species (Alagawany et al. 2021). Increased proportions of circulating n-3PUFA produce a shift in fatty acid composition of many body compartments, namely cell membranes, that favours eicosanoid synthesis towards anti-inflammatory and pro-resolving states (Serhan 2014). In, dairy cows supplementation with n-3PUFA interferes with the animal's physiology in different functions, namely reproductive and immune functions (Moallem 2018). It is hypothesised that feeding rumen-protected n-3PUFA sources would increase the blood n-3PUFA concentrations, resulting in enhanced endometrial homeostasis thus minimizing the detrimental effects of SCE in dairy systems.

The work here presented comprises a series of studies that aimed to: (**1**) describe and validate adipokines as biomarkers with diagnostic and prognosis applications in the management of SCE in postpartum dairy cows; (**2**) elucidate the mechanisms of endometrial inflammation persistence by deciphering the cell-specific transcription regulation exerted by progesterone and transient and persistent SCE in the endometrium of postpartum dairy cows; and (**3**) assess if feeding a rumen-protected n-3PUFA enriched fat supplement could ameliorate both the energetic deficit and immune status of postpartum dairy cows thus contributing to the control of SCE in postpartum dairy cows.

These studies resulted in 4 manuscripts, submitted to international peer-reviewed and indexed journals, which were converted in the 4 chapters of the experimental work in this thesis and 2 public databases with the transcriptomics data.

1. **Adipokines as biomarkers of postpartum subclinical endometritis in dairy cows.**
   G Pereira, R Bexiga, J Chagas e Silva, E Silva, C Ramé, J Dupont, Y Guo, P Humblot, L Lopes-da-Costa. 2020. Reproduction, 160, 417-430. https://doi.org/10.1530/REP-20-0183
2. **Progesterone differentially affects the transcriptomic profiles of cow endometrial cell types.**
   G Pereira, Y Guo, E Silva, C Bevilacqua, G Charpigny, L Lopes-da-Costa, P Humblot. 2022. *BMC Genomics*, 23, 82. https://doi.org/10.1186/s12864-022-08323-z
3. **Subclinical endometritis differentially affects the transcriptomic profiles of endometrial glandular, luminal and stromal cells of postpartum dairy cows.**
   G Pereira, Y Guo, E Silva, MF Silva, C Bevilacqua, G Charpigny, L Lopes-da-Costa, P Humblot. 2022. Under review in *Journal of Dairy Science*.

4. **Effects of feeding rumen-protected linseed fat to postpartum dairy cows on plasma n-3 polyunsaturated fatty acid concentrations and metabolic and reproductive parameters.**
   G Pereira, P Simões, R Bexiga, E Silva, L Mateus, T Fernandes, S P Alves, R J B Bessa, L Lopes-da-Costa. 2022. *Journal of Dairy Science*, 105, 361-374. https://doi.org/10.3168/jds.2021-20674
5. Database deposited in NCBI's Gene Expression Omnibus and accessible through GEO Series accession number GSE182932 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182932).
6. Database deposited in NCBI's Gene Expression Omnibus and accessible through GEO Series accession number GSE192545 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192545).

CHAPTER 2 – LITERATURE REVIEW

2.1 Context and background

Predictions for the upcoming decades are that world population and dairy consumption will continue to increase, leading to more modernised dairy farms and doubling global cow milk production by 2067 (Britt et al. 2018). Due to their limited potential to expand grassland areas and the need to reduce nitrogen leaching and greenhouse gas emissions, European Union countries are hence expected to shift to production systems with fewer cows and higher yields (EC 2018). To meet the expected rise in milk production, it is paramount to improve dairy cows' fertility as reproductive management is crucial for the dairy industry due to the necessity of a cow to calve regularly to produce milk and provide economic sustainability (Inchaisri et al. 2010).

In order to foster optimal fertility after parturition and placenta expulsion, dairy cows are required to: effectively control bacteria in the uterus; rapidly return to ovarian cyclical activity with ovulation of competent oocytes; and have a swift involution of the uterus with the restoration of a receptive endometrium (Sheldon et al. 2019). Uterine involution is the process by which the uterus returns to the pre-gravid state and comprises reduction in diameter (Mateus et al. 2002), weight (Sheldon et al. 2019) and regeneration of the epithelium (Sheldon et al. 2020). Ideally, the uterus weight decreases from 9 kg at parturition to 1 kg by 30 days postpartum (Sheldon et al. 2019). Moreover, like all ruminants, cows exhibit syndesmochorial placentation, meaning that following parturition and placental detachment, the caruncular regions will be devoided of epithelium, and these "wounds" will be exposed to any bacteria that might be present in the uterine lumen (Pascottini and LeBlanc 2020). Consequently, to restore the uterus receptivity, a well-timed and effective reparative inflammation process is of the utmost importance.

2.2 Postpartum uterine disease

Postpartum uterine diseases are the leading cause of reproductive inefficiency in dairy cattle (Pascottini 2016) costing the EU dairy industry €1.4 billion/year (Sheldon et al. 2009a). After parturition, most dairy cows readily experience intra-uterine bacterial contamination (Sheldon et al. 2009a). However, the development of uterine diseases is dependent on the balance between host defence mechanisms and the pathogenicity of the bacteria (Sheldon et al. 2009a). The modern dairy cow is the result of intense genetic selection for milk yield, often at the expense of increased incidence of uterine disease after parturition (Dobson et al. 2007; Sheldon et al. 2019). Due to the increased energy requirements for milk production, diminished dry matter intake, and resulting negative energy balance, postpartum dairy cows undergo a state of metabolic stress that impairs their immune response (Sheldon et al. 2018). This metabolic stress threatens the balance between host defence mechanisms and

bacteria pathogenicity compromising dairy cows' resilience to uterine infection. Additionally, the ability of bacteria to overwhelm the host immune system depends on the burden as well as the bacteria involved (Sheldon et al. 2019). It was common belief that the uterus was sterile during pregnancy becoming contaminated with nonspecific bacteria following parturition. However, there is now evidence that the endometrium has its own microbiome and that the uterus of virgin heifers as well as pregnant cows is not sterile and a microbiome including three phyla, *Firmicutes*, *Bacteroides*, and *Proteobacteria* was described (Moore et al. 2017). Moreover, the microbiome structure between cows that develop metritis and healthy cows is identical until 2 days postpartum, after which the structure of metritis cows deviates in favor of greater abundance of *Bacteroidetes* and *Fusobacteria* and lesser relative abundance of *Proteobacteria* and *Tenericutes* (Galvão et al. 2019). *Escherichia coli* is the most prevalent bacterium isolated during the first week postpartum from the uterus of cows that developed metritis (Williams et al. 2007). Its presence then establishes a suitable environment for *Fusobacterium necrophorum*, which was strongly associated with metritis (Galvão et al. 2019), and for *Trueperella pyogenes* in postpartum weeks 2 and 3, which in turn has been associated with chronic postpartum endometritis (Bicalho et al. 2012; Pascottini et al. 2020). These bacteria act synergistically to increase the likelihood and severity of disease with *T. pyogenes* acting as an opportunistic pathogen to cause pathology of the endometrium once the protective epithelium is lost as endometrial stromal cells are particularly sensitive to pyolysin (main virulence factor of *T. pyogenes)*, compared with endometrial epithelial cells or immune cells (Sheldon et al. 2019). Moreover, the more prevalent *E. coli* clonal types recovered from the uterus of postpartum dairy cows were present both in healthy and clinical metritis cows, indicating that other factors, namely the presence of other bacteria or the cow's defense mechanisms, could dictate the establishment and persistence of uterine infection (Silva et al. 2009). Furthermore, Pascottini et al. (2020) found no differences in the uterine microbiome between healthy and SCE cows, supporting the hypothesis that the cow's defense mechanisms, namely inflammation regulation, play an essential role in the development of postpartum uterine disease.

Postpartum uterine disease comprises several different clinical presentations and here the clinical definitions suggested by Sheldon et al. (2006) have been adopted.

2.2.1 Clinical metritis

Clinical metritis has been defined as a systemic illness characterized by an enlarged uterus, with a purulent uterine discharge ranging from watery red-brown fluid to thick off-white pus, often with a fetid odor. It usually occurs within ten days of parturition and, in severe cases, leads to pyrexia, anorexia, reduced milk yield, dehydration and depression (Sheldon et al.

2006). The diagnosis is straightforward based on the observation of fetid uterine discharge and systemic signs. Additionally, clinical metritis severity has been categorized according to the cows' health signs in grades 1, 2, and 3 (Sheldon et al. 2009a). Animals with grade 1 metritis exhibit an abnormally enlarged uterus with purulent uterine discharge but without any systemic signs of ill health. Grade 2 metritis is characterized by an abnormally enlarged uterus and purulent uterine discharge, with additional signs of systemic illness, such as decreased milk yield, dullness, and fever. Grade 3 metritis, also called puerperal metritis or toxic metritis, includes animals with toxemia signs, such as reduced appetite, cold extremities, and depression. Lactation incidence of metritis varies between breed, country, and herd, but it has been reported to be 20 to 40% (Sheldon et al. 2008). Clinical metritis is often associated with risk factors linked to metabolic status in the prepartum period and increased uterine trauma and bacterial contamination triggered by retained placenta, dystocia, stillbirth, or twins (Giuliodori et al. 2013). Regarding its consequences on fertility, metritis delays and reduces conception rates to the first insemination and increases the calving-to-conception interval (Fourichon et al. 2000).

2.2.2 Pyometra

Pyometra, defined as the accumulation of purulent material within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix (Sheldon et al. 2006), is relatively rare, comprising <5% of clinical cases of uterine disease (Sheldon et al. 2008). This condition develops when cows ovulate while there is still ongoing uterine infection, and the ensuing corpus luteum produces progesterone responsible for the cervix closure (Olson et al. 1984). Diagnosis can be achieved by rectal palpation or ultrasound examination, by detecting a corpus luteum in the ovaries, accumulation of mixed echogenicity fluid in the uterine lumen and distension of the uterus (Sheldon et al. 2006).

2.2.3 Clinical endometritis

Clinical endometritis is defined as the presence of a purulent or mucopurulent uterine discharge detectable in the vagina at 21 days or more postpartum, which is not accompanied by systemic signs (Sheldon et al. 2006). The incidence of clinical endometritis is about 20%, with variations between breed, country and herd (Sheldon et al. 2008; 2017). The diagnosis is accomplished by examining the vagina employing a vaginoscope, a gloved hand, or a metricheck device. However, it has become clear that the endometritis designation was somehow inaccurate as the abnormal vaginal discharge may also result from cervicitis or vaginitis. Consequently, nowadays, the presence of a purulent or mucopurulent discharge in the vagina is referred to as "Purulent vaginal discharge". Risk factors for purulent vaginal discharge include factors that increase uterine trauma and bacterial contamination, such as

dystocia, twins, and metritis (Dubuc et al. 2010). This disease is significant because even after the clinical resolution of the disease, a history of endometritis increases the interval to first insemination and delays conception compared with healthy cows (Fourichon et al. 2000; Pascottini 2016; Sheldon et al. 2019).

2.2.4 Subclinical endometritis

Subclinical endometritis is characterized by inflammation of the endometrium's superficial layer without clinical signs of endometritis. It became relevant when the cytological evidence of endometritis was associated with impaired fertility (Kasimanickam et al. 2004). Subclinical endometritis is usually diagnosed through endometrial cytology from samples collected by flushing the uterine lumen or a cytobrush device. Consequently, it is also known as cytological endometritis. Subclinical endometritis is highly prevalent, affecting 30–35% of dairy cows between 4 and 9 weeks postpartum (LeBlanc 2008). However, it is often overlooked, unlike clinical presentations of postpartum uterine disease. It is considered that a cow has SCE when the percentage of neutrophils in endometrial cytology exceeds operator-defined thresholds. These thresholds depend on the amount of time encompassed since parturition, ranging from 18% at 20-33 days postpartum (Kasimanickam et al. 2004) to 5% at 40-60 days postpartum (Gilbert et al. 2005). Risk factors that reflect immune suppression or metabolic imbalance in the peripartum period, such as low body condition score or ketosis, have been linked to SCE (Cheong et al. 2011; Senosy et al. 2012). Moreover, the uterine microbiome is not different between healthy and SCE cows, supporting the concept that SCE embodies a problem of persistent dysregulated "sterile" inflammation (Pascottini and LeBlanc 2020), and that it results from the failure to regulate the inflammation resolution.

2.2.4.1 Diagnostic challenges

Due to the absence of clinical signs, supplemental examinations are necessary to diagnose SCE accurately. Among these examinations are uterine biopsy with subsequent histopathology, ultrasonography, and the most used, endometrial cytology. Ultrasonography has shown the potential to diagnose SCE by allowing the detection of fluid in the uterine lumen, which is associated with impaired fertility (Kasimanickam et al. 2004). However, despite being an easy and fast method of diagnosing SCE, it is not precise enough to give a final diagnosis when done alone (Pascottini 2016). Uterine biopsy with subsequent histopathology is considered the "gold standard" for the endometritis diagnosis in mare (Buczkowska et al. 2014). However, due to the invasive nature of the sampling technique, veterinary skills required, time-consuming logistics, and cost, its use in diagnosing SCE in dairy cows has been reserved for research purposes (Figure 1A). Endometrial cytology is the most used technique in both field and research setups. There are two different techniques for

sampling the endometrium, low volume lavage and cytobrush. Low volume lavage consists of infusing a low volume (20-50ml) of a sterile physiologic solution in the uterine lumen followed by a gentle massage of the uterus before collecting the physiologic solution into a sterile container. Subsequently, the fluid is centrifuged in the lab, and a smear is done from the cellular pellet before staining. This technique has the drawback of taking the uterine flushing back to the lab for centrifugation before the smear is done. Additionally, some degree of cellular distortion is observed following centrifugation (Kasimanickam et al. 2005a). The cytobrush technique consists of adapting a brush intended to collect human cervical samples to an AI gun (Figure 1B and 1C), which is transrectally guided to the uterine body. Then, the brush is pushed out, gently rolled against the endometrium, and once retrieved, rolled along a glass slide. Despite being easier to perform, the cytobrush technique has the limitation of sampling only a small area of the endometrium, unlike the low volume lavage, which samples the luminal layer of the whole endometrium (Kasimanickam et al. 2005a). In both cases, slides are stained (e.g. with modified Wright-Giemsa® stain), and the percentage of polymorphonuclear neutrophils (PMN) is assessed under microscopic visualization (Figure 1D and 1E). Moreover, regardless of the technique used, SCE should be diagnosed in a time frame that allows for any therapeutic decision to take effect before the cows are bred. Nevertheless, cows should be given enough time to complete their normal uterine involution, which is an inflammatory process, before this examination is performed. Otherwise, there is a risk of finding false-positive results. Overall, there are still issues of the practicality of the sampling technique and the best moment to perform a reliable diagnostic while allowing enough time for any therapeutic interventions to work before the cows are bred.

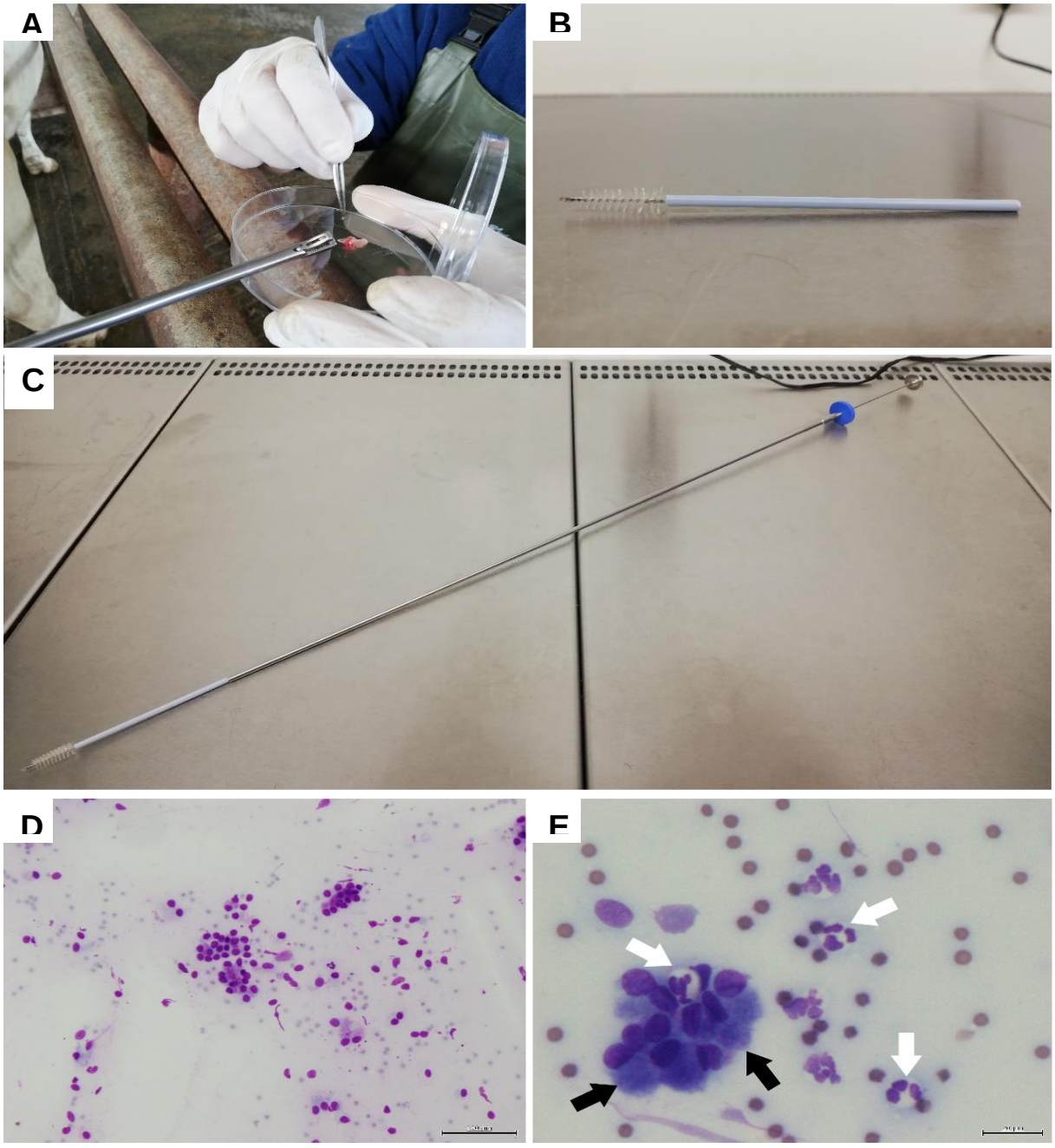

Figure 1. Endometrial biopsy and cytology. (A) Endometrial biopsy collection for research purposes. (B) Brush intended to collect cervical samples in humans. (C) Brush adapted to an AI gun for endometrial sampling in dairy cows. (D) Endometrial cytology smears stained with Diff Quick (100x). (E) Endometrial cytology smears stained with Diff Quick (400x). Black arrows pointing to epithelial cells and white arrows pointing to polymorphonuclear neutrophils.

2.2.4.2 Link between energy balance and immune function

In the past decades has become evident that the adipose tissue not only serves a role in lipid storage but also has a crucial endocrine function secreting many hormones termed adipokines (Kershaw and Flier 2004). Despite being described as “adipose tissue-derived

hormones", different cell types outside adipose tissue depots have also been described as primary sources of these mediators (Kurowska et al. 2018). Among other functions, adipokines have been implicated in energy metabolism, insulin resistance, inflammation, immunity, and reproductive function (Reverchon et al. 2014a). Adiponectin, the most abundant adipokine in human plasma (Barbe et al. 2019), plays a significant role in suppressing systemic and tissue inflammation due to its anti-inflammatory properties (Fang and Judd 2018). On the contrary, chemerin has mostly pro-inflammatory effects acting as a leukocyte attractant and exhibiting broad-spectrum antimicrobial properties (Zabel et al. 2014). However, depending on the enzymatic proteolysis of the chemerin precursor, chemotactic chemerins (pro-inflammatory) or anti-inflammatory chemerins can be generated (Du and Leung 2009). Serine proteases from neutrophils originate chemotactic chemerins, whereas cysteine proteases from activated macrophages originate chemerins with anti-inflammatory effects, thus suggesting that chemerin may be implicated in the initiation and resolution of inflammation (Du and Leung 2009). Visfatin is an adipokine with an immunomodulatory function involved in obesity, inflammation and insulin resistance (Zhang et al. 2019). Moreover, in cows, visfatin was proposed to predict retained placenta and other inflammatory diseases (Fadden and Bobe 2016). In sum, owing to the regulation of metabolism and inflammatory responses by adipokines, these molecules have promising potential to elucidate the link between energy balance and immune function in postpartum dairy cows.

2.2.4.3 Transcriptional changes associated with SCE

Subclinical endometritis exerts a profoundly detrimental effect on subsequent reproductive performance (Gilbert et al. 2005). It promotes an adverse uterine environment responsible for suboptimal conditions for sperm cell transportation and storage, oocyte maturation and ovulation, fertilization, embryo development, implantation, and embryonic and fetal growth (Pascottini et al. 2017; Sheldon et al. 2019). The presence of PMN in the uterine lumen reflects the activation of an immune response responsible for a pro-inflammatory milieu and is characterized by altered transcriptomic profiles of the endometrial cells. Postpartum primiparous dairy cows with clinical endometritis and SCE exhibited similar inflammatory responses by displaying similar upregulation of cytokine coding genes (*IL1A*, *IL6*, *IL17A*, *TNF-α*, and PG enzymes *PGHS2* and *PGES*) (Johnson et al. 2015). On the other hand, Hoelker et al. (2012) revealed that SCE is also associated with changes in endometrial gene transcription, including decreased transcription of genes related to cell adhesion (*GPLD1* and *VCAM1*), increased transcription of *PSPIP2,* which protects the autologous tissue against the activated immune system, and *VCAM1*, *ARHGEF*, and *BOLA-DQA5* that were found to be downregulated and may be associated with the low immune competence in cows with SCE.

Moreover, Raliou et al. 2019 evidenced that peripheral and endometrial responses to SCE are distinct in terms of biological pathways and genes that are affected, with the endometrium displaying an increased transcription of factors related to tissue remodeling, acute phase response, and lipopolysaccharide (LPS) signaling. Fagundes et al. (2019) showed that SCE increases *CCL5*, *CXCL8*, *IL6*, and *IL1B* gene transcription. It also evidenced that gene transcription was increased in samples collected by cytology relative to those derived from biopsies. This difference in gene transcription from the two different sampling techniques was also observed by Chapwanya et al. (2010), who suggested that the difference is due to a higher concentration of inflammatory cells in the more superficial layers sampled by the cytology technique. Salilew-Wondim et al. 2016 showed that SCE alters the endometrial transcriptome related to response to stimulus, immune system processes, G-protein couple receptor signaling, and chemotaxis. However, since their analysis was conducted in whole tissue biopsies, the authors stated that it was unclear whether the gene transcription alterations were specific to epithelial or stromal cells. In sum, samples obtained from cytology overlook the information regarding deeper layers of the endometrium, whereas biopsy specimens are likely to have a lower concentration of inflammatory cells as these are diluted by a larger amount of cells.

2.2.4.3.1 Immunomodulatory effects of steroid hormones

The inhibition of uterine innate and adaptive immune responses by steroid hormones, namely progesterone, was reviewed in human and animal models (Beagley and Gockel 2003). Moreover, in the cow, during the follicular phase of the oestrus cycle, the endometrium is more resistant to infection, whereas, during the luteal phase, the progesterone-induced downregulation of immune functions prevents the uterus from resisting infection (Lewis 2003, 2004; Herath et al. 2006; Raliou et al. 2019). Progesterone downregulates uterine eicosanoid synthesis, namely prostaglandin F2α (PGF2α), a critical mediator for mounting endometrial immune responses as it increases the uterus ability to resist or resolve infections through the up-regulation of pro-inflammatory cytokines production and various neutrophil functions (Lewis 2003, 2004). Additionally, progesterone suppresses lymphocyte proliferation, and this effect was also associated with the inability of the uterus to prevent the development of infections (Lewis 2003). Furthermore, Herath et al. (2006) found that progesterone can increase the susceptibility of the postpartum endometrium to infection by suppressing the immune response to LPS in endometrial cells *in vitro*. However, the molecular mechanisms underlying the immunosuppression exerted by progesterone on endometrial cells are not fully elucidated (Sheldon et al. 2009b), and Saut et al. (2014) found no effect of the stage of the oestrus cycle, or exogenous ovarian steroids on

key cytokine and chemokine responses to *E. coli* or LPS, challenging the dogma that steroids suppress endometrial immune function.

2.2.4.4 Prevention and therapeutics

Motivated by the understanding that, in dairy cows, postpartum uterine diseases are the result of uterine bacterial contamination coupled with dysregulation of uterine inflammation mechanisms, researchers have been making efforts to develop preventive and alternative therapies so the harmful effects of endometritis in dairy cows' fertility can be prevented and endometrial health restored. Moreover, given the global threat of antimicrobial resistance dissemination (Roca et al. 2015) and the acknowledgment that SCE is often a problem of persistent "sterile" inflammation (Pascottini and LeBlanc 2020), the development of alternative therapies is of particular interest in order to decrease the antimicrobial usage in the dairy industry.

Due to increased metabolic demands to support lactation and diminished feed intake, dairy cows are exposed to a metabolic imbalance during the postpartum period, which is responsible for reducing and impairing neutrophil function (LeBlanc 2020). Granulocyte colony-stimulating factor (G-CSF) is an endogenous hematopoietic growth factor that stimulates the production and differentiation of neutrophils by progenitor cells in the bone marrow (Kehrli et al. 1991). Pegbovigrastim is a recombinant bovine G-CSF that has been tested to treat peripartum immunosuppression on commercial dairy farms (Pascottini and LeBlanc 2020). Studies confirmed that Pegbovigrastim consistently causes substantial, supra-physiologic increases in the number of circulating neutrophils (Kimura et al. 2014; Canning et al. 2017), with modest increases in per cell function (McDougall et al. 2017), but these effects did not translate into reduced incidence of diseases thought to be related to neutrophil function, namely uterine diseases (Zinicola et al. 2018).

Interleukin-8 is a potent chemotactic and activating factor for neutrophils (Mitchell et al. 2003). Due to its potential to modulate innate immune function, Bicalho et al. (2019) purified recombinant bovine IL-8 and performed *in vitro* and *in vivo* experiments. Recombinant bovine IL-8 was biologically active and successfully induced neutrophil activation, chemotaxis, and diapedesis in both experiments. The same research group also reported that the uterine infusion of recombinant bovine IL-8 within 24 hours postpartum reduced the incidence of puerperal metritis in multiparous cows while it did not affect the incidence of retained placenta or purulent vaginal discharge (Zinicola et al. 2019). These studies confirmed the immunomodulatory potential of recombinant bovine IL-8. However, its preventive effect on SCE remains to be evaluated.

Regarding therapy, the two most classically used approaches to endometritis are intrauterine (e.g. cephapirin) or parenteral antibiotics and intramuscular injection of PGF2α. Cephapirin, a first-generation cephalosporin antibiotic, has been the antibiotic of choice for intrauterine infusion thanks to its activity against *T. pyogenes*, which, combined with the absence of milk withholding time, makes it of particular interest for the treatment of uterine infections in dairy cattle. The rationale behind using intrauterine antibiotics is to reduce the burden of pathogens in the uterus and consequently diminish uterine inflammation and facilitate the repair of the endometrium (Lefebvre and Stock 2012). However, the usage of antibiotics to treat clinical and SCE has produced inconsistent results. While some authors reported improved reproductive performance of cows with SCE following antimicrobial treatment (Kasimanickam et al. 2005b; Denis-Robichaud and Dubuc 2015), others claim that although antibiotics help resolve the clinical signs of postpartum uterine disease, there is still reduced fertility (Sheldon et al. 2009a; Haimerl and Heuwieser 2014; Sheldon et al. 2020).

The rationale behind the administration of PGF2α to dairy cows with endometritis is that in cyclic cows, the PGF2α would induce luteolysis of the corpus luteum (if responsive), favoring the clearance of uterine contents and improving the uterine defenses by temporarily increasing estrogen and decreasing progesterone concentrations in plasma, thus improving uterine health and reproductive performance (Kasimanickam et al. 2005b; Dubuc et al. 2011a). However, data regarding the benefit of PGF2α administration for improving uterine health and reproductive performance have been conflicting (Dubuc et al. 2011a), and in one meta-analysis and respective update, authors concluded that a positive effect of a PGF2α treatment in case of clinical endometritis could not be shown (Haimerl et al. 2013; 2018).

The lack of indisputable evidence that antibiotics or PGF2α are valid therapeutic approaches for improving uterine health in dairy cows affected with SCE prompted researchers to suggest new therapies. Due to the acknowledgment that metabolic imbalance and impaired immune function are predisposing factors for SCE, some molecules responsible for the modulation of immune function have been put forward as potential therapeutic options (Pascottini and LeBlanc 2020).

Dairy cows are exposed to systemic excessive or persistent inflammatory stimuli during the postpartum period, affecting the normal uterine involution. For that reason, reducing systemic inflammation with non-steroidal anti-inflammatory drugs (NSAID) has been suggested to control the excessive or persistent inflammation and eventually control SCE. However, the administration of carprofen, an NSAID, between 21 and 31 days postpartum, did not affect the PMN percentage at 42 days postpartum (Priest et al. 2013). Despite the absence of effect on PMN percentage on endometrial cytology, carprofen treatment increased

pregnancy rates in high-PMN cows, highlighting the potential of NSAIDs for control of systemic dysregulated inflammation with fertility consequences.

Moreover, daily administrations of meloxicam, another NSAID, at early postpartum, improved indicators of energy metabolism and PMN function but without effect on the endometrial inflammatory status (Pascottini et al. 2020). These results validate NSAIDs as therapeutic options to control systemic inflammation in postpartum dairy cows. However, to control the inflammation dysfunction responsible for persistent SCE, route of drug administration, dosages, timing, and duration of treatment need to be further investigated.

2.2.4.4.1 Nutraceutical role of n-3 polyunsaturated fatty acids

In the past decades, fat supplementation became a common strategy to increase the energy density of dairy cows' diets with beneficial effects minimizing postpartum negative energy balance (Palmquist and Jenkins 2017; Bionaz et al. 2020). PUFA supplementation can also have adverse side effects by reducing dry matter intake (DMI), disturbing rumen function, and originating fatty acid (FA) isomers that depress milk fat (Palmquist and Jenkins 2017; Lopreiato et al. 2020). Nevertheless, these adverse effects can be reduced using rumen-protected fats, which pass through the rumen partly intact and are subsequently released for absorption in the small intestine (Moallem 2018).

The beneficial effects of PUFA of the n-3 and n-6 families go beyond their nutritional value, as reviewed on human health (Djuricic and Calder 2021). Alpha-linolenic acid (ALA) is the shortest chain member of the n-3PUFA family and is considered an essential fatty acid because mammals cannot de novo synthesize it (Djuricic and Calder 2021). ALA is the precursor of longer n-3PUFA (eicosapentaenoic acid - EPA, docosapentaenoic acid - DPA, and docosahexaenoic acid - DHA), which have beneficial effects in reproductive and immune functions (Moallem 2018). On the other hand, linoleic acid (LA) is the shortest member of the n-6 PUFA family and is also considered an essential fatty acid (Djuricic and Calder 2021). The linoleic acid is the precursor of longer n-6 PUFA like the arachidonic acid (ARA), and both play key roles as inflammation modulators in postpartum dairy cows (Contreras et al. 2017; Putman et al. 2019).

In humans, increased proportions of circulating n-3 and n-6 PUFA produce a shift in FA composition of many body compartments, namely cell membranes, that favours eicosanoid synthesis towards an anti-inflammatory or pro-inflammatory state, respectively (Wall et al. 2010). Eicosanoids are bioactive signaling lipids derived from ARA, EPA, and DHA. While ARA is the substrate for prostaglandins and leukotrienes with pro-inflammatory properties,

EPA and DHA are substrates for the biosynthesis of specialised pro-resolving mediators (SPM) which have anti-inflammatory and pro-resolving properties (Serhan 2014).

Specialised pro-resolving mediators are biosynthesised during the initial phases of acute inflammatory responses and trigger a pro-resolving cascade of events (Figure 2) that includes epithelial and inflammatory cell regulation, inhibiting further infiltration of neutrophils into the tissue, inducing neutrophil apoptosis, enhancing neutrophil efferocytosis by macrophages, shifting macrophage program (M1 to M2 types), promoting the return of non-apoptotic cells to the lymphatics and initiating tissue repair mechanisms and healing, while also lowering antibiotic requirements for bacterial clearance (Chiang et al. 2012; Serhan and Levy 2018).

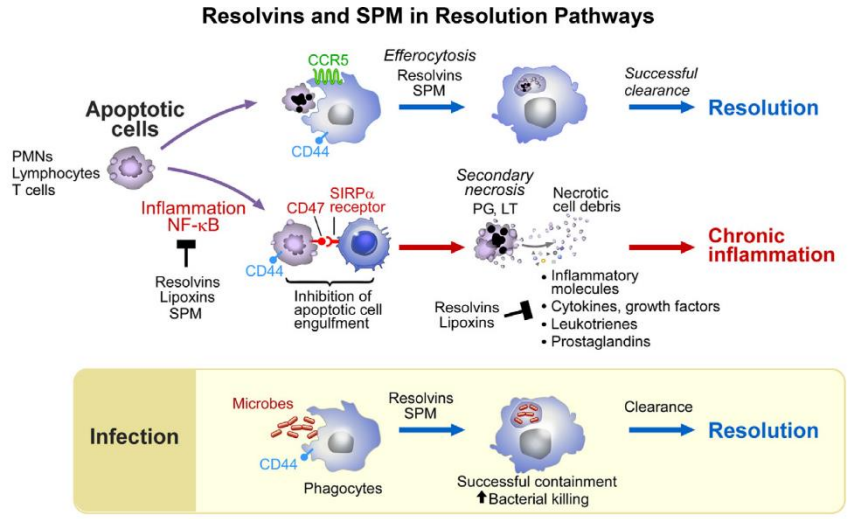

Figure 2. Schematic representation of SPMs in the cellular resolution pathway. From Serhan (2017).

Reproductive tract inflammatory diseases in postpartum dairy cows result from altered innate immune function (Sheldon et al. 2019), as a well-regulated immune response is paramount to avoid excessive or persistent inflammatory conditions in the reproductive tract of dairy cows (Pascottini and LeBlanc 2020). Despite the n-3PUFA enriched diets' known beneficial effects on health of dairy cows (Moallem 2018), the role of n-3PUFA in promoting pro-resolving mechanisms and thus limiting the negative effects of SCE in postpartum dairy cows remains unknown.

2.3 Laser capture microdissection

Laser capture microdissection emerged as a powerful research tool to isolate cell populations from a mixed population under microscopic visualization for further molecular analyses (Bevilacqua and Ducos, 2018). This technique allows the identification of new expression profiles in small and single-cell samples, in what is named "microgenomics"

(Taylor et al. 2004). In heterogeneous tissues, quantification of gene expression from the whole tissue may not reflect the specific transcription of different cell types, additionally, interactions between different cell types may be disregarded resulting in an imperfect interpretation of molecular data. Other techniques, like flow cytometry, also allow the isolation of small and single-cell samples, however, unlike LCM, these techniques require a cell dissociation step which can influence RNA integrity and induce expression artefacts (Vrtacnik et al. 2014). One of the advantages of LCM, is that small and single-cell samples are obtained within the context of their tissue microenvironment (Espina et al. 2006), resulting in a more reliable gene transcription profile of the isolated cells. Since LCM is performed under microscopic control, the quality of histologic sections is a key for the successful isolation of the cell population of interest. However, conventional histologic techniques like formalin fixation and paraffin embedding are not ideal as these modify nucleic acids challenging the isolation of high-quality RNA for genetic profiling (Wimmer at al. 2018). Following a review of the available approaches for tissue fixation before LCM by Bevilacqua and Ducos (2018), embedding in a cryoprotector, like optimal cutting temperature (OCT), followed by cryofixation in isopentane at -80 °C, was elected the method of choice for the preservation of tissue morphology and RNA integrity. In addition, in order to preserve the integrity of RNA, slide preparation, fixation (allows LCM to be performed at room temperature) and staining are also critical steps in the LCM workflow and must be performed in stringent RNAse-free conditions (Espina et al. 2006; Bevilacqua and Ducos 2018). Ethanol is the fixative of election as it eliminates the water within the tissue hence stopping enzymatic activity (Goldsworthy et al. 1999). A gradient of ethanol stabilizes the tissue during LCM thus increasing the time available for cutting tissues without compromising RNA integrity (Bevilacqua and Ducos 2018). Staining time and nature of the dye are also important parameters impacting the success of LCM (DeCarlo et al. 2011). Stains dissolved in water proved to be more efficient in terms of tissue morphology. However, as aqueous solutions allow a certain degree of sample rehydration, these stains proved inferior in terms of RNA integrity. In a review of the available staining dyes, Bevilacqua and Ducos (2018) elected 1% Cresyl violet ethanol solution as an acceptable choice for all tissue studied by the research team in terms of tissue morphology and RNA integrity.

Laser microdissection depends on pulsed infrared (IR) or ultraviolet (UV) lasers for capturing or cutting cells, respectively (Bevilacqua and Ducos, 2018). Laser capture microdissection relies on an infrared laser that melts a polymer fitted to the region of interest, this polymer constitutes a tool termed “cap” which. The cells of interest adhere to the melted polymer in such a way that when the cap is removed from the slide these are successfully isolated from the remaining tissue preparation and can be channeled for nucleic acid extraction (Figure 3).

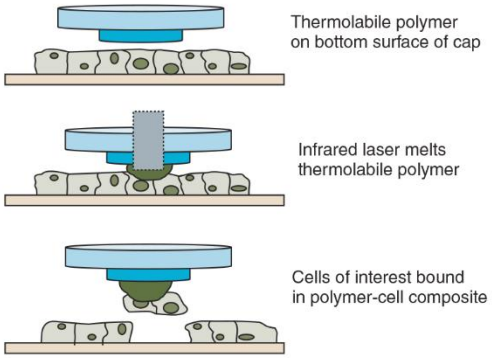

Figure 3. Principle of Laser Capture Microdissection. Infrared laser melts a polymer fitted to the region of interest, cells of interest adhere to the melted polymer and are isolated from the remaining tissue preparation. Adapted from Espina et al. (2006).

In postpartum dairy cows, LCM was recently used to describe the specific molecular signatures of the different endometrial cells (luminal epithelial - LE), glandular epithelial - GE, and stromal cells - ST), as well as the effect of negative energy balance on cell-specific transcriptomic profiles (Chankeaw et al. 2021a; 2021b). These studies highlight the potential of LCM to increase the understanding of cell-specific molecular regulation at the endometrium level in postpartum dairy cows.

EXPERIMENTAL WORK

CHAPTER 3 - Adipokines as biomarkers of postpartum subclinical endometritis in dairy cows

G. Pereira, R. Bexiga, J. Chagas e Silva, E. Silva, C. Ramé, J. Dupont, Y. Guo, P. Humblot and L. Lopes-da-Costa.

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3.1 Abstract

Adipokines emerged as regulators of metabolism and inflammation in several scenarios. This study evaluated the relationship between adipokines (adiponectin, chemerin and visfatin) and cytological (subclinical) endometritis, by comparing healthy (without), transient (recovered by 45 days postpartum (DPP) and persistent (until 45 DPP) endometritis cows (n = 49). Cows with persistent endometritis had higher adiponectin concentrations in plasma (at 21 DPP, $P < 0.05$ and at 45 DPP, $P < 0.01$) and in uterine fluid (at 45 DPP, $P < 0.001$), and higher chemerin concentrations in plasma ($P < 0.05$) and uterine fluid ($P < 0.01$) at 45 DPP, than healthy cows. Cows with persistent endometritis had higher gene transcription in the cellular pellet of uterine fluid and protein expression in the endometrium of these adipokines and their receptors, than in healthy cows. Adiponectin plasma concentrations allowed to discriminate healthy from persistent endometritis cows, in 87% (21 DPP) and 98% (45 DPP) of cases, and adiponectin and chemerin uterine fluid concentrations at 45 DPP allowed for this discrimination in 100% of cases. Cows with concentrations above the cutoff were a minimum of 3.5 (plasma 21 DPP), 20.4 (plasma 45 DPP), and 33.3 (uterine fluid 45 DPP) times more at risk of evidencing persistent endometritis at 45 DPP than cows with concentrations below the cutoff. Overall, results indicate a relationship between adipokine signaling and the inflammatory status of the postpartum uterus of dairy cows, evidencing that adipokines represent suitable biomarkers of subclinical endometritis, able to predict the risk of persistence of inflammation.

**Text adapted from the original published paper (Annex I)*

3.2 Introduction

Cow fertility is critical for the sustainable worldwide increasing demand of dairy products and the profitability of the dairy industry (Inchaisri et al. 2010, Britt et al. 2018). A main factor impairing fertility is the occurrence of postpartum endometritis, which disrupts ovarian and endometrial function leading to a delay in conception and failure in pregnancy establishment (Mateus et al. 2002, Sheldon et al. 2018). The diagnosis of subclinical endometritis, also termed CYTO, which affects 30-35% of dairy cows between 4 and 9 weeks postpartum (LeBlanc 2008), remains a challenge (Raliou et al. 2019). Cytological endometritis is an inflammatory state of the endometrium, detected by histology or cytology, in the absence of purulent vaginal discharge and other clinical signs (Sheldon et al. 2006). Due to the invasive nature of the sampling technique, veterinary skills required, time-consuming logistics and cost of the uterine biopsy and swab techniques, the development of reliable, non-invasive biomarkers for the early diagnosis and prognosis of endometritis has been the scope of recent research (Adnane et al. 2017, Mayasari et al. 2019). The early identification of biomarkers that trigger and/or signal the pathological inflammation of the endometrium would enable to predict uterine health status, administer appropriate prophylactic therapy (Adnane et al. 2017) and better manage time of first insemination during the post-partum period.

The adipose tissue serves not only as a depot for lipid storage but also as an endocrine gland that secretes many mediators generally named adipokines (Reverchon et al. 2014a), including hormone-like mediators as adiponectin, chemerin and visfatin (Kurowska et al. 2018). Although adipokines are mainly produced by adipocytes and immune cells found in the stromal vascular fraction of adipose tissue, different cell types outside adipose tissue depots have also been described as primary sources of these mediators (Mancuso 2016, Kurowska et al. 2018). Among other functions, adipokines regulate energy metabolism, glucose homeostasis, angiogenesis, reproductive function, immunity and inflammation (Reverchon et al. 2014a, Mancuso 2016).

Mainly produced by white adipose tissue (Reverchon et al. 2014a), adiponectin (ADIPOQ) is the most abundant adipokine in human plasma (Barbe et al. 2019). Its primary physiological function is to increase insulin sensitivity, but it is also thought that ADIPOQ plays a major role in suppressing systemic and tissue inflammation due to its anti-inflammatory properties (Fang and Judd 2018). Its effects are mainly mediated by two seven-transmembrane domain receptors termed ADIPOR1 and ADIPOR2 (Parker-Duffen et al. 2014). These receptors as well as ADIPOQ are expressed in the uterus of humans (Takemura et al. 2006), sows (Smolinska et al. 2014) and cows (Astessiano et al. 2017). In cows, blood ADIPOQ concentrations show a defined pattern, with the nadir around the time of parturition and a

progressive increase over the following few weeks of lactation (Barbe et al. 2019), and Kasimanickam et al. (2013) reported increased ADIPOQ serum concentrations in cows with metritis and clinical endometritis compared to heathy cows.

Chemerin, also known as tazarotene-induced gene 2 protein or retinoic acid receptor responder protein 2 (RARRES2), is a pro-inflammatory adipokine produced by both adipose tissue and liver (Zabel et al. 2014). It is secreted as the inactive precursor prochemerin, which becomes active following cleavage at the C-terminus by extracellular proteases (Mattern et al. 2014), and contributes to the regulation of adipogenesis, insulin secretion and the inflammatory process (Kurowska et al. 2018). In the latter, RARRES2 works as a chemoattractant for monocytes and dendritic cells (Mancuso 2016), and its blood concentrations correlate with those of TNFα, IL-6 and C-reactive protein (Rourke et al. 2013). Chemerin binds to three seven-transmembrane domain receptors - CMKLR1 (Chemokine like receptor 1), CCRL2 (C-C chemokine receptor-like 2) and GPR1 (G Protein-Coupled Receptor 1) (Mariani and Roncucci 2015). The activation of CMKLR1 by RARRES2 induces the migration of macrophages and dendritic cells *in vitro*, which supports the pro-inflammatory role of the RARRES2/CMKLR1 axis (Yoshimura and Oppenheim 2011). In cows, RARRES2 was referred as a mediator, linking metabolism and ovarian function, and plasma concentrations are positively correlated with body fat mobilization and milk yield (Mellouk et al. 2019).

Visfatin, also known as pre-B-cell colony enhancing factor or nicotinamide phosphoribosyl-transferase (NAMPT), is predominantly expressed in visceral adipose tissue but also in muscle, bone marrow, liver, lymphocytes and foetal membranes (Reverchon et al. 2014a, Dupont et al. 2015). Visfatin has an immunomodulatory function and is involved in obesity, inflammation and insulin resistance (Zhang et al. 2019). In cows, peripartal serum concentrations of NAMPT were proposed as predictive indicators of retained placenta and other inflammatory diseases (Fadden and Bobe 2016).

Puerperal dairy cows are under metabolic stress, which is related to uterine inflammation (Hammon et al. 2006, Guo et al. 2019). Owing to the regulation of metabolism and inflammation by adipokines, these molecules may represent promising biomarkers for the early diagnosis and prognosis of postpartum SCE. The objective of this study was to evaluate the relationship between adipokines (adiponectin, chemerin, visfatin and their receptors) and postpartum endometritis in dairy cows. The hypothesis behind this study was that adipokine signalling may be active in the cow uterus, and that this signalling pattern, and adipokine concentrations (blood and uterine) may be related to the inflammatory status of the uterus.

3.3 Material and Methods

3.3.1 Ethics statement

The experiments were conducted in compliance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº129/92 and Portaria nº1005/92, DR nº245, série I-B, 4930-42) and with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU). The research protocol was approved by the Institutional Animal Care and Use Committee (Reference CEIE nº37/2019). All animal technical procedures were performed by licenced veterinarians, all of which are accredited as FELASA category C scientists or equivalent.

3.3.2 Animals, sampling and experimental design

Sample collection was performed in a 400 milking Holstein-Friesian dairy cow herd, with a three daily milking routine and a milk yield average of 11500 L/cow/305 days of lactation. After calving, cows were monitored daily for signs of puerperal disease. Cows that experienced dystocia, retained foetal membranes, puerperal metritis or mastitis, clinical hypocalcaemia, ketosis and lameness were not included in the study, and enrolled cows did not receive antibiotic or anti-inflammatory therapy throughout the study. Selected cows (n= 49) were examined and sampled at 21 (21 ± 0.4) and 45 (44 ± 0.7) DPP. At 21 DPP, cows were blood sampled, the score of the vaginal discharge was assessed, the genital tract was evaluated through transrectal palpation and ultrasonography, and an endometrial swab was taken for cytological examination. At 45 DPP the above procedures were repeated. In addition, as described in details under, a small volume flush and an endometrial biopsy were collected from both uterine horns. Once the voluntary waiting period (50 DPP) was over, cows were submitted to artificial insemination (AI) at the ensuing estrus (detected by visual observation plus pedometer measured walking activity). At the entrance of the milking parlour, an electronic collar identified cows and milk yield and body weight were automatically recorded after each milking. Data including milk yield and body weight (three times daily), estrus and insemination dates, and pregnancy status (at 35-42 and 90 days post-AI) were retrieved from the herd management software, until completion of lactation (305 DPP).

The experimental design considered the comparison between three groups, with cows being retrospectively allocated to: i) control healthy cows, without SCE at 21 and 45 DPP (group HH; n = 19); ii) cows with SCE at 21 DPP that had recovered by 45 DPP (group EH; n = 19); and iii) cows with SCE at 21 DPP, which persisted at 45 DPP (group EE; n = 11). A further refinement of group HH considered two subsets of cows, including cows pregnant at first AI

(subset HHP; n = 6) and cows pregnant at subsequent AIs (n = 13). The subset HHP was considered for “extreme cases” comparison (healthy pregnant versus endometritis) in endometrial immunolocalization and transcription analysis of the cellular pellet of uterine flushing.

3.3.3 Blood sampling

Blood samples were aseptically collected by venipuncture of the coccygeal vein into 10 mL dry tubes and 10 mL tubes containing K3 EDTA (13060, Vacutest KIMA, Arzegrande, Italy). Tubes were immediately centrifuged (2,000 g for 15 min), plasma and serum were aliquoted into 1.5 mL Eppendorfs and stored at -20 °C until subsequent analysis for adipokines, Non Esterified Fatty Acids (NEFA) and progesterone concentrations.

3.3.4 Genital tract evaluation

The genital tract was palpated per-rectum to assess the size of the cervix and uterus, and the symmetry and tonus of uterine horns. An ensuing ultrasound examination (ExaGo, Echo Control Medical, France) evaluated the uterine content (intrauterine fluid volume and echogenicity) and identified and measured the ovarian structures. The vaginal discharge score was graded in a 0-3 scale according to Williams et al. (2005), following collection with a Metricheck device (EndoControl Sampler, Minitube, La Selva del Camp, Spain).

3.3.5 Endometrial cytology

Endometrial swabs were performed using an adapted cytobrush technique. A cervical brush (Bastos Viegas SA, Penafiel, Portugal) was aseptically adapted to the inner stylet of, and retracted into an AI gun (IMV technologies, L'Aigle, France) covered with an AI sheath (IMV technologies), and the swab obtained as described by Pascottini et al. (2016). The brush was then gently rolled along the length of two glass microscope slides, which were subsequently labelled and air-dried. Slides were stained with a modified Wright-Giemsa® stain (Diff-Quick, MAIM SL, Barcelona, Spain) and the percentage PMN was assessed from 400 cells (200 in each slide). At 21 DPP a ≥ 18% PMN cut-off was chosen (Kasimanickam et al. 2004), and cows with a PMN percentage below this cut-off were considered without SCE. At 45 DPP, a ≥ 5% PMN cut-off was chosen (Gilbert et al. 2005) and, similarly, cows with a PMN percentage below this cut-off were considered without SCE.

3.3.6 Uterine flushing

Both uterine horns were flushed independently with sterile Phosphate-Buffered Saline (PBS; Millipore Corp, Billerica, USA), using a sterile silicone-coated latex Foley catheter (Rusch Gold, Rusch, Perak, Malaysia) aseptically mounted in a metal stylet and covered with a

sanitary sheath (IMV technologies). At the cervical external opening, the sanitary sheath was retracted, the catheter guided to one of the uterine horns past the external bifurcation level, and the cuff inflated. 50 mL PBS were infused into each uterine horn, which was gently massaged per-rectum, before allowing collection of the fluid into a 50 mL centrifuge tube. Samples were transported to the laboratory at 4 °C, filtered with a mesh to remove mucus and debris, centrifuged (5,000 g for 15 minutes), and the supernatant and cellular pellet independently stored at -80 °C until assayed, respectively for adipokines' concentrations and transcription of adipokine signalling genes.

3.3.7 Endometrial biopsy

Endometrial biopsies were independently collected from both uterine horns by using Kervokian-Younge endometrial biopsy instrument (Alcyon, Paris, France), according to procedures described by Chapwanya et al. (2009, 2010). Briefly, a low epidural anaesthesia was performed with 200 mg of Procaine hydrochloride + 0.18 mg of Epinephrine tartrate (Pronestesic, Fatro, Bologna, Italy) injected in the epidural cavity at the first coccygeal space. After hygiene of the vulva, the sterile biopsy instrument, guarded in a protective sheath, was introduced into the vagina and advanced to the external cervical orifice, where the protective sheath was ruptured. The biopsy instrument was then guided into the first third of the uterine horn, and an endometrial sample of about 1.5 cm^2 recovered. The endometrial sample was cut into three equal size portions, one of which was immediately fixed in 4% Paraformaldehyde (PFA) and paraffin embedded within 24-48 hours for immunohistochemistry analysis. The remaining portions were processed for other analysis.

3.3.8 Progesterone Assay

Serum progesterone was assayed by a chemiluminescent immunoassay in an IMMULITE 1000 analyser (Siemens Healthcare Diagnostics, Eschborn, Germany), using a commercial kit (IMMULITE 1000 Progesterone Kit, Siemens Healthcare Diagnostics). The analytical sensitivity of the assay was 0.2 ng/mL and the inter-assay coefficient of variation was < 10%. Cows were classified as in luteal phase (at 21 and 45 DPP) when progesterone concentration was > 0.5 ng/mL and an ovarian luteal structure (corpus luteum) was detected during the ultrasound examination.

3.3.9 Non Esterified Fatty Acids Assay

Serum NEFA concentrations were determined by a colorimetric method (kit no. FA 115, Randox, Crumlin, UK) using a Randox RX Daytona equipment (Randox). Each sample was analysed in duplicate and the quality control of the assay was performed with the Randox Acusera Assayed Chemistry Premium Plus Control (Assayed Chemistry Premium Plus Level

2 and 3, Randox). The analytical sensitivity of the assay was 0.072 mmol/L and the inter-assay coefficient of variation was < 5%.

3.3.10 Adipokine Assays

Plasma and uterine fluid total ADIPOQ, RARRES2 and NAMPT concentrations were measured by using ELISA kits (E11A0125 and E11C0104 from BlueGene, Shanghai, China and EK-003-80 from Phoenix France, SAS, Strasbourg, France, respectively), as previously described (Fadden and Bobe 2016, Mellouk et al. 2017, 2019). The intra-assay coefficients of variation for total ADIPOQ, RARRES2 and NAMPT were 6%, 4.5% and 6.5%, respectively. The inter-assay coefficients of variation for total ADIPOQ, RARRES2 and NAMPT were 6.2%, 6.5% and 6%, respectively. Adipokine uterine fluid measurements (as well as quantitative real time RT-PCR of the uterine fluid cell pellet; see below) were performed in samples recovered from the uterine horn contralateral to the corpus luteum, or to a randomly selected uterine horn if no corpus luteum was present.

3.3.11 Immunohistochemistry

Endometrial samples from three cows of each group (HHP, EH and EE) were used for protein immunolocalisation. Paraffin embedded endometrial samples were serially sectioned at a thickness of 4 µm, deparaffinised and hydrated. Quenching of endogenous peroxidase activity, antigen retrieval (EnVision™ FLEX Target Retrieval Solution, DM829; Dako, Glostrup, Denmark) and nonspecific background elimination were performed according to Maillard et al. (2010). Twin sections were incubated overnight at 4 °C with control rabbit or mouse IgG and the different primary antibodies (Annex II) diluted in PBS with 5% lamb serum. Sections were then washed four times in PBS for 10 min and incubated for 30 min at room temperature with a ready-to-use labelled polymer-HRP (EnVision™ FLEX/HRP detection reagent, SM802; Dako). After washing twice in PBS for 10 min, immunoreactivity was revealed by incubation with DAB chromagen/DAB buffer at room temperature, according to manufacturer instructions (Envision™ FLEX, K8000; Dako). Finally, the slides were counterstained with Meyers hematoxylin (Merck, Darmstadt, Germany), then dehydrated and mounted with Entellan® (Merck). Staining intensity and cell type identification were independently assessed by two observers, at 100x and 1000x magnification. Intensity was classified as – (absent); +/- (weak); + (moderate); ++ (strong). From each cow, staining was assessed from a minimum of three optical fields from twin slides for each antibody.

3.3.12 Quantitative Real Time RT-PCR analysis of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2, CMKLR1 and GPR1 in the cellular pellet of the uterine flushing

Total RNA of the cellular pellet of the uterine flushing performed at 45 DPP was extracted using the RNeasy Midi kit (Quiagen®, Courtaboeuf, France), according to manufacturer instructions. Concentration and purity of RNA were determined with a NanoDrop Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany), RNA integrity evaluated on 1.25% agarose-formaldehyde gels, and total RNA quality assessed with an Agilent Bioanalyzer 2100, using a RNA 6000 pico kit (Agilent Technologies). The 260/280 ratio (1.7 - 1.9) and RIN value (7.2 - 8.5) evidenced a suitable RNA quality for analysis (Annex III). The cDNA was generated by reverse transcription (RT) of total RNA (1µg) in a mixture comprising: 0.5mM of each deoxyribonucleotide triphosphate (dATP, dGTP, dCTP, DTTP), 2M of RT buffer, 15µg/µL of oligodT, 0.125 U of ribonuclease inhibitor, and 0.05 UMMLV (Moloney murine leukemia virus reverse transcriptase), for one hour at 37 °C. Real-time PCR was performed using the MyiQ Cycle device (Bio-Rad, Marnes-la-Coquette, France), in a mixture of SYBR Green Supermix 1X reagent (Bio-Rad), 250nM specific primers (Invitrogen ™ by Life Technologies ™, Villebon sur Yvette, France) (Annex IV), and 5µl of cDNA diluted 1:5, in a total volume of 20µl. The amplification protocol started with an incubation for 2 min at 50 °C and a denaturation step of 10 min at 95 °C, followed by 40 cycles (30s at 95 °C, 30s at 60 °C, 30s at 72 °C), and acquisition of the melting curve. Each sample was analysed in duplicate in the same plate, and amplification with water, instead of cDNA, was performed systematically as a negative control. Gene transcription was calculated according to primer efficiency (E) and quantification cycle (Cq), where transcription = E-Cq. Then, relative expression of the target gene was analyzed following standardization of the level of mRNA expression for the geometric mean of three reference genes (GAPDH, ACTB and PPIA) which were reported as accurate normalisation factors (Vandesompele et al. 2002).

3.3.13 Statistical Analysis

Data were managed in Microsoft Excel and statistical analyses were conducted using SPSS for Windows 26.0 (IBM Corp., North Castle, USA). The normality of the distribution of the data was tested with the Shapiro-Wilk-Test. Values are reported as median and interquartile range for non-normally distributed variables, and mean ± SEM for those normally distributed (Annex V). Significant differences between groups were determined using a one way ANOVA or the non-parametric Kruskal-Wallis-Test with Dunns post-test and Bonferroni correction for multiple tests, respectively for normally and non-normally distributed variables. The level of significance was set at $P < 0.05$. Correlation analysis used the Spearman's rank test. The

predictive accuracy of the different adipokines to discriminate cows with SCE was evaluated by Receiver Operating Characteristic (ROC) curve analysis, testing the probability that the Area Under Curve (AUC) differed significantly from random (AUC = 0.5). ROC curve coordinates allowed Youden Index calculation, best cut-off and odds ratio (OR) determination for ADIPOQ and RARRES2 concentrations. Cohen's kappa statistic was used to assess the agreement between the vaginal discharge Metricheck score and the endometrial cytology PMN percentage.

3.4 Results

3.4.1 Endometrial cytology PMN percentage and vaginal discharge Metricheck score

Endometrial cytology PMN percentage reflects the retrospective allocation of cows to groups, based on endometrial cytology cut-off (Table 1). At 21 DPP, the endometrial cytology PMN percentage was lower in group HH than in groups EH and EE ($P < 0.001$). However, at 45 DPP, the endometrial cytology PMN percentage was similar in groups HH and EH, and lower than in group EE ($P < 0.001$). At 21 DPP, the vaginal discharge Metricheck score was lower ($P = 0.001$) in group HH than in group EE but at 45 DPP, the vaginal discharge Metricheck score was similar in all groups. At 21 DPP, there was a moderate level of agreement (Cohen’s Kappa = 0.47; $P < 0.01$) between the vaginal discharge Metricheck score and the endometrial cytology PMN percentage (Annex VI). However, at 45 DPP, there was no agreement between the above parameters (Cohen’s Kappa = -0.10; $P = 0.46$) (Annex VI).

Table 1. Metabolic and fertility parameters in healthy cows (group HH), cows with SCE at 21 DPP but that recovered by 45 DPP (group EH), and cows with persistent SCE until 45 DPP (group EE).

Parameters	HH (n=19)	EH (n=19)	EE (n=11)
Lactation number*	1 (1 – 2)	2 (1 – 2)	2 (1 – 3)
BWL Calving - 21 DPP (%)*	2.2 (0 – 5.4)	1.9 (-1.2 – 5.8)	7.5 (-0.2 – 9.0)
BWL Loss Calving - 45 DPP (%)*	4.1 ± 1.8	2.5 ± 1.4	3.9 ± 1.8
BWL Calving - 60 DPP (%)*	3.6 (1.6 – 7.8)	1.3 (-1.9 – 5.0)	4.9 (-1.1 – 10.2)
Milk yield by 21 DPP (L)**	863.5 ± 38.7	839.8 ± 33.0	874.0 ± 48.6
Milk yield by 45 DPP (L)**	1716.8 ± 76.5	1710.0 ± 64.7	1806.7 ± 95.0
Milk yield by 60 DPP (L)**	2375.2 ± 102.1	2392.6 ± 88.0	2512.4 ± 133.7
NEFA at 21 DPP (mmol/L)*	0.28 (0.17 - 0.50)	0.25 (0.17 - 0.60)	0.26 (0.20 - 0.43)
NEFA at 45 DPP (mmol/L)*	0.27 (0.17 - 0.50)	0.28 (0.14 - 0.43)	0.17 (0.13 - 0.34)
Cows in luteal phase at 21 DPP (n (%))	14 (74) [a]	5 (26) [b]	6 (55) [a,b]
Cows in luteal phase at 45 DPP (n (%))	18 (95) [a]	10 (53) [b]	7 (64) [b]
Metricheck Score at 21 DPP*	0 (0 – 1) [a]	1 (0 – 2) [a,b]	2 (2 – 2) [b]
Metricheck Score at 45 DPP*	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
PMN% in Cytobrush at 21 DPP*	4.0 (1.0 – 8.0) [a]	25.5 (20 - 41.5) [b]	61 (35 – 86) [b]
PMN% in Cytobrush at 45 DPP*	1 (0.0 - 1.0) [a]	1 (0 – 1) [a]	11 (7 – 17) [b]
Calving – first AI interval (days)*	61 (56 - 67)	61 (58 – 80)	64 (50 – 94)
Cows pregnant at first AI (n (%))	6 (32)	0	0
Cows conceiving within 305 DPP (n (%))	19 (100)	14 (74)	7 (64)
Calving – conception interval (days)*	136 (64 - 232) (n=19)	110 (98 – 157) (n=14)	94 (85 – 236) (n=7)
AI number/conception*	4 (1 – 6) (n=19)	3 (2 - 4) (n=14)	2 (2 – 7) (n=7)

BWL = Body Weight Loss; DPP = days postpartum
*Values reported as median and (interquartile range) for non-normally distributed data.
**Values reported as mean ± SEM for normally distributed data.
Different letters indicate significant differences between groups (level of significance $p < 0.05$) determined with ANOVA and the non-parametric Kruskal-Wallis-Test with Dunns post-test for normally and non-normally distributed variables, respectively.

3.4.2 Metabolic and fertility parameters

As shown in Table 1, at 21 DPP and 45 DPP, there were no significant differences between groups in mean milk yield, body weight loss and serum NEFA concentrations. There were no significant differences for the calving to first AI interval, calving to conception interval and AI number/conception between the three groups. However, the latter two parameters of groups EH and EE were favoured by only including pregnant cows within 305 DPP (as five and four cows were culled for non-conception at 305 DPP, respectively). Six of 19 (32%) HH cows conceived at first AI and the remaining were pregnant within 305 DPP. Also, at 45 DPP there were more cows in luteal phase in group HH than in groups EH and EE ($P = 0.01$), evidencing an earlier resumption of ovarian activity of HH cows, compared to EH and EE cows.

3.4.3 Adipokine concentrations in plasma and uterine fluid

Plasma and uterine fluid concentrations of ADIPOQ, RARRES2 and NAMPT are presented in Figure 4 and Annex VII. ADIPOQ concentrations in plasma at 21 ($P < 0.05$) and 45 DPP ($P < 0.01$) and uterine fluid ($P < 0.001$) were higher in EE cows than in HH and EH cows. At 45 DPP, plasma concentrations of RARRES2 were higher ($P < 0.05$) in EE than in HH cows, and uterine fluid concentrations were higher ($P < 0.01$) in group EE than in groups HH and EH. In contrast, uterine fluid concentrations of NAMPT were similar in the three groups. Nevertheless, plasma NAMPT concentrations of EH cows were lower ($P < 0.01$) than of EE cows at 21 DPP, and of both HH and EE cows at 45 DPP ($P < 0.01$). Plasma concentrations of ADIPOQ, RARRES2 and NAMPT at 21 DPP and 45 DPP were similar in luteal phase and follicular / anoestrous cows of groups HH, EH and EE. This was also observed in uterine concentrations at 45 DPP, except in HH group, where only ADIPOQ concentrations were higher ($P< 0.01$) in luteal than in follicular / anoestrous cows (data not shown).

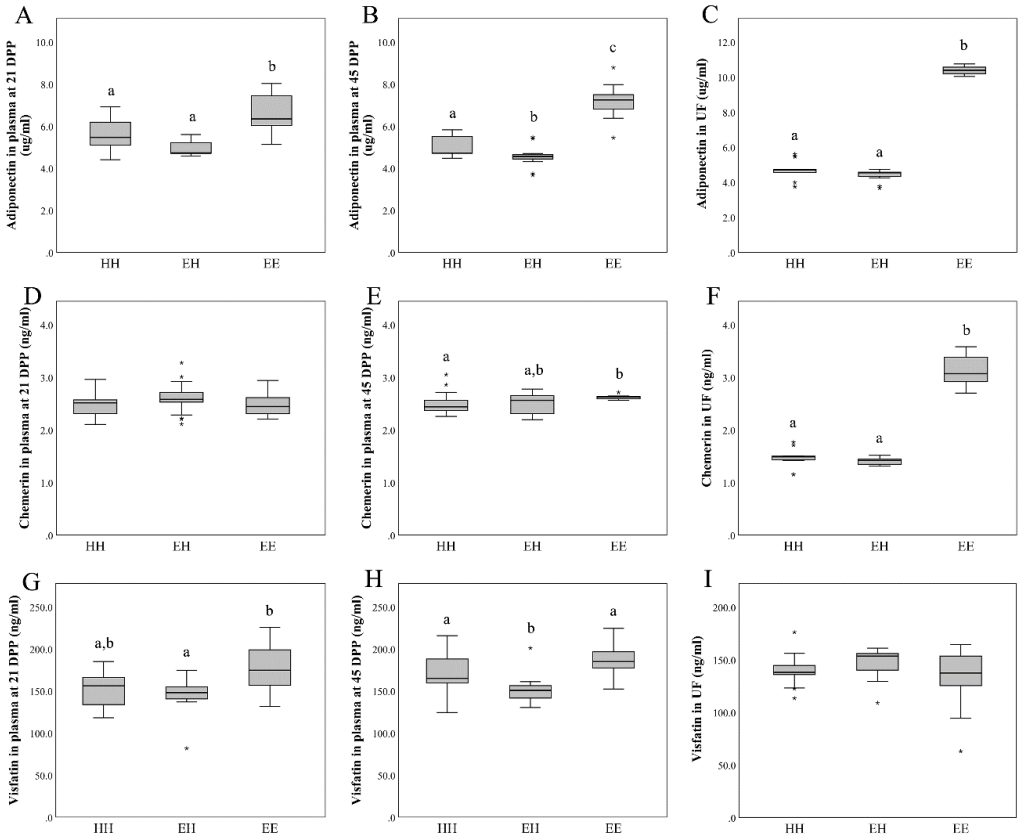

Figure 4. Plasma and uterine fluid concentrations of adiponectin (A, B and C), chemerin (D, E and F) and visfatin (G, H and I) in healthy cows (group HH), cows with SCE at 21 DPP but recovered by 45 DPP (group EH), and cows with persistent SCE until 45 DPP (group EE). Different letters indicate a significant difference at $P < 0.05$. Horizontal black lines indicate median, boxes extend from the 25th to the 75th percentile and vertical lines indicate values within 1.5 interquartile range of the 25th and 75th percentile. Asterisks indicate outliers.

3.4.4 Relationship between adipokines' concentrations and PMN percentage from endometrial cytology

Table 2 displays a summary of Spearman correlations between adipokines' concentrations and PMN percentage from endometrial cytology. Uterine fluid concentrations of ADIPOQ and RARRES2 showed a significant positive correlation with the endometrial cytology PMN percentage at 45 DPP. In addition, concentrations of ADIPOQ in plasma significantly correlated with those in uterine fluid, and at 45 DPP with the endometrial cytology PMN percentage. RARRES2 uterine fluid levels did not correlate with its plasma levels at 45 DPP.

Table 2. Spearman's correlations between the plasma (21 and 45 DPP) and uterine fluid (UF; 45 DPP) adipokines' concentrations and the PMN percentage from endometrial cytology (n = 49).

	ADIPOQ 21 DPP	ADIPOQ 45 DPP	ADIPOQ UF	RARRES2 45 DPP	RARRES2 UF	PMN% 21 DPP	PMN% 45 DPP
ADIPOQ 21 DPP		0.599**	0.631**	0.349*	0.457**	0.087^{ns}	0.281^{ns}
ADIPOQ 45 DPP			0.704**	0.216^{ns}	0.720**	0.087^{ns}	0.436**
ADIPOQ UF				0.093^{ns}	0.782**	0.244^{ns}	0.537**
RARRES2 45 DPP					-0.019^{ns}	0.231^{ns}	0.153^{ns}
RARRES2 UF						0.218^{ns}	0.640**
PMN% 21 DPP							0.507**

DPP = days postpartum; ns - not significant; * P < 0.05 and ** P < 0.01

Figure 5 represents the ROC analysis of ADIPOQ and RARRES2 concentrations, to discriminate cows with SCE at 45 DPP. Plasma ADIPOQ at 21 DPP (AUC = 0.872; P < 0.001), plasma ADIPOQ at 45 DPP (AUC = 0.981; P < 0.001), and uterine fluid ADIPOQ and RARRES2 at 45 DPP (AUC = 1.000; P < 0.001) showed high AUC values. In contrast, plasma RARRES2 at 21 DPP (AUC = 0.423; P = 0.443) and at 45 DPP (AUC = 0.738; P = 0.017) showed either no ability or a low ability to discriminate cows with SCE. The risk of endometritis persistence at 45 DPP (Annex VIII) was higher for cows with plasma ADIPOQ concentrations at 21 DPP above 5.9 µg/mL (OR = 19.9; 95% CI 3.5-113.3; P < 0.001) than for cows with concentrations below the cutoff. At 45 DPP, plasma and uterine fluid ADIPOQ concentrations showed a high discriminatory power for diagnosis of endometritis. Cows with plasma and uterine fluid ADIPOQ concentrations above 6.1 and 7.8 µg/mL, respectively, had increased risk of being diagnosed with endometritis (OR = 539; 95% CI 20.4-14218.7; P < 0.001 and OR = 1771; 95% CI 33.3 – 94301.3; P < 0.001, respectively) than cows with concentrations below the cutoff. For plasma concentrations only one of 39 cows with concentrations below the cutoff showed endometritis, and all 10 cows with concentrations above the cutoff presented endometritis. For uterine fluid concentrations, all cows with concentrations below the cutoff were healthy, whereas all cows with concentrations above the cutoff presented endometritis. This was also observed in the case of uterine fluid RARRES2 concentrations (cutoff = 2.2 ng/mL).

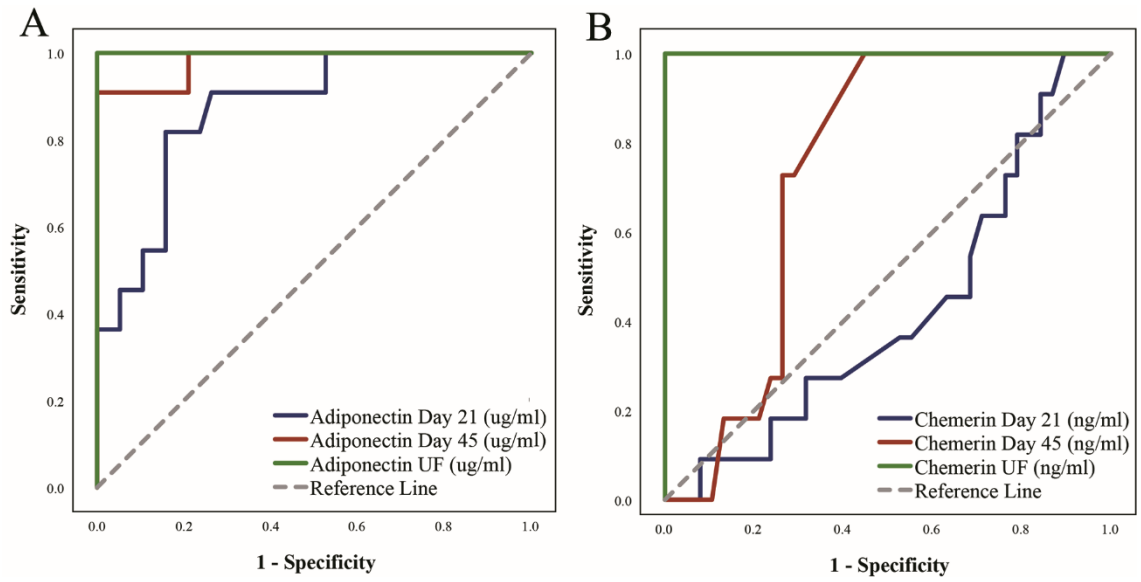

Figure 5. Receiver Operating Characteristic curve analysis of Adiponectin (A) and Chemerin (B) to discriminate cows with SCE at 45 days postpartum. Concentrations were measured in plasma at 21 and 45 days postpartum, and in uterine fluid (UF) at 45 days postpartum.

As concentrations in plasma and uterine fluid showed significant differences between healthy (HH) and endometritis cows (EE) only for ADIPOQ and RARRES2, the analysis of the endometrial immunolocalisation and of the gene expression in cellular pellets from uterine flushings was performed only for these two adipokines and their receptors. Also, to further refine the evaluation of differences between healthy and endometritis cows ("extreme cases"), the above analysis considered a subset of the HH group, consisting of healthy cows that became pregnant at first AI (group HHP; n = 6).

3.4.5 Immunolocalisation of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2 and CMKLR1 in the bovine endometrium

Positive immunostaining for ADIPOQ was observed in the luminal and glandular epithelial cells, and in endothelial, stromal and inflammatory cells of all cows. However, cows of group EE exhibited a stronger staining in the luminal epithelial cells than cows of HHP and EH groups (Figure 6A, B and C; Table 3). Positive staining for ADIPOQ was observed in endothelial cells (Figure 6D) and macrophages (Figure 6F) within the stroma, whereas the infiltrated PMN were negative (Figure 6E). Positive immunostaining for ADIPOR1 and ADIPOR2 was observed in luminal and glandular epithelial cells. Additionally, ADIPOR2 staining was observed in stromal and inflammatory cells (Figure 6G, H, I, J, K and L). Both these receptors displayed a stronger staining in EE cows than in HHP and EH cows (Table 3).

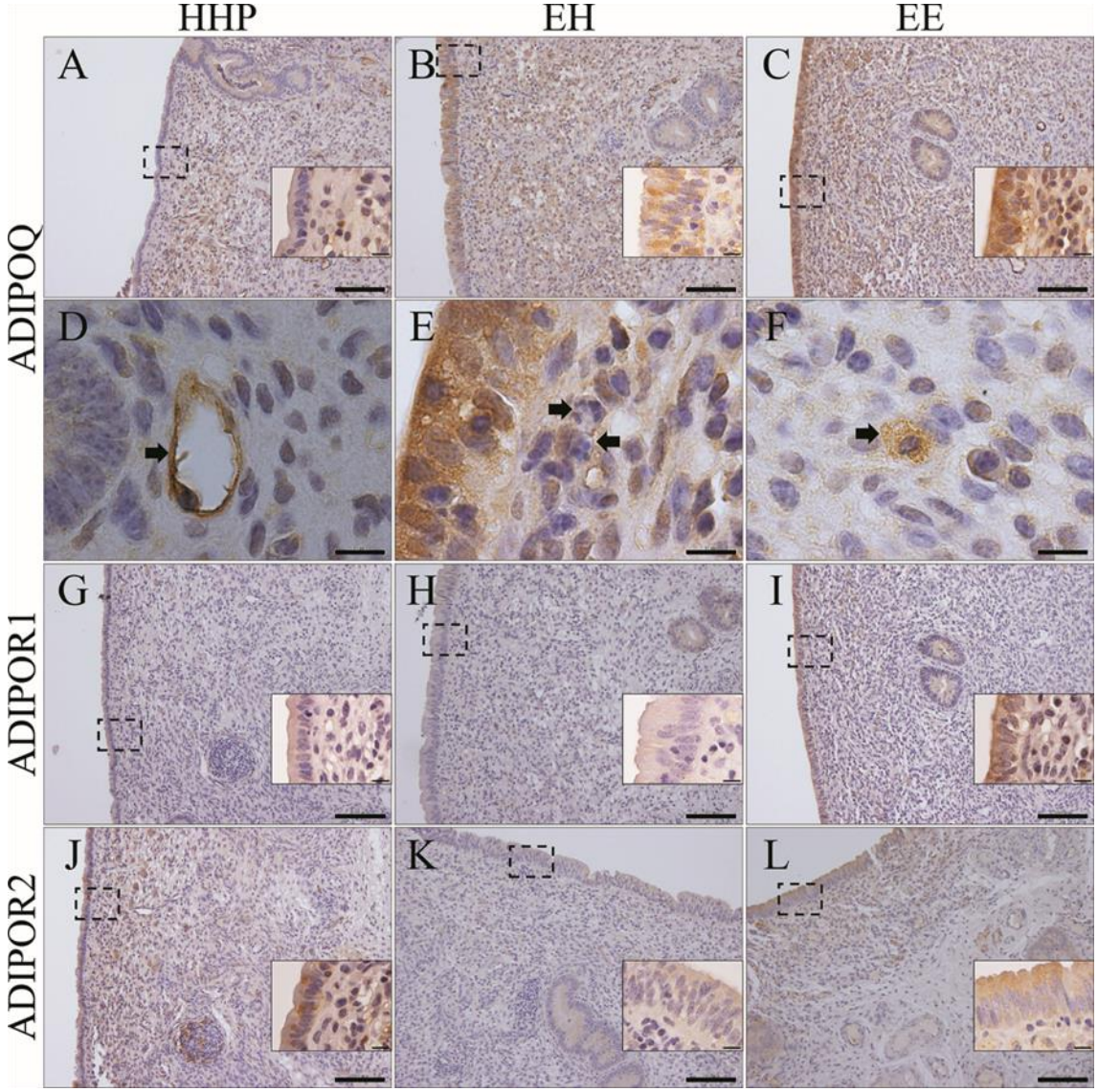

Figure 6. Representative photomicrographs of endometrial sections from biopsies recovered at 45 days postpartum from a subset of healthy cows pregnant at first AI (group HHP), cows with SCE at day 21 postpartum but that recovered by day 45 postpartum (group EH), and cows with persistent SCE until day 45 postpartum (group EE). Immunostaining for ADIPOQ (A, B, C, D, E, F), for ADIPOR1 (G, H, I) and for ADIPOR2 (J, K, L). In D, arrow pointing to stained endothelial cell; in E, arrows pointing to non-stained PMN; in F, arrow pointing to stained macrophage. Scale bar 10 µm (D, E, F and all the insets) and 100 µm (A, B, C, G, H, I, J, K and L).

Positive immunostaining for RARRES2 was detected in the luminal and glandular epithelia, and in stroma and inflammatory cells of all cows (Figure 7A, B and C), and the level of staining was stronger in EE group than in HHP and EH groups (Table 3). Positive immunostaining for CMKLR1 was observed in the luminal epithelial cells, and in stroma and inflammatory cells (Figure 7D, E and F). However, in the luminal epithelial cells of HHP and EH cows, the staining was mostly found in the apical membrane of the cells (Figure 7D and E) whereas in EE cows a strong staining is present throughout the cytoplasm (Figure 7F).

Table 3. Level of immunostaining of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2 and CMKLR1 in different cell populations of endometrial biopsies taken at 45 DPP in healthy cows (group HHP, n = 3), cows with SCE at 21 DPP but that recovered by 45 DPP (group EH, n = 3), and cows with persistent SCE until 45 DPP (group EE, n = 3).

		HHP	EH	EE
ADIPOQ	LE	+/-	+	++
	GE	+	+	+
	ST	+	+	+
	ST-IC	+/-	+	+
	ST-LF	-	-	+/-

		HHP	EH	EE
ADIPOR1	LE	+/-	+	++
	GE	+	+	+
	ST	-	-	-
	ST-IC	-	-	-
	ST-LF	-	-	-

		HHP	EH	EE
ADIPOR2	LE	+/-	+	++
	GE	+	+	+
	ST	+	+	+
	ST-IC	+	+	+
	ST-LF	+	+/-	+

		HHP	EH	EE
RARRES2	LE	+	+	++
	GE	+	+/-	+
	ST	+	+	+
	ST-IC	+	+	+
	ST-LF	+	+/-	+/-

		HHP	EH	EE
CMKLR1	LE	+	+	++
	GE	-	-	-
	ST	+/-	+/-	+
	ST-IC	+/-	+/-	++
	ST-LF	+/-	-	+/-

DPP = days postpartum; LE - luminal epithelial cells; GE - glandular epithelial cells; ST - stroma cells; ST-IC - inflammatory cells in stroma; ST-LF - lymphoid follicles in stroma; level of staining: – (absent); +/- (weak); + (moderate); ++ (strong).

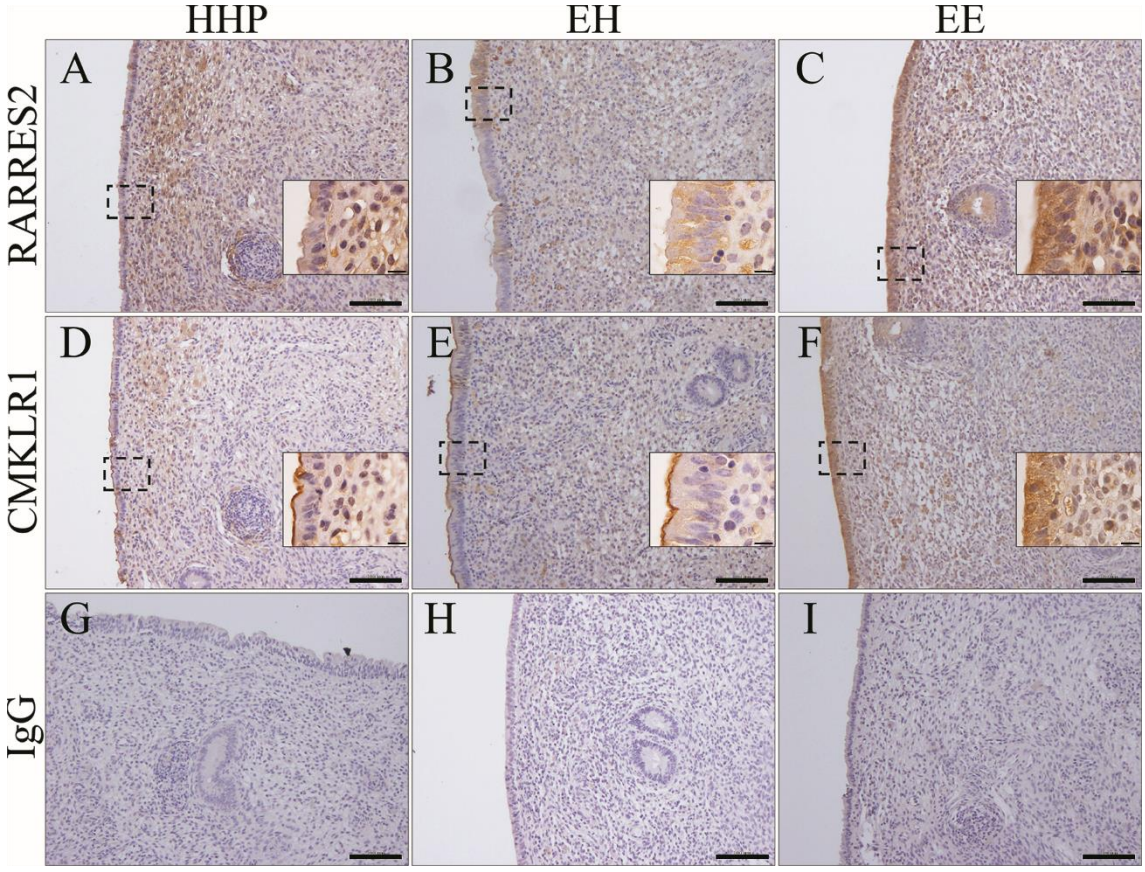

Figure 7. Representative photomicrographs of endometrial sections from biopsies recovered at 45 days postpartum from a subset of healthy cows pregnant at first AI (group HHP), cows with SCE at day 21 postpartum but that recovered by day 45 postpartum (group EH), and cows with persistent SCE until day 45 postpartum (group EE). Immunostaining for RARRES2 (A, B, C), CMKLR1 (D, E, F) and negative control (G, H, I). Scale bar 10 µm (insets) and 100 µm (A, B, C, D, E, F, G, H and I).

3.4.6 Transcription levels of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2, CMKLR1, GPR1 and CCRL2 in the cellular pellet of the uterine flushing

Transcription levels of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2, CMKLR1 and GPR1 are presented in Figures 8 and 9. Transcription levels of ADIPOQ and ADIPOR2 were higher ($P < 0.001$) in group EE than in groups HHP and EH. In contrast, mRNA levels of ADIPOR1 were lower ($P < 0.05$) in EE cows than in HHP cows. Transcription levels of RARRES2, CMKLR1 and GPR1 were also higher ($P < 0.001$, $P < 0.001$ and $P = 0.001$, respectively) in group EE than in groups HHP and EH, whereas transcription levels of CCRL2 were similar in the three groups (data not shown).

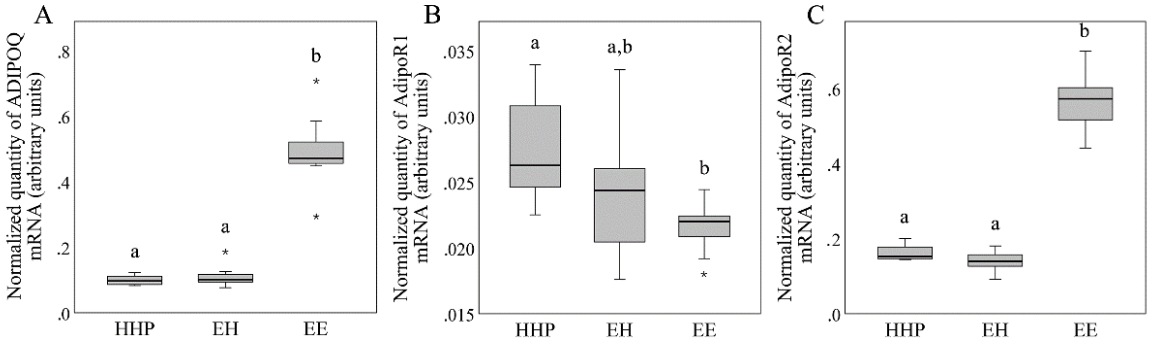

Figure 8. Transcript abundance of ADIPOQ (A), ADIPOR1 (B) and ADIPOR2 (C) standardized to the geometric mean of GAPDH, β-ACTIN and PPIA in uterine cell pellets collected at 45 days postpartum. Data analysed using Kruskal-Wallis-Test with Dunns post-test. Different letters indicate a significant difference at $P < 0.05$. Groups HHP – healthy cows; EH – cows with SCE at 21 days postpartum but that recovered by 45 days postpartum; EE – cows with persistent SCE until 45 days postpartum. Horizontal black lines indicate median, boxes extend from the 25th to the 75th percentile and vertical lines indicate values within 1.5 interquartile range of the 25th and 75th percentile. Asterisks indicate outliers.

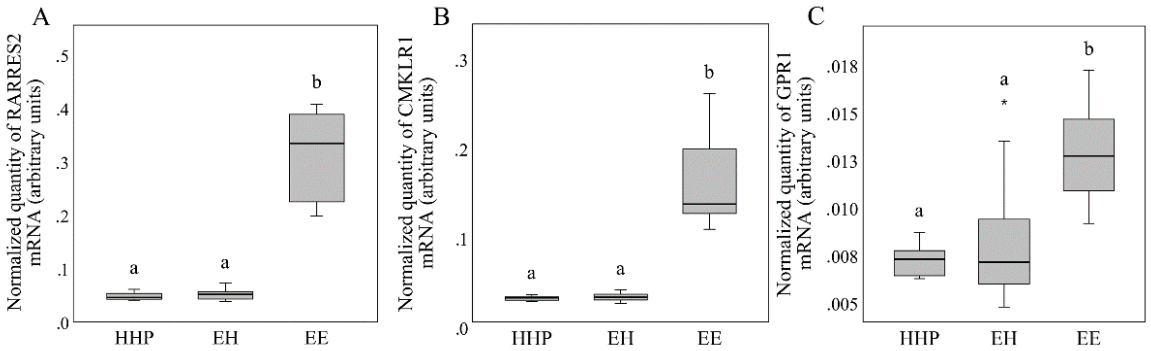

Figure 9. Transcript abundance of RARRES2 (A), CMKLR1 (B) and GPR1 (C) standardized to the geometric mean of GAPDH, β-ACTIN and PPIA in uterine cell pellets collected at 45 days postpartum. Data analysed using Kruskal-Wallis-Test with Dunns post-test. Different letters indicate a significant difference at $p < 0.05$. Groups HHP – healthy cows; EH – cows with SCE at 21 days postpartum but that recovered by 45 days postpartum; EE – cows with persistent SCE until 45 days postpartum. Horizontal black lines indicate median, boxes extend from the 25th to the 75th percentile and vertical lines indicate values within 1.5 interquartile range of the 25th and 75th percentile. Asterisks indicate outliers.

3.5 Discussion

There is a wide range in the reported prevalence of SCE in dairy cows, but it is frequently pointed around 30-35% between 4 to 9 weeks postpartum (LeBlanc 2008). Here, in cows without puerperal disease, the prevalence of SCE was 61% (30 out of 49) at 21 DPP and 22% (11 out of 49) at 45 DPP. This evidences that over 60% of early affected cows have recovered spontaneously by the end of the voluntary waiting period expressing then transient endometritis. This also shows that from the total number of cows initially recruited at 21 DPP, 22% still suffer from persistent endometrial inflammation at 45DPP.

At 21 DPP, 83% of cows with SCE showed a positive vaginal discharge Metricheck score, indicating the subclinical nature of endometrial inflammation in about 20% of cows. In contrast, at 45 DPP, only 9% of cows with SCE showed a positive vaginal discharge Metricheck score, indicating the subclinical nature of endometritis in about 90% of cows. These figures, as reported by McDougall et al. (2011) demonstrates a moderate level of agreement between the vaginal discharge Metricheck score and the endometrial cytology PMN percentage at 21DPP and no agreement at 45DPP. This may be partially explained by, or may reflect the presence of cervicitis or vaginitis instead of endometritis (Westermann et al. 2010). By contrast, the onset of postpartum ovarian activity promotes cervix closure under the influence of progesterone, which may block the flux of uterine contents into the vagina.

Therefore, for descriptive purposes, herein cows with SCE until 45 DPP are also referred as persistent SCE cows, whereas cows that recovered of SCE by 45 DPP are also referred as transient SCE cows.

Milk yield, body weight loss and blood NEFA concentrations were not associated to persistent or transient SCE. These findings are in agreement with Dubuc et al. (2011b) and reinforce the subclinical nature of this condition. In dairy cows, plasma concentrations of adipokines are affected by negative energy balance status (Mellouk 2017), variations in white adipose tissue quantities (Reverchon et al. 2014a) and milk yield (Kafi et al. 2015). However, no differences were observed here between uterine health groups regarding these parameters. Therefore, it is unlikely that they have contributed to the observed between-group differences in adipokines' gene transcription, protein expression and production described under.

This study evidenced that cows affected by persistent endometritis exhibited increased concentrations of ADIPOQ at 21 and 45 DPP in plasma, and at 45 DPP in uterine fluid, compared to healthy and transient endometritis cows. The ROC curve analysis showed that the chance of discriminating a healthy from a persistent endometritis cow, at 45 DPP, was

87% and 98% based on plasma concentrations at 21 and 45 DPP, respectively, and 100% based on uterine fluid concentrations at 45 DPP. In addition, the OR analysis showed that cows with concentrations above the cutoff were a minimum of 3.5 (plasma 21 DPP), 20.4 (plasma 45 DPP) and 33.3 (uterine fluid 45 DPP) times more at risk of evidencing persistent endometritis at 45 DPP than healthy cows. These observations evidence that ADIPOQ is a promising biomarker, with putative high discriminatory power for the identification of persistent endometritis cows at 45 DPP. In contrast with these results, Kasimanickam et al. (2013) although reporting increased concentrations of serum ADIPOQ in cows affected by metritis and clinical endometritis, found no differences between cows affected by SCE and healthy cows. However, the above study (Kasimanickam et al. 2013) only included six SCE cows, and four cows of the healthy cow group (n = 13) were subsequently found to develop clinical endometritis two weeks later. Therefore, the inconsistency between the two studies may be explained by differences in population size and categorization of the healthy cow group.

The increased plasma and uterine fluid ADIPOQ concentrations observed in endometritis cows can result from white adipose tissue production or it can originate from local expression in the reproductive tissues, which would indicate, besides an endocrine function, also autocrine or paracrine effects (Reverchon et al. 2014a). In this study, the putative uterine origin of ADIPOQ was addressed through the evaluation of gene transcription in the cellular pellet of uterine fluid, and the endometrial protein immunolocalisation. Transcription of ADIPOQ, ADIPOR1 and ADIPOR2 was detected in the cellular pellet of uterine flushings, and persistent endometritis increased local transcription of ADIPOQ and its receptors. This may indicate that the presence of ADIPOQ in the uterine lumen is at least partially explained by local transcription. The above findings were confirmed by the immunolocalisation of ADIPOQ, ADIPOR1 and ADIPOR2 in the cow endometrium, and by the increased immunostaining of these proteins in persistent endometritis cows. Overall, our data indicate that the ADIPOQ signalling system is present in all types of endometrial cells, and is related to the inflammatory status of the uterus.

A relationship between ADIPOQ concentrations and the inflammatory status was also observed in the mammary gland of cows following a LPS challenge (Singh 2014), and in human patients with inflammatory and immune-mediated diseases (Fantuzzi 2013). ADIPOQ may express anti-inflammatory properties by inducing the switch of macrophage phenotype to an anti-inflammatory state, decreasing the expression of Toll-like receptor 4 (TLR4) and by suppressing the endothelial inflammatory response (Fang and Judd, 2018). The cow innate immune response to LPS is dependent on TLR4 signalling in the epithelial and stromal cells of the endometrium (Herath et al. 2006, Sheldon and Roberts 2010, Piras et al. 2017). In

humans, increased TLR4 expression levels were associated with disease progression in patients with chronic endometritis (Ju et al. 2014), and in the bitch, pyometra caused by *E. coli*, lead to a significantly increased TLR4 transcription and protein expression in all endometrial compartments, including inflammatory cells (Silva et al. 2010). Therefore, endometrial cis and/or trans ADIPOQ signalling may be up-regulated following LPS injury, which may convey early predictive information regarding the establishment of endometritis. Expression of ADIPOQ and its receptors in endometrial endothelial cells is also increased in persistent endometritis cows. Therefore, the anti-inflammatory action of ADIPOQ may be also related with the suppression of the endothelial inflammatory response (Fang and Judd 2018).

This study also evidenced that cows with persistent endometritis exhibited higher plasma and uterine fluid concentrations of RARRES2 at 45 DPP than healthy and transient endometritis cows. Consistently with the above, cows with persistent endometritis showed up-regulated transcription of RARRES2, CMKLR1 and GPR1 in the cellular pellet of uterine fluid, and an increased expression of RARRES2 and CMKLR1 in the endometrium. These data indicate a relationship between RARRES2 signalling and the inflammatory status of the postpartum cow uterus, and a local production of this mediator. However, unlike ADIPOQ, RARRES2 ROC curve and OR analysis revealed that only uterine fluid RARRES2 concentrations were suitable to discriminate persistent endometritis cows at 45 DPP. In fact, cows with uterine fluid concentrations at 45 DPP above the cutoff were a minimum of 33.3 times more at risk of evidencing endometritis at 45 DPP than healthy cows, and concentrations above the cutoff were able to discriminate all healthy from persistent endometritis cows at 45 DPP. Although adipose tissue and liver are considered the major sites of RARRES2 expression, its production has been detected in other tissues including the reproductive tract (Bongrani et al. 2019). This protein is expressed in several epithelial cell types and participates in the host defence mechanisms, as a broad-spectrum antimicrobial protein and as a leukocyte attractant, evidencing pro-inflammatory properties (Zabel et al. 2014). In fact, local RARRES2 concentrations were positively associated with metabolic and inflammatory diseases (Dranse et al. 2015). A systemic repercussion of a local inflammatory status, as evidenced in this study, is consistent with data from Weigert et al. (2010) showing increased levels of serum RARRES2 in human patients affected by inflammatory conditions.

Chemerin (RARRES2) is secreted as a precursor termed prochemerin, which is converted into the active form under conditions such as coagulation, fibrinolysis, inflammatory and complement cascades activation (Zabel et al. 2014). These conditions are present in the uterine lumen of cows affected with endometritis, due to the presence of PMNs. These inflammatory cells are the first to be recruited, and once activated degranulate and unleash a

set of proteases capable of activating prochemerin into RARRES2 (Mariani and Roncucci 2015). The observed significant positive correlation between RARRES2 concentrations in the uterine fluid, and the endometrial cytology PMN percentage at 45 DPP, is in accordance with the above mechanism. Also, the lack of correlation between plasma and uterine concentrations of RARRES2 at 45 DPP is in accordance with a local activation and inactivation of RARRES2 by proteolytic processes (Bondue et al. 2011). The immunolocalisation of RARRES2 in the endometrial luminal and glandular epithelial cells as well as in the stroma and inflammatory cells lead to the suggestion that RARRES2 also act in the cow endometrium as a player of the pro-inflammatory cascade.

Its receptor CMKLR1 is expressed by the luminal epithelial cells, either at the apical membrane (groups HHP and EH) or throughout the cytoplasm (group EE). As observed in other scenarios (Zhou et al. 2014, De Henau et al. 2016), this may reflect the receptor's internalisation after binding with RARRES2, which appeared here more pronounced in cows with persistent endometritis than in other groups. Plasma and uterine fluid NAMPT concentrations were similar in healthy and persistent endometritis cows. This finding was unexpected since NAMPT was suggested as an indicator of inflammatory disease in cows (Fadden and Bobe 2016), and to be involved in the modulation of the uterine LPS-induced inflammatory response in rats (Yang et al. 2015).

A significant positive correlation between ADIPOQ and RARRES2 concentrations in the uterine fluid was observed. This may represent the two faces of a balanced inflammatory response, combining anti-inflammatory (ADIPOQ) and pro-inflammatory (RARRES2) characteristics. This could act through a mechanism by which ADIPOQ could induce CMKLR1 expression, and consequently activation of the RARRES2/CMKLR1 system, as proposed in humans following reduction of weight (Shin et al. 2017).

3.6 Conclusion

In conclusion, postpartum dairy cows affected with persistent endometritis presented increased plasma and uterine fluid concentrations of ADIPOQ and RARRES2, up-regulation of transcription of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2, CMKLR1 and GPR1 in the cellular pellet of uterine fluid, and increased expression of ADIPOQ, ADIPOR1, ADIPOR2, RARRES2 and CMKLR1 in the endometrium. Plasma and uterine fluid ADIPOQ and RARRES2 concentrations were positively correlated with the endometrial cytology PMN percentage, and the ROC curve and OR analysis showed that both plasma and uterine fluid ADIPOQ concentrations, and uterine fluid RARRES2 concentrations were able to identify a persistent endometrial inflammation at 45 DPP. These data indicate a relationship between adipokine signalling and the inflammatory status of the postpartum uterus of dairy cows,

where ADIPOQ and RARRES2 systems are potentially involved in a balanced anti-inflammatory and pro-inflammatory response, respectively. Therefore, ADIPOQ and RARRES2 represent suitable biomarkers able to provide an early diagnosis of SCE and predict the risk of persistence of uterine inflammation. This would be of particular interest for discriminating cows elective for timely therapeutic approaches, as affected cows could be identified early in the postpartum period and allocated to appropriate therapy. Further studies are necessary to determine the role of these adipokines in the establishment of SCE.

CHAPTER 4 - Progesterone differentially affects the transcriptomic profiles of cow endometrial cell types

G. Pereira[1], Y. Guo[1], E. Silva, C. Bevilacqua, G. Charpigny, L. Lopes-da-Costa and P. Humblot.

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[1]These authors contributed equally to this work

4.1 Abstract

The endometrium is a heterogeneous tissue composed of luminal epithelial (LE), glandular epithelial (GE), and stromal cells (ST), experiencing progesterone regulated dynamic changes during the estrous cycle. In the cow, this regulation at the transcriptomic level was only evaluated in the whole tissue. This study describes specific transcription in the three types of cells isolated from endometrial biopsies following laser capture microdissection, and the transcriptome changes induced by progesterone in GE and ST cells.

Endometrial LE, GE, and ST cells show specific transcriptomic profiles. Most of the differentially expressed genes (DEGs) in response to progesterone are cell type-specific (96%). Genes involved in cell cycle and nuclear division are less transcribed in the presence of progesterone in GE, highlighting the anti-proliferative action of progesterone in epithelial cells. Elevated progesterone concentrations are also associated with decreased transcriptionof genes encoding estrogen receptor 1 (*ESR1*) in GE and oxytocin receptor (*OXTR*) in GE and ST cells. In ST cells, genes encoding transcription factors such as *SOX17* and *FOXA2*, known to regulate uterine epithelial-stromal cross-talk conveying to endometrial receptivity, display increased transcription under progesterone influence.

The results from this study show that progesterone regulates endometrial function in a cell type-specific way, which is independent of the transcription of its main receptor *PGR*. These novel insights into uterine physiology present the cell compartment as the physiological unit rather than the whole tissue.

**Text adapted from the original published paper (Annex IX)*

4.2 Introduction

The bovine endometrium is composed of different cell compartments, comprising luminal epithelial, glandular epithelial, and stromal cells, which is submitted to intense tissue remodelling during the estrous cycle, embryo implantation and puerperal involution (Bauersachs et al. 2005; Sheldon et al. 2019). Progesterone released by the corpus luteum (CL) plays a key role regulating endometrial function and remodelling (Geisert et al. 1992; Spencer et al. 2004). At the transcriptomic level, this P4 regulation has been evaluated so far, only from the whole endometrial tissue, following luteolysis (Forde et al. 2012), comparing different oestrus cycle stages (Mitko et al. 2008) or status (dioestrus versus anoestrus) and type of ovulation (single versus multiple ovulation) (Madoz et al. 2020). However, as in other heterogeneous tissues, quantification of gene expression from the whole endometrium may not reflect the specific transcription of the cell compartments, which should be preferably analysed from homogeneous cell populations. Laser capture microdissection emerged as a research tool to isolate cell populations for further molecular analyses (Bevilacqua and Ducos, 2018). This method has been used to study the interactions between the endometrial epithelial and stromal compartments with seminal plasma in a murine model (Field et al. 2015), and the conceptus induced regulation of endometrial function in the porcine (Zeng et al. 2018), ovine (Brooks et al. 2016) and equine (Scaravaggi et al. 2018) species. In the cow, recent studies based on this approach described the specific molecular signatures of endometrial stromal, glandular and luminal epithelial cells, as well as the effect of negative energy balance on the transcriptomic profiles of endometrial compartments in the mid-luteal phase (Chankeaw et al. 2021a; 2021b). The above experiments revealed that the different endometrial cell types show distinct transcription patterns. The main objective of this study was to evaluate the effects of P4 on the transcription patterns of the three main bovine endometrial cell types which, to our knowledge have not been documented so far.

4.3 Material and Methods

4.3.1 Ethics statement

All animal procedures were conducted by licenced veterinarians, in compliance with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU), and the research protocol was approved by the Institutional Animal Care and Use Committee (Reference CEIE nº37/2019).

4.3.2 Animals

The animal handling and sampling procedures of cows enrolled in this study were published (Pereira et al. 2020). In brief, high-yielding dairy cows of the Holstein-Friesian breed from a single herd were submitted to blood sampling, genital ultrasonography, uterine cytology and biopsy at 44 ± 0.7 DPP. The uterine status of all the cows included in this study (n = 13) was assessed as healthy, with no clinical signs of endometritis and a percentage of polymorphonuclear leukocytes measured by cytology from 400 cells < 5% (Gilbert et al. 2005, Pereira et al. 2020). Additionally, endometrial tissue was confirmed to be healthy as no contamination by immune cells was perceived on histology analysis performed retrospectively. The presence of a CL and plasma P4 concentrations were used to categorise cows in high P4 (n = 4) and low P4 (n = 9). Ovarian structures as observed by ultrasonography are presented in Table 4. A functional CL was defined as a luteal structure > 23 mm in diameter (Bicalho et al. 2008). Based on heat observation, ovarian ultrasonography and plasma P4 concentrations at 21 and 44 DPP, among the 9 low P4 cows, 4 were still in anoestrus at 44 DPP, and 5 were cyclic at a peri estrus stage of the cycle. From the 4 high P4 cows, 2 had a functional CL arising from the first ovulation postpartum and the other 2 had a functional CL arising from the second ovulation postpartum.

Table 4 Cow groups according to clinical characterisation and progesterone concentrations.

Characteristics	Cows with Low Progesterone concentrations	Cows with High Progesterone concentrations
Number of cows	n = 9	n = 4
Lactation number[1]	1.8 ± 0.3	1.7 ± 0.4
Results of ovarian ultrasonography		
Dominant follicle	n = 5	n = 0
Non-functional CL	n = 4	n = 0
Functional CL	n = 0	n = 4
Progesterone concentrations (ng/mL)[1]	0.5 ± 0.1	5.7 ± 1.4
Range	0.20-0.84	1.8-9.3

[1]mean ± standard error of the mean

4.3.3 Progesterone assay

Blood was collected by venipuncture of the coccygeal vein into 10 mL dry vacutainers (Becton-Dickinson), allowed to clot and centrifuged (2000 g for 15 min) within 30 min of collection. Serum samples were transferred to the laboratory at 4 °C and then stored at -20 °C until analysis. Progesterone concentrations were measured by a chemiluminescent immunoassay in an IMMULITE 1000 analyser (Siemens Healthcare Diagnostics) using a commercial kit (IMMULITE 1000 Progesterone Kit, Siemens Healthcare Diagnostics). The

assay's sensitivity was 0.2 ng/mL, and the inter-assay coefficient of variation was <10%. The cut-off value used to define cows with high P4 concentrations was 1 ng/mL. Means and range of values for the groups of cow with high and low P4 concentrations are shown in Table 4.

4.3.4 Endometrial biopsy

Endometrial biopsies were collected with a Kervokian–Younge endometrial biopsy instrument (Alcyon), according to procedures previously described by Pereira et al. (2020). The biopsy instrument was guided into the first third of one uterine horn, and an endometrial sample of about 0.5-1 cm^2 and 3-5 mm thick recovered. The endometrial samples were immediately frozen in dry ice cold isopentane (2-Methylbutane, Sigma Aldrich) for 60 seconds and embedded in a cryomold with optimal cutting temperature compound (Tissue-Tek OCT Compound, Sakura Finetek). Cryomolds were transferred to the laboratory on dry ice, then kept at -80ºC until tissue processing.

4.3.5 Endometrial tissue processing and staining

Serial sections (8 µm thick) were cut from the tissue blocks on a cryostat (Cryotome FSE, Thermo Scientific) set at -20ºC, mounted on glass slides at 4ºC and immersed for 60 seconds in 75% ethanol inside the cryostat chamber (- 20ºC). Slides were then stained with Cresyl Violet (1% in 50% ethanol) and dehydrated at room temperature as described by Bevilacqua et al. (2010). When taken out of the cryostat chamber, the slides were transferred to 75% ethanol for 20 seconds, stained with 1% Cresyl violet in ethanol (25 sec), rinsed successively with 75% ethanol (30 sec), 95% ethanol (2 x 1 min), 100% ethanol (2 x 1 min), and finally, pure xylene (M-xylene, Sigma-Aldrich; 2 x 5 min). In order to ensure appropriate dehydration, new bottles of absolute ethanol and pure xylene were opened every day immediately before use. Stained and dehydrated tissue sections were air-dried to remove xylene residues before microdissection.

4.3.6 Laser Capture Microdissection and RNA extraction

The endometrial cell types (LE, GE and ST) were isolated from the whole tissue sections using an ARCTURUS XT™ Laser Capture Microdissection System and software (Applied Biosystems®, Arcturus). Although cells isolated from the ST compartment may comprise a combination of fibroblasts, immune and endothelial cells, ST is hereafter referred to as a "cell type". A previous report showed that contamination of micro-dissected ST samples by other cell types was negligible (Chankeaw et al. 2021a). Laser capture was performed either under 200× or 400× magnification and infrared settings (power, duration and intensity) were adjusted for each field of view, to maximise the size of the laser spot without contaminating

the sample with undesired cells. Following capture, each LCM plastic cap (CapSure®Macro LCM Caps, Arcturus) was examined at the quality control (QC) station and if necessary, undesired cells were removed from the cap by low power UV laser. For a given tissue section, the full microdissection processing did not last more than 90 minutes to preserve RNA integrity. Examples of histology slides of each endometrial cell type before and after capture with LCM are presented in Figure 10. After microdissection, total RNA from LE, GE and ST cells was extracted using the PicoPureTM RNA Isolation Kit (Arcturus) following the manufacturer's protocol. A DNAse I (Qiagen) treatment step was added according to the protocol and eluted in 15 µl of Elution buffer. The RNA quantity and quality [RNA Integrity Number (RIN)] were assessed with the Agilent Bioanalyzer 2100 system (Agilent Technologies) and the RNA 6000 pico Chip Kit. Due to difficulty in harvesting enough RNA with eligible RIN value (≥ 7) for gene transcription measurements (Bevilacqua et al. (2010), from the initial 39 samples from 13 cows, only 7 LE, 10 GE, and 12 ST samples were analysed by RNA sequencing (Table 5).

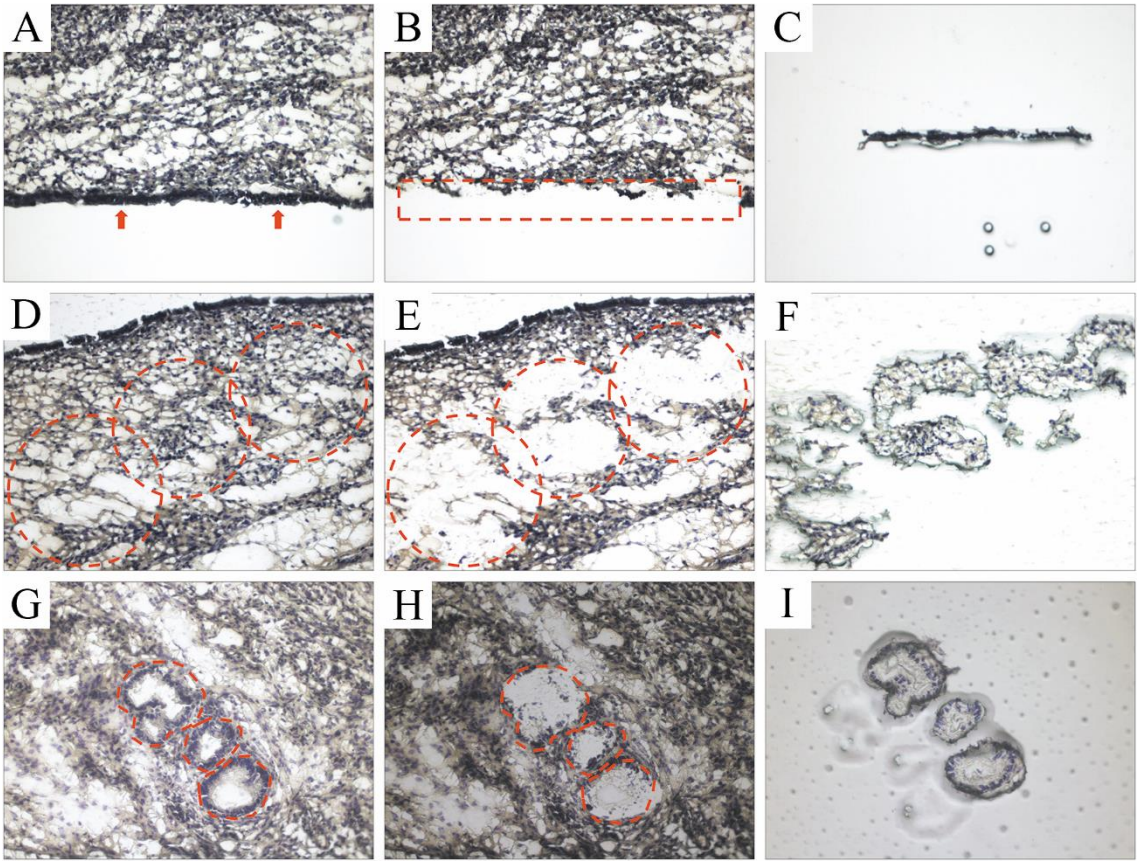

Figure 10 Histologic aspect of endometrial cell types before and after Laser Capture Microdissection (LCM). A, arrows pointing to luminal epithelial (LE) cells before LCM; B, dashed rectangle highlighting the place where the LE cells were on the slide; C, captured LE cells in the LCM plastic caps; D, dashed circles highlighting the stromal (ST) cells; E, dashed circles highlighting the place where the ST cells were on the slide; F, captured ST cells in the LCM plastic caps; G, dashed shapes highlighting the glandular epithelial (GE) cells; H, dashed shapes highlighting where the GE cells were on the slide; I, captured GE cells in the LCM plastic caps. (400× magnification).

Table 5 Number of samples used for each cell type and cow group with respective mean RIN values.

Endometrial cell types	Cow sub-group		RIN[1]
	Low P4	High P4	
Stromal cells	9	3	7.41 (± 0.24)
Glandular epithelial cells	7	3	7.23 (± 0.17)
Luminal epithelial cells	6	1	7.54 (± 0.19)

[1]RNA Integrity Number, values reported as mean ± SEM

4.3.7 RNA sequencing and data analysis

RNA sequencing libraries from 29 samples were prepared and sequenced on the GenomEast Platform (IGBMC, Cedex, France; http://genomeast.igbmc.fr/). Full-length cDNA was generated from 2.5 ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio Europe, Ozyme) according to manufacturer's instructions, with 10 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Then, 600 pg of pre-amplified cDNA was used as input for Tn5 transposon tagmentation using the Nextera XT DNA Library Preparation Kit (Illumina), followed by 12 cycles of library amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter), the size and concentration of libraries were assessed by capillary electrophoresis. Sequencing was performed on an Illumina HiSeq 4000 with 100 bp paired-end reads. Image analysis and base calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14. The sequencing depth of RNA-seq libraries was in the range of 72 to 83 million reads per sample and all samples had a quality score over 30, meaning that the base call accuracy was 99.9%, in at least 90% of the sequenced bases. Gene level exploratory analysis and differential transcription analysis were performed using the RNAseq workflow described by Love et al. (2015) (update version https://bioconductor.org/help/course-materials/2017/CSAMA/labs/2-tuesday/lab-03-rnaseq/rnaseqGene_CSAMA2017. html). The Salmon method (Patro et al. 2017) was used to quantify transcript abundance. Tximport method (Soneson et al. 2015) (R package version 1.8.0) was then used to import Salmon's transcript-level quantifications. The cDNA sequence database for *Bos taurus* was obtained from Ensembl (release-98; Bos_taurus.ARS-UCD1.2.cdna.all.fa) and was used to build a reference index for the bovine transcriptome (see details in Patro et al. 2017). Power analysis was performed using the method described by Bi and Liu (2016) and compiled in the R package ssizeRNA (version 1.3.2). Calculated at an FDR of 0.05, power was 15%, 58%, 87% to detect 1.5, 2 and 3 log2 fold change in GE cells, and 24%, 63%, 87% to detect 1.5, 2 and 3 log2 fold change in ST cells.

4.3.8 Gene transcription analysis

Following quantification of RNA-seq data, transcripts whose average value computed from biological replicates was less than 10 TPM (transcripts per million) were regarded as biological background noise and were not considered to identify the number of genes specifically transcribed by each cell type. Principal component analysis (PCA) was performed with DESeq2 and FactoMineR (R package, version 1.4.1) using the variance stabilising transformation output files from DESeq2. Venn diagrams were plotted with VennDiagram package (1.6.20). The downstream DESeq2 package (R package, version 1.26.0) was used for the analysis of differential expressed genes (DEGs) with the corresponding statistical methods (Love et al. 2015) including tests for differential transcription by use of negative binomial generalised linear models. The following terms were added in the design formula (cell_type + progesterone_group + cell_type : progesterone_group) to test the main effects of endometrial cell types and P4 concentration groups, as well as their interaction with the false discovery rate adjusted p-value of 0.05 used for the identification of DEGs. False discovery rate adjustment was performed using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). In all comparisons, ratios for fold change are expressed as mean TPM from cows with elevated P4 / mean TPM from cows with low P4. Cell-specific genes were defined as genes with average TPM ≥ 10 for a given cell population and average TPM < 10 for the other cell populations. Data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE182932 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182932).

4.3.9 Gene ontology (GO) and pathway enrichment analysis

Significant GO terms of the "Biological Process" domain were found with the GO-TermFinder software (Boyle et al. 2004), summarised with similarity coefficient at low or medium level, and visualised in semantic space by REVIGO (http://revigo.irb.hr/) (Supek et al. 2011). When analysing the lists of specific cell type genes, GO-TermFinder settings were set to $P < 0.01$. The lists of DEGs in LE, GE and ST cells between elevated and low P4 cows were analysed with Bonferroni adjustment and FDR settings at $P < 0.01$. Analysis of DEGs possibly involved in "hormonal regulation", "uterine receptivity", and "pregnancy" terms was based on GeneCards database (http://www.genecards.org/), as previously described (Guo et al. 2019).

4.3.10 Construction of protein-protein interaction (PPI) networks

The interaction networks among proteins encoded by DEGs of LE, GE and ST cells between elevated and low P4 cows were constructed with STRING database v11.0 (http://string-db.org) (Szklarczyk et al. 2019). All PPI networks were generated at a confidence score of

0.9 with “non/query protein only”, and the sources of active interaction were all selected (Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, and Co-occurrence). Then the networks were sent to Cytoscape v 3.8.2 and were visualised by yFiles layout algorithms for the Cytoscape app.

4.4 Results

This study was performed initially while considering the main effects of cell type, P4 concentrations, and their interaction, on the transcriptomic profile of endometrial cells of postpartum cows.

Due to the fact that only one sample from LE cells was associated with high progesterone, transcriptomic changes induced by P4 and their interaction with cell type were studied only from GE and ST cells. From the full list of DEGs (GE plus ST cells), where the main effects was significant (FDR adjusted P-value ≤ 0.05), the interaction was only significant for 1% of these (6 / 591). Furthermore, for most of these genes, significance was mainly associated with a low level of transcription. For three genes, the interaction resulted from progesterone being associated with an effect in one cell type but not in the other cell type. For three other genes, the significant interaction resulted from opposite effects of P4 in GE and ST cells. Due to the very low number of genes for which a significant interaction was detected, the results presented and discussed below address principally the main effects of cell type and P4 concentrations.

4.4.1 Overall gene expression and differentially expressed genes between the three endometrial cell types

The total number of genes with more than 10 transcripts per million (TPM) was 15420, 15555, and 15308 for LE, GE, and ST cells, respectively. From these, 274 (1.78%) were LE specific, 280 (1.80%) were GE specific and 346 (2.26%) were ST specific (Figure 11A). Among genes specifically transcribed by LE cells, *TM4SF4*, *C29H11orf86*, *CSF2*, *CAPN14*, *SERPINB10*, *CA1*, *SLC5A5*, *SLC6A12*, and two uncharacterized genes (*ENSBTAG00000036102*, *ENSBTAG00000055111*) had the highest level of average transcription. Among GE-specific genes, *BTG4*, *IBSP*, *TMEM212*, *C4BPA*, *DPP6*, *ANKS4B*, *PROC*, *CFAP58*, *LDLRAD1* and *GAS2L2* were the most transcribed, whereas, among the ST-specific genes, the most transcribed were *CHRNA2*, *KLK5*, *SST*, *DMRT2*, *GABRA4*, *NFASC*, *CDH9*, *DLK1*, and two uncharacterized genes (*ENSBTAG00000054090*, *ENSBTAG00000045630*). The full lists of genes specifically transcribed by each cell type with respective transcription levels are provided in Supplementary File 1 (https://data.mendeley.com/datasets/97cznzzcvg/1, Annex X).

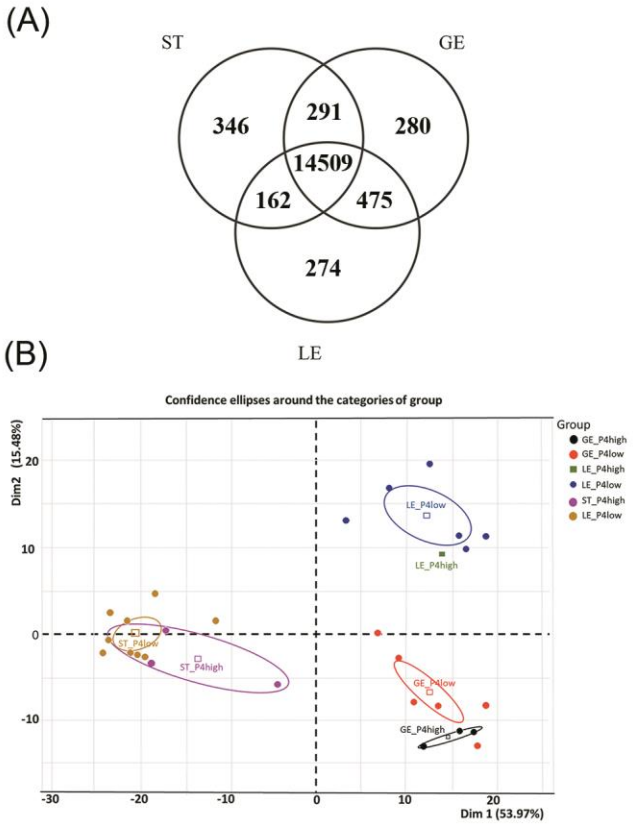

Figure 11. Transcriptome of endometrial cell types and effect of different progesterone concentrations of cows from which samples were issued. (A) Venn diagram with genes expressed (>10 transcripts per million (TPM)) in endometrial cells (stromal cells (ST), glandular epithelium (GE), and luminal epithelium (LE) (numbers of identified genes are indicated). (B) Principal component analysis (PCA) of cell types (ST, GE and LE) among cow groups (High progesterone, P4high and low progesterone, P4low).

Although a very high proportion of total genes were transcribed over 10TPM in all three cell types (14398/16274; 88.5%; Figure 11A), their level of transcription was not the same in the different cell types. This is shown by the PCA analysis revealing a clear separation of the samples from the three cell types. The two first dimensions explain 70% of the variability (Figure 11B), the first one allowing the distinction of epithelial from stromal cells, whereas the second differentiates GE from LE cells. The lists of genes most correlated ($P < 0.01$) with each dimension are provided in Supplementary File 2 (https://data.mendeley.com/datasets/bvs2r75sk9/1, Annex X). The DESeq2 analysis revealed 4045 DEGs between GE and LE cells, 7974 between GE and ST cells, and 7839 between LE and ST cells.

4.4.2 Gene ontology enrichment analysis of cell-specific genes

Enriched GO terms in each cell-specific genes list were visualized using the REVIGO algorithm to reduce term redundancy and identified 64, 30, and 79 GO terms clusters in LE, GE, and ST cells, respectively (Annex XI). The most significant over-represented terms in LE cells included transport processes, immune system process, cytokine production and

regulation, response to stimulus and cell surface receptor signalling, whereas for GE cells, these terms included cilium movement, regulation of triglyceride biosynthetic process, regulation of peptide secretion and transport, regulation of glucose transmembrane transport, and complement activation. For ST cells, the most significant over-represented terms included signalling, cell communication, multiple developmental and biological regulation processes. The complete list of over-represented GO terms is provided in Supplementary File 3 (https://data.mendeley.com/datasets/b7m2kch89p/1, Annex X).

4.4.3 Differentially expressed genes between elevated and low progesterone cows

Following microdissection, only 1 high RNA quality LE sample collected from cows with high progesterone concentrations was available. Therefore, differences in the transcriptomic profiles in response to progesterone will be presented and further discussed here from lists of DEGs obtained for GE and ST cells (Supplementary File 4 (https://data.mendeley.com/datasets/k6yfhfzg34/1, Annex X)). However, given its potential interest for future studies, the list of putative DEGs from LE samples is also present in Supplementary File 4 (https://data.mendeley.com/datasets/k6yfhfzg34/1, Annex X). The number of DEGs found between samples collected from cows with elevated or low P4 concentrations were 386 and 205 in GE and ST cells, respectively. From these DEGs, 365 (95%) and 184 (90%) were cell type-specific (Figure 12A). Twenty one DEGs were common to GE and ST cells (*TNC*, *ADAMTS18*, *P4HA2*, *APEX1*, *PNPLA2*, *SOSTDC1*, *TUBB*, *FBLN7*, *MAPK4*, *EEF1G*, *TNFRSF13B*, *ENSBTAG00000015493*, *B9D1*, *RACK1*, *OXTR*, *RPL8*, *C5AR2*, *ENSBTAG00000050840*, *ENSBTAG00000052405*, *TP53INP1*, *ENSBTAG00000040367*). When considering the above 21 DEGs, the regression analysis of the log2 fold change in response to progesterone from the two cell types showed a similar effect in the two cell types (regression slope = 0.72; adjusted R-squared 0.975; Figure 12B). The slope coefficient lower than 1 indicates that the magnitude of response was only slightly higher in ST than in GE cells which is consistent with the average log2 fold change for all DEGs observed in ST and GE cells, 3.81 and 2.95, respectively. Moreover, elevated P4 was most often associated with decreased transcription of genes in GE (280/386; 73%) and increased transcription in ST (118/205; 58%) (Figure 13).

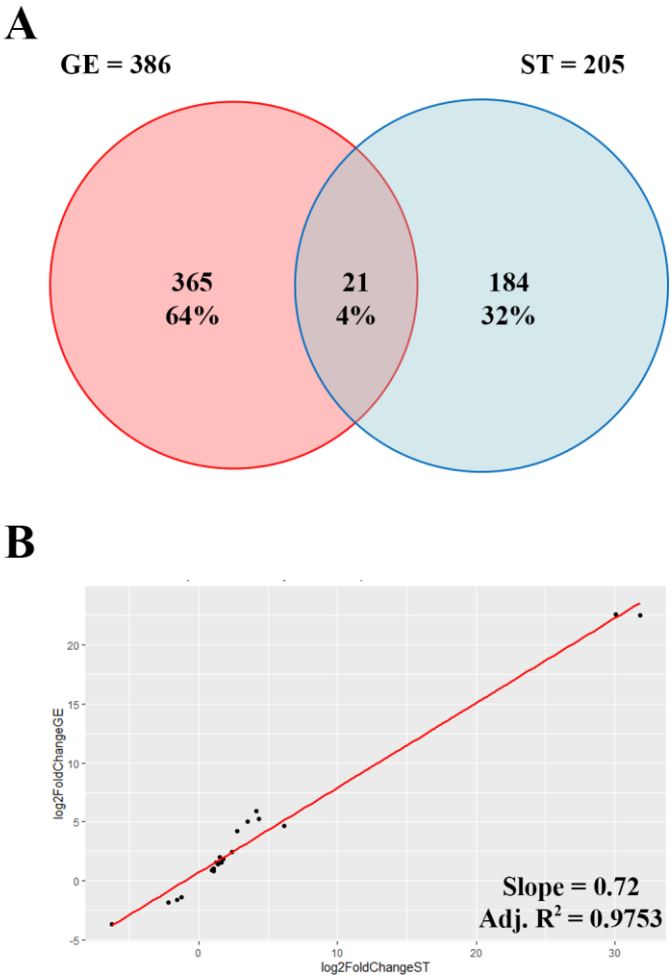

Figure 12. Differentially expressed genes (DEGs) between high and low progesterone cows A) Venn diagram from DEGs between samples issued from cows of the high and low progesterone groups in glandular epithelial (GE) and stromal (ST) endometrial cells. (Numbers and percentage of DEGs are indicated). **B) Linear regression analysis of the fold changes of the 21 common DEGs from ST (x-axis) and GE (y-axis) cells.**

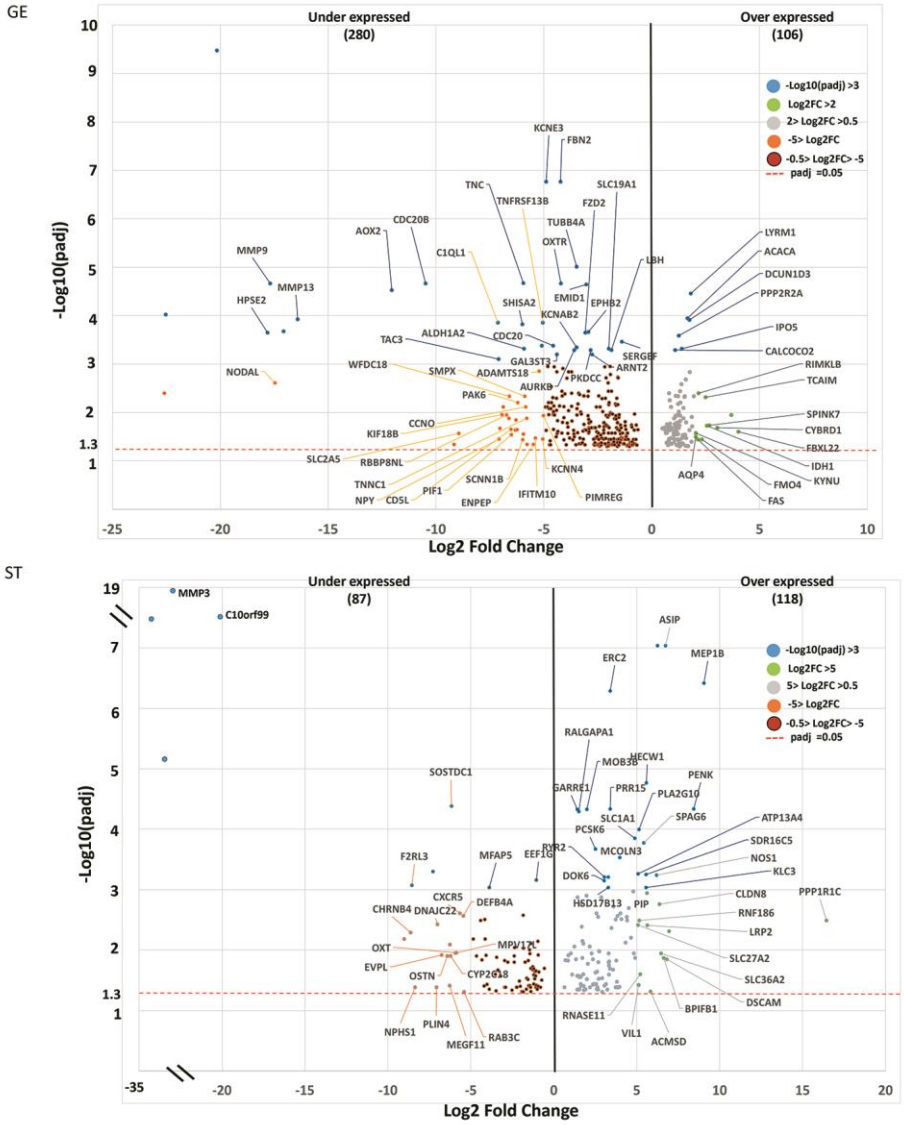

Figure 13. Volcano plots with the distribution of differentially expressed genes between high and low progesterone cows for glandular epithelial (GE) and stromal (ST) endometrial cells.

4.4.4 Gene ontology enrichment analysis of samples from cows with elevated and low progesterone concentrations

Enriched GO terms in the DEGs lists of elevated and low P4 cows are presented in Figure 14, and the corresponding lists of genes in Supplementary File 5 (https://data.mendeley.com/datasets/nxbwdmh99x/1, Annex X). In GE cells, genes with increased and decreased transcription under the effect of P4 relate to 42 and 62 enriched GO terms, respectively. Genes with increased transcription are mostly categorized in cellular metabolic processes (GO:0044237) (n = 49), response to organic substances (GO:0010033) (n = 19) and regulation of diverse biological processes like response to stimulus (GO:0048583) (n = 20), cell communication (GO:0010646) (n = 18) and signalling (GO:0023051) (n = 18). Genes with decreased transcription are overrepresented in cell cycle

(GO:0007049) (n = 31), nuclear division (GO:0000280) (n = 14), nuclear chromosome segregation (GO:0098813) (n = 14), mitotic cell cycle (GO:0000278) (n = 20), localisation (GO:0051179) (n = 58), response to stimulus (GO:0050896) (n = 7), response to stress (GO:0006950) (n = 36) and cell-cell signalling (GO:0007267) (n = 23).

In ST, the analysis revealed 22 and 2 enriched GO terms corresponding to genes with increased and decreased transcription, respectively. Genes with increased transcription relate to cell motility (GO:0048870) (n = 14), movement of cell or subcellular component (GO:0006928) (n = 18), locomotion (GO:0040011) (n = 18), animal organ morphogenesis (GO:0009887) (n = 15), localization (GO:0051179) (n = 37) and regulation of localization (GO:0032879) (n = 18), whereas genes with decreased transcription are associated with anatomical structure development (GO:0048856) (n = 21) and developmental processes (GO:0032502) (n = 22).

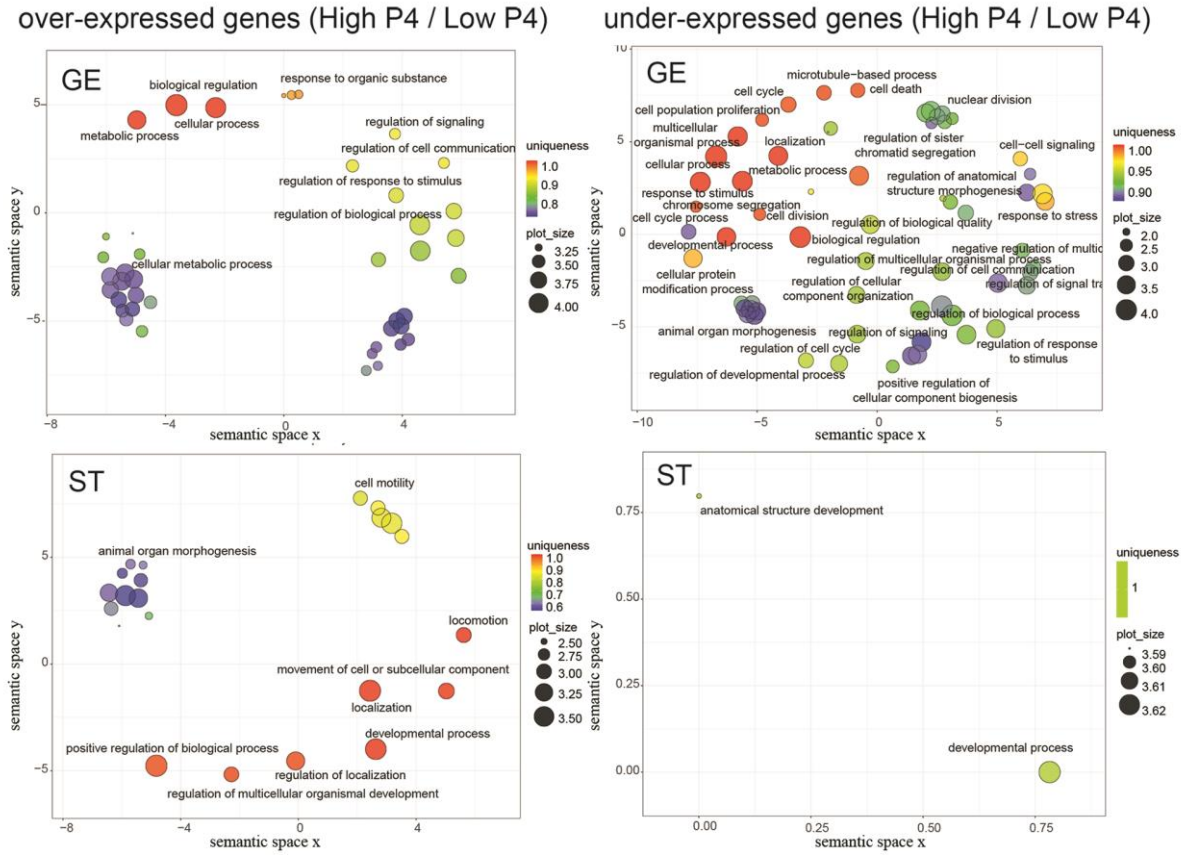

Figure 14. Scatterplot representation of enriched GO terms in semantic space using REVIGO (Supek et al. 2011), from lists of over-expressed and under-expressed genes in glandular epithelial (GE) and stromal (ST) cells, between high and low progesterone cows. Circle size represents the frequency of the GO term in the underlying GOA database (bubbles of more general terms are larger) and colour indicates the uniqueness value.

4.4.5 GeneCards and protein-protein interaction (PPI) network analysis

The comparison between the genes of the GeneCards database (http://www.genecards.org) corresponding to “hormonal regulation”, “uterine receptivity” and “pregnancy”, and the DEGs identified in the present study concerning the presence of P4 is shown in Figure 15. For GE and ST cells, a considerable proportion of DEGs is involved in hormonal regulation (> 60%) and pregnancy (>40%), and all DEGs participating in uterine receptivity relate also to the above terms. A more thorough analysis of the present DEGs involved in “hormonal regulation”, “pregnancy” and “uterine receptivity” revealed that some of the genes important for these processes exhibit differential transcription under progesterone in both cell types (Figure 16). For instance, *ESR1* is less transcribed under the effect of P4 in GE, *OXTR* is less transcribed under the effect of P4 in GE and ST, and transcription factors such as *SOX17* and *FOXA2* as well as interferon related genes which are known to regulate uterine epithelial–stromal crosstalk conveying to endometrial receptivity, are more transcribed under the effect of P4 in ST whereas no change is observed in GE cells.

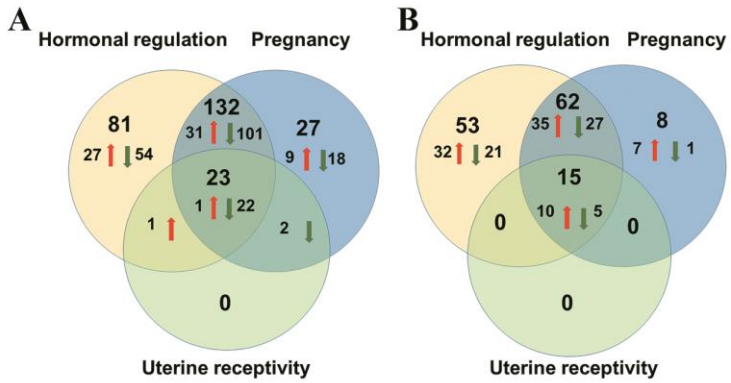

Figure 15. Comparison of differentially expressed genes in glandular epithelial (A) and stromal (B) cells to GeneCards database. Numbers and sense of variation (arrows) of DEGs participating in hormonal regulation, pregnancy, and uterine receptivity. From the 386 DEGs identified in GE, 237 participate in hormonal regulation, 184 in pregnancy and 26 in uterine receptivity. From the 205 DEGs that emerged in ST, 130 participate in hormonal regulation, 85 in pregnancy and 15 in uterine receptivity.

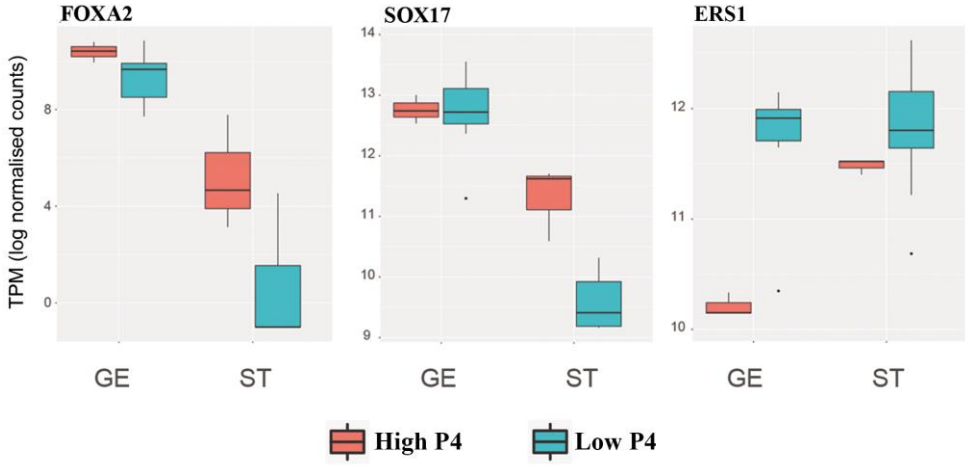

Figure 16. Log normalized transcripts per million (TPM) of *ESR1*, *FOXA2* and *SOX17* genes for glandular epithelial (GE) and stromal (ST) endometrial cells issued from high (red) and low (blue) progesterone cows. Horizontal black lines indicate median; boxes extend from the 25th to the 75th percentile and vertical lines indicate values within 1.5 interquartile range of the 25th and 75th percentiles. Dots indicate outliers.

In high P4 cows, the STRING-generated protein interaction network obtained from GE DEGs revealed 11 clusters of genes with decreased transcription including one of a very large size (Figure 17A) and 5 clusters of genes with increased transcription (Figure 17B), whereas, in ST cells, the analysis revealed 6 clusters of genes with decreased transcription (Figure 17C) and 5 clusters of genes with increased transcription (Figure 17D).

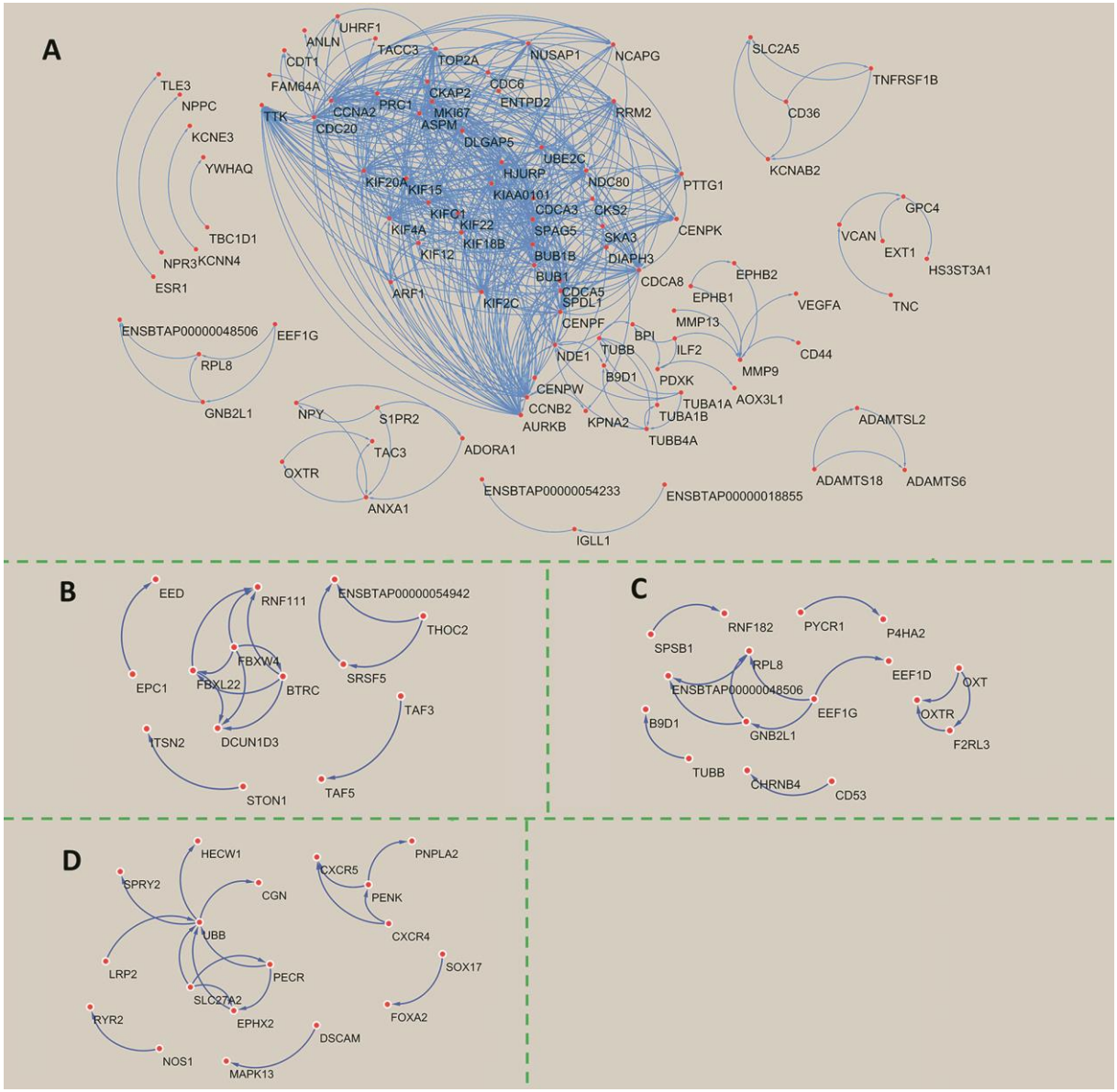

Figure 17. STRING-generated protein-protein networks from differentially expressed genes of glandular (GE), and stromal (ST) endometrial cells between high and low progesterone cows. A) Under-expressed in GE; B) Over-expressed in GE; C) Under-expressed in ST; D) Over-expressed in ST.

4.5 Discussion

This study combined LCM to isolate endometrial cell compartments from uterine biopsies and RNAseq to analyze their full transcriptomes, identifying changes induced by P4. This approach provides novel information regarding cell-specific gene transcription that remains undetected when analyzing whole tissue samples. This is particularly relevant for epithelial cells for which the cell type's representability is low, as specific gene transcription data is diluted in the average of the whole tissue. Furthermore, as observed here and recently also reported in another model (Chankeaw et al. 2021b), genes may only be differentially transcribed in a specific cell type and remain unaffected in the others.

This study confirms the difficulty in capturing samples with good quality RNA from LE. As in Chankeaw et al. (2021a; 2021b), despite preparation of a high number of slides from biopsies, the number of sequenced samples was lower for LE than for GE and ST cells.

Of the three different endometrial samples (LE, GE, and ST), ST samples are the most heterogeneous, comprising the *stratum compactum* and *stratum spongiosum*, and including blood vessel and migrating immune cells. By carefully selecting the captured areas, contamination with blood vessel cells was avoided, and contamination with migrating immune cells was minimized by including only healthy cows, as assessed by endometrial cytology and histology. Variation regarding the different strata that compose endometrial ST was addressed by capturing samples from the most superficial layers (*stratum compactum*) as these may induce paracrine interactions with the neighbouring epithelial cells.

The design model here applied revealed strong main effects of cell type and P4 concentrations, but showed lack of significant interaction for most of the genes influenced by progesterone. The fact that *ERP27*, *GK* and *ENSBTAG00000003408*) exhibit opposite changes in transcription under P4 in GE and ST cells, would deserve further investigations in relation with endometrial function.

4.5.1 Transcriptome of endometrial cell types

The PCA from full RNA-seq data corroborates previous work (Chankeaw et al. 2021a) documenting that LE, GE, and ST cells of the bovine endometrium exhibit different molecular signatures. The full description of the different cell types' transcriptome is beyond the scope of this chapter (lists are set in Supplementary File 1 (https://data.mendeley.com/datasets/x5kkf369kn/1, Annex X).

A low percentage (around 2%) of cell type-specific genes were identified, which is in agreement with data from the porcine endometrium (Zeng et al. 2018). These cell-specific

transcribed genes encode proteins that putatively support specialized functions of each cell type, which is supported by the overrepresentation of different enriched GO terms in the cell-specific gene lists (Annex XI). Discussion of the compared function of these proteins is beyond the scope of this chapter, but some relevant examples are summarized below.

LE specific genes encode proteins involved in processes of transport and uptake across epithelial surfaces (solute carrier family 5 member 5, solute carrier family 6 member 12, transmembrane 4 L six family member 4). SLC6 has been shown to be differentially transcribed through different phases of the human oestrus cycle (Chi et al. 2020) and stimulated by INF-Tau in the cow (Baldwin 2019). These comprise also an enzyme responsible for maintaining acid-base homeostasis (carbonic anhydrase 1), a serine peptidase inhibitor (serpin family B member 10), an embryokine (colony stimulating factor 2), and a member of the calpain family (calpain 14).

Carbonic anhydrase 1 is a member of the large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide and plays a pivotal role maintaining acid-base homeostasis (Quade et al. 2021) possibly impacting in the endometrium, sperm fertilization capacity, embryo transport, development and implantation (López-Albors et al. 2021).

Colony stimulating factor 2 (CSF2) is among the most studied embryokines, being secreted into the bovine uterine lumen (Tríbulo et al. 2018). This cytokine is involved in the recruitment, differentiation and function of neutrophils, when secreted by mouse uterine epithelial cells, following stimulation with TLR agonists (Soboll et al. 2006). CFS2 treatment during the preimplantation period improved the development and survival of bovine embryos (Loureiro et al. 2009).

In postpartum dairy cows, LE cells are a primary line of defence against bacteria, and an important component of the innate immune system (Sheldon et al. 2019). This is here evidenced with the over-representation of genes related to immune response and interleukin production, especially interleukin-23 which was shown to be involved in human endometrial immune regulation (Uz et al. 2010). Also, the secretory role of LE cells is evidenced by the overrepresentation of processes regarding multiple transport processes (ion, chloride, oxalate, bicarbonate) (Supplementary File 3 (https://data.mendeley.com/datasets/b7m2kch89p/1, Annex X). Overall, gene data from LE cells highlight their putative specialized functions, such as the regulation of uterine fluid composition, providing favourable microenvironments for sperm and embryos, and the immune response against potential pathogens.

GE specific genes encode proteins involved in cell cycle regulation (BTG anti-proliferation factor 4), adhesion processes (integrin binding sialoprotein), immune response (complement

component 4 binding protein alpha, protein C), localized in brush border (ankyrin repeat and sterile alpha motif domain containing 4B, cilia and flagella associated protein 58, low density lipoprotein receptor class A domain containing 1, growth arrest specific 2 like 2) and transmembrane proteins associated with voltage-gated potassium channels (Dipeptidyl peptidase-like protein 6).

The transcription of genes encoding proteins related to microvilli adhesion and assembly support that bovine endometrial GE cells form a cluster of tightly packed microvilli, as observed in rat endometrial GE cells (Elgamal et al. 2016). As example, low density lipoprotein receptor class A domain containing 1 is a membrane receptor already identified in GE cells in previous work (Chankeaw et al. 2021a) and also expressed by mature ciliated cells in airway epithelium (He et al. 2020). Cow endometrial GE cells have specialized functions regarding synthesis, transport and secretion of substances into the uterine lumen (Spencer et al. 2019), here illustrated by the overrepresentation of biological processes of regulation of peptide secretion and cilium movement, the latter believed to be essential for moving secretory products across the surface of GE cells into the uterine lumen (Afzelius et al. 1978). In addition, the role of GE cells in innate and adaptive immune responses is highlighted by the overrepresentation of genes involved in complement activation, as observed in woman's endometrial GE cells (Fonzar-Marana et al. 2006). Complement component 4 binding protein alpha is a protein that controls the activation of the complement cascade and is upregulated in the bovine endometrium following exposure to seminal plasma components, likely involved in the regulation of peri-implantation events (Recuero et al. 2020). Protein C is a potent anticoagulant and anti-inflammatory molecule which regulates the functions of different epithelial barriers by controlling inflammation (Nakamura et al. 2005; Vetrano et al. 2011). Dipeptidyl peptidase-like protein 6 is a transmembrane protein that binds to voltage-gated potassium channels from the Kv4 family (Kaulin et al. 2009), associated with uterine capacity for pregnancy and fertility in beef heifers (Galliou et al. 2020), and relevant for uterine function (Neupane et al. 2017).

The overrepresented processes in ST cells are more numerous and diverse than in GE and LE cells. Most of them relate to regulatory processes, which is consistent with the ST regulatory role exerted on adjacent epithelial cells in co-culture systems (Arnold et al. 2001), or within the cow endometrium (Chankeaw et al. 2021a). This regulatory role of ST cells also emerges from the overrepresentation of cell communication and signalling processes, which are paramount for the coordination of cellular responses. ST specific genes encode, among others, extracellular proteases (Kallikrein related peptidase 5) and cell adhesion molecules. In women, kallikrein related peptidase 5 is an extracellular protease expressed in endometrial GE cells, suggested to play a role in host defense (Shaw et al. 2008). This

protein is also involved in remodelling and repair of epithelial barriers (Lenga et al. 2021) and able to generate plasmin indicating a role in wound healing (Nauroy and Nyström 2019). Different collagens were described as substrates for kallikrein-related peptidase 5, hinting a role in extracellular matrix remodelling and cell migration (Nauroy and Nyström 2019).

The specific roles of some of the most correlated genes to each PCA dimension, which were previously found to be involved in endometrial function or associated to pathologies, are also explored below. In the first PCA dimension, which separates stromal from epithelial cells, the most specific ST cells' gene was the Wilms' tumor suppressor gene (*WT1*), previously reported as specifically expressed in woman endometrial ST cells (Makrigiannakis et al. 2001). This was also the case of smooth muscle cell markers (*TAGLN*; *ACTA2*; *CNN1*; *TPM2*) also specifically detected in the endometrial stromal compartment of healthy women (Queckbörner et al. 2021). Additionally, *STRA6* and *SFRP1*, encoding a receptor for retinol uptake and a soluble modulator of Wnt signalling, respectively, were highly transcribed in ST cells, as previously described in woman's endometrium (Cheng et al. 2007; Pavone et al. 2011).

The most specific GE and LE genes were those encoding an epithelial splicing regulatory protein (ESRP1), which is a regulator of FGFR2 splicing (Warzecha et al. 2009), the keratinocyte differentiation factor (KDF1), strongly expressed in the dental epithelium of mouse embryos (Zeng et al. 2019), and the Msh Homeobox 1 (MSX1), with strong nuclear localization in GE and LE cells of fertile woman's endometrium (Bolnick et al. 2016). In addition, a large set of genes involved in epithelial cell differentiation, epithelium development, and cell adhesion (*CLDN3*, *SYNE4*, *LRP2*, *F2RL1*, *DLX6*, *ELF3*, *SPINT1*, *PHGDH*, *OVOL1*, *TACSTD2*, *ST14*, *EHF*, *MSX1*, *EPCAM*, *ST14*, *KDF1*, *IRF6*, *TJP3*, *SLC44A4*, *RAB25*, *DSP*, *MCOLN3*) were identified, also previously correlated with epithelial cells (GE + LE) in the endometrium of dairy cows (Chankeaw et al. 2021a).

In the second PCA dimension, which separates GE cells from LE cells, the most specific GE cells' genes were *PPP1RB*, which encodes DARPP-32, a phosphoprotein expressed in ciliary epithelia (Stone et al. 1986), and *CCDC146*, a ciliated cell marker (Haider et al. 2019). Although both LE and GE contain ciliated cells (Hyttel, 1985), the number of these cells is expected to be lower in LE than in GE, as documented in women (Masterton et al. 1975). Also as in the human endometrium (Delforce et al. 2017), the angiotensinogen coding gene (*AGT*), was highly transcribed in GE cells. A set of genes involved in axonemal dynein complex assembly and cilium movement processes (*CCDC65*, *DRC1*, *DRC3*, *DAW1*, *CFAP45*, *DNAH5*, *DNAH9*) was associated to GE, as previously reported (Chankeaw et al. 2021a).

LE cells were correlated with *SMOC2*, encoding an extracellular glycoprotein recognized as an endometrial cancer stem cell signature (Lu et al. 2019). Stem cells were identified in the epithelial and stromal compartments of human endometrium, where they are said to be responsible for its remarkable regenerative capacity (Figueira et al. 2011). The endometrium of postpartum dairy cows experiences intense tissue remodeling and re-epithelialization, suggesting *SMOC2* as a putative uterine stem cell maker. The desmin coding gene (*DES*) was also specific of LE cells, despite being identified as a smooth muscle cell marker (Liu et al. 2013). However, desmin has also been used to distinguish epithelial cells undergoing epithelial-mesenchymal transition (EMT) (Zhao et al. 2016), and there is evidence of EMT participation in endometrial regeneration and re-epithelialization (Owusu-Akyaw et al. 2018; Whitby et al. 2020). This indicates desmin transcription to be a putative EMT marker of LE cells. *BPIFB1*, a gene encoding an innate defence protein identified in other epithelial barriers, such as human airways (De Smet et al. 2017) was also specific of LE cells.

4.5.2 Impact of progesterone on the transcription profile of endometrial cell types

For GE and ST cells, the PCA analysis did not identify outliers and individual samples clustered nicely, showing similar gene transcription profiles within each P4 group. Overall, data on number of DEGs and overrepresentation of biological processes indicate that the response to elevated P4 was more significant in GE than in ST cells. As documented in the methods section, the average log2 fold change of DEGs in GE and in ST cells (of 2.95 and 3.81, respectively) are associated with a good power to detect differences. A low percentage of common DEGs in GE and ST cells was observed, highlighting the cell type-specific effect of P4 on endometrial gene transcription. Interestingly, the lack of impact of P4 on the gene transcription of P4 receptors (main *PGR* or *PGRMC1*, *PGRMC2*, *NR2F2*, and *SRD5A2*; data not shown) suggests that the above specific effects are not mediated by the differential binding of P4 to this family of receptors and that other mechanisms should be explored.

As evidenced by the GeneCards analysis, most DEGs in response to P4 effect participate in hormonal regulation and pregnancy mechanisms and all DEGs involved in uterine receptivity also fall within the two previously mentioned categories. This agrees with the known role of P4 in modulating the transcriptomic profile of the endometrium and modifying the composition of the histotroph for the establishment of uterine receptivity during the pre-implantation period (Forde et al. 2009; Forde and Lonergan 2012; Spencer et al. 2016; Madoz et al. 2020). Progesterone is responsible for major changes governing the establishment of uterine receptivity between day 7 and 13 post-estrus (Spencer et al. 2016). Changes in endometrial gene expression elicit modifications in the histotroph, including an increase in specific amino acids, glucose, cytokines, and growth factors that support the

survival and growth of the conceptus (Spencer et al. 2016). As reported before, the changes in GE cells include gene upregulation of meprin A subunit beta (*MEP1B*), a zinc metalloendopeptidase, hypothesized to regulate proteins involved in elongation of the trophectoderm (Forde et al. 2012). In addition, this gene exhibited increased transcription in ST cells of cows with elevated P4, suggesting an alternative role in the cleavage of extracellular matrix proteins, as earlier proposed (Forde et al. 2012). Also, the results of our study showing that both *ESR1* and *VEGFA* are less transcribed in GE cells of cows with elevated P4 are consistent with their expression decline during the elongation stage of the conceptus development (from 13 to 20 days post-estrus; review by Forde and Lonergan (2012)).

In cows, follicular phase E2 promotes epithelial cell proliferation and endometrial growth, whereas diestral P4 inhibits estrogen-driven epithelial proliferation and promotes differentiation (Espejel and Medrano, 2017; Sá Filho et al. 2017). The antiproliferative action of P4 in endometrial epithelia of other species (Franco et al. 2008) is evidenced here in GE cells by the decreased transcription of genes involved in cell cycle processes. The coordinated and intimate interplay between epithelia and stroma is essential for endometrial response to estradiol (E2) and P4 stimulation (reviewed in humans, Kim et al. 2013; and mice, Diep et al. 2015). In mice, Kurita et al. (1998) demonstrated that P4 receptors in ST cells are essential for the P4-driven inhibition of epithelial proliferation. There is strong evidence that this inhibition occurs through paracrine interactions and Li et al. (2011) observed that under P4 influence, the transcription factor HAND2 is expressed in stromal cells suppressing the production of several fibroblast growth factors, which are responsible for epithelial proliferation. Moreover, Diep et al. (2015) suggested that P4 inhibit Wnt signalling in ST cells, resulting in inhibition of cell cycle progression. In this study, elevated P4 was associated with decreased transcription of *WNT2* in ST cells. However, ST transcription of *HAND2* was not altered by P4, suggesting a different paracrine loop in bovine endometria.

Increased transcription of several genes related with ubiquitin-dependent protein catabolic processes was observed under P4 influence in GE (*RNF111*, *FBXW4*, *BTRC*, *DCUND1D3*, *FBXL22*) and ST cells (*HECW1*, *UBB*, *SPRY2*). In women, ubiquitin expression changes along the menstrual cycle and modulates steroid receptor concentrations and endometrial development (Bebington et al. 2001). In this study, elevated P4 was associated with a strong decreased transcription of estrogen receptor alpha coding gene (*ESR1*) in GE. This is in accordance with the finding that ESR1 mediating the proliferative role of E2, present its lowest concentrations during the mid-luteal phase (Sá Filho et al. 2017). Unlike ESR1, uterine estrogen receptor beta (ESR2) expression is positively associated with increasing P4

concentrations (Sá Filho et al. 2017). This association is here supported by the *ESR2* gene increased transcription in ST cells of cows with elevated P4. In addition to its role on ESR1, the present results showing that elevated P4 associated with decreased transcription of the oxytocin receptor (*OXTR*) gene in GE and ST cells, and *OXT* gene in ST cells, are in full agreement with former studies describing the role of OXT in luteolysis, as reviewed by Skarzynski et al. (2001). During diestrus, P4 regulates the endometrial expression of OXTR by suppressing E2 signaling (Bishop, 2013), and in pregnant ruminants the conceptus trophoblast produces interferon tau (IFNT), which downregulates the transcription of *ERS1* and *OXTR* to block the endometrial luteolysis mechanism (Spencer and Bazer, 2004).

In GE, genes encoding Tachykinin Precursor 3 (*TAC3*) and Annexin A1 (*ANXA1*), which are OXTR interacting proteins, were also less transcribed under elevated P4. TAC3 mediates the contractibility of the non-pregnant women uterus (Patak et al. 2003). This raises the hypothesis that in cows, P4 can modulate uterine contractibility through the *TAC3* gene. Annexin A1 is a pro-resolving mediator involved in the clearance of apoptotic cells (Maderna et al. 2005; Serhan et al. 2007). By down-regulating *ANXA1* gene transcription, elevated P4 induces innate immune response suppression (Hall and Klein, 2017). *ANXA2*, another member of the Annexin A protein family, was also less transcribed in ST cells of cows with elevated P4. Annexin A2 promotes the formation of phagophores, an essential step in the process of autophagy (Xi et al. 2020) thus contributing to host immunity during bacterial infection (Li et al. 2015). This result is consistent with work from (Bauersachs et al. 2005) showing this gene was under-expressed in full tissue biopsies from intercaruncular endometrium of cows at a high P4 stage of the estrus cycle when compared with cows at a low P4 stage of the estrus cycle.

Moreover, genes encoding Leukocyte surface antigen (*CD53*), a tetraspanin involved in regulation of immune cell function (Dunlock, 2020), and Cholinergic Receptor Nicotinic Beta 4 Subunit (*CHRNB4*) were less transcribed under P4 influence in the ST compartment. Since both genes constitute the GO term “neutrophil degranulation” their under-expression under the effect of P4 may also contribute to the higher susceptibility to uterine infections during diestrus in the cow (Lewis, 2003).

Elevated P4 was associated with increased transcription of genes encoding proteins involved in endocytosis processes (*ITSN2*, *STON1*) in GE. Endometrial endocytosis occurs in pregnant and non-pregnant cows, mainly during stages at which circulating P4 concentrations are high (Guillomot et al. 1986) and during the implantation window in the woman (Bartosch et al. 2011). Although the endometrial role of endocytosis remains unknown, it may be involved in the embryo-endometrium crosstalk during the preimplantation

period. In the present study, the overrepresentation of regulation of signalling, cell communication and response to stimulus processes observed in the GE cells of elevated P4 cows are consistent with the above information suggesting that P4 stimulates endocytosis.

As reported before from full tissue biopsies (Bauersachs et al. 2005), elevated P4 was associated with increased transcription of transcription factors SRY-box transcription factor 17 (*SOX17*) and forkhead box A2 (*FOXA2*) in ST samples from this study. In humans, *FOXA2* is a P4-induced gene involved in transcriptional regulation in endometrial stromal cells (Lin et al. 2018), and both *SOX17* and *FOXA2* were found to regulate endometrial epithelial-stromal crosstalk related to endometrium receptivity and embryo implantation (Wang et al. 2018). As *SOX17* suppresses E2 signalling (Wang et al. 2018; Rubel et al. 2016), the network formed with FOXA2 may represent a mechanism by which transcription of *ESR1* is downregulated in GE of elevated P4 cows (Figure 16).

In ST, two genes encoding proteins involved in proline metabolism (P4HA2, PYCR1) exhibited decreased transcription under elevated P4. This is consistent with the downregulation of *P4HA2* by progestins in human patients with endometrial hyperplasia (Orbo et al. 2009). In addition, the knockdown of both *P4HA2* and *PYCR1* reduced cell proliferation of cervical and liver cancer cells, respectively (Cao et al. 2020; Ding et al. 2020). Taken together, these data suggest that downregulation of *P4HA2* and *PYCR1* may be an additional mechanism by which P4 exerts its endometrial anti-proliferative action in postpartum cows.

Progesterone is also a known inhibitor of cell death, a function supported by the under-expression of tumor necrosis factor receptors when comparing cyclic and non-cyclic cows at 5 weeks postpartum (Moore et al. 2019). The results of our study further illustrate this role of progesterone as 4 members of the TNF receptor superfamily exhibited decreased transcription under elevated P4 conditions (*TNFRSF13B* in GE and ST cells, *TNFRSF1B* in GE cells, *TNFRSF9* and *TNFSF8* in ST cells).

In addition, consistently with what was reported before from full tissue biopsies (Bauersachs et al. 2005), cows with elevated P4 displayed increased transcription of *EED*, *IDH1*, *SGK3* in GE cells, and *ARHGDIB*, *BCAT1*, *EPHX2*, *LRP2*, *MCOLN3*, *NDRG4*, *PENK*, *PLA2G10* in ST cells. On the contrary, transcription of *ACP5*, *CLDN10*, *FBLN7*, *GJA1*, *PRDX2*, *TNC*, *TUBA1A*, *TUBA1B*, *TUBB* was decreased in GE cells, and *EEF1G*, *FBLN7*, *MFAP5*, *TNC*, *TUBB* was decreased in ST cells. The present results confirm the former study while allowing a more precise compartment characterization of the effect.

4.6 Conclusion

This study evidences that endometrial cell types have different transcriptome signatures, which are differentially regulated by P4. Decreased transcription of genes in GE cells by elevated P4 mainly affected cell cycle processes, denoting an anti-proliferative action of P4 in epithelial compartments. In contrast, the elevated P4 regulation of the transcriptomic profile of ST cells is mainly related to the epithelial-stromal cross-talk. Altogether, this study reflects an intricate cell-specific regulation of biological processes in endometrial compartments, which were unnoticed from whole tissue approaches. These results may open paths to understand better the mechanisms regulating endometrial function and their roles with the establishment of pregnancy.

CHAPTER 5 - Subclinical endometritis differentially affects the transcriptomic profiles of endometrial glandular, luminal and stromal cells of postpartum dairy cows

G. Pereira[1], Y. Guo[1], E. Silva, M. F. Silva, C. Bevilacqua, G. Charpigny, L. Lopes-da-Costa and P. Humblot.

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[1]These authors contributed equally to this work

5.1 Abstract

In postpartum dairy cows, subclinical endometritis (SCE) is characterized by persistent endometrial inflammation, which exerts profound detrimental effects on subsequent reproductive performance. So far, transcriptomic studies related to this condition were either based on biopsy-derived whole endometrium tissue or endometrial swab/cytobrush samples, thus masking effects of disease on cell type-specific gene transcription. This study tested the hypothesis that different endometrial health statuses are associated with distinct transcription profiles of endometrial stromal, glandular and luminal epithelial cells.

At 44 days postpartum (DPP), endometrial biopsies were taken from dairy cows (n = 24) classified as Healthy, Recovered from SCE, and affected by Persistent SCE, according to endometrial cytology taken at 21 and 44 DPP. Stromal, glandular and luminal epithelial cells were isolated from the whole tissue biopsy by laser capture microdissection, and the cell-specific transcription profiles were determined by RNAseq. Differential gene expression was analyzed with DESeq2.

Results demonstrate that the global transcriptomic profiles and the corresponding lists of differentially expressed genes between cows with different health statuses were distinct among cell types. Results also evidence that despite Healthy and Recovered cows present a similar “endometrial clinically healthy phenotype” at 44 DPP, the previous presence of immune cells is still impacting the transcriptome of endometrial cells at this stage, delaying the complete functional recovery. Recovery or persistence of inflammation was associated with gene expression patterns involved not only in immune function but also in tissue remodelling, cell adhesion and uterine receptivity in a cell type-specific manner.

**Text adapted from the original submitted paper*

Identifying these signatures may prove instrumental to develop novel diagnostic and therapeutic targets, either to prevent persistence or improving speed of recovery from endometrial inflammation, thus helping to restore the fertility of postpartum dairy cows.

5.2 Introduction

In postpartum dairy cows, uterine involution, characterized by the elimination of bacterial contamination and regeneration of the endometrial tissue, is essential to restore uterine receptivity and establishment of pregnancy (Sheldon, 2004). However, due to impaired immune response, cows are not always successful in eliminating uterine pathogens quickly (Sheldon et al. 2006; LeBlanc, 2008). This is associated with persistent inflammatory responses in endometrial tissue, which are sources of uterine disease such as endometritis (Johnson et al. 2015; Raliou et al. 2019). Clinical endometritis is easily diagnosed. On the contrary, despite being highly prevalent and exerting profound detrimental effects on subsequent reproductive performance (Gilbert et al. 2005; LeBlanc, 2008), subclinical endometritis (SCE), given the absence of clinical signs (Sheldon et al. 2006), is often undiagnosed. At present, such cases are identified by an increased proportion of polymorphonuclear neutrophils (PMN) revealed by endometrial cytology or biopsy (Sheldon et al. 2006; Madoz et al. 2014; Pascottini et al. 2016). SCE is characterized by an uncontrolled persistent inflammation associated with tissue damage leading to the release of damage-associated molecular patterns, further stimulating inflammation and contributing to its persistence (Sheldon et al. 2019).

The analysis of the full transcriptome following exposure of bovine endometrial mixed epithelial and stromal cells (Oguejiofor et al. 2015) or isolated epithelial cells (Guo et al. 2019) to lipopolysaccharide (LPS), showed that this virulence factor triggered the differential expression of a large number of genes, including critical ones for the establishment of pregnancy such as those encoding proteins involved in the control of tissue remodeling, immune-tolerance, uterine receptivity, and pregnancy establishment. When using the same *in vitro* model as Guo et al. (2019), changes in proteins related to the above pathways were also observed (Piras et al. 2017). *In vivo*, cows with SCE present increased local gene transcription of pro-inflammatory cytokines characteristic of acute inflammatory processes (Kasimanickam et al. 2014; Johnson et al. 2015; Fagundes et al. 2019, Pereira et al. 2020). In addition, cows affected by endometritis exhibit a large number of genes involved in immune response, cell adhesion, chemotaxis, apoptosis and G-protein coupled receptor signaling pathways differentially expressed when compared to healthy cows (Salilew-Wondim et al. 2016). These changes are also associated with an increased expression of

molecules involved in LPS signaling, tissue remodeling, and acute phase response (Raliou et al. 2019). Overall, these reports highlight that SCE triggers not only an inflammatory response but also alters the transcription of networks of genes encoding proteins involved in the control of tissue remodeling, immune tolerance, and pregnancy establishment.

However, so far, transcriptomic studies aiming to analyze the impacts of SCE from *in vivo* material were either based on biopsy-derived whole endometrium tissue (Johnson et al. 2015; Salilew-Wondim et al. 2016, Raliou et al. 2019) or endometrial swab/cytobrush samples (Hoelker et al. 2012, Kasimanickam et al. 2014; Fagundes et al. 2019), without discriminating between endometrial cell types. The bovine endometrium is a complex and heterogeneous tissue, and quantification of gene transcription from the whole endometrium may not reflect cell-specific transcription. In postpartum dairy cows, stromal (ST), glandular epithelial (GE) and luminal epithelial (LE) endometrial cells isolated by laser capture microdissection (LCM) exhibited very distinct molecular signatures (Chankeaw et al. 2021a), differentially influenced by progesterone (Pereira et al. 2022) and negative energy balance (Chankeaw et al. 2021b).

The present study was designed to test the hypothesis that SCE differentially affects gene transcription profiles of the three endometrial cell types (ST, GE and LE). In addition, as former studies aiming at identifying differences between healthy and SCE cows included only two groups of animals for which the uterine health status was determined at a single time point, the transcriptomic profiles of the above three cell types were here compared between healthy cows, cows which apparently recovered from subclinical endometritis, and cows with persistent subclinical endometritis.

Differences between the transcriptomic profiles of these uterine health phenotypes provide key information to understand the mechanisms associated with the persistence of endometrial inflammation and subsequent consequences on the establishment of pregnancy.

5.3 Material and Methods

5.3.1 Ethics statement

The project was approved by the Institutional Animal Care and Use Committee (reference CEIE nº37/2019). All clinical procedures were conducted in compliance with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU).

5.3.2 Animals

Animals entering this study correspond to a subset of those included in a formerly published study from our group, where the experimental design, animal handling and sampling procedures are described in detail (Pereira et al. 2020) (Table 6). Briefly, sample collection was performed in a single dairy farm in Benavente, Portugal, where postpartum dairy cows (n = 24), without signs of puerperal disease and antibiotic or anti-inflammatory therapy, were submitted to a gynecological examination and endometrial cytology at 21 ± 0.4 and 44 ± 0.7 days postpartum (DPP), plus a uterine biopsy at 44 DPP. Based on genital tract evaluation and endometrial cytology, cows were classified as Healthy (n = 6), Recovered (n = 7) or Persistent (n= 11). This classification, initially based on cytology results (Pereira et al. 2020), was confirmed by retrospective histological analysis (Figure 18).

Table 6. Clinical characterization of cow groups (Healthy, Recovered, and Persistent SCE).

Characteristics	Healthy	Recovered	Persistent SCE
Number of cows	6	7	11
Milk yield (kg) by 44 DPP[1]	1770 ± 92	1743 ± 77	1807 ± 91
Body weight loss (%) by 44 DPP[1]	4.2 ± 1.9	4.7 ± 1.7	3.9 ± 1.7
Lactation number[1]	2.0 ± 0.5	1.6 ± 0.2	2.5 ± 0.4
21 DPP			
PMN%[1]	6.3 ± 1.5[a]	52.0 ± 6.4[b]	60.7 ± 7.2[b]
Range of PMN%	2.5 - 12.5	25.5 - 71	25 - 90
44 DPP			
PMN%[1]	1.3 ± 0.3[a]	1.1 ± 0.4[a]	13.5 ± 2.3[b]
Range of PMN%	0.5 - 2.5	0 - 3.5	6 - 33

[1]mean ± standard error of the mean
DPP - days postpartum
PMN - polymorphonuclear neutrophils

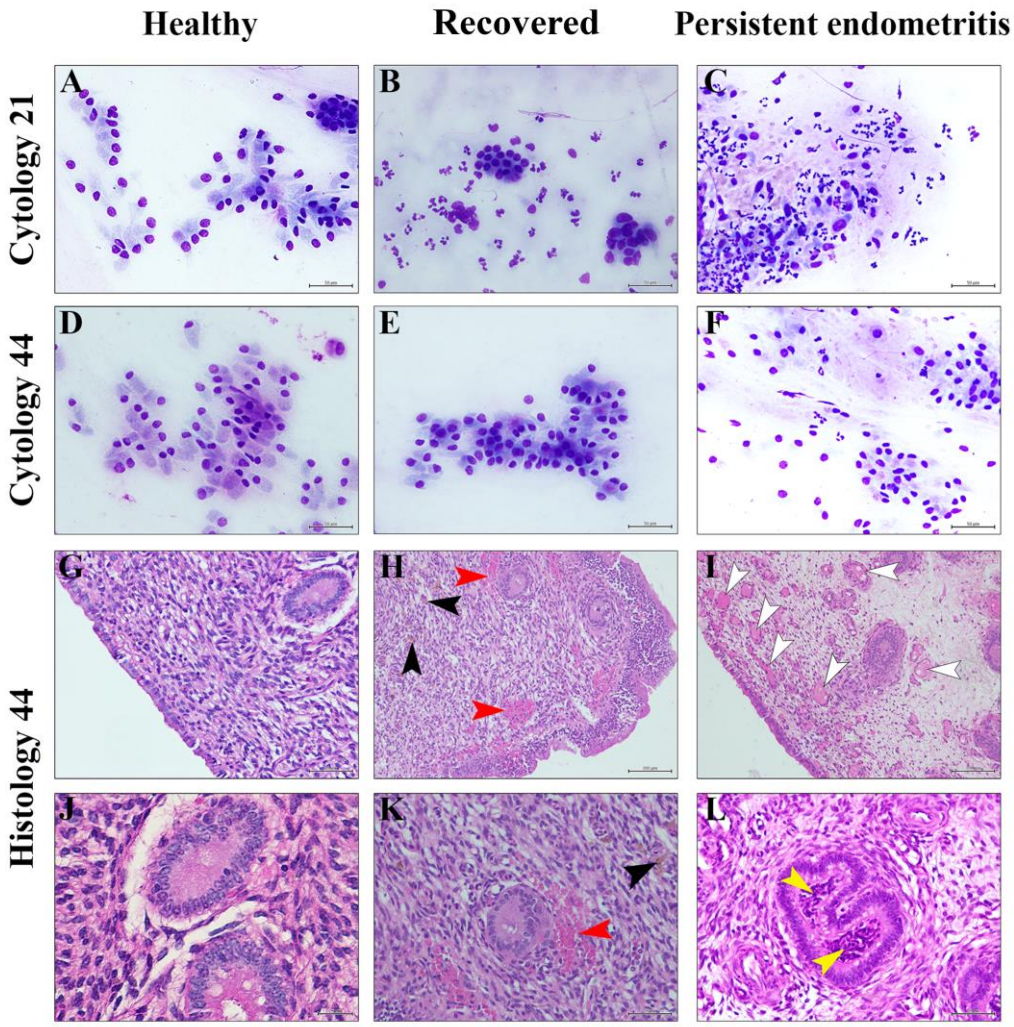

Figure 18. Representative photomicrographs of endometrial cytology (21 and 44 DPP) and biopsy (44 DPP) from healthy, recovered, and persistent subclinical endometritis cows. Endometrium of recovered cows displays areas of hemorrhage (red arrows) and infiltration of macrophages with hemosiderin (black arrows). The endometrium of persistent endometritis cows exhibits areas of vascular congestion (white arrows) and accumulation of inflammatory cells in the lumen of glands (yellow arrows). Scale bar 100 µm (H, I), 50 µm (A, B, C, D, E, F, G, K, L), and 20 µm (J).

5.3.3 Genital tract evaluation and endometrial cytology

The vaginal discharge was graded (Williams et al. 2005) following collection with a Metricheck device (EndoControl Sampler, Minitube, La Selva del Camp, Spain), and animals with vaginal discharge score ≥ 1 were excluded from further exploration. In addition, the genital tract was examined by rectal palpation and visualized by ultrasonography. The endometrial cytology was performed with the cytobrush technique (Pereira et al. 2020), and the percentage of PMN was assessed by counting 400 cells. Cows were regarded as healthy when PMN percentage was < 18% (Kasimanickam et al. 2004) or < 5% (Gilbert et al. 2005) at 21 and 44 DPP, respectively. Cows from the Healthy group had a healthy uterus at both examinations, whereas Recovered cows had a healthy uterus only at 44 DPP, and Persistent cows presented PMN percentages higher than the above cut-offs at 21 and 44 DPP (Figure 18, Table 6).

5.3.4 Collection of Biopsies

As previously described, endometrial biopsies were collected with a Kervokian–Younge instrument (Alcyon, Paris, France) (Pereira et al. 2020). The biopsy instrument was guided into the first third of one uterine horn, where an endometrial sample of 0.5-1 cm^2 and 3-5 mm thick was recovered. The endometrial samples were immediately frozen in dry ice cold isopentane (2-Methylbutane, Sigma Aldrich) for 60 seconds and embedded in a cryomold with optimal cutting temperature compound (Tissue-Tek OCT Compound, Sakura Finetek). Cryomolds were transferred to the laboratory on dry ice, then kept at -80ºC until tissue processing.

5.3.5 Endometrial tissue processing, staining, microdissection, and RNA extraction

Endometrial tissue processing and staining procedures were performed as recently described (Chankeaw et al. 2021; Pereira et al. 2022). Briefly, 8 µm thick sections were cut from the tissue blocks on a cryostat (Cryotome FSE, Thermo Scientific) set at -20ºC, mounted on glass slides at 4ºC and immersed for 60 seconds in 75% ethanol inside the cryostat chamber (-20ºC). Then, at room temperature, slides were transferred to 75% ethanol for 20 seconds, stained with Cresyl Violet (1% in 50% ethanol for 25 sec) and dehydrated, by rinsing successively with 75% ethanol (30 sec), 95% ethanol (2 x 1 min), 100% ethanol (2 x 1 min), and xylene (M-xylene, Sigma-Aldrich; 2 x 5 min), as described by Bevilacqua et al. (2010). Slides were then air-dried to remove xylene residues before microdissection. The endometrial cell types (LE, GE and ST) were isolated from the whole tissue sections using an ARCTURUS XT Laser Capture Microdissection System and software (Applied Biosystems), as recently described (Chankeaw et al. 2021; Pereira et al. 2022). Following

capture, each LCM plastic cap (CapSure LCM macrocaps, Applied Biosystems) was examined at the quality control (QC) station and, if necessary, undesired cells were removed from the cap by a low power UV laser. The full microdissection processing of each sample took less than 90 minutes to preserve RNA integrity. After microdissection, total RNA from LE, GE and ST cells was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems) following the manufacturer's protocol, and total RNA was eluted in 15 µL of elution buffer. The RNA quantity and quality (RNA Integrity Number (RIN)) were assessed with the Agilent Bioanalyzer 2100 system (Agilent Technologies) and the RNA 6000 pico Chip Kit. Due to difficulty in harvesting enough RNA with eligible RIN value (≥ 7) for gene transcription measurements, from the initial 72 samples from 24 cows, only 13 LE, 20 GE, and 22 ST samples were forwarded for RNA sequencing (Table 7).

Table 7. Number and respective RNA Integrity Number (RIN) values of samples used for sequencing from each cell type of healthy (H), recovered (R) and persistent SCE (PE) cows (Total, n = 55).

Endometrial cell types	Cow sub-group			RIN[1]
	H	R	PE	
Stromal cells	5	7	10	7.32 ± 0.15
Glandular epithelial cells	4	6	10	7.35 ± 0.15
Luminal epithelial cells	2	5	6	7.18 ± 0.22

[1]RNA Integrity Number, values reported as mean ± SEM

5.3.6 RNA sequencing and data analysis

RNA sequencing libraries from 55 samples were prepared and sequenced on the GenomEast Platform (IGBMC, Cedex, France; http://genomeast.igbmc.fr/). Full-length cDNA was generated from 2.5 ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio Europe, Ozyme) according to manufacturer's instructions, with 10 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Then, 600 pg of pre-amplified cDNA was used as input for Tn5 transposon tagmentation using the Nextera XT DNA Library Preparation Kit (Illumina), followed by 12 cycles of library amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter), the size and concentration of libraries were assessed by capillary electrophoresis. Sequencing was performed on an Illumina HiSeq 4000 with 100 bp paired-end reads. Image analysis and base calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14. The sequencing depth of RNA-seq libraries was in the range of 71 to 100 million reads per sample, and all samples had a quality score over 30, meaning that the base call accuracy was 99.9%, in at least 90% of the sequenced bases.

All steps of the gene level exploratory analysis and differential transcription analysis were carried out as described (Pereira et al. 2022), using the RNAseq workflow described by Love et al. (2015) (updated version https://bioconductor.org/help/course-materials/2017/CSAMA/labs/2-tuesday/lab-03-rnaseq/rnaseqGene_CSAMA2017.html). The Salmon method (Patro et al. 2017) was used to quantify transcript abundance, and the Tximport method (Soneson et al. 2015) (R package version 1.8.0) was then used to import Salmon's transcript-level quantifications. The cDNA sequence database for *Bos taurus* was obtained from Ensembl (release-98; Bos_taurus.ARS-UCD1.2.cdna.all.fa) and used to build a reference index for the bovine transcriptome (Patro et al. 2017). Power analysis was performed using the method described by Bi et al. (2016) and compiled in the R package ssizeRNA (version 1.3.2). Calculated at a false discovery rate (FDR) of 0.05, power was 58%, 83%, 93% to detect 1.5, 2 and 3 log2 fold change respectively.

Principal component analysis (PCA) was performed with DESeq2 (R package, version 1.26.0) and FactoMineR (R package, version 1.4.1) using the variance stabilizing transformation output files from DESeq2. The downstream analysis of differentially expressed genes (DEGs) was performed by DESeq2 with the corresponding statistical methods (Love et al. 2015), including tests for differential transcription by use of negative binomial generalized linear models. The following terms for main effects and interaction were added in the model (cell_type (LE, GE, ST) + cow_group (H, PE, R) + cell_type:cow_group), with the FDR adjusted p-value of 0.05 (using the method of Benjamini and Hochberg, 1995) for the identification of DEGs. Following the use of this model, the group effect was analyzed within each cell type, and corresponding lists of DEGs were produced. Data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE192545 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192545).

5.3.7 Gene ontology (GO) and DEGs list exploration

Significant GO terms of the "GO-slim Biological Process" domain were found using the PANTHER classification system (Protein ANalysis THrough Evolutionary Relationships version 16.0, http://pantherdb.org). Identified GO terms were summarized by removing redundant GO terms with dispensability at medium level, using REVIGO (http://revigo.irb.hr/) (Supek et al. 2011). In a second step, the exploration of DEGs encoding proteins involved in inflammatory status, tissue remodeling, cell adhesion, and interferon-mediated signaling was performed considering significant differences when the adjusted $P \leq 0.10$.

5.3.8 Construction of protein-protein interaction (PPI) networks

The PPI networks were assembled with STRING database v11.0 (http://string-db.org) (Szklarczyk et al. 2019), generated with “non/query protein only”, and the sources of active interaction selected (Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, and Co-occurrence). Initially, only the highest confidence interactions (> 0.9) were included, but if none were detected, high (> 0.7) and medium (> 0.4) confidence interactions were also considered. Then, networks were analyzed in Cytoscape v 3.8.2 and visualized by yFiles layout algorithms for the Cytoscape app.

5.4 Results

5.4.1 Differences in transcriptomic patterns in relation to the health status of the endometrium

The PCA evidenced that the three cell types exhibit distinct gene transcription profiles in Healthy, Recovered and Persistent cows (Figure 19). Regarding GE cells, Healthy and Recovered cows exhibit similar transcriptomic profiles, which are clearly distinct from Persistent cows along the PCA horizontal and vertical axis. By contrast, LE cells of Recovered cows exhibit a similar gene transcription profile to Persistent cows and both are distinguishable from Healthy cows along the vertical axis. In addition, there is a large variation in Persistent cows as shown by the wide confidence ellipse for this group. The situation looks more complex in ST cells where Persistent cows differ from Healthy and Recovered cows on the vertical axis, whereas Persistent and Healthy cows differ from Recovered cows along the horizontal axis.

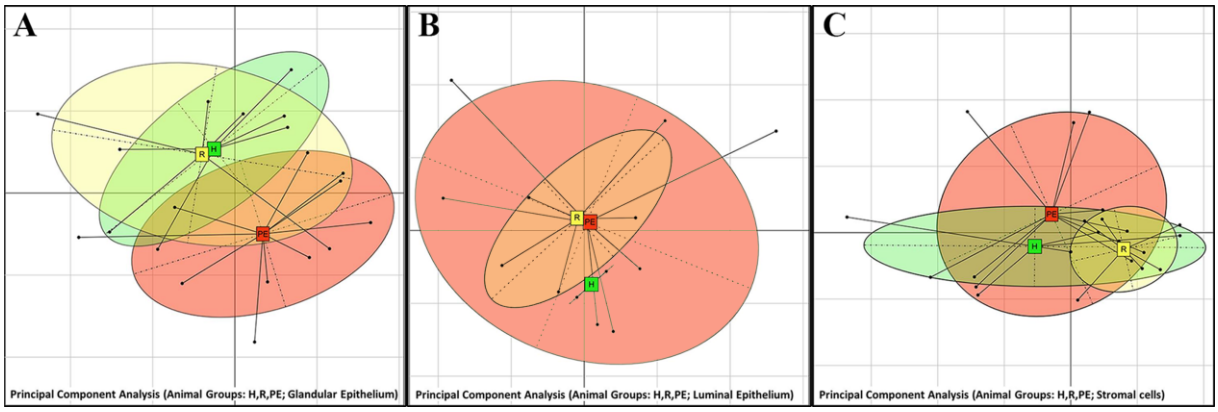

Figure 19. Principal component analysis (PCA) of the three cell types among healthy (H), recovered (R), and persistent SCE (PE) cows. A) Glandular Epithelial. B) Luminal Epithelial. C) Stromal cells.

The number of DEGs obtained through the two per two contrasts between the three types of uterine health status in each cell type (GE, LE and ST) are shown in Table 8. Globally, when compared to Healthy cows, Recovered cows presented a higher proportion of genes with decreased transcription in ST (82%), but not in GE (32%) and LE (45%) cells. In contrast, in comparison to Healthy cows, Persistent cows presented a large proportion of genes with increased transcription in GE (69%), LE (77%), and ST (78%) cells. The same trend was observed when comparing Persistent to Recovered cows. The number of DEGs common between the different comparisons (Persistent vs Healthy, Persistent vs Recovered, Recovered vs Healthy), in each cell type group (GE, LE, ST) are presented in Annex XII. In general, a low proportion of DEGs are commonly affected in all three cell types by endometrial health status. These patterns highlight that in epithelial compartments (GE +

LE), Healthy cows display a fair proportion of DEGs, which differ both in Recovered and Persistent cows, and that ST cells of Persistent cows exhibit a set of DEGs which are commonly affected in comparison with both Recovered and Healthy cows.

Table 8. Differently expressed genes (DEGs) either with decreased (<0) or increased (>0) transcription between cows with different uterine health statuses (H- healthy; R- recovered; PE- persistent SCE,) for each cell type (GE- glandular epithelial; LE- luminal epithelial; ST- stromal).

	GE			LE			ST		
	<0	>0	total	<0	>0	total	<0	>0	total
PE vs H	60	134	194	19	65	84	54	197	251
PE vs R	22	24	46	17	44	61	99	364	463
R vs H	12	26	38	27	33	60	119	27	146

The full lists of genes with increased and decreased transcription in GE, LE and ST and between Healthy, Recovered and Persistent cows are given in Supplementary File 6 (https://data.mendeley.com/datasets/x5kkf369kn/2, Annex X). In most cases (95%), when genes were differentially expressed in common between two or the three cell types the sense of deregulation was the same.

5.4.2 Analysis of differential gene transcription between cows with persistent SCE and healthy cows

The majority of DEGs resulting from the comparison between Healthy and Persistent cows, were cell type-specific but 3 were found common in GE and LE, 19 in GE and ST, 7 in LE and ST, and 2 were common between the 3 cell types (Figure 20a). In GE cells, 4 overrepresented GO terms were identified from the full list of DEGs (Supplementary File 7 (https://data.mendeley.com/datasets/922v7859tg/2, Annex X)). According to biological process classification, these DEGs are related to immune effector processes (GO:0002252), immune system processes (GO:0002376), response to virus (GO:0009615), and response to stress (GO:0006950). No GO enrichment was identified from DEGs of LE cells, whereas 22 enriched GO terms were revealed from the list of DEGs of ST cells. These terms include response to virus (GO:0009615), muscle system process (GO:0003012), muscle contraction (GO:0006936), response to biotic stimulus (GO:0009607), defense response (GO:0006952), and other biological processes (Supplementary File 7 (https://data.mendeley.com/datasets/922v7859tg/2, Annex X)).

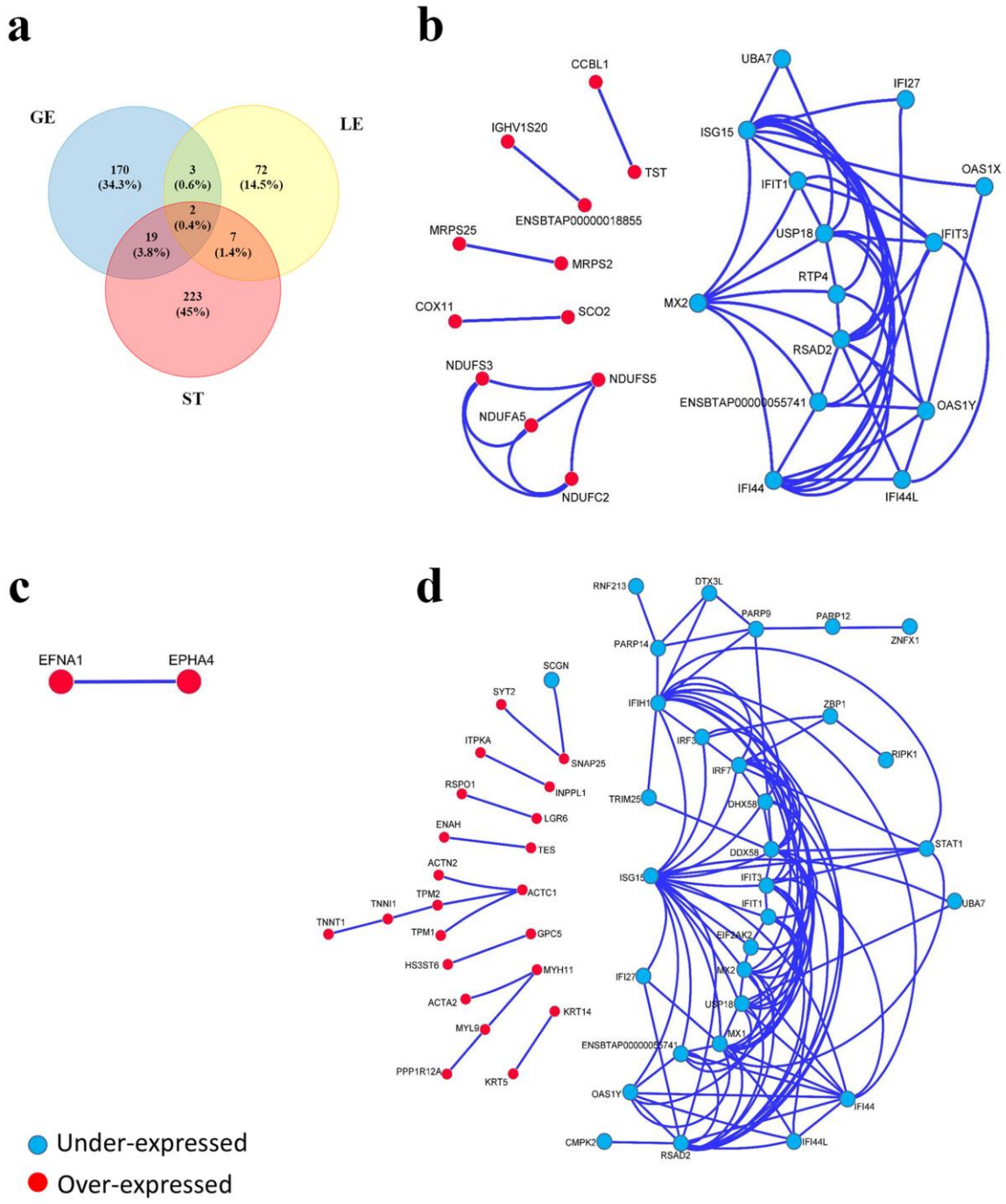

Figure 20. Differentially expressed genes (DEGs) between cows with persistent SCE and healthy cows. a) Venn diagram with DEGs identified in glandular epithelial (GE), luminal epithelial (LE) and stromal (ST) cells. b) Protein–protein interaction (PPI) network analysis of DEGs identified in GE cells. c) PPI network analysis of DEGs identified in LE cells. d) PPI network analysis of DEGs identified in ST cells.

Proteins encoded by the DEGs between Persistent and Healthy cows were further explored through a PPI network analysis based on STRING database. In GE cells, 6 modules were identified (Figure 20b), 1 connecting genes with decreased transcription and 5 linking genes with increased transcription. The module produced from genes with decreased transcription

includes numerous genes encoding Interferon (IFN) dependent or stimulated proteins. The modules from genes with increased transcription include genes encoding proteins related to NADH-Ubiquinone oxidoreductase complex (*NDUFS3, NDUFS5, NDUFA5, NDUFC2*), ATP synthesis and defense against oxidative stress (*SCO2, COX11*), enzymes involved in amino acid metabolism (*TST, CCBL1*), mitochondrial ribosomal proteins (*MRPS25, MRPS2*), and immunoglobulins (*IGHV1S20, ENSBTAP00000018855*). In LE cells only one module was identified from genes with increased transcription encoding proteins involved in Eph/ephrin signaling (*EPHA4, EFNA1*) (Figure 20c). In ST cells, 9 modules were identified (Figure 20d), 1 connecting genes with decreased transcription, 7 connecting genes with increased transcription, and 1 linking 1 gene with decreased transcription and 2 genes with increased transcription. As in GE cells, the large module corresponding to genes with decreased transcription included interferon-induced or stimulated genes. The modules with genes with increased transcription encoded proteins of the keratin family (*KRT14, KRT5*), modulating cell adhesion and motility (*TES, ENAH*), smooth muscle cells and contractile function (*ACTA2, MYH11, MYL9, PPP1R12A, ACTC1, ACTN2, TPM1, TPM2, TNNT1, TNNI1*), enzymes of inositol phosphate metabolism (*INPPL1, ITPKA*), members of the R-spondin family of Wnt modulators (*RSPO1, LGR6*), and members of the heparan sulfate proteoglycans or involved in its biosynthesis (*GPC5, HS3ST6*). The module with genes with increased and decreased transcription included genes encoding proteins involved in secretion events and vesicle trafficking (*SYT2, SNAP25, SCGN*).

5.4.3 Analysis of differential gene transcription between cows with persistent SCE and recovery cows

When comparing Persistent to Recovered cows, 3 DEGs were found common in GE and LE, 7 in GE and ST, 4 in ST and LE, and 2 were common between the 3 cell types (Figure 21a). In GE and LE cells, no GO enrichment was detected from DEGs lists, whereas in ST cells, overrepresentation analysis revealed 1 enriched GO term composed of genes involved in ion homeostasis (GO:0050801) (Supplementary File 7 (https://data.mendeley.com/datasets/922v7859tg/2, Annex X)).

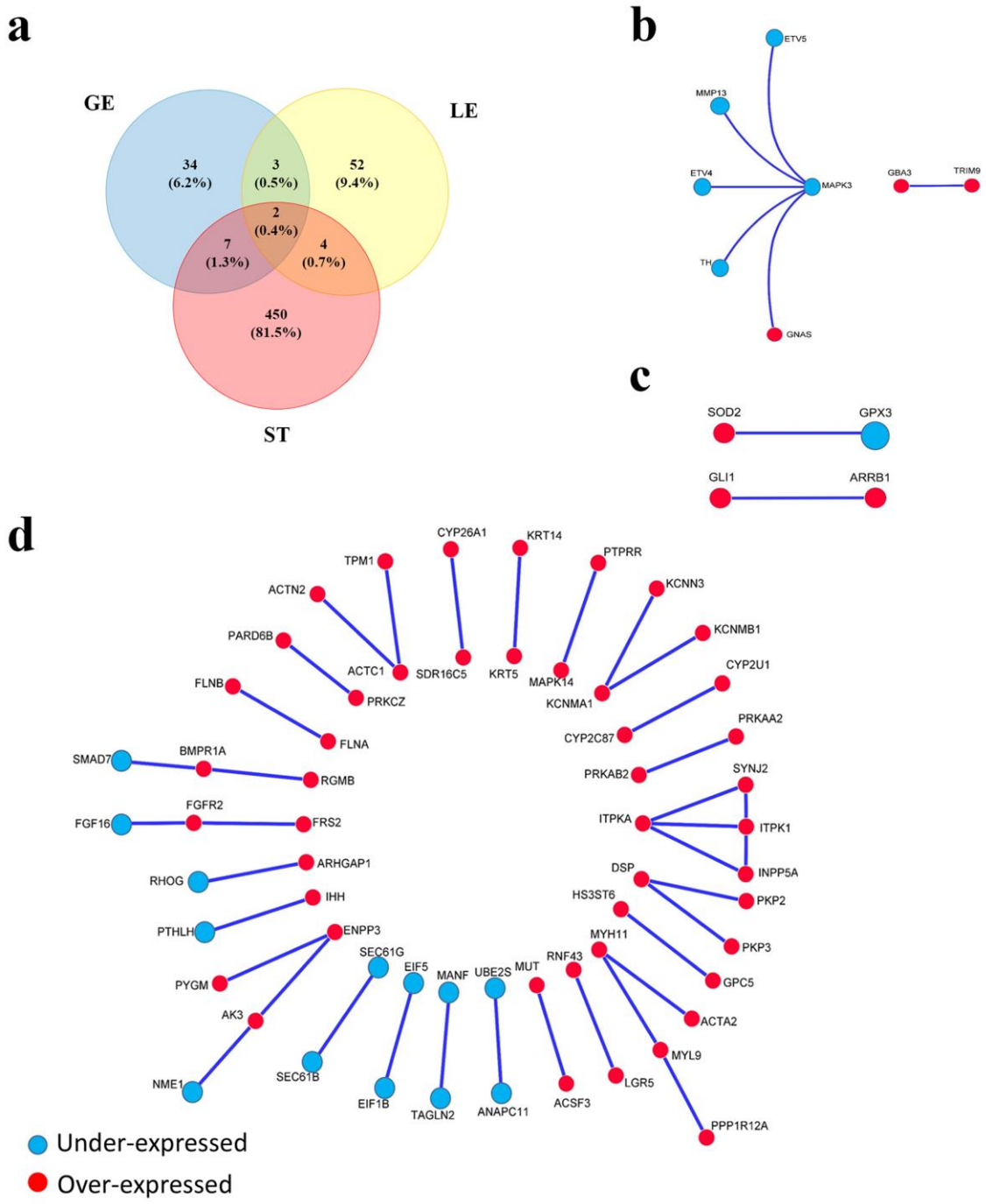

Figure 21. Differentially expressed genes (DEGs) between cows with persistent SCE and recovered cows. a) Venn diagram with DEGs identified in glandular epithelial (GE), luminal epithelial (LE) and stromal (ST) cells. b) Protein–protein interaction (PPI) network analysis of DEGs identified in GE cells. c) PPI network analysis of DEGs identified in LE cells. d) PPI network analysis of DEGs identified in ST cells.

In GE cells, the PPI network analysis identified 2 modules (Figure 21b), one linking 1 gene with increased transcription and 5 genes with decreased transcription encoding proteins related to mitogen-activated protein kinase signaling (*MAPK3, TH, ETV4, MMP13, ETV5, GNAS*) and one linking genes with increased transcription encoding glycosides hydrolyzing enzyme (*GBA3*) and E3 ubiquitin ligase (*TRIM9*). In LE cells, 2 modules were identified (Figure 21c), one involving genes with increased and decreased transcription encoding enzymes involved in protection against oxidative damage (*SOD2, GPX3*), and one relating genes with increased transcription encoding proteins associated to Hedgehog-GLI (*HH-GLI*) signaling (*ARRB1, GLI1*). By contrast, a large number of modules was identified from the DEGs list of ST cells (Figure 21d). Four modules linking genes with decreased transcription, encoding proteins related with translation initiation (*EIF5, EIF1B*), protein translocation (*SEC61B, SEC61G*), the ubiquitin-proteasome system (*ANAPC11, UBE2S*), and immune modulation (*TAGLN2, MANF*). Fifteen modules corresponded to genes with increased transcription encoding proteins of epithelial cell apico-basolateral polarization (*PARD6B, PRKCZ*), retinoic acid (*CYP26A1, SDR16C5*), fatty acid (*ACSF3, MUT*) and inositol polyphosphate (*INPP5A, ITPK1, ITPKA, SYNJ2*) metabolism, mitogen-activated protein kinase signaling (*MAPK14, PTPRR*), of the filamin (*FLNA, FLNB*) and cytochrome P450 CYP2 (*CYP2C87, CYP2U1*) families, members of the R-Spondin family of proteins (*RNF43, LGR5*), subunits of potassium calcium-activated channels (*KCNMB1, KCNMA1, KCNN3*), subunits of AMP-activated protein kinase (*PRKAA2, PRKAB2*) and desmosome related proteins (*PKP3, PKP2, DSP*). Five modules comprised genes with increased and decreased transcription encoding proteins of bone morphogenetic protein (*RGMB, BMPR1A, SMAD7*) and fibroblast growth factor (FGF) (*FRS2, FGFR2, FGF16*) signaling, enzymes involved in energy sensing, generation, and utilization (*AK3, ENPP3, PYGM, NME1*), and members of the Rho GTPase (*ARHGAP1, RHOG*) and Indian Hedgehog–PTH-related protein (Ihh-PTHrP) (*PTHLH, IHH*) pathways.

5.4.4 Analysis of differential gene transcription between samples from recovery cows and healthy cows

The number of DEGs emerging from the comparison between Recovered and Healthy cows is lower than for previous comparisons including Persistent cows. However, similarly, the number of DEGs per cell type is higher in ST than in GE and LE cells and the majority of DEGs were also cell type-specific (Figure 22a), except for 2 common between GE and LE, 5 common between GE and ST, and 4 common between ST and LE.

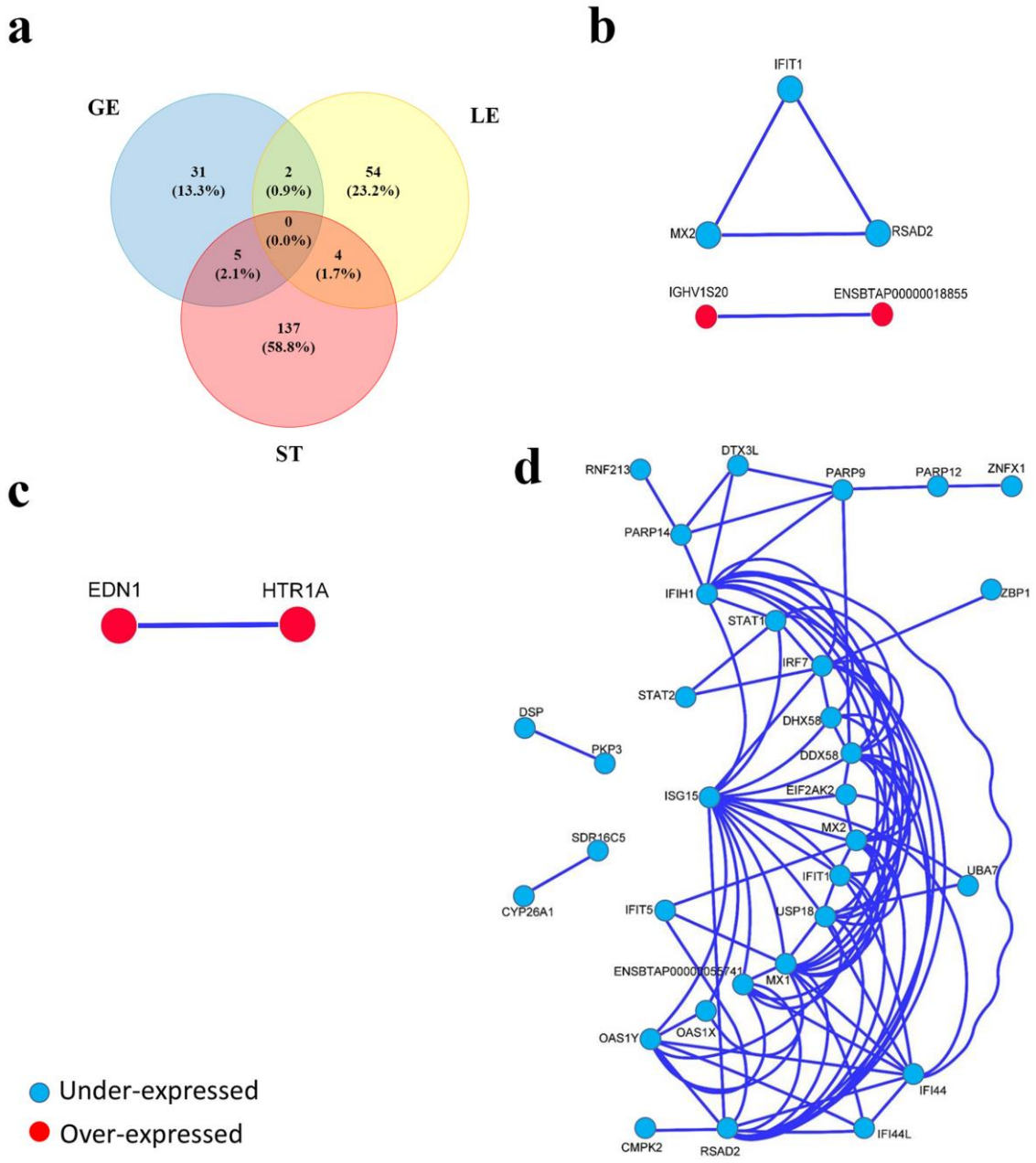

Figure 22. Differentially expressed genes (DEGs) between recovered and healthy cows. a) Venn diagram with DEGs identified in glandular epithelial (GE), luminal epithelial (LE) and stromal (ST) cells. b) Protein–protein interaction (PPI) network analysis of DEGs identified in GE cells. c) PPI network analysis of DEGs identified in LE cells. d) PPI network analysis of DEGs identified in ST cells.

Among DEGs observed in GE and LE cells, no GO enrichment was identified. In ST samples, overrepresentation analysis revealed 17 enriched GO terms. The detected genes are related to response to virus (GO:0009615), defense response (GO:0006952), immune effector process (GO:0002252), immune system process (GO:0002376), positive regulation of cytokine production (GO:0001819), and other biological processes (Supplementary File 7 (https://data.mendeley.com/datasets/922v7859tg/2, Annex X)).

Proteins encoded by the DEGs between Recovered and Healthy cows were also further explored through PPI network analysis. In GE cells, 2 modules were identified (Figure 22b), one involving genes with decreased transcription encoding interferon dependent or stimulated proteins (*RSAD2, IFIT1, MX2*), and one connecting genes with increased transcription encoding immunoglobulins (*IGHV1S20, ENSBTAP00000018855*). In LE cells, 1 module was identified that relates genes with increased transcription encoding proteins involved in smooth muscle contraction and vascular function (*HTR1A, EDN1*) (Figure 22c). In ST cells, 3 modules connecting genes with decreased transcription were identified (Figure 22d), encoding desmosome related proteins (*PKP3, DSP*), enzymes of the retinoic acid metabolism (*CYP26A1, SDR16C5*), and several interferon dependent or stimulated proteins.

5.4.5 Comparisons between the three groups of cows with different endometrial health status

To further evaluate differences between the three types of cows on a functional basis, the presence of DEGs encoding proteins involved in inflammatory status (Supplementary File 8 (https://data.mendeley.com/datasets/cpb5vznzhn/3, Annex X)), tissue remodeling, and cell adhesion (Supplementary File 9 (https://data.mendeley.com/datasets/d36t3d438g/3, Annex X)) is described below.

5.4.6 Inflammatory status

This evaluation considered genes encoding proteins within the cytokine family (transforming growth factor beta (TGF-β) family, tumor necrosis factor (TNF) superfamily, chemokines family, and interleukins) and the class of pattern recognition receptors, such as Toll-like receptors, which are responsible for the recognition of pathogen-associated molecular patterns (PAMPs).

In GE cells, when compared to Healthy cows, both Recovered and Persistent cows display decreased transcription of transforming growth factor beta receptors (*TGFBR1* in Recovered and *TGFBR2* in Persistent cows). In addition, Persistent cows exhibit decreased transcription of interleukin 10 receptor subunit alpha (*IL10RA*) and 2 interleukin 1 receptor associated kinases (*IRAK3, IRAK4*). Recovery cows display increased transcription of TNF receptor superfamily member 12A (*TNFRSF12A*) when compared with Persistent cows.

When considering LE cells, compared with both Healthy and Recovered cows, Persistent cows display increased transcription of chemokine ligand 21 (*CCL21*). Increased transcription of chemokine receptor 10 (*CCR10*) and chemokine ligand 19 (*CCL19*) was also observed in Persistent cows compared with Healthy and Recovered cows, respectively. No

DEGs encoding proteins of immune response were found when comparing Healthy and Recovered cows.

In ST cells, Persistent cows exhibit increased transcription of chemokine ligand 11 (*CCL11*), and decreased transcription of interleukin 1 receptor type 1 (*IL1R1*) and chemokine receptor 1 (*CCR1*) when compared to Healthy cows. In addition, Persistent cows display increased transcription of interleukin 17B and 17D (*IL17B, IL17D*) when compared with Healthy and Recovered cows. When compared with Recovered cows, Persistent cows show increased transcription of interleukin 13 receptor subunit alpha 1 (*IL13RA1*), and decreased transcription of interleukin 17 receptor A (*IL17RA*), interleukin 1 beta (*IL1-β*), 2 members of the TNF superfamily (TNF receptor superfamily member 12A (*TNFRSF12A*) and TNF alpha induced protein 6 (*TNFAIP6*, also known as *TSG6*)). The comparison between Healthy and Recovered cows revealed only decreased transcription of transforming growth factor beta receptor 1 (*TGFBR1*) in Recovered cows.

5.4.7 Tissue remodeling

Numerous DEGs between the three groups of cows were identified in this category. In GE cells, when compared with Healthy cows, Persistent cows exhibit increased transcription of genes encoding homeobox A5 and A6 and histone deacetylase 10 (*HDAC10*), and decreased transcription of genes encoding matrix metalloproteinases 13 (*MMP-13*) (also found when comparing Persistent to Recovered cows) and 19 (*MMP-19*). In LE cells, Persistent cows exhibit increased transcription of genes encoding 2 members of the collagen family (*COL11A1, COL4A6*) when compared to Healthy cows and in ST cells increased transcription of genes encoding histone deacetylase 9 (*HDAC9*), *FGFR2*, and ADAM metalloproteinase domains 11 and 20. The comparison between ST cells of Persistent and Recovered cows revealed increased transcription of the HDAC9 coding gene, as well as differential transcription of a series of metalloproteinases (MMP-3 and ADAM with thrombospondin type 1 motif 14, decreased transcription, and ADAM with thrombospondin type 1 motif 6, ADAMTS like 3 and ADAM metalloproteinase domain 11, increased transcription). The comparison between Persistent and Recovered cows evidenced that most DEGs encoding proteins related to tissue remodeling were identified in ST cells. In ST cells, changes observed in networks of genes encoding proteins associated with smooth muscle cells and contractile function, and proteins related to heparan sulfate are also noticeable with increased transcription observed in Persistent cows when compared to both Healthy and Recovered cows (Figure 20d and 21d).

5.4.8 Cell adhesion

In GE cells, compared to Healthy cows, both Persistent and Recovered cows display decreased transcription of integrin subunit alpha V coding gene, and Persistent cows also exhibit decreased transcription of integrin subunit alpha 5 coding gene. When compared to Recovered cows, ST cells of Persistent cows exhibit a consistent increased transcription of numerous genes encoding protein members of the cadherin family, namely cadherin 1, transmembrane O-mannosyltransferase targeting cadherin 1, FAT atypical cadherin 3, cadherin EGF LAG seven-pass G-type receptor 2, and Cadherin like and PC-esterase domain containing 1. In LE cells, both Persistent and Recovered cows display increased transcription of integrin-binding sialoprotein coding gene when compared to Healthy cows. In addition, when compared with Healthy cows, Persistent cows exhibit increased transcription of genes encoding FAT atypical cadherin 4 and protocadherin gamma subfamily A in LE cells and, Recovered cows exhibit decreased transcription of the cadherin 1 coding gene in ST cells.

5.4.9 Interferon dependent or stimulated genes

When compared to Healthy cows, Persistent and Recovered cows show a striking decreased transcription of a large common set of genes belonging to this family in GE and ST cells (Figure 20c-d and 22c-d).

5.5 Discussion

Following an early diagnosis of SCE during the postpartum period, it is suggested that half of the cases develop inflammatory processes without the presence of pathogens (Madoz et al. 2014; Pascottini and LeBlanc, 2020). This condition is regarded as an unresolved inflammation following the immune activation triggered by the initial bacterial assault (Brewer et al. 2020). The inflammatory response comprises the induction phase, associated with fast and robust immune activation, and the resolution phase which is critical to limit the acute response and restore tissue homeostasis (Schett and Neurath, 2018). In early postpartum, a temporal switch from a pro-inflammatory gene transcription profile to a regenerative profile was identified in healthy cows (Foley et al. 2012). In contrast, in cows that develop SCE, this transition is arrested or delayed leading to sub-optimal restoration of homeostasis (Foley et al. 2015). Even when an apparent clinical cure of endometritis takes place, these cows still have reduced fertility (Sheldon et al. 2020).

The present study aimed to describe the transcriptomic profiles associated to different endometrial cell types in cows with contrasted health statuses. Confirming the results from former studies showing that endometrial cell types present very different transcriptomic

signatures (Chankeaw et al. 2021a; Pereira et al. 2022), LCM was used here successfully to isolate cell types and study their specific response to persistent inflammation of the endometrium and/or recovery. The relevance of the approach is evidenced by the different transcriptomic profiles between groups of cows with contrasted health status showing that the majority (> 90%) of DEGs between Healthy, Recovered and Persistent cows was cell-type specific.

Overall, this study shows that endometrial ST cells are the most affected by persistent or transient inflammation (Persistent and Recovered cows, respectively), as depicted by the higher number of DEGs and enriched GO terms, compared with GE and LE cells. However, this finding is also influenced by the higher statistical power arising from the increased number of ST samples, compared to GE and LE samples. In fact, the main limitation of the present study is the amount of LE samples with enough RNA and eligible for RNAseq. Due to this, the lists of DEGs identified from LE samples, although accurate, are truncated since they would be more complete with a higher number of samples from this cell type.

Interestingly, the PCA revealed different gene transcription patterns between cows with contrasted health status in each cell type. Such differences were expected between the samples issued of cows from extreme phenotypes Healthy and Persistent (Johnson et al. 2015; Salilew-Wondim et al. 2016, Raliou et al. 2019). However, the present study clearly reveals, that despite Healthy and Recovered cows present a similar “healthy phenotype” (as shown by the low percentage of immune cells) at biopsy sampling, the previous presence of immune cells is possibly associated with other mechanisms still impacting the transcriptome of endometrial cells at a later stage. This finding confirms an earlier report that past microbial exposure is responsible for long-term effects in the endometrial transcriptome of postpartum dairy cows (Moore et al. 2019).

This is especially true for LE and ST cells showing very distinct transcriptomic profiles between Healthy and Recovered cows. Overall, differences in gene transcription between these two groups are less prominent in GE cells, but there are still many individual genes differentially transcribed in response to former health status.

Due to the above findings and the vast number of individual genes differentially transcribed in our model, we developed the discussion under, focusing essentially on various functions related to immune response, cell adhesion and tissue remodeling, and the subsequent establishment of pregnancy and how these respond to contrasted uterine health phenotypes.

5.5.1 Impact of endometrial health status on gene transcription related to immune response

In GE cells, both Persistent and Recovered cows exhibit decreased transcription of genes encoding TGF-β receptors, and Persistent cows also display decreased transcription of *IL10RA*. TGF-β regulates the onset and resolution of inflammation, co-acting with IL10 to ensure a controlled inflammatory response through the regulation of T cells (Li and Flavell, 2008a; 2008b). In epithelial cells, TGF-β and IL10RA prevent LPS-driven damage, eliciting anti-inflammatory and tolerogenic responses (Jarry et al. 2008; Mallikarjunappa et al. 2020). Moreover, an association between endometrial IL10 mRNA and protein expression by uterine immune cells and conceptus survival was found in dairy heifers (Vasudevan et al. 2017). In GE cells, Persistent cows also exhibit decreased transcription of IRAK3 and IRAK4 coding genes, which regulate TLR and IL-1 signaling (Jain et al. 2014; Singer et al. 2018), with IRAK3 promoting anti-inflammatory effects through a paradoxical “second wave” of NF-κB activation (Jain et al. 2014). Therefore, results from the present study showing decreased transcription of TGF-β receptors and *IL10RA*, and *IRAK3* and *IRAK4* confirm that crucial pathways in the regulation of innate immunity and inflammation are affected in GE cells of cows with persistent endometrial inflammation.

In LE cells, the increased transcription of *CCR10* and *CCL21* in samples from Persistent cows compared to Healthy cows, and *CCL21* and *CCL19*, compared to Recovered cows, is consistent with the roles of these chemokines/receptors in the local recruitment of circulating immune cells (Xiong et al. 2012; Hjortø et al. 2016). Epithelial CCR10 and its ligands attract leukocytes, such as B cells, T cells, and eosinophils (Choi et al. 2016; Cha et al. 2011; Eckel and Ametaj, 2020). CCL21 enhances mature dendritic cells receptor-mediated endocytosis (Kikuchi et al. 2005) and regulates T cells migration and activation (Flanagan et al. 2004). Moreover, both CCL21 and CCL19 are involved in chemotaxis and activation of naive T cells and antigen-presenting dendritic cells (Marsland et al. 2005; Hjortø et al. 2016), and CCL21 can be involved in the regulation of ovarian function (Mellouk et al. 2019). Thus the increased transcription of CCL21 in Persistent cows can contribute to the already extensively explored link between endometrial inflammation and impaired ovarian function in postpartum dairy cows (Mateus et al. 2002; Sheldon et al. 2002).

The comparison between Persistent and Healthy cows from LE cells also reveals the increased transcription of ephrin signaling genes (*EPHA4* and *EFNA1*). These genes reported to be involved in proliferation, protection against endoplasmic reticulum stress, and inflammatory responses in bovine endometrial and mammary epithelial cells (Kang et al. 2018; Lim et al. 2019) are associated here with persistent inflammation.

In ST cells, one of the major changes observed in Persistent cows compared to other groups is the increased transcription of genes from the IL17 inflammatory cascade (*IL17B* and *IL17D*). IL17B and IL17D were associated with tissue regeneration, but also with the recruitment of immune cells and the progression of inflammation (Bie et al. 2017, Liu et al. 2020). Our results are consistent with Foley et al. (2015), showing a persistent increased transcription of *IL17D* in cows with SCE.

In addition, Persistent cows display increased transcription of genes encoding members of the R-Spondin family of proteins (*RSPO1* and *LGR6* compared to Healthy cows and *RNF43* and *LGR5* compared to Recovered cows) known for their capacity to amplify β-catenin/Wnt signaling which controls cell fate, tissue development, growth and homeostasis (Chen et al. 2013; Nagano, 2019). These results may be related to uncomplete tissue repair in these cows as LGR6, LGR5 and RNF43 are receptors for R-spondins (Chen et al. 2013). Moreover, LGR6 is known to promote tissue repair and regeneration when activated by Maresin 1, a potent immune resolving agent enhancing macrophage uptake of apoptotic PMN and limiting PMN infiltration (Chiang et al. 2019). Compared to Recovered cows, Persistent cows also exhibit increased transcription of CYP2C87 and CYP2U1 (members of cytochrome P450, CYP2 family), involved in biosynthesis and inactivation of pro-inflammatory and pro-resolving lipid-mediators (Divanovic et al. 2013), and CYP26A1 and SDR16C5, which respectively regulate the cellular level of retinoic acid (Stevison et al. 2015), and its biosynthesis (Wu et al. 2019). Retinoic acid is essential for the normal regeneration of mucosal barriers damaged by infection as well as for the function of neutrophils, macrophages, and natural killer cells (Stephensen et al. 2001).

Taken together, our results showing the increased transcription of genes involved in the resolution of inflammation and mucosal barrier regeneration, still present at 44 DPP may indicate that the above processes are still active and delayed in Persistent cows compared to Recovered and Healthy cows.

In ST cells, Persistent cows transcribe less *IL1R1* (compared to Healthy cows), which encodes the receptor for IL1-β and IL1-α, responsible for eliciting a pro-inflammatory response (Dinarello 2018). They also display decreased transcription of *IL1-β*, *TNFRSF12A* and *TSG6* coding genes compared with Recovered cows. This was unexpected since both IL1-β and TNF are pro-inflammatory cytokines (Lukens et al. 2012), with increased concentrations in cows with endometritis (Galvão et al. 2011; LeBlanc 2012). TNFRSF12A is up-regulated in epithelial and Natural Killer (NK) cells in diseased tissues (Dohi and Burkly 2012; Qi et al. 2016) and acts as receptor of TWEAK (TNFSF12), eliciting signaling related to tissue remodeling (Dohi and Burkly 2012). The transient activation of TWEAK/TNFRSF12A

pathway may be beneficial for tissue repair after acute injury (Burkly et al. 2011), whereas excessive or sustained activation mediates pathological tissue damage and remodeling (Burkly 2014). Therefore, the results from the present study suggest that Persistent cows, contrary to Recovered cows, do not elicit a TNFRSF12A-dependent beneficial tissue repair program. Persistent cows also exhibit decreased transcription of TSG6, a member of the TNF superfamily up-regulated upon exposure to inflammatory mediators (Watanabe et al. 2018). TSG6 is released from neutrophils, mast cells, macrophages and stromal cells and displays anti-inflammatory and tissue-protective properties (Watanabe et al. 2018; Day and Milner 2019). These include inhibition of neutrophil migration (Dyer et al. 2014), polarisation of macrophages to an anti-inflammatory M2 phenotype (Mittal et al. 2016) and modulation of endometrial matrix turnover and organization (Capp et al. 2014; Day and Milner 2019). The present results lead to the hypothesis that decreased transcription of TSG6 in ST cells of Persistent cows, may be a relevant marker for the persistence of endometrial inflammation and/or a putative therapeutic target.

Finally, when compared to both Healthy and Recovered cows, Persistent cows exhibit increased transcription of *HDAC9*, which is known to inhibit the development and function of T regulatory cells (Shakespear et al. 2011). This result suggests that the increased transcription of this enzyme by ST cells of Persistent cows may be associated with alterations of immune tolerance mechanisms (Okeke and Uzonna, 2019).

5.5.2 Impact of endometrial health status on gene transcription related to tissue remodeling and cell adhesion

In GE cells, when compared with Healthy cows, Persistent cows exhibit decreased transcription of the *MMP-19* gene, encoding a basement membrane-degrading protease involved in tissue remodeling, wound healing, and epithelial cell migration (Cui et al. 2017). However, contrary to most MMPs, MMP-19 is also expressed in healthy epithelial cells while maintaining the epithelial barrier function and regulating innate immune response, especially the influx of neutrophils (Brauer et al. 2016). As found before in a mice model of epithelial barrier damage (Brauer et al. 2016), the decreased transcription of *MMP-19* in Persistent cows suggests that adequate epithelial expression of MMP-19 is necessary for effective uterine involution and resolution of inflammation. Moreover, Persistent cows also display increased transcription of the *HDAC10* gene in GE cells. Members of the corresponding family of enzymes remove acetyl groups from lysine residues of histones, leading to transcriptionally silenced chromatin (Lawlor and Yang, 2019). Changes in gene expression of this group have been associated with alterations of endometrial remodeling and pathologies such as cancer, endometriosis, and infertility (Gujral et al. 2020). Downregulation of histone

deacetylases coupled with TGF-β stimulation was favourable to epithelial barrier regeneration (Friedrich et al. 2019). In addition, genes of the integrin family (*ITGAV* and *ITGA5*) were also less transcribed in Persistent cows. ITGAV integrins regulate inflammation in epithelial cells through activation of TGF-β (Munger et al. 1999; Mu et al. 2002). Considering the above, results of the present study (combination of *HDAC10* increased transcription coupled with *ITGAV* decreased transcription leading to lower transcription of *TGFBR2* in GE cells of Persistent cows) suggest that these changes may lead to delayed epithelial regeneration and alter immunotolerant mechanisms in Persistent cows.

In ST cells, Persistent cows show decreased transcription of *MMP-3* compared with Recovered cows and no difference was found with Healthy cows. Apparently, these results contrast with those of literature where endometrial MMP-3 mRNA and protein levels were positively correlated with the severity of endometritis and the inflammatory response to LPS in an *in vitro* model of endometrial epithelial cells (Zhang et al. 2021). Differences may be related to differences in cell type or the postpartum stage.

A member of FGF signaling pathway (*FGFR2*), which regulates tissue repair and repair-related angiogenesis through cell migration, proliferation, differentiation, and survival (Xie et al. 2020), was more transcribed in ST cells of Persistent cows when compared to Recovered and Healthy cows. The active role of FGFR2 has been evidenced in postpartum goats, where its high protein expression in fibroblasts was associated with epithelial and stromal cell regeneration (Sánchez et al. 2002). The results obtained here support the idea that *FGFR2* increased transcription is related to delayed endometrial involution and that Persistent cows are still under active repair processes.

PE cows show increased transcription also in ST cells of genes encoding proteins associated with smooth muscle cells and contractile function (*ACTA2, MYH11, MYL9, PPP1R12A, ACTC1, ACTN2, TPM1, TPM2, TNNT1, TNNI1*). Most of these genes were also identified from GO terms enrichment related to muscle system process, myofibroblasts, smooth muscle cells and muscle contraction, and expression of their encoded proteins is restricted to smooth muscle cells and myofibroblasts (Rockey et al. 2013; Queckbörner et al. 2021). The differentiation of fibroblasts into myofibroblasts and building of the extracellular matrix are critical components for wound healing and tissue repair (Rockey et al. 2013), contributing to cessation of haemorrhage, restoration of barrier integrity, and re-establishment of tissue function (Klingberg et al. 2013). As mentioned before for other sets of genes, the increased transcription of these genes in Persistent cows may reflect the continuing endometrial tissue repair.

Finally, Persistent cows show increased transcription of *GPC5* and *HS3ST6* in ST cells compared to Recovered and Healthy cows. Heparan sulfate is found on the surface of most cell types, and its expression is induced by LPS and TNF-α in endometrial and endothelial cells, respectively (Oguejiofor et al. 2015; Collins and Troeberg, 2019), where is involved in the formation of perivascular chemokine gradients contributing to the transendothelial recruitment of leukocytes from the circulation to the site of inflammation (Kumar et al. 2015). Moreover, heparan sulfate regulates angiogenesis by playing a proangiogenic role (Fuster and Wang, 2010), and given that IL-1 regulates inflammation and angiogenesis (Healy et al. 2014), the increased transcription of these heparan sulfate-related genes and the decreased transcription of *IL1R1* in ST cells of Persistent cows may result in differential regulation of angiogenesis and tissue remodeling. In addition, decreased transcription of *IL1R1* may be detrimental for subsequent fertility of Persistent cows, as this system was shown to be involved in establishment of pregnancy in humans, pigs, and cattle (Bellehumeur et al. 2009; Seo et al. 2012; Correia-Álvarez et al. 2015).

5.5.3 Impact of endometrial health status on genes related to uterine receptivity and pregnancy establishment

A large number of genes encoding regulators of interferon signaling were less transcribed in Persistent and Recovered cows compared to Healthy cows in both GE and ST cells. Most of them are identified in enriched GO terms related to immune system or effector processes, virus response, and stress response. Interferons are regulators of the neutrophil-based inflammatory response (Glennon-Alty et al. 2021), and *in vitro* LPS stimulation of bovine endometrial cells induced the up-regulation of interferon-stimulated genes (Oguejiofor et al. 2015; Guo et al. 2019). Type I IFN activates the innate immune response (Kovarik et al. 2016; Kopitar-Jerala 2017), and depending on the context, may either enhance or inhibit immune response (Kovarik et al. 2016; Fox et al. 2020). When inflammation results from failure to repair and regenerate damaged tissues, rather than inefficient pathogen clearance, type I IFN is regarded as protective as it suppresses IL-1β expression and neutrophil chemo-attractants (Kovarik et al. 2016). In the present study, the decreased transcription of interferon-stimulated genes in Persistent and Recovered cows may lead to failure of the protective role of IFN signaling. This agrees with Iyer (2013), who defined a type I IFN, IL-27 and IL-10 gene program required to resolve an acute inflammatory response and protect against tissue injury.

Embryonic IFNT, the pregnancy recognition signal in ruminants, induces the same IFN-stimulated genes as type I IFN (Schabmeyer et al. 2021). Most of these genes, such as MX1, MX2, RSAD2, USP18, OAS1, RNF213 and ISG15, have been found over-expressed in the

bovine endometrium at the time of maternal recognition of pregnancy (D16) as a consequence of IFNT production by the conceptus (Forde et al. 2011; Forde et al. 2012; Hansen et al. 2017). The enhanced expression of these IFN-stimulated genes is hypothesized to regulate uterine receptivity as well as conceptus elongation and implantation (Spencer et al. 2015). Therefore, decreased transcription of the corresponding set of genes in GE and ST cells of Persistent and Recovered cows may prove detrimental for the success of embryo-maternal crosstalk.

5.6 Conclusion

This study showed that the transcriptomic signatures of the endometrial compartments are altered in postpartum dairy cows suffering from sub-clinical endometritis. Results also reveal the lack of complete functional recovery at 44 DPP in cows presenting a high percentage of immune cells in endometrial cytology at an earlier stage. The analysis of global transcriptomic profiles revealed that functional recovery appears quicker in glandular cells than in other cell types. The lists of differentially expressed genes corresponding to contrasts between health status groups were different between cell types, and very few were commonly altered by the persistence of inflammation. The results of this study evidenced that recovery or persistence of inflammation is associated with gene transcription patterns involved not only in immune function but also in tissue remodeling and uterine receptivity in a cell type-specific manner. However, cows from the recovery type were close to healthy ones when considering the transcription of genes related to immunity and tissue remodelling whereas strong differences were still present especially in stromal cells for genes related to cell adhesion and interferon dependant or stimulated genes. In addition to these genes revealing main alterations of endometrial function, many differentially transcribed genes were poorly annotated. The present data may represent a base for future studies aiming to decipher their roles and how they interfere with the above processes. The changes in transcriptomic signatures associated with the persistence of inflammation may pave the way for further work to develop novel diagnostic and therapeutic targets to prevent the persistence of endometrial inflammation and possibly restore postpartum fertility.

CHAPTER 6 - Effects of feeding rumen-protected linseed fat to postpartum dairy cows on plasma n-3 polyunsaturated fatty acids concentrations, metabolic and reproductive parameters

G. Pereira, P. Simões, R. Bexiga, E. Silva, L. Mateus, T. Fernades, S. Alves, R. Bessa, and L. Lopes-da-Costa

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6.1 Abstract

High-yielding dairy cows experience a negative energy balance (NEB) and inflammatory status during the transition period. Fat supplementation increases diet energy density, and plasma n-3PUFA were proposed to improve immune function. This study tested the hypothesis that dietary supplementation with a rumen-protected and n-3PUFA enriched fat could ameliorate both the energetic deficit and immune status of postpartum high-yielding dairy cows, improving overall health and reproductive efficiency. At 11 days in milk (DIM), cows were randomly allocated to groups i) n-3PUFA (n = 29) - encapsulated linseed oil supplying additional up to 64 g/d (mean 25 ± 4 g/d) of α-linolenic acid and (ii) Control (n = 31) – hydrogenated palm oil without ALA content. Fat supplements of groups n-3PUFA and Control were available through an automated, off-parlor feeding system, and intake depended on the cow's feeding behavior. Plasma ALA concentrations were higher in n-3PUFA than Control cows, following a linear relation with supplement ingestion, resulting in a lower n-6/n-3 ratio in plasma. Metabolic parameters (BCS, glucose, and BHB blood concentrations) were unaffected, but milk yield improved with increased intake of fat supplements. Plasma total adiponectin concentrations were negatively correlated with the ingestion of n-3PUFA enriched fat supplement, following a linear relation with intake. Conception rate to first AI increased with higher intake of both fats, but a decrease of calving to conception interval only occurred in n-3PUFA cows. Postpartum ovarian activity and endometrial inflammatory status at 45 DIM were unaffected. In conclusion, this study evidenced a positive linear relation between rumen-protected linseed fat intake and plasma n-3PUFA concentrations, which modulated adiponectin expression and improved reproductive parameters.

**Text adapted from the original published paper (Annex XIII)*

6.2 Introduction

The high-yielding dairy cow transition period is characterized by a state of NEB, which adversely affects cow health, fertility, and milk yield (Raboisson et al. 2014; Abdelli et al. 2017; Sheldon et al. 2019). Fat supplementation became a common strategy to increase the energy density of diets and minimize the effects of NEB (Palmquist and Jenkins 2017; Bionaz et al. 2020). However, this may reduce DMI, disturb rumen function, and originate fatty acid isomers that depress milk fat (Chamberlain and DePeters 2017; de Souza and Lock 2019; Manriquez et al. 2019). As FA, namely PUFA, have essential physiologic roles, the beneficial effects of fat supplementation may rely more on FA type than on increased energy intake (Herrera-Camacho et al. 2011). In this context, the PUFA from n-3 (α-linolenic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid) and n-6 (linoleic acid, arachidonic acid) families receive special attention due to their roles in reproductive and immune function (Moallem 2018; Moallem et al. 2020). Increased intake of n-6PUFA increases the proportion of LA and ARA in cell membranes, favoring eicosanoid synthesis towards a pro-inflammatory state (Wolf et al. 2019). In contrast, increased intake of n-3PUFA increases the proportion of EPA and DHA in cell membranes, which favors eicosanoid synthesis towards an anti-inflammatory state (Greco et al. 2015; Wolf et al. 2019).

Transition dairy cows are good candidates for the modulation of inflammation through nutrition since they experience a physiologic inflammatory state during the puerperal period (Bradford et al. 2015). A pro-inflammatory state characterizes initial uterine involution directed to elimination of endometrial pathogens by immune cells (LeBlanc 2012). Then, it switches to a pro-resolving state where restauration of endometrial homeostasis occurs. Failure to regulate this shift results in persistent chronic endometrial inflammation (subclinical or cytological endometritis) (Pascottini and LeBlanc 2020). Recently a link was unveiled between the metabolic and immune function at the endometrial level through adipokine signaling (adiponectin and chemerin) (Pereira et al. 2020). In neonatal calves, transfer of n-3PUFA into the plasma may also take place via placenta during late gestation or via colostrum (Uken et al. 2021a), and n-3PUFA supplementation of colostrum increases plasma n-3PUFA content, with beneficial effects on the inflammatory response (Opgenorth et al. 2020) and metabolic and endocrine development (Uken et al. 2021b).

This study evaluated the effects of feeding a rumen-protected linseed fat supplement to postpartum high-yielding dairy cows on metabolic and reproductive parameters. The hypothesis to be tested was that feeding a rumen-protected linseed fat rich in ALA would increase the blood n-3PUFA concentrations, resulting in enhanced endometrial homeostasis at the end of the voluntary waiting period and increased fertility.

6.3 Material and Methods

6.3.1 Ethics statement

The project was approved by the Institutional Animal Care and Use Committee (reference CEIE nº36/2019). All clinical procedures were conducted in compliance with the European Union legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU).

6.3.2 Animals and Experimental Design

The field study took place in a commercial dairy herd milking 550 Holstein-Friesian cows three times per day, with an average milk yield of 13,800 L/cow/305 days (d) of lactation. Once calved, cows were blood sampled to assess their total calcium (at 0 DIM) and NEFA (at 0, 7, 14, and 21 DIM). Cows experiencing dystocia, retained fetal membranes, puerperal metritis or mastitis, clinical hypocalcemia, clinical ketosis, or severe lameness until 11 DIM were removed from the study. At 11 ± 0.3 DIM cows (n = 60) were stratified by parity and randomly assigned to one of two groups, receiving different fat supplements during 9 wk: i) group n-3PUFA (n = 29; 14 primiparous and 15 multiparous) – received a rumen-protected n-3 fat (linseed oil encapsulated with a proprietary coating technology by SIPENA, Saint Malo, France) mixed with excipient (by Eurocereal, Malveira, Portugal); and ii) group Control (n = 31; 16 primiparous and 15 multiparous) – received an isoenergetic supplement with saturated FA (hydrogenated palm oil mixed with the same excipient). The number of enrolled cows was chosen to allow detection of a 25% increase in plasma ALA concentrations (α = 0.05), and operators were blinded to cow allocation to groups. Milk samples were collected weekly until 11 weeks postpartum (WPP) and analyzed for milk composition. Body condition score (BCS) was evaluated, and blood samples collected once a week, from 11 DIM until 11 WPP (Figure 23) for analysis of fatty acids, glucose, β-hydroxybutyric acid (BHB) and progesterone P4 concentrations. At 23 ± 0.5 and 44 ± 0.6 DIM, vaginal discharge scoring, ultrasound genital tract evaluation, and endometrial cytology were performed, and blood samples were addressed for total adiponectin concentrations (Figure 23).

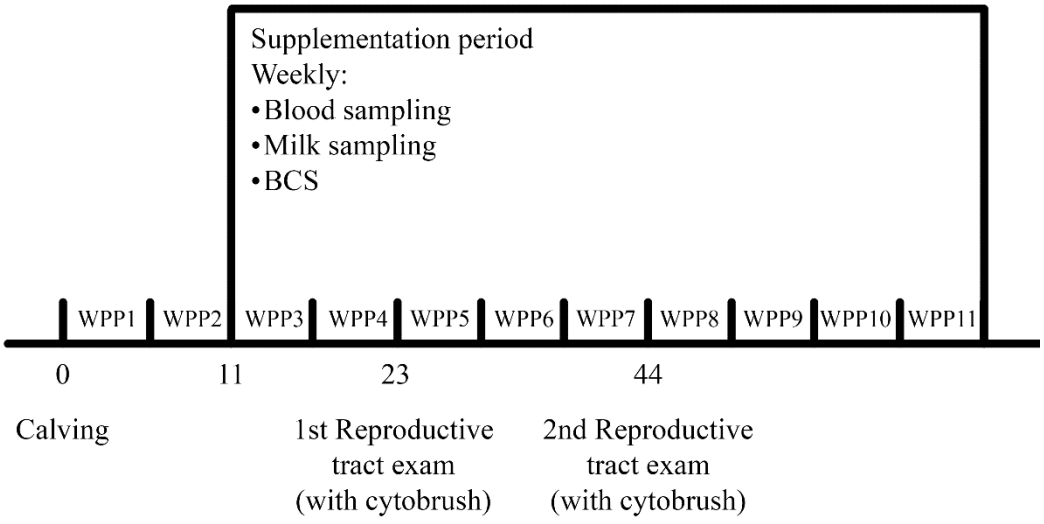

Figure 23. Experimental Design. WPP - week postpartum.

6.3.3 Diet, fat supplements and DMI

Cows were kept as a single group and offered a total mixed ration (TMR) diet, with an estimated DMI of 25.0 kg/cow/d. Ingredients of TMR as well as TMR nutrient concentrations were analyzed by near-infrared spectroscopy (Table 9). Individual supplementation was supplied through an automated, off-parlor feeding system (DairyFeed C-8000, GEA Farm Technologies, Bönen, Germany), allowing 1.25 kg/d of supplement in 4 feeding windows, and the daily intake recorded by the cow-collar electronic system. The basal TMR diet supplied 38 g/d/cow of ALA, and n-3PUFA cows received up to 64 g/d (mean 25 ± 4 g/d) of additional ALA, whereas Control cows received no additional ALA.

Table 9. Ingredient composition and nutrient concentrations of the total mixed ration and fat supplements delivered to cows enrolled in the supplementation trial.

		Fat supplements	
Ingredients in % of Dry Matter (DM)	**TMR**	**Control**	**n-3PUFA**
Corn silage	29.1	-	-
Ryegrass hay	12.6	-	-
Barley grain	27.8	-	-
Brewers yeast	2.1	-	-
Soybean meal	13.9	-	-
Beet molasses	3.0	-	-
Rice bran	3.5	-	-
Palm kernel meal	3.9	-	-
Palmitic acid 98%	1.1	-	-
Mineral and vitamin premix	2.9	-	-
Nutrient concentrations[1] (% of DM)			
% DM	51.6	90.7	91
Crude protein	16.9	14.4	13.5
Crude fat	4.3	15.6	14.6
Starch	26.4	18.4	20.6
Neutral detergent fiber (NDF)	32.5	21.4	20
Acid detergent fiber (ADF)	18.9	-	-
Acid detergent lignin (ADL)	2.9	-	-
Ash	8.6	10.2	11.4
NE_L (Mcal/kg of DM)	1.702	-	-
Fatty acids (g/100 g of total fatty acids)			
14:0	0.91	1.5	0.1
16:0	43.4	46.5	5.3
18:0	3.63	47.5	30.8
18:1 cis-9	17.8	4.5	11.8
18:1 cis-11	1.38	-	0.4
18:2 n-6	28.5	-	10.5
18:3 n-3	3.32	-	40
20:0	0.38	-	0.6
Others	0.59	-	0.5

[1]Analyzed by Near-infrared spectroscopy.

6.3.4 Blood and milk sampling

Blood samples were aseptically collected by venipuncture of the coccygeal vein into 10 mL dry tubes and 10 mL tubes containing K3 EDTA (13060, Vacutest KIMA, Arzegrande, Italy). Tubes were centrifuged (2000 g for 15 min) within 30 min of collection, serum and plasma were aliquoted into 1.5 mL tubes and stored at −80 °C until analysis. Composite milk samples were automatically collected during the afternoon milking, dispensed into a 50 mL tube with bronopol, and stored at 4ºC until milk composition analysis.

6.3.5 Plasma fatty acid profile

Fatty acid methyl esters (FAME) from freeze-dried plasma samples were prepared by transesterification with sodium methoxide in methanol (0.5 M; at 50ºC for 30 min), followed

by HCl in methanol (1.25 M; at 80ºC for 20 min), with the addition of methyl nonadecanoate (1 mL of a 1 mg/mL solution) as internal standard (Alves et al. 2017). FAME were analyzed by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m × 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA) capillary column. The chromatographic conditions were as follows: injector and detector temperatures were set at 220 and 250ºC, respectively; helium was used as the carrier gas at 1 mL/min constant flow; the initial oven temperature of 50ºC was held for 1 min, increased at 50ºC/min to 175ºC and held for 35 min, increased at 2ºC/min to 220ºC and held for 30 min. Identification of FAME was achieved by comparison of the FAME retention times with those of commercial standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA) and by mass spectrometry using a GC-MS Shimadzu 2010-Plus (Shimadzu, Kyoto, Japan). Integration of peaks was performed using GCsolution Version 2.41.00 (Shimadzu, 2000-2011). Theoretical relative FID response correction factors for FAME were used to correct peak areas, according to Ackman (2002).

6.3.6 BCS and energy balance indicators

Cows were scored for BCS using a 1 to 5 scale with 0.25 increments (Ferguson et al. 1994). Within 30 minutes of blood collection, glucose and BHB concentrations were assessed with a hand-held device (Precision Xtra, Abbott Diabetes Care, Abingdon, UK) in EDTA-added blood. Serum NEFA concentrations were determined by a colorimetric method (kit no. FA 115, Randox, Crumlin, UK) using Randox RX Daytona equipment (Randox, Crumlin, UK) as described by Pereira et al. (2020). The analytical sensitivity of the assay was 0.072 mmol/L, and the inter-assay coefficient of variation was <5%. Serum total calcium concentrations at calving were determined by a colorimetric method (kit no. CA 3871, Randox), with a 0.09 mmol/L analytical sensitivity.

6.3.7 Adiponectin assay

Plasma total adiponectin concentrations were assessed as previously described (Mellouk et al. 2017; 2019; Pereira et al. 2020) with an ELISA commercial kit (E11A0125, BlueGene, Shanghai, China). The intra- and inter-assay coefficients of variation were 4.2% and 15%, respectively.

6.3.8 Milk yield and composition

Milk yield was retrieved from the milking parlor recording system (3×/d). Milk composition, including fat mass percentage (% m/m) and crude protein mass percentage (% m/m), were analyzed by Mid Infra Red (MIR; MilkoScan 7, Foss, Hilleroed, Denmark), and SCC (cells/ml*1000) was analyzed by flow cytometry (Fossomatic, Foss, Hilleroed, Denmark)

within 24 h of collection. The fat (4%) and protein (3.3%) corrected milk (FPCM) was calculated according to Kok et al. (2017) as follows: FPCM = milk (kg) x (0.337 + 0.116 × fat% + 0.06 × crude protein%).

6.3.9 Genital tract evaluation and endometrial cytology

Vaginal fornix discharge was collected through a Metricheck device, and the score was evaluated as described by Williams et al. (2005). Then, the genital tract was assessed through transrectal palpation and ultrasonography, and an endometrial swab was performed to evaluate endometrial cytology, as described by Pereira et al. (2020). Briefly, a cervical brush (Bastos Viegas SA, Penafiel, Portugal) was aseptically adapted to the inner stylet of an AI gun (IMV technologies, L'Aigle, France) and gently rolled against the endometrium in the uterine body. Slides were stained with a modified Wright-Giemsa® stain (Diff-Quick, MAIM SL, Barcelona, Spain), and the percentage of PMN was assessed from 400 total cells. PMN cut-offs for identification of SCE were set at ≥ 18% (Kasimanickam et al. 2004) and ≥ 5% (Gilbert et al. 2005), at 23 and 44 DIM, respectively.

6.3.10 Progesterone assay

Plasma progesterone concentrations were assayed by a chemiluminescent immunoassay, using an IMMULITE 1000 analyzer (Siemens Healthcare Diagnostics) and a commercial kit (IMMULITE 1000 Progesterone Kit, Siemens Healthcare Diagnostics), as validated for bovine samples by Martin et al. (2007). The analytical sensitivity of the assay was 0.2 ng/mL, and the inter-assay coefficient of variation was <10%. The onset of postpartum ovarian luteal activity was considered to occur at the first P4 measurement > 1 ng/mL (Colazo et al. 2008), and postpartum ovarian patterns were defined as described by Lamming and Darwash (1998).

6.3.11 Reproductive management

Cows were inseminated at the first observed estrus after 45 DIM. Cows not exhibiting estrus by 75 DIM were enrolled in an OvSynch plus FTAI protocol. Pregnancy was detected by ultrasound at 39 d post AI.

6.3.12 Statistical analysis

Data were compiled using Excel® 2013 (Microsoft Corporation, Redmond, WA) spreadsheets before being imported into SAS (SAS 9.4, SAS Institute Inc., Cary, NC) for data analysis. The continuous response variables with repeated measures (FPCM, milk fat and protein percentages, BCS, glucose, BHB, NEFA, FA, and adiponectin) were analyzed by fitting linear mixed models with the PROC MIXED (SAS). Serum calcium at calving and total

fat supplement ingestion were analyzed by fitting linear models with the procedure PROC GLM (SAS). Binary response variables (SCE at 23 and 44 DIM, pregnancy status after first AI, and postpartum P4 pattern) were analyzed by fitting generalized linear mixed models with a logit link and a binomial distribution using the procedure PROC GLIMMIX (SAS). As recommended by Lean et al. 2016, intervals from calving to onset of postpartum ovarian luteal activity and from calving to conception (CCI) were analyzed by multivariable survival analysis using Cox proportional hazard regression (PROC PHREG; SAS).

The repeated measures models had the cow specified as subject and included parity (Primiparous/Multiparous), fat supplement (n-3PUFA/Control), conception at first AI (Yes/No), pregnant at 150 DIM (Yes/No), SCE at 23 and 44 DIM (Yes/No) as fixed effects, and the amount of fat supplement ingested weekly (kg) and DIM as covariates. The fat supplement intake was required because animals from both treatments presented intake values that varied continuously from zero to the maximum allowed. Thus, the effect of fat supplementation was evaluated by the interaction term of “supplement type*supplement ingested weekly”. The model of serum calcium at calving included the fixed effects of parity, fat supplement, conception at first AI, and SCE at 23 and 44 DIM. This model was generated to evaluate putative effects of calcemia in uterine health and fertility, as suboptimal serum calcium concentrations at calving can be detrimental to uterine health (Martinez et al. 2012), fertility (Caixeta et al. 2017), and alter FA metabolism (Chamberlin et al. 2013) of postpartum dairy cows. The conception at first AI and postpartum P4 pattern generalized linear mixed models, and the interval from calving to the onset of postpartum ovarian luteal activity and CCI survival analysis models, included the fixed effects of parity, fat supplement, SCE at 23 and 44 DIM, the total fat supplement ingested (kg) as a covariate, and the “supplement type*total supplement ingested” interaction term. For the survival analysis, cows were right-censored if not diagnosed pregnant at 240 DIM or removed from the herd.

As suggested by Heinze and Dunkler (2017), manual backward elimination with a P-value criterion of 0.157 without a preceding univariable prefiltering was performed to reach the final models. The assumption of homoscedasticity was evaluated visually using the plots of studentized residuals against predicted values, and the assumption of normality of residuals was evaluated using the Q-Q plots. To satisfy these assumptions, milk SCC, plasma adiponectin, and blood BHB concentrations were natural log-transformed. For models with repeated measures, the covariance structure that resulted in the lowest Akaike information criteria was selected. In the final models, differences were considered significant when $P \leq 0.05$, whereas a tendency was defined as $0.05 < P \leq 0.10$.

6.4 Results

6.4.1 Fat supplement intake and plasma fatty acid profile

Cows from group n-3PUFA had higher (P = 0.004; Table 10) total fat supplement intake than cows of group Control. Mean total supplement intake was 33.2 ± 3.8 kg and 17.7 ± 3.7 kg for groups n-3PUFA and Control, respectively. Plasma concentrations of total, n-3, and n-6PUFA increased with DIM (P ≤ 0.001). Plasma total FA concentrations were higher (P = 0.041) in multiparous than in primiparous cows, and were also affected by fat supplement ingestion (P = 0.001), increasing 1.26 ± 0.39 mg/dL per kg ingested. Plasma n-3PUFA concentrations were higher in group n-3PUFA than in group Control, increasing (P ≤ 0.001; Table 11) 0.142 ± 0.026 mg/dL per kg of n-3PUFA supplement ingested, whereas no increase (P = 0.292) was observed in group Control. Plasma n-6PUFA concentrations were increased (P = 0.002) by 0.676 ± 0.212 mg/dL per kg of supplement ingested, irrespective of fat source. The n-6/n-3 ratio decreased with DIM (P = 0.001), also decreasing 0.183 ± 0.023 units per n-3PUFA kg ingestion (P ≤ 0.001; Figure 24). Plasma ALA concentrations increased 0.124 ± 0.021 mg/dL with n-3PUFA ingestion (P ≤ 0.001; Figure 24) and DIM (P ≤ 0.001). Increased intake of Control fat produced no effect on the n-6/n-3 ratio (P = 0.988) or ALA (P = 0.393) plasmatic concentrations. Similarly to total n-6 PUFA, plasma LA concentrations increased with DIM (P ≤ 0.001) and fat supplement ingestion (P = 0.002), with no difference between the two fat supplements (Figure 24). Plasma EPA and DPA concentrations also increased with DIM (P ≤ 0.001) and supplement ingested (P = 0.004 and P = 0.033 for EPA and DPA, respectively), whereas DHA concentrations were not affected either by DIM or supplement ingested. Average concentrations of plasma FA at the end of supplementation period (11 WPP) solved for 1 kg/d ingestion of fat supplements are supplied in Table 12.

Table 10. Final models with statistical significance of parity, DIM, treatment (group), fat supplement ingestion, SCE and conception to first AI.

Dependent variables	*P*-value							
	Parity	DIM	Trt[1]	Fat sup. intake	Trt x Fat sup. intake[1]	SCE at 23 DIM	SCE at 44 DIM	Conception 1st AI
Total Fat Supplement Ingested (kg)	-	-	0.004	-	-	-	-	-
FPCM Yield (kg/wk)	< 0.001	0.042	-	0.042	-	-	-	-
Milk Fat percentage (%)	-	< 0.001	-	-	-	-	-	-
Milk Protein percentage (%)	-	0.003	-	-	-	-	-	-
Milk SCC (x1000 cells/mL)	-	-	-	-	-	-	-	-
BCS	-	< 0.001	-	-	-	-	-	-
Blood Glucose (mmol/L)	< 0.001	-	-	-	-	-	0.024	-
Blood BHB (mmol/L)	-	-	-	-	-	-	-	-
Plasma Adiponectin (ug/mL)	-	0.002	-	0.072	< 0.001	-	0.040	-
Serum Calcium at calving (mmol/L)	< 0.001	-	-	-	-	-	-	-
NEFA (mmol/L)	0.048	0.088	-	-	-	-	-	0.098
Total FA (mg/dL)	0.041	< 0.001	-	0.001	-	-	-	-
Total n-3 FA (mg/dL)	0.066	< 0.001	-	0.001	0.010	-	-	-
Total n-6 FA (mg/dL)	0.045	< 0.001	-	0.002	-	-	-	-
n-6 / n-3 FA ratio	-	0.001	-	< 0.001	< 0.001	-	-	-
Linoleic Acid (mg/dL)	0.068	< 0.001	-	0.002	-	-	-	-
α-linolenic (mg/dL)	0.020	< 0.001	-	< 0.001	0.005	-	-	0.054
EPA[2] (mg/dL)	-	< 0.001	-	0.004	-	-	-	-
DPA[3] (mg/dL)	-	< 0.001	-	0.033	-	-	-	-
DHA[4] (mg/dL)	-	-	-	-	-	-	-	-

Variables not included in the final models are represented with the symbol "-".
[1]Treatment.
[2]Eicosapentaenoic acid.
[3]Docosapentaenoic acid.
[4]Docosahexaenoic acid.

Table 11. Effects of Control and n-3PUFA fat supplement ingestion on plasma concentrations of fatty acids in postpartum dairy cows.

Plasma fatty acids (mg/dL)[1]	Control	n-3PUFA	*P*-value for differences
Linoleic Acid	0.565*	0.640*	0.8516
α-linolenic	0.024†	0.124*	0.0052
EPA[2]	0.008†	0.011*	0.6037
DPA[3]	0.006†	0.009†	0.7387
DHA[4]	0.000†	0.000†	0.5866
Total n-3 FA	0.035†	0.142*	0.0110
Total n-6 FA	0.657*	0.694*	0.9339
n-6/n-3 FA ratio	0.000†	-0.183*	<.0001
Total FA	1.090*	1.377*	0.7250

[1]Values are slopes for 1 unit change in ingestion, kg/wk.
[2]Eicosapentaenoic acid.
[3]Docosapentaenoic acid.
[4]Docosahexaenoic acid.
† slope does not differ from zero (P ≥ 0.10); *slope differs from zero (P < 0.10).

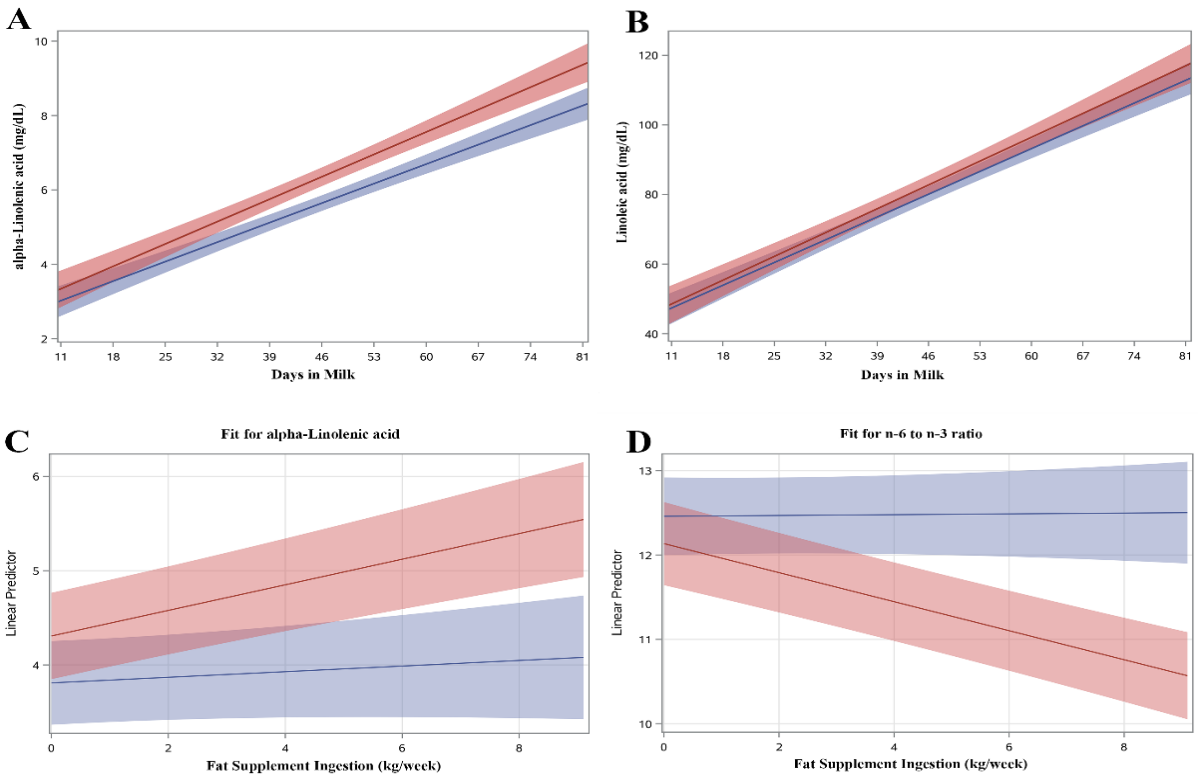

Figure 24. Plot of regression lines with respective 95% confidence limits of plasma α-linolenic acid concentrations (A), linoleic acid concentrations (B), effect of varying rates of weekly fat supplement ingestion on plasma α-linolenic acid concentrations (C), and plasma n-6 to n-3 ratio (D) in postpartum dairy cows ingesting control (blue) or n-3PUFA (red) fat supplements.

Table 12. Least Squares Means (± SEM) and P-values for the differences of plasma fatty acid concentrations of dairy cows at 11 weeks postpartum ingesting 1 kg/d of fat supplements CONTROL and n-3PUFA.

FA (mg/dL)	CONTROL	n-3PUFA	*P*-value for differences
12:0	0.22±0.02	0.26±0.02	0.1532
14:0	1.69±0.07	1.79±0.06	0.2656
iso 15:0	0.49±0.02	0.49±0.02	0.9032
anteiso 15:0	1.08±0.06	1.11±0.05	0.6766
14:1 c9	0.65±0.06	0.51±0.06	0.0813
15:0	1.69±0.09	1.59±0.08	0.3825
16:0 DMA	0.37±0.11	0.50±0.11	0.3667
iso 16:0	0.32±0.02	0.34±0.02	0.4728
16:0	28.82±1.06	28.04±0.99	0.5404
16:1t	0.44±0.03	0.44±0.02	0.9583
iso 17:0	1.07±0.04	1.09±0.04	0.6929
16:1c7	1.16±0.13	1.11±0.13	0.7678
16:1c9	3.46±0.20	3.51±0.20	0.8021
anteiso 17:0	0.03±0.01	0.02±0.01	0.1643
17:0	0.99±0.05	1.00±0.04	0.7964
18:0 DMA	0.45±0.03	0.48±0.03	0.4280
iso 18:0	0.03±0.01	0.03±0.01	0.4391
17:1c9	0.52±0.04	0.58±0.04	0.2208
18:0	31.00±1.24	32.31±1.14	0.3504
18:1t6t7t8	0.20±0.02	0.20±0.02	0.8540
18:1t9	0.27±0.03	0.29±0.02	0.6546
18:1t10	0.55±0.09	0.71±0.08	0.1732
18:1t11	0.44±0.03	0.45±0.03	0.7324
18:1t12	0.33±0.03	0.38±0.02	0.1466
18:1-t13/-t14	1.31±0.06	1.41±0.06	0.1577
18:1c9	20.32±1.0	19.07±0.9	0.3139
18:1t15	0.09±0.01	0.08±0.01	0.6228
18:1c11	1.65±0.07	1.62±0.07	0.7689
18:1c12	0.91±0.06	0.98±0.05	0.3604
18:1c13	0.07±0.01	0.05±0.01	0.2176
18:1-t16/-c14	0.19±0.02	0.16±0.01	0.2719
18:1c15	0.022±0.004	0.017±0.003	0.2884
18:2tt	0.08±0.01	0.08±0.01	0.6738
18:2 tcct	0.49±0.04	0.53±0.03	0.2761
18:1c16	0.16±0.02	0.14±0.02	0.5605
18:2t9c12	0.14±0.02	0.13±0.01	0.6314
18:2-t11c15/-t10c15	0.09±0.02	0.08±0.02	0.6814
18:2 n-6	107.46±4.24	109.31±4.02	0.6781
20:0	0.07±0.01	0.06±0.01	0.7779
18:3 n-6	2.22±0.12	2.20±0.11	0.8846
20:1c11	0.027±0.007	0.022±0.005	0.4471
18:3 n-3	8.06±0.37	8.97±0.35	0.0100
c9,t11-CLA	0.05±0.01	0.06±0.01	0.2271
tt CLA	0.24±0.02	0.28±0.02	0.0510
20:2 n-6	0.036±0.008	0.031±0.007	0.5267
18:3c9t11c15/20:3n-9	0.23±0.02	0.24±0.02	0.5829
22:0	0.041±0.009	0.034±0.006	0.5313
20:3 n-6	5.23±0.26	5.40±0.25	0.5405
20:3 n-3	0.011±0.006	0.021±0.004	0.2004
20:4 n-6	4.90±0.28	4.63±0.28	0.4319
20:5 n-3	1.00±0.07	1.12±0.06	0.1339
22:4 n-6	0.61±0.05	0.64±0.05	0.6137
22:5 n-3	1.08±0.07	1.16±0.07	0.3288
22:6 n-3	0.018±0.008	0.012±0.006	0.5109

Table 13 (cont). Least Squares Means (± SEM) and P-values for the differences of plasma fatty acid concentrations of dairy cows at 11 weeks postpartum ingesting 1 kg/d of fat supplements CONTROL and n-3PUFA.

FA (mg/dL)	CONTROL	n-3PUFA	*P*-value for differences
Total n-3 FA	10.14±0.46	11.08±0.44	0.0316
Total n-6 FA	120.25±4.69	122.06±4.47	0.7145
n6/n3 ratio	12.19±0.31	10.66±0.28	0.0002
Total FA	231.82±8.39	234.12±7.99	0.8037

6.4.2 Milk yield and metabolic variables

Fat (4%) and protein (3.3%) corrected milk yield increased (P = 0.042) with DIM, and was lower (P ≤ 0.001) in primiparous than multiparous cows (Annex XIV). Both milk fat (P ≤ 0.001) and protein (P = 0.003) percentages decreased with DIM. Intake of both fat supplements (Control and n-3PUFA) increased FPCM yield by 1.95 kg per kg ingested (P = 0.042), whereas milk fat and protein percentages remained unaffected.

Serum total calcium concentrations at calving (2.21 ± 0.06 mmol/L versus 1.96 ± 0.05 mmol/L, P < 0.001) and plasma glucose concentrations throughout the study were higher (P ≤ 0.001) in primiparous than multiparous cows. Plasma NEFA concentrations were lower (P = 0.048) in primiparous than multiparous cows, and BCS decreased (P < 0.001) with DIM (Annex XIV). Plasma total adiponectin concentrations displayed a negative linear relation with intake of n-3PUFA supplement (P = 0.001), being 0.93 times lower per each kg of n-3PUFA supplement ingested on the week of sampling (Figure 25a). Plasma total adiponectin concentrations were increased in cows with SCE at 44 DIM (P = 0.040, Figure 25b).

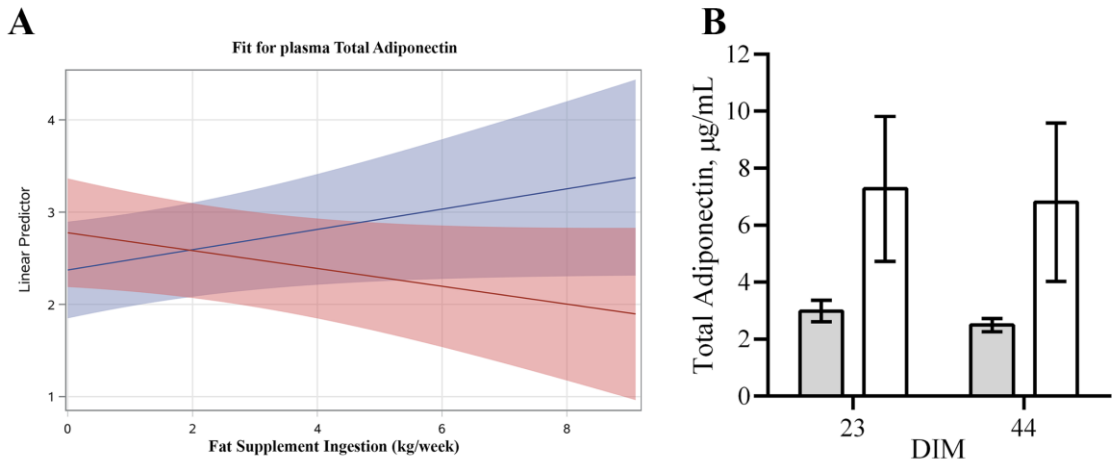

Figure 25. Adiponectin plots (A) Plot of regression lines with respective 95% confidence limits of varying rates of weekly fat supplement ingestion effect on plasma total adiponectin concentrations in postpartum dairy cows ingesting control (blue) or n-3 PUFA (red) fat supplements. (B) Mean (± SE) plasma concentrations of total adiponectin at 23 and 44 DIM of healthy cows (gray bars) and cows with SCE (white bars).

6.4.3 Reproductive variables

Final reproductive outcome models are presented in Table 13. Fat supplementation had no effect on postpartum P4 profiles, which were normal in 28 of 60 (47%) cows, whereas the remaining 32 cows presented atypical profiles (delayed ovulation or persistent luteal phase). The interval from calving to onset of postpartum ovarian luteal activity was not affected by fat supplementation and averaged 34.5 ± 1.5 d. Diagnosis of SCE at 23 DIM affected the interval from calving to onset of postpartum ovarian luteal activity (SCE cows had an ovulation hazard ratio of 0.425 compared to healthy cows). The conception rate at first AI was 25% (15 of 60 cows) and increased with fat supplementation (P = 0.015). The odds of pregnancy at first AI increased 1.037 times for each kg of fat supplement ingested irrespective of fat supplement. Regarding the CCI, primiparous cows had a higher (P =0.034) hazard ratio of conception (1.832; 95% Wald confidence limits: 1.045 – 3.211) than multiparous cows. After adjusting for the effect of parity, the hazard ratio of conception for cows ingesting n-3PUFA increased (P = 0.004) 1.009 (95% Wald Confidence Limits: 0.996 – 1.022) times for each kg ingested, whereas cows ingesting increased amounts of Control fat supplement tended (P =0.062) to exhibit a lower hazard ratio of conception (0.977; 95% Wald confidence limits: 0.954 – 1.001).

Table 14. Reproductive outcome models with statistical significance.

	P-value					
Outcome variables	Parity	Trt[1]	Fat sup. intake[1]	Trt × Fat sup. intake[2]	SCE at 23DIM	SCE at 44DIM
P4 pattern (Normal/Abnormal)	-	-	-	-	-	-
Calving to 1st ovulation (Days)	-	-	-	-	0.004	-
Conception 1st AI (Yes/No)	-	-	0.015	-	-	-
Calving to conception (Days)	0.034	-	0.062	0.004	-	-
SCE at 23 DIM (Yes/No)	-	-	-	-	NA	NA
SCE at 44 DIM (Yes/No)	-	-	-	-	NA	NA

Variables not included in the final models are represented with the symbol "-".
NA - Not Applicable.
[1]Treatment group (n-3PUFA/Control).
[2]Effect of the intake of fat supplement, 1 unit = 1kg.

6.5 Discussion

This study evaluated the effects of feeding a rumen-protected n-3PUFA enriched fat supplement on metabolic and reproductive indicators of high-yielding dairy cows. Treatment increased n-3PUFA plasmatic concentrations, modulated plasma adiponectin concentrations, and shortened the calving to conception interval.

6.5.1 Fat supplement intake

There was a large individual variation in intake of both fat supplements, but n-3PUFA had higher total ingestion than Control fat supplement. In fat supplemented cows, DMI is affected by forage to concentrate ratio, supplement inclusion rate, palatability, chain length and saturation of FA (Bionaz et al. 2020). Since the forage to concentrate ratio and supplement inclusion rate were kept constant, the differences in supplement intake observed between groups were probably due to palatability and/or FA chain length and saturation. The irregular intake pattern of both supplements resulted in the planned daily and total dosage not being attained uniformly. Although this jeopardized the magnitude of the metabolic and reproductive effects in several cows, it allowed the evaluation of the interaction between type of supplement and amount ingested.

6.5.2 Effects on plasma FA composition

Total plasma FA concentrations increased as lactation progressed, an expected consequence of higher DMI, resulting in a higher outflow of FA from the rumen, as observed by others (Penner and Oba 2009; Elis et al. 2016). Corroborating this, primiparous cows with lower DMI capacity (Neave et al. 2017) also had lower plasma total FA concentrations than multiparous cows. As projected, the plasma FA profile was affected by fat supplement intake. Dietary ALA from the n-3PUFA supplement was successfully transferred into plasma following a linear relation with intake, which agrees with studies using other n-3PUFA sources (Gonthier et al. 2005; Zachut et al. 2010; Moallem et al. 2013). As a consequence of increased plasma ALA and total n-3FA concentrations, the n-6/n-3 ratio was lower in n-3PUFA than in the Control group. Both supplements increased plasma total n-6 and LA concentrations. This was unexpected because, unlike the n-3PUFA supplement, the Control supplement had no PUFA content. Since dairy cows do not synthesize LA, the increase in plasma LA concentrations in the Control group resulted from an increased TMR intake. This contrasts with the common observation that feeding fat to dairy cows decreases the DMI (Moallem 2018). Nevertheless, palmitic acid supplements have been reported to increase DMI (Mosley et al. 2007; de Souza and Lock 2019). In fact, palmitic acid accounted for almost half of the FA present in the Control fat supplement. Thus, Control cows that ingested more fat supplement probably also ingested more TMR, which likely was the cause for their elevated total n-6 FA and LA in plasma. In addition, both fat supplements equally increased EPA and DPA plasma concentrations, which was unexpected since only n-3PUFA fat supplement increased ALA plasma concentrations, and both EPA and DPA result from the elongation of ALA (Saini and Keum 2018). This increase in EPA and DPA plasma concentrations in cows of the Control group might also be attributed to an increased TMR

intake, which contained ALA. In mammals, elongation of ALA and LA to the respective n-3 and n-6 long-chain PUFA families share the same enzymatic pathways, and many tissues convert dietary ALA to EPA and DHA by desaturation and elongation combined with peroxisomal chain shortening (Cook and McMaster 2002). One explanation could be that due to a limited capacity of dairy cows converting ALA to an n-3 family long PUFA, excess ALA would remain in circulation. Alternatively, since the distribution of dietary n-3PUFA is organ-specific (Wolf et al. 2019), blood plasma might not be the best tissue to detect putative increased EPA and DHA availability. In mammals, the pathway of ALA elongation, which has DHA as the final product, is considered highly inefficient, and ALA supplementation frequently fails to affect plasma DHA concentrations (Brenna et al. 2009). Therefore, the absence of the effect of fat supplementation on DHA concentrations was somehow expected.

6.5.3 Effects on metabolic parameters

Fat supplementation increased milk yield but did not affect BCS. Additional energy intake can be partitioned either towards body adipose depots or towards milk production. Energy channelling towards adipose tissue occurs when there is a lack of glucose or amino acids to support increased milk synthesis (Weiss and Pinos-Rodríguez 2009) or when the metabolic limits (growth hormone and insulin) for milk production are reached (Bines and Hart, 1982). Weiss and Pinos-Rodríguez (2009) suggested that in a 40% forage diet, as in this study (42%), the supplemental fat-induced increase in energy intake was largely partitioned to milk synthesis. This is in accordance with results from this study, where fat supplementation did not affect glucose and BHB blood concentrations, supporting the concept that when energy is a limiting factor, additional energy intake from fat supplementation is directed towards additional milk synthesis (Zachut et al. 2010; Haubold et al. 2020). The saturated or unsaturated nature of FAs from supplement had no effect on BCS, blood glucose, BHB, and plasma NEFA concentrations, which is in accordance with other studies (Petit et al. 2007; Zachut et al. 2010; Hutchinson et al. 2012; Dirandeh et al. 2013).

Total adiponectin plasma concentrations decreased in association with n-3PUFA fat supplementation in a dose-dependent manner. Supplementing ALA and conjugated linoleic acid to mid-lactation dairy cows also decreased adiponectin plasma concentrations (Haubold et al. 2020), and n-3PUFA supplemented cows displayed increased transcription of the adiponectin gene in the adipose tissue compared with n-6PUFA supplemented cows (Elis et al. 2016). Taken together, these data indicate that n-3PUFA affects the adiponectin pathway, namely through adipose tissue, opening opportunities to modulate adipokine expression through feeding. However, as adiponectin promotes insulin sensitivity (Haubold et al. 2020), by reducing adiponectin plasma concentrations, n-3PUFA supplementation may have the

undesirable effect of extending a state of insulin resistance and lipid mobilization (Singh et al. 2014). Plasma total adiponectin concentrations were unaffected by BCS, which, together with data from other studies (Krumm et al. 2017; Mann et al. 2017) indicates that plasma adiponectin concentrations are remarkably irresponsive to BCS in postpartum dairy cows. In contrast, De Koster et al. (2017) found that adiponectin serum concentrations were negatively associated with BCS during the dry period. Altogether, this information favors the concept that in postpartum dairy cows, the relation between body fat reserves and adiponectin might be uncoupled, maintaining insulin resistance and adipose tissue mobilization (Singh et al. 2014). The endometrial inflammatory status at 44 DIM affected plasma total adiponectin concentrations, with SCE cows displaying increased concentrations. This is consistent with a previous study (Pereira et al. 2020), evidencing that postpartum increased adiponectin expression is a biomarker of persistent uterine inflammation.

6.5.4 Effects on FPCM yield

Feeding n-3FA originated conflicting results regarding milk yield effects, some studies reporting increased yields (Moallem 2009; Zachut et al. 2010), whereas others found no effect (Ferlay et al. 2013; Neveu et al. 2013). Reviewing the subject, Moallem (2018) suggested that contradictory results might result from different levels of fat intake and varying degrees of rumen protection. Also, Ariza et al. (2019) suggested that contrary to high-fat diets, supplementing moderate quantities of PUFA (extruded linseed) may increase the diet's energy density without lowering DMI or reducing fiber digestibility. Supplementation with saturated FA from palm oil also originated contrasting results, again some authors reporting increased milk yield (Mosley et al. 2007; Piantoni et al. 2013), whereas others found no effect (de Souza et al. 2016), which may reflect the basal diet composition or fat supplement characteristics (de Souza et al. 2016). These reports highlight the dose-dependent effect of fat supplementation in dairy cows' milk yield. At a lower dose, fat supplementation increases energy density and consequently milk yield, but above a certain level of supplementation, fat depresses feed intake and milk yield (Palmquist and Jenkins 2017). In this study, both fat supplements increased FPCM yield and had no adverse effect on milk fat. A reduction in milk fat percentage can be observed following n-3FA supplementation if the rumen biohydrogenation pathways shift to the production of 18:1 trans-10, 18:2 trans-10,cis-12, and other related intermediates known to depress milk fat (Moallem 2018).

6.5.5 Effects on Reproductive Parameters

Despite decreasing n-6/n-3 ratio and increasing n-3PUFA plasma concentrations, n-3PUFA supplementation did not affect endometrial inflammation status at 23 and 44 DIM. Following PUFA supplementation, tissue concentrations take longer to increase than plasma

concentrations (Cook and McMaster 2002), making the time required to incorporate dietary n-3FA into target tissues an important point when developing supplementation approaches (Moallem 2018). Persistent SCE can result from an unbalanced pro-inflammatory/anti-inflammatory cytokine production during the first WPP (Arias et al. 2018), when reduced activation of pro-inflammatory cytokines impair the clearance of endometrial bacteria, which coupled with persistent pro-inflammatory stimuli during later stages maintain a hostile environment for the embryo (Wagener et al. 2017; Sheldon et al. 2019; Pascottini and LeBlanc 2020). Since PUFA supplementation started at 11 DIM, one may hypothesize that it was not effective in modulating cytokine production. This could potentially be achieved by beginning fat supplementation either in the prepartum or at calving, allowing for essential PUFA to accumulate in the endometrium. Additionally, a higher dietary inclusion of PUFA, namely ALA, could prove beneficial, as daily values here registered (102 g/d) are lower than those reported by others (162 g/d, as per Moallem 2009; 402 g/d, as per Zachut et al. 2010; 131 g/d, as per Moallem et al. 2013). However, the calving to first ovulation interval was affected by SCE at 23 DIM. Uterine bacterial infection impairs early postpartum follicular development, as LPS and tumor necrosis factor-α suppress follicular growth and estradiol production, making affected cows less likely to ovulate the first dominant follicle (Sheldon et al. 2002; Gilbert 2012).

Fat supplementation did not affect the onset of postpartum ovarian luteal activity, nor postpartum progesterone profiles. Dietary n-6 to n-3PUFA ratio can modulate prostaglandin synthesis and metabolism (Greco et al. 2018), and prostaglandins play a relevant role in uterine involution and onset of postpartum estrous cycles. Again, starting supplementation at 11 DIM might have jeopardized a beneficial effect on endometrial prostaglandin synthesis.

First service conception rate increased in association with higher intake of both fat supplements, which agrees with data reviewed by Rodney et al. (2015). This improvement in conception rate to first AI following PUFA supplementation was related to increased LA concentrations, which would inhibit prostaglandin synthesis in the presence of trophoblast interferon, thus preventing early embryonic mortality (McNamara et al. 2003). As both n-3PUFA and Control fat supplements increased plasma LA concentrations, this could explain the observed increase in the first AI conception rate. However, cows that conceived to first AI had higher plasma concentrations of ALA, suggesting that n-3PUFA supplement may have exerted an additional positive effect. Interestingly, the impact on the first AI conception rate was reflected in a shorter CCI in n-3PUFA cows but not in Control group cows. This effect in n-3PUFA cows was proportional to ingested supplement. Although n-3PUFA are believed to weaken the uterine inflammatory status, leading to higher chances of embryo survival (Leroy

et al. 2014), the effects of n-6 and n-3PUFA supplementation on progesterone and prostaglandin metabolism are inconsistent (Otto et al. 2014).

6.6 Conclusion

Supplement feeding with PUFA or saturated FA of postpartum high-yielding dairy cows increased FPCM yield. Although PUFA and saturated FA supplements increased plasma LA, only n-3PUFA supplement increased plasma ALA concentrations and regulated adiponectin expression. Fat supplementation increased first AI conception rate, and n-3PUFA supplement shortened the CCI in a mechanism independent of early return to postpartum ovarian activity, postpartum progesterone patterns, or persistent endometrial inflammation. These findings highlight the potential of n-3PUFA to improve high-yielding dairy cows' fertility by favouring early conception in the postpartum period. However, in order to prevent postpartum persistent endometrial inflammation, alternative n-3PUFA supplementation regimens must be developed, namely with rumen-protected EPA and DHA sources, due to the inefficient elongation of ALA. Moreover, the mechanisms behind the improvement of fertility following n-3PUFA supplementation warrant further studies.

CHAPTER 7 – GENERAL DISCUSSION

Postpartum uterine disease impairs fertility of dairy cows by disrupting ovarian and endometrial functions, resulting in conception failure and delay in pregnancy establishment (Mateus et al. 2002, Sheldon et al. 2018). Since Kasimanickam et al. (2004) established a link between cytological evidence of endometritis and impaired fertility, efforts have been made to develop early and accurate diagnostic tools that would allow the correct identification of the 30-35% of dairy cows which are affected by this subclinical disease between 4 and 9 weeks postpartum (LeBlanc 2008). Subclinical endometritis is an inflammatory state of the endometrium without any clinical signs, only detected by histology or cytology (Sheldon et al. 2006). Given the invasive nature of the sampling technique, veterinary skills required, time-consuming logistics and cost of the uterine biopsy and swab techniques, the diagnosis of SCE in commercial dairy farms is extremely limited. The impracticality of the general adoption of endometrial cytology in commercial dairy farms resulted in the search for non-invasive biomarkers for the early diagnosis and prognosis of SCE (Miller et al. 2019).

In this work, the first experiment (Chapter 3) was designed to elucidate if adipokines, hormone-like mediators known to regulate immunity and inflammation, are suitable biomarkers with diagnostic and prognosis applications in the management of SCE in postpartum dairy cows. Our results demonstrated that cows with SCE at 45 DPP had higher ADIPOQ concentrations in plasma at 21 and 45 DPP and in uterine fluid at 45 DPP. Moreover, we also observed that at 45 DPP, RARRES2 concentrations were higher in cows with SCE than in healthy cows, both in plasma and uterine fluid. Following ROC analysis, ADIPOQ plasma and uterine fluid and RARRES2 uterine fluid concentrations showed a high ability to discriminate cows with SCE at 45 DPP, whereas RARRES2 plasma concentrations showed either no ability (21 DPP) or a low ability (45 DPP) to discriminate cows with SCE. Therefore, in our study, ADIPOQ and RARRES2 represent suitable biomarkers able to provide an early diagnosis of SCE and predict the risk of persistence of uterine inflammation. However, to be applicable in commercial dairy farms, it is not enough to accurately discriminate between diseased and healthy cows. Three technical attributes contribute to a “good” biomarker, to be as specific as possible for the disease in question, easy to detect or quantify in affordable and robust assays, and must be present in peripheral body tissue and/or fluid (e.g., blood, urine, saliva) for convenience of sample collection (Biomarkers on a roll 2010). Considering these 3 criteria, only plasma total ADIPOQ has the potential to serve as “good” biomarker of SCE in postpartum dairy cows for adoption in commercial dairy farms, given that the use of uterine fluid lacks practicality of sampling procedure. However, the specificity of plasma total ADIPOQ needs to be further validated in larger field trials. De Koster et al. (2017) found that ADIPOQ serum concentrations were negatively associated

with BCS during the dry period. If this was also the case during the postpartum period, it could impair the diagnostic of SCE based on plasma total ADIPOQ. However, in Chapter 3, percentage of body weight change was similar between groups, making it unlikely that it contributed to the observed between-group differences in total ADIPOQ concentrations. Moreover, in Chapter 6, plasma total ADIPOQ concentrations were unaffected by BCS or BCS variation from calving, thus suggesting they maintain their diagnostic abilities even in the presence of different metabolic states during the postpartum period. The transcription of ADIPOQ and respective receptor ADIPOR2 coding genes was increased in the cellular pellet of the uterine flushing from cows affected with SCE suggesting a local origin of these molecules. However, since cows that had experienced other forms of puerperal disease were not included in the study, care must be taken before asserting that total plasma ADIPOQ is specific for SCE and that other postpartum inflammatory conditions cannot impair this molecule's diagnostic abilities. Following the confirmation of its reliability as a SCE biomarker in a larger cohort, plasma ADIPOQ can significantly improve the early diagnosis of SCE, allowing for a timely therapeutic intervention, thus maximizing herd health, improving reproductive efficiency and increasing profits in dairy operations.

Moreover, further studies are necessary to determine if there is a role of ADIPOQ in the establishment of SCE. Adipokines inform the host regarding long-term energy storage and integrate systemic metabolism with immune function. In situations of negative energy balance, anti-inflammatory adipokines like ADIPOQ increase, thus contributing to the suppression of immune function (Mancuso 2016). Given the anti-inflammatory role of ADIPOQ, it may suppress the endometrial inflammatory response, thus prompting an impaired immune response. However, in our studies there were no variations in percentage of body weight loss or BCS among healthy and disease groups, suggesting that, if this adipose tissue-induced regulation is taking place there is the need to develop better tools to assess the metabolic state of postpartum dairy cows. Alternatively, increased expression of ADIPOQ in affected cows may represent a failed attempt to control local inflammatory stimuli. This determination is of particular importance given the evidence in Chapter 5 that cows affected by SCE exhibit an endometrial transcriptome profile supporting a delay in resolution programs compared to healthy cows.

The second experiment (Chapter 4) evidenced that the different cell types (LE, GE, ST) comprising the bovine endometrium exhibit different molecular signatures with cell-specific gene transcription profiles, encoding proteins that putatively support specialized functions of each cell type. These specialized functions comprise regulation of uterine fluid composition, providing favorable microenvironments for sperm and embryos, and immune response against potential pathogens in LE cells. For GE cells, specialized functions include cell cycle

regulation, adhesion processes, innate and adaptive immune responses through complement activation, transport and secretion of substances into the uterine lumen through peptide secretion and cilium movement regulation. In ST cells, specialized functions comprise multiple regulatory, cell communication, and signaling processes, which are paramount for coordinating cellular responses.

Moreover, Chapters 4 and 5 confirmed that progesterone and SCE impact endometrial transcription in a cell type-specific way, confirming recent reports from our research team (Chankeaw et al. 2021a; 2021b) and highlighting the cell compartment as the physiological unit rather than the whole tissue.

When exposed to increased P4 concentrations, GE cells displayed under-expression of genes involved in cell cycle and nuclear division, highlighting the anti-proliferative effect of P4 in epithelial cells. This effect is of particular importance in the context of epithelial repair since signals triggered by tissue damage, DAMPs, orchestrate repair by promoting epithelial cell migration and proliferation in order to replace damaged epithelial cells and restore tissue homeostasis (Lara et al. 2017; Brazil et al. 2019). Moreover, when exposed to high LPS concentrations, endometrial epithelial cells exhibit decreased proliferation (Chanrot et al. 2017; Lim et al. 2018). Given the decreased proliferation following LPS exposure and the anti-proliferative action of P4 in epithelial cells, P4 may impair endometrial healing mechanisms, thus contributing to delayed epithelial repair adding to the already characterized mechanisms of endometrial immune function suppression.

Additionally, genes encoding annexin A1 (ANXA1) and annexin A2 (ANXA2) were found less transcribed in cows with elevated P4 in GE and ST cells, respectively. These 2 members of the Annexin A protein family are expressed in monocytes, macrophages, neutrophils and epithelial cells (Leoni and Nusrat 2016; Xi et al. 2020). Annexin A1 is a pro-resolving mediator, promoting the nonphlogistic phagocytosis of apoptotic PMNs, thus sparing tissue from exposure to the harmful and immunogenic contents of necrotic cells (Maderna et al. 2005). Moreover, annexin A1 inhibits neutrophil tissue accumulation by reducing leukocyte infiltration and recent evidence has suggested it could induce macrophage reprogramming toward a resolving phenotype, thus reducing pro-inflammatory cytokines production and increasing the release of pro-resolving molecules (Sugimoto et al. 2016). Annexin A2 also contributes to host immunity during bacterial infection, as it promotes the formation of phagophores, thus regulating the process of autophagy (Xi et al. 2020). Moreover, Annexin A2 deficiency impairs bacterial clearance by macrophages and intensifies cytokine production and tissue injury (Li et al. 2015). Overall, the decreased transcription of *ANXA1*

and *ANXA2* when P4 concentrations are elevated may also be a contributing factor to delayed endometrial resolution programs.

In addition, endometrial ST samples of cows with elevated P4 concentrations also exhibited decreased transcription of the leukocyte surface antigen (CD53) coding gene. CD53 is a member of the tetraspanin superfamily, expressed exclusively on the surface of immune cells, such as B cells, T cells, dendritic cells, macrophages, and natural killer cells (Dunlock 2020). CD53 has been linked to leukocyte adherence, extravasation, and aggregation to endothelia at sites of inflammation, as well as to the activation of immune cells (Dunlock 2020).

Altogether, elevated circulating P4 concentrations in postpartum dairy cows were associated with decreased endometrial transcription of genes involved in immunoresolvent pathways, thus impairing healing processes and possibly contributing to the establishment of chronic endometrial inflammatory conditions.

Interestingly, this P4-associated transcription regulation on endometrial cells was not associated with altered transcription of P4 receptors (PGR or PGRMC1, PGRMC2, NR2F2, and SRD5A2), thus suggesting that non classical P4 signalling, as suggested by Garg et al. (2017), may mediate the above-described effects and that other mechanisms should be explored.

In the context of SCE, the work described in Chapter 5 allowed the characterization of the transcription profiles in GE, LE and ST endometrial cells associated with recovery or persistence of inflammation. These transcription profiles associated with recovery or persistence of endometrial inflammation comprised genes involved in immune function, tissue remodelling, cell adhesion and uterine receptivity in a cell type-specific manner.

This work also highlights that, when compared with healthy cows, recovery and persistent SCE cows display a fair proportion of common DEGs in epithelial compartments (LE and GE). Conversely, compared to recovery and healthy cows, ST cells of persistent SCE cows exhibit a considerable set of common DEGs in the two comparisons.

Following apparent recovery from SCE at 44 DPP, as assessed by a low percentage of immune cells in endometrial cytology, cows with a recovery phenotype exhibit a profile similar to healthy cows considering the transcription of genes related to immunity and tissue remodelling. However, despite this similarity to healthy cows, recovered cows still display altered transcription of genes related to immune tolerance mechanisms (*TGFBR1*) and interferon dependant or stimulated genes (e.g. *ISG15*, *IFIT5*, *IFIT1*, *IFIH1*, *STAT2*, *STAT1*, *MX2*, *MX1*). These altered transcription profiles from cows with a recovery phenotype, are

exhibited in GE and especially in ST cells, where they may prove detrimental for the success of embryo-maternal crosstalk and pregnancy establishment.

Compared to healthy and recovered cows, ST cells of cows exhibiting persistent subclinical endometrial inflammation at 44 DPP, display increased transcription of genes involved in inflammation resolution and mucosal barrier regeneration, proving that the endometrium of these animals is still under active repair.

This endometrial repair process comprises, among others, increased transcription of genes encoding members of the R-Spondin family of proteins, such as the ligand R-spondin 1, the receptor ring finger protein 43 and coreceptors leucine-rich repeat-containing G-protein coupled receptors 5 and 6. This signalling pathway stimulates regeneration in a variety of tissues (Zhang et al. 2020) and was suggested that these molecules could be used as therapeutic targets in scenarios of tissue damage (Nagano, 2019). Moreover, the leucine-rich repeat-containing G-protein coupled receptor 6 is activated by Maresin 1, a SPM, which stimulates resolution of acute inflammation and organ protection (Chiang, 2019). When Maresin 1 activates the LGR6 receptor it promotes phagocyte immunoresolvent functions (Chiang, 2019). In fact, it has been suggested that the therapeutic use of Maresin 1 may be useful for the treatment of acute and chronic inflammation by reducing inflammation-induced damage (Li et al. 2020). In this context, it is also noteworthy that Persistent cows exhibit increased transcription of genes (CYP2C87 and CYP2U1) encoding enzymes of the P450 family CYP2, which is involved in biosynthesis and further inactivation of lipid mediators (Divanovic et al. 2013; Nebert et al. 2013).

Also compared to healthy and recovered cows, ST cells of cows exhibiting persistent subclinical endometrial inflammation, show increased transcription of the *FGFR2* gene. This gene encodes fibroblast growth factor receptor 2, a member of FGF signaling pathway which regulates tissue repair, regeneration and inflammation (Xie et al. 2020). The active role of FGFR2 has been evidenced in postpartum goats, where its high protein expression in fibroblasts was associated with epithelial and stromal cell regeneration (Sánchez et al. 2002). Our results support the idea that FGFR2 over-expression is related to delayed endometrial involution and that ST cells of Persistent cows are still under active repair processes. Moeover, the FGF/FGFR signaling pathway has been considered a privileged target for the therapeutic approach, both with “pro-FGF signaling” and “anti-FGF signaling” therapeutics (Xie et al. 2020). Overall, this result lays the foundation for further studies aiming to elucidate how the FGF/FGFR signaling pathway is affected in cows exhibiting persistent subclinical endometrial inflammation, thus creating new therapeutic opportunities to improve endometrial inflammation modulation and tissue repair.

Compared to recovery cows, ST cells of cows with persistent subclinical endometrial inflammation also display increased transcription of genes (*CYP26A1* and *SDR16C5*) encoding enzymes involved in the biosynthesis and regulation of the cellular level of retinoic acid (Stevison et al. 2015; Wu et al. 2019). The retinoic acid is a Vitamin A metabolite which is essential for the normal regeneration of mucosal barriers damaged by infection and is crucial to both immunological tolerance and the elicitation of adaptive immune responses in diferent mucosae (Stephensen et al. 2001; Hall et al. 2011; Oliveira et al. 2018). However, there are also reports describing a possible association between pharmacological retinoic acid treatment and spontaneous development of inflammatory mucosal disease, suggesting that retinoic acid can also be an instigator of chronic inflammation (Hall et al. 2011). Therefore, a greater understanding of how retinoic acid modulates endometrial inflammation in postpartum dairy cows is essencial in order to assess the validity of a retinoic acid-based therapeutic approach for this condition.

Moreover, compared to recovered cows, ST cells of cows with persistent subclinical endometrial inflammation exhibit decreased transcription of the *TSG6* gene, which encodes the TNF alpha induced protein 6. This protein is up-regulated upon exposure to inflammatory mediators (Watanabe et al. 2018), being released from neutrophils, mast cells, macrophages and stromal cells prompting anti-inflammatory and tissue-protective responses (Watanabe et al. 2018; Day and Milner 2019). These responses include the inhibition of neutrophil migration (Dyer et al. 2014) and the polarisation of macrophages to an anti-inflammatory M2 phenotype (Mittal et al. 2016). Given these immune-regulatory effects of the TNF alpha induced protein 6, its potential therapeutic application was assessed in lung injury (Foskett et al. 2014), brain injury (Bertling et al. 2016), intestinal mucosal wound repair (Sammarco et al. 2019), gingival wound healing (Beltran et al. 2015), colitis (Sala et al. 2015), inflammation-mediated dry eye syndrome (Lee et al. 2015), and skin wound healing models (Qi et al. 2014). In all these reports TNF alpha induced protein 6 produced beneficial effects ameliorating inflammation-induced injury and improving tissue healing. Unlike other molecular pathways involved in tissue repair, like FGF/FGFR signaling pathway, that are upregulated in Persistent cows as a consequence of delayed resolution programs, TNF alpha induced protein 6 is still down regulated in Persistent cows, suggesting this animals are unable to mount this type of response. In sum, this finding lays the foundation for the study of the therapeutic application of TNF alpha induced protein 6 in postpartum dairy cows affected by persistent subclinical endometrial inflammation.

Overall, among the large number of altered pathways identified between healthy, recovered and persistent SCE cows in Chapter 5, which would be impractical to discuss in full, the molecular pathways here discussed in depth (FGF/FGFR, TNF alpha induced protein 6,

specialised pro-resolving mediators and retinoic acid signalling pathways) comprise new putative therapeutic targets, either to prevent persistence or to stimulate recovery of endometrial inflammation, therefore helping to restore the fertility of postpartum dairy cows.

Moreover, among the genes with altered transcription in the endometrium of healthy, recovered and persistent SCE cows there are several genes which are still uncharacterized (Supplementary File 6) which may interfere with the above processes.

Since SPMs derived from the PUFA n-3 family, influence some of the inflammation resolution programs identified in Chapter 5, in Chapter 6 we assessed if supplementing a rumen-protected source of n-3PUFA (linseed fat) could modulate both the energetic and immune status of postpartum dairy cows. In this chapter the beneficial effects of supplementing rumen-protected linseed fat were assessed through the evaluation of endometrial inflammation by endometrial cytology and respective reproductive performance of postpartum dairy cows.

Compared to cows from control group, cows fed n-3PUFA had increased plasma concentrations of n-3 PUFA, namely ALA. This plasmatic enrichment in fatty acids from the n-3 family also resulted in a lower n-6/n-3 ratio compared to cows from control group. Despite the successful transfer of dietary ALA into the blood stream, EPA and DHA concentrations from cows fed n-3PUFA were similar to the ones exhibited by cows from the control group. Regarding endometrial inflammation and reproductive outcome, cows fed n-3PUFA exhibited shorter calving to conception intervals, whereas postpartum ovarian activity and endometrial inflammatory status remained unaffected.

The lack of effect in EPA and DHA concentrations in cows fed n-3PUFA agrees with Brenna et al. (2009) which reviewed the ALA conversion to long chain n-3PUFA in humans and other mammals. Overall, in mammals, the pathway of ALA elongation, which has EPA as an intermediate product and DHA as the final product, is considered inefficient (Brenna et al. 2009). This inefficiency, is the result of competition among fatty acids of the n-3 (ALA) and n-6 (LA) families for desaturation and chain elongation enzymes (Cook and McMaster, 2002) with an excess of one family causing a significant decrease in the conversion of the other (Schmitz and Ecker 2008). Thus, the plasmatic n-6/n-3 ratio strongly affects the enzymatic enlongation of ALA and LA to longer PUFA. In the work presented in Chapter 6, the plasmatic n-6/n-3 ratio was around 10 in cows fed n-3PUFA and around 12 in cows from control group (Table 12). This high n-6/n-3 ratio is the result of the fatty acids composition of the TMR, which was rich in fatty acids of the n-6 family, namely LA. As a consequence, given the high dietary n-6/n-3 ratio (even in cows fed n-3 PUFA) cows were extremely inefficient in the synthesis of EPA and DHA.

Therefore, in order to successfully modulate SPM biosynthesis through n-3PUFA supplementation in dairy cows, a better solution would be to provide the long chain n-3PUFA (EPA and DHA) as these don't require any enzymatic process of elongation and are bioactive by themselves.

Moreover, following n-3PUFA supplementation, tissue concentrations take longer to increase than plasma concentrations (Cook and McMaster 2002), making the time required to incorporate dietary n-3PUFA into target tissues an important point when developing supplementation approaches (Moallem 2018). Given the original concept by Serhan et al. (2005) that "the beginning programs the end", this lag between start of supplementation and increased tissue concentrations is of particular importance when trying to influence SPM-modulated inflammation resolution programs. This concept in inflammation resolution suggests that an active coordinated program initiates in the first hours after an inflammatory response begins (Serhan et al. 2005). In the context of postpartum uterine diseases in dairy cows, this inflammatory response begins at parturition. It is in that moment that the physiological uterine involution process starts as well as the elimination of bacteria which are ubiquitous in the bovine uterus after parturition (Sheldon et al. 2019). With this timetable in mind, in order to effectively modulate the endometrial inflammation resolution of postpartum dairy cows through dietary long n-3 PUFA, it would be necessary to achieve increased EPA and DHA concentrations in endometrial tissue at parturition day. In order to achieve this, EPA and DHA would have to be supplied to prepartum dairy cows for a period allowing their accumulation in endometrial tissues. Alternatively, in laboratory animals, it was shown that the direct administration of SPMs in diferent scenarios, either locally (Rajasagi et al. 2011; Van Dyke 2017; Wang et al. 2020; Saito-Sasaki et al. 2018) or intraperitoneally (Cezar et al. 2019) protects against inflammation-induced tissue damage and promotes healing. Following these reports, and the fact that SPMs enhance bacterial clearance while lowering antibiotic requirements (Chiang et al. 2012), in the context of persistent subclinical endometrial inflammation in postpartum dairy cows, topic application of SPMs into the endometrium may prove beneficial protecting against tissue damage, promoting healing, and enhancing bacterial clearance.

Despite the absence of effect on PMN percentage on endometrial cytology, cows fed n-3PUFA exhibited shorter calving to conception intervals. One justification for this result is that by lowering the n-6/n-3 ratio in plasma, the n-3PUFA supplementation helped control systemic inflammation which also has negative fertility consequences (Bertoni et al. 2008; Cheong et al. 2017; Dolecheck et al. 2019). Alternatively the beneficial effects of feeding n-3PUFA may be the result of an altered state of endometrial cell viability or molecular milleu

that are overlooked on endometrial cytology, and may lead a higher chance of embryo survival.

CHAPTER 8 – CONCLUSION

The work presented in this PhD thesis highly contributes to a better understanding of aspects related with diagnosis, physiopathology and putative preventive/therapeutic approaches to subclinical endometrial inflammation in postpartum dairy cows. Regarding diagnosis, this work demonstrates that adipokines are differentially expressed in cows affected by SCE, with plasma adiponectin levels showing a promising potential application as a biomarker for this disease. Moreover, this work also provides new knowledge about the modulation of the endometrial cell-specific transcriptome induced by progesterone and SCE. In the context of SCE, among a large number of altered pathways, this work uncovers molecular pathways involved in the persistence or recovery from endometrial inflammation like FGF/FGFR, TNF alpha induced protein 6, specialised pro-resolving mediators and retinoic acid signalling pathways. Moreover, the present work also clarifies that recovered and persistent SCE cows exhibit altered transcription of genes involved not only in immune response but also in tissue remodelling, cell adhesion and interferon dependant or stimulated genes which are involved in endometrial tissue specialized functions. Therefore, this study contributes to the design of alternative therapeutic strategies to modulate endometrial inflammation in postpartum dairy cows and restore endometrial homeostasis. Additionaly, as some of the genes affected by endometrial inflammation in the present study are still uncharacterized, this work stimulates further research with the objective of determining the contribution of these uncharacterized genes in the physiopathology of SCE. The results of the present work also clarifiy some aspects of the nutraceutical use of n-3PUFA in postpartum dairy cows with the purpose of improving the restoration of endometrial homeostasis and reproductive outcome. In sum, cows fed n-3PUFA exhibited shorter calving to conception intervals, whereas endometrial inflammatory status, as assessed by endometrial cytology, remained unaffected. However, further research is needed for the development of efficacious and practical strategies to modulate endometrial inflammation through the use of n-3PUFA and/or derived immunomodulatory molecules.

REFERENCES

Abdelli A, Raboisson D, Kaidi R, Ibrahim B, Kalem A, Iguer-Ouada M. 2017. Elevated non-esterified fatty acid and beta-hydroxybutyrate in transition dairy cows and their association with reproductive performance and disorders: A meta-analysis. Theriogenology. 93:99–104. https://doi.org/10.1016/j.theriogenology.2017.01.030.

Ackman RG. 2002. The gas chromatograph in practical analyses of common and uncommon fatty acids for the 21st century. Anal Chim Acta. 465:175-192. https://doi.org/10.1016/S0003-2670(02)00098-3.

Adnane M, Chapwanya A, Kaidi R, Meade KG, O'Farrelly C. 2017. Profiling inflammatory biomarkers in cervico-vaginal mucus (CVM) postpartum: Potential early indicators of bovine clinical endometritis? Theriogenology. 103:117-122. https://doi.org/10.1016/j.theriogenology.2017.07.039.

Afzelius BA, Camner P, Mossberg B. 1978. On the function of cilia in the female reproductive tract. Fertil Steril. 29(1):72-4. https://doi.org/10.1016/s0015-0282(16)43041-4.

Alagawany M, Elnesr SS, Farag MR, El-Sabrout K, Alqaisi O, Dawood MAO, Soomro H, Abdelnour SA. 2021. Nutritional significance and health benefits of omega-3, -6 and -9 fatty acids in animals. Anim Biotechnol. 20:1-13. https://doi.org/10.1080/10495398.2020.1869562.

Alves SP, Francisco A, Costa M, Santos-Silva J, Bessa RJB. 2017. Biohydrogenation patterns in digestive contents and plasma of lambs fed increasing levels of a tanniferous bush (Cistus ladanifer L.) and vegetable oils. Anim Feed Sci Technol. 225:157-172. https://doi.org/10.1016/j.anifeedsci.2017.01.018.

Arias LAQ, Fernández MV, González JJB, López MB, Herradón PJG, Martínez AIP. 2018. Subclinical Endometritis in Dairy Cattle. Pages 79-97 in New Insights into Theriogenology. R. Payan-Carreira, ed. Intech, Rijeka, Croatia. https://doi.org/10.5772/intechopen.80229.

Ariza JM, Meignan T, Madouasse A, Beaudeau F, Bareille N. 2019. Effects on milk quantity and composition associated with extruded linseed supplementation to dairy cows diets. Sci Rep. 9:17563. https://doi.org/10.1038/s41598-019-54193-z.

Arnold JT, Kaufman DG, Seppälä M, Lessey BA. 2001. Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. Hum Reprod. 16(5):836-45. https://doi.org/10.1093/humrep/16.5.836.

Astessiano AL, Carriquiry M, Mattiauda DA, Adrien ML, Chilibroste P, Meikle A. 2017. Endometrial gene expression in primiparous dairy cows at the end of the voluntary waiting period is affected by nutrition: Total mixed ration vs increasing levels of herbage allowance. Reprod Domest Anim. 52(5):798-805. https://doi.org/10.1111/rda.12981.

Baldwin HL. 2019. Investigation of bovine conceptus galectins and the effect of bovine conceptus secretory proteins on the endometrial epithelial transcriptome [master's thesis on the Internet]. Morgantown (WV): West Virginia University [cited 2021 Nov 10]. Available from: https://researchrepository.wvu.edu/etd/4080.

Barbe A, Bongrani A, Mellouk N, Estienne A, Kurowska P, Grandhaye J, Elfassy Y, Levy R, Rak A, Froment P et al. 2019. Mechanisms of adiponectin action in fertility: An overview from gametogenesis to gestation in humans and animal models in normal and pathological conditions. Int J Mol Sci. 20(7):1526. https://doi.org/10.3390/ijms20071526.

Bartosch C, Lopes JM, Beires J, Sousa M. 2011. Human endometrium ultrastructure during the implantation window: a new perspective of the epithelium cell types. Reprod Sci. 18(6):525-39. https://doi.org/10.1177/1933719110392055.

Bauersachs S, Ulbrich SE, Gross K, Schmidt SEM, Meyer HHD, Einspanier R, Wenigerkind H, Vermehren M, Blum H, Sinowatz F et al. 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. J Mol Endocrinol. 34(3):889-908. https://doi.org/10.1677/jme.1.01799.

Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. FEMS Immunol Med Microbiol. 2003 Aug 18;38(1):13-22. https://doi.org/10.1016/S0928-8244(03)00202-5.

Bebington C, Doherty FJ, Fleming SD. 2001. The possible biological and reproductive functions of ubiquitin. Hum Reprod Update. 7(1):102-11. https://doi.org/10.1093/humupd/7.1.102.

Bellehumeur C, Blanchet J, Fontaine JY, Bourcier N, Akoum A. 2009. Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum Reprod. 24:2193-204. https://doi.org/10.1093/humrep/dep192.

Beltran SR, Svoboda KK, Kerns DG, Sheth A, Prockop DJ. 2015. Anti-inflammatory protein tumor necrosis factor-α-stimulated protein 6 (TSG-6) promotes early gingival wound healing: an in vivo study. J. Periodontol. 86(1):62–71. https://doi.org/10.1902/jop.2014.140187.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Royal Stat Soc Ser B. 57:289–300.

Bertling F, Bendix I, Drommelschmidt K, Wisniewski HG, Felderhoff-Mueser U, Keller M, Prager S. 2016. Tumor necrosis factor-inducible gene 6 protein: A novel neuroprotective factor against inflammation-induced developmental brain injury. Exp. Neurol. 279:283–289. https://doi.org/10.1016/j.expneurol.2016.03.005.

Bertoni G, Trevisi E, Han X, Bionaz M. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci. 91(9):3300-3310. https://doi.org/10.3168/jds.2008-0995.

Bevilacqua C, Ducos B. 2018. Laser microdissection: A powerful tool for genomics at cell level. Mol Aspects Med. 59:5-27. https://doi.org/10.1016/j.mam.2017.09.003.

Bevilacqua C, Makhzami S, Helbling JC, Defrenaix P, Martin P. 2010. Maintaining RNA integrity in a homogeneous population of mammary epithelial cells isolated by Laser Capture Microdissection. BMC Cell Biol. 11:95. https://doi.org/10.1186/1471-2121-11-95.

Bi R, Liu P. 2016. Sample size calculation while controlling false discovery rate for differential expression analysis with RNA-sequencing experiments. BMC Bioinformatics. 17(1):146. https://doi.org/10.1186/s12859-016-0994-9.

Bicalho ML, Machado VS, Oikonomou G, Gilbert RO, Bicalho RC. 2012. Association between virulence factors of Escherichia coli, Fusobacterium necrophorum, and Arcanobacterium pyogenes and uterine diseases of dairy cows. Vet Microbiol. 157(1-2):125-31. https://doi.org/10.1016/j.vetmic.2011.11.034.

Bicalho MLS, Zinicola M, Machado VS, Lima FS, Teixeira AGV, Narbus C, Xavier MR, Higgins H, Bicalho RC. 2019. Effects of recombinant bovine interleukin-8 (rbIL-8) treatment on health, metabolism, and lactation performance in Holstein cattle I: Production and functional characterization of rbIL-8 in vitro and in vivo. J Dairy Sci. 102(11):10304-10315. https://doi.org/10.3168/jds.2019-16334.

Bicalho RC, Galvão KN, Guard CL, Santos JE. 2008. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. Theriogenology. 70(2):199-207. https://doi.org/10.1016/j.theriogenology.2008.03.015.

Bie Q, Jin C, Zhang B, Dong H. 2017. IL-17B: A new area of study in the IL-17 family. Mol Immunol. 90:50-56. https://doi.org/10.1016/j.molimm.2017.07.004.

Bines JA, Hart IC. 1982. Metabolic limits to milk production, especially roles of growth hormone and insulin. J Dairy Sci. 65:1375-89. https://doi.org/10.3168/jds.S0022-0302(82)82358-8.

Bionaz M, Vargas-Bello-Pérez E, Busato S. 2020. Advances in fatty acids nutrition in dairy cows: from gut to cells and effects on performance. J Anim Sci Biotechnol. 11(1):110. https://doi.org/10.1186/s40104-020-00512-8.

Bishop CV. 2013. Progesterone inhibition of oxytocin signaling in endometrium. Front Neurosci. 7:138. https://doi.org/10.3389/fnins.2013.00138.

Bolnick AD, Bolnick JM, Kilburn BA, Stewart T, Oakes J, Rodriguez-Kovacs J, Kohan-Ghadr HR, Dai J, Diamond MP, Hirota Y et al. 2016. Reduced homeobox protein MSX1 in human endometrial tissue is linked to infertility. Hum Reprod. 31(9):2042-50. https://doi.org/10.1093/humrep/dew143.

Bondue B, Wittamer V, Parmentier M. 2011. Chemerin and its receptors in leukocyte trafficking, inflammation and metabolism. Cytokine Growth Factor Rev. 22(5-6):331-338. https://doi.org/10.1016/j.cytogfr.2011.11.004.

Bongrani A, Mellouk N, Rame C, Cornuau M, Guérif F, Froment P, Dupont J. 2019. Ovarian expression of adipokines in polycystic ovary syndrome: A role for chemerin, omentin, and apelin in follicular growth arrest and ovulatory dysfunction? Int J Mol Sci. 20(15):3778. https://doi.org/10.3390/ijms20153778.

Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G. 2004. GO::TermFinder--open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics. 20(18):3710-5. https://doi.org/10.1093/bioinformatics/bth456.

Bradford BJ, Yuan K, Farney JK, Mamedova LK, Carpenter AJ. 2015. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. J Dairy Sci. 98:6631-6650. https://doi.org/10.3168/jds.2015-9683.

Brauer R, Tureckova J, Kanchev I, Khoylou M, Skarda J, Prochazka J, Spoutil F, Beck IM, Zbodakova O, Kasparek P, et al. 2016. MMP-19 deficiency causes aggravation of colitis due to defects in innate immune cell function. Mucosal Immunol 9:974–985 https://doi.org/10.1038/mi.2015.117.

Brazil JC, Quiros M, Nusrat A, Parkos CA. 2019. Innate immune cell-epithelial crosstalk during wound repair. J. Clin. Investig. 129(8):2983–2993. https://doi.org/10.1172/JCI124618.

Brenna JT, Salem NJ, Sinclair AJ, Cunnane SC. 2009. Alpha-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. Prostaglandins Leukot Essent Fatty Acids. 80:85-91. https://doi.org/10.1016/j.plefa.2009.01.004.

Brewer A, Cormican P, Lim JJ, Chapwanya A, O'Farrelly C, Meade KG. 2020. Qualitative and quantitative differences in endometrial inflammatory gene expression precede the development of bovine uterine disease. Sci Rep. 10:18275. https://doi.org/10.1038/s41598-020-75104-7.

Britt JH, Cushman RA, Dechow CD, Dobson H, Humblot P, Hutjens MF, Jones GA, Ruegg PS, Sheldon IM, Stevenson JS. 2018. Invited review: Learning from the future-A

vision for dairy farms and cows in 2067. J Dairy Sci. 101(5):3722-3741. https://doi.org/10.3168/jds.2017-14025.

Brooks K, Burns GW, Moraes JG, Spencer TE. 2016. Analysis of the uterine epithelial and conceptus transcriptome and luminal fluid proteome during the Peri-implantation period of pregnancy in sheep. Biol Reprod. 95(4):88. https://doi.org/10.1095/biolreprod.116.141945.

Buczkowska J, Kozdrowski R, Nowak M, Raś A, Staroniewicz Z, Siemieniuch MJ. 2014. Comparison of the biopsy and cytobrush techniques for diagnosis of subclinical endometritis in mares. Reprod. Biol. Endocrinol. 12:27. https://doi.org/10.1186/1477-7827-12-27.

Burkly LC, Michaelson JS, Zheng TS. 2011. TWEAK/Fn14 pathway: an immunological switch for shaping tissue responses. Immunol Rev. 244:99-114. https://doi.org/10.1111/j.1600-065X.2011.01054.x.

Burkly LC. 2014. TWEAK/Fn14 axis: the current paradigm of tissue injury-inducible function in the midst of complexities. Semin Immunol. 26:229-36. https://doi.org/10.1016/j.smim.2014.02.006.

Caixeta LS, Ospina PA, Capel MB, Nydam DV. 2017. Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows. Theriogenology. 94:1-7. https://doi.org/10.1016/j.theriogenology.2017.01.039.

Canning P, Hassfurther R, TerHune T, Rogers K, Abbott S, Kolb D. 2017. Efficacy and clinical safety of pegbovigrastim for preventing naturally occurring clinical mastitis in periparturient primiparous and multiparous cows on US commercial dairies. J Dairy Sci. 100(8):6504-6515. https://doi.org/10.3168/jds.2017-12583.

Cao Y, Han Q, Li J, Jia Y, Zhang R, Shi H. 2020. P4HA2 contributes to cervical cancer progression via inducing epithelial-mesenchymal transition. J Cancer. 11(10):2788-2799. https://doi.org/10.7150/jca.38401.

Capp E, Milner CM, Williams J, Hauck L, Jauckus J, Strowitzki T, Germeyer A. 2014. Modulation of tumor necrosis factor-stimulated gene-6 (TSG-6) expression in human endometrium. Arch Gynecol Obstet. 289:893-901. https://doi.org/10.1007/s00404-013-3080-9.

Cezar T, Martinez RM, Rocha CD, Melo C, Vale DL, Borghi SM, Fattori V, Vignoli JA, Camilios-Neto D, Baracat MM, Georgetti SR, Verri Jr WA, Casagrande R. 2019. Treatment with maresin 1, a docosahexaenoic acid-derived pro-resolution lipid, protects skin from inflammation and oxidative stress caused by UVB irradiation. Sci. Rep. 9(1):3062. https://doi.org/10.1038/s41598-019-39584-6.

Cha HR, Ko HJ, Kim ED, Chang SY, Seo SU, Cuburu N, Ryu S, Kim S, Kweon M. 2011. Mucosa-associated epithelial chemokine/CCL28 expression in the uterus attracts CCR10+ IgA plasma cells following mucosal vaccination via estrogen control. J Immunol. 187:3044-52. https://doi.org/10.4049/jimmunol.1100402.

Chamberlain MB, DePeters EJ. 2017. Impacts of feeding lipids supplements high in palmitic acid or stearic acid on performance of lactating dairy cows. J Appl Anim Res. 45:126–135. https://doi.org/10.1080/09712119.2015.1124327.

Chamberlin WG, Middleton JR, Spain JN, Johnson GC, Ellersieck MR, Pithua P. 2013. Subclinical hypocalcemia, plasma biochemical parameters, lipid metabolism, postpartum disease, and fertility in postparturient dairy cows. J Dairy Sci. 96:7001-7013. https://doi.org/10.3168/jds.2013-6901.

Chankeaw W, Lignier S, Richard C, Ntallaris T, Raliou M, Guo Y, Plassard D, Bevilacqua C, Sandra O, Andersson G, Humblot P, Charpigny G. 2021a. Analysis of the

transcriptome of bovine endometrial cells isolated by laser micro-dissection (1): specific signatures of stromal, glandular and luminal epithelial cells. BMC Genomics. 22 451(2021). https://doi.org/10.1186/s12864-021-07712-0.

Chankeaw W, Lignier S, Richard C, Ntallaris T, Raliou M, Guo Y, Plassard D, Bevilacqua C, Sandra O, Andersson G, Humblot P, Charpigny G. 2021b. Analysis of the transcriptome of bovine endometrial cells isolated by laser micro-dissection (2): impacts of post-partum negative energy balance on stromal, glandular and luminal epithelial cells. BMC Genomics. 22 450(2021). https://doi.org/10.1186/s12864-021-07713-z.

Chanrot M, Guo Y, Dalin AM, Persson E, Båge R, Svensson A, Gustafsson H, Humblot P. 2017. Dose related effects of LPS on endometrial epithelial cell populations from dioestrus cows. Anim. Reprod. Sci. 177:12–24. https://doi.org/10.1016/j.anireprosci.2016.12.002.

Chapwanya A, Meade KG, Doherty ML, Callanan JJ, Mee JF, O'Farrelly C. 2009. Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: toward an improved understanding of uterine innate immunity. Theriogenology. 71(9):1396-407. https://doi.org/10.1016/j.theriogenology.2009.01.006.

Chapwanya A, Meade KG, Narciandi F, Stanley P, Mee JF, Doherty ML, Callanan JJ, O'Farrelly C. 2010. Endometrial biopsy: a valuable clinical and research tool in bovine reproduction. Theriogenology. 73(7):988-94. https://doi.org/10.1016/j.theriogenology.2009.11.015.

Cheng CW, Smith SK, Charnock-Jones DS. 2007. Transcript profile and localization of Wnt signaling-related molecules in human endometrium. Fertil Steril. 90(1):201-4. https://doi.org/10.1016/j.fertnstert.2007.05.077.

Cheong SH, Nydam DV, Galvão KN, Crosier BM, Gilbert RO. 2011. Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. J Dairy Sci. 94(2):762-70. https://doi.org/10.3168/jds.2010-3439.

Cheong SH, Sá Filho OG, Absalon-Medina VA, Schneider A, Butler WR, Gilbert RO. 2017. Uterine and systemic inflammation influences ovarian follicular function in postpartum dairy cows. PloS one. 12(5):e0177356. https://doi.org/10.1371/journal.pone.0177356.

Chi RA, Wang T, Adams N, Wu SP, Young SL, Spencer TE, et al. 2020. Human Endometrial Transcriptome and Progesterone Receptor Cistrome Reveal Important Pathways and Epithelial Regulators. J Clin Endocrinol Metab. 105(4):e1419–39. https://doi.org/10.1210/clinem/dgz117.

Chiang N, Libreros S, Norris PC, de la Rosa X, Serhan CN. 2019. Maresin 1 activates LGR6 receptor promoting phagocyte immunoresolvent functions. J Clin Invest. 129:5294-5311. https://doi.org/10.1172/JCI129448.

Choi Y, Seo H, Han J, Yoo I, Kim J, Ka H. 2016. Chemokine (C-C motif) Ligand 28 and Its Receptor CCR10: Expression and Function at the Maternal-Conceptus Interface in Pigs. Biol Reprod. 95:84. https://doi.org/10.1095/biolreprod.116.141903.

Colazo MG, Ambrose DJ, Kastelic JP, Small JA. 2008. Comparison of 2 enzyme immunoassays and a radioimmunoassay for measurement of progesterone concentrations in bovine plasma, skim milk, and whole milk. Can J Vet Res. 72:32-36.

Collins LE, Troeberg L. 2019. Heparan sulfate as a regulator of inflammation and immunity. J Leukoc Biol. 105:81-92. https://doi.org/10.1002/JLB.3RU0618-246R.

Contreras GA, Strieder-Barboza C, de Souza J, Gandy J, Mavangira V, Lock AL, Sordillo LM. 2017. Periparturient lipolysis and oxylipid biosynthesis in bovine adipose tissues. PLoS One. 12(12):e0188621. https://doi.org/10.1371/journal.pone.0188621.

Cook HW, McMaster CR. 2002. Fatty acid desaturation and chain elongation in eukaryotes. New Compr Biochem. 36:181-204. https://doi.org/10.1016/S0167-7306(02)36009-5.

Correia-Álvarez E, Gómez E, Martín D, Carrocera S, Pérez S, Otero J, Peynot N, Giraud-Delville C, Caamaño JN, Sandra O, et al. 2015. Expression and localization of interleukin 1 beta and interleukin 1 receptor (type I) in the bovine endometrium and embryo. J Reprod Immunol. 110:1-13. https://doi.org/10.1016/j.jri.2015.03.006.

Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM. 2012. Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. Biol Reprod. 86(2):51. https://doi.org/10.1095/biolreprod.111.092718.

Cui N, Hu M, Khalil RA. 2017. Biochemical and Biological Attributes of Matrix Metalloproteinases. Prog Mol Biol Transl Sci. 147:1-73. https://doi.org/10.1016/bs.pmbts.2017.02.005.

Day AJ, Milner CM. 2019. TSG-6: A multifunctional protein with anti-inflammatory and tissue-protective properties. Matrix Biol. 78-79:60-83. https://doi.org/10.1016/j.matbio.2018.01.011.

De Henau O, Degroot GN, Imbault V, Robert V, De Poorter C, Mcheik S, Galés C, Parmentier M, Springael JY. 2016. Signaling properties of chemerin receptors CMKLR1, GPR1 and CCRL2. PLoS One. 11(10):e0164179. https://doi.org/10.1371/journal.pone.0164179.

De Koster J, Urh C, Hostens M, Van den Broeck W, Sauerwein H, Opsomer G. 2017. Relationship between serum adiponectin concentration, body condition score, and peripheral tissue insulin response of dairy cows during the dry period. Domest Anim Endocrinol. 59:100–104. https://doi.org/10.1016/j.domaniend.2016.12.004.

De Smet EG, Seys LJ, Verhamme FM, Vanaudenaerde BM, Brusselle GG, Bingle CD, Bracke KR. 2017. Association of innate defense proteins BPIFA1 and BPIFB1 with disease severity in COPD. Int J Chron Obstruct Pulmon Dis. 13:11-27. https://doi.org/10.2147/COPD.S144136.

de Souza J, Lock AL. 2019. Effects of timing of palmitic acid supplementation on production responses of early-lactation dairy cows. J Dairy Sci. 102:260-273. https://doi.org/10.3168/jds.2018-14976.

de Souza J, Preseault CL, Lock AL. 2016. Short communication: Lactational responses to palmitic acid supplementation when replacing soyhulls or dry ground corn. J Dairy Sci. 99:1945–1950. https://doi.org/10.3168/jds.2015-10367.

Delforce SJ, Lumbers ER, Corbisier de Meaultsart C, Wang Y, Proietto A, Otton G, Scurry J, Verrills NM, Scott RJ, Pringle KG. 2017. Expression of renin-angiotensin system (RAS) components in endometrial cancer. Endocr Connect. 6(1):9-19. https://doi.org/10.1530/EC-16-00829.

Denis-Robichaud J, Dubuc J. 2015. Randomized clinical trial of intrauterine cephapirin infusion in dairy cows for the treatment of purulent vaginal discharge and cytological endometritis. J Dairy Sci. 98(10):6856-64. https://doi.org/10.3168/jds.2014-9129.

Diep CH, Daniel AR, Mauro LJ, Knutson TP, Lange CA. 2015. Progesterone action in breast, uterine, and ovarian cancers. J Mol Endocrinol. 54(2):R31-53. https://doi.org/10.1530/JME-14-0252.

Dinarello CA. 2018. Overview of the IL-1 family in innate inflammation and acquired immunity. Immunol Rev. 281:8-27. https://doi.org/10.1111/imr.12621.

Ding Z, Ericksen RE, Escande-Beillard N, Lee QY, Loh A, Denil S, Steckel M, Haegebarth A, Wai Ho TS, Chow P et al. 2020. Metabolic pathway analyses identify proline biosynthesis pathway as a promoter of liver tumorigenesis. J Hepatol. 72(4):725-735. https://doi.org/10.1016/j.jhep.2019.10.026.

Dirandeh E, Towhidi A, Zeinoaldini S, Ganjkhanlou M, Ansari Pirsaraei Z, Fouladi-Nashta A. 2013. Effects of different polyunsaturated fatty acid supplementations during the postpartum periods of early lactating dairy cows on milk yield, metabolic responses, and reproductive performances. J Anim Sci. 91:713-721. https://doi.org/10.2527/jas.2012-5359.

Diskin MG, Lonergan P. 2014. Editorial: International Cow Fertility Conference 'New Science – New Practices' in Westport, Ireland, in 2014. Animal. 8(S1):1-4. https://doi.org/10.1017/S1751731114000846.

Divanovic S, Dalli J, Jorge-Nebert LF, Flick LM, Gálvez-Peralta M, Boespflug ND, Stankiewicz TE, Fitzgerald JM, Somarathna M, Karp CL, et al. 2013. Contributions of the three CYP1 monooxygenases to pro-inflammatory and inflammation-resolution lipid mediator pathways. J Immunol. 191:3347-57. https://doi.org/10.4049/jimmunol.1300699.

Djuricic I, Calder PC. 2021. Beneficial Outcomes of Omega-6 and Omega-3 Polyunsaturated Fatty Acids on Human Health: An Update for 2021. Nutrients. 13(7):2421. https://doi.org/10.3390/nu13072421.

Dobson H, Smith R, Royal M, Knight Ch, Sheldon I. 2007. The high-producing dairy cow and its reproductive performance. Reprod Domest Anim. 42(Suppl 2):17-23. https://doi.org/10.1111/j.1439-0531.2007.00906.x.

Dohi T, Burkly LC. 2012. The TWEAK/Fn14 pathway as an aggravating and perpetuating factor in inflammatory diseases: focus on inflammatory bowel diseases. J Leukoc Biol. 92:265-79. https://doi.org/10.1189/jlb.0112042.

Dolecheck KA, García-Guerra A, Moraes LE. 2019. Quantifying the effects of mastitis on the reproductive performance of dairy cows: A meta-analysis. J Dairy Sci. 102(9):8454-8477. https://doi.org/10.3168/jds.2018-15127.

Dranse HJ, Rourke JL, Stadnyk AW, Sinal CJ. 2015. Local chemerin levels are positively associated with DSS-induced colitis but constitutive loss of CMKLR1 does not protect against development of colitis. Physiol Rep. 3(8):e12497. https://doi.org/10.14814/phy2.12497.

Du XY, Leung LL. 2009. Proteolytic regulatory mechanism of chemerin bioactivity. Acta biochimica et biophysica Sinica. 41(12):973–979. https://doi.org/10.1093/abbs/gmp091.

Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. 2010. Risk factors for postpartum uterine diseases in dairy cows. J Dairy Sci. 93(12):5764-71. https://doi.org/10.3168/jds.2010-3429.

Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. 2011a. Randomized clinical trial of antibiotic and prostaglandin treatments for uterine health and reproductive performance in dairy cows. J. Dairy Sci. 94:1325–1338. https://doi.org/10.3168/jds.2010-3757.

Dubuc J, Duffield TF, Leslie KE, Walton JS, Leblanc SJ. 2011b. Effects of postpartum uterine diseases on milk production and culling in dairy cows. J Dairy Sci. 94(3):1339-46. https://doi.org/10.3168/jds.2010-3758.

Dunlock VE. 2020. Tetraspanin CD53: an overlooked regulator of immune cell function. Med Microbiol Immunol. 209(4):545-552. https://doi.org/10.1007/s00430-020-00677-z.

Dupont J, Pollet-Villard X, Reverchon M, Mellouk N, Levy R. 2015. Adipokines in human reproduction. Horm Mol Biol Clin Investig. 24(1):11-24. https://doi.org/10.1515/hmbci-2015-0034.

Dyer DP, Thomson JM, Hermant A, Jowitt TA, Handel TM, Proudfoot AE, Day AJ, Milner CM. 2014. TSG-6 inhibits neutrophil migration via direct interaction with the chemokine CXCL8. J Immunol. 192:2177-85. https://doi.org/10.4049/jimmunol.1300194.

Eckel EF, Ametaj BN. 2020. Bacterial Endotoxins and Their Role in Periparturient Diseases of Dairy Cows: Mucosal Vaccine Perspectives. Dairy. 1:61-90. https://doi.org/10.3390/dairy1010006.

Elgamal DA, Othman ER, Ahmed SF. 2016. Ultrastructural Features of Eutopic Endometrium in a Rat Model of Endometriosis. J Microsc Ultrastruct. 4(1):20-27. https://doi.org/10.1016/j.jmau.2015.10.002.

Elis S, Desmarchais A, Freret S, Maillard V, Labas V, Cognié J, Briant E, Hivelin C, Dupont J, Uzbekova S. 2016. Effect of a long-chain n-3 polyunsaturated fatty acid–enriched diet on adipose tissue lipid profiles and gene expression in Holstein dairy cows. J Dairy Sci. 99:10109-10127. https://doi.org/10.3168/jds.2016-11052.

Espejel MC, Medrano A. 2017. Histological Cyclic Endometrial Changes in Dairy Cows: An Overview. JDVS. 2 555577. https://doi.org/10.19080/JDVS.2017.02.555577.

Espina V, Wulfkuhle JD, Calvert VS, VanMeter A, Zhou W, Coukos G, Geho DH, Petricoin EF, Liotta LA. 2006. Laser-capture microdissection. Nat. Protoc. 1 586e603. https://doi.org/10.1038/nprot.2006.85.

European Commission. 2018. EU agricultural outlook for markets and income, 2018-2030. Brussels: DG Agriculture and Rural Development.

Fadden AN, Bobe G. 2016. Serum visfatin is a predictive indicator of retained placenta and other diseases in dairy cows. J Vet Sci Med Diagn. 5(1). https://doi.org/10.4172/2325-9590.1000188.

Fagundes NS, Rezende AL, Alvarenga PB, Magalhães LQ, Santos RM, Headley SA, Silva MJB, Beletti ME, Saut JPE. 2019. Short communication: Proinflammatory gene expression relative to the collection technique of endometrial samples from cows with and without subclinical endometritis. J Dairy Sci. 102:5511-5517. https://doi.org/10.3168/jds.2018-15212.

Fang H, Judd RL. 2018. Adiponectin regulation and function. Compr Physiol. 8(3):1031-1063. https://doi.org/10.1002/cphy.c170046.

Fantuzzi G. 2013. Adiponectin in inflammatory and immune-mediated diseases. Cytokine. 64(1):1-10. https://doi.org/10.1016/j.cyto.2013.06.317.

Ferguson JD, Galligan DT, Thomsen N. 1994. Principal descriptors of body condition score in Holstein cows. J Dairy Sci. 77:2695–2703. https://doi.org/10.3168/jds.S0022-0302(94)77212-X.

Ferlay A, Doreau M, Martin C, Chilliard Y. 2013. Effects of incremental amounts of extruded linseed on the milk fatty acid composition of dairy cows receiving hay or corn silage. J Dairy Sci. 96:6577-6595. https://doi.org/10.3168/jds.2013-6562.

Field SL, Cummings M, Orsi NM. 2015. Epithelial and stromal-specific immune pathway activation in the murine endometrium post-coitum. Reprod. 150(2):127-38. https://doi.org/10.1530/REP-15-0087.

Figueira PG, Abrão MS, Krikun G, Taylor HS. 2011. Stem cells in endometrium and their role in the pathogenesis of endometriosis. Ann N Y Acad Sci. 1221(1):10-7. https://doi.org/10.1111/j.1749-6632.2011.05969.x.

Flanagan K, Moroziewicz D, Kwak H, Hörig H, Kaufman HL. 2004. The lymphoid chemokine CCL21 costimulates naive T cell expansion and Th1 polarisation of non-regulatory CD4+ T cells. Cell Immunol. 231:75-84. https://doi.org/10.1016/j.cellimm.2004.12.006.

Foley C, Chapwanya A, Callanan JJ, Whiston R, Miranda-CasoLuengo R, Lu J, Meijer WG, Lynn DJ, O' Farrelly C, Meade KG. 2015. Integrated analysis of the local and systemic changes preceding the development of post-partum cytological endometritis. BMC Genomics. 16:811. https://doi.org/10.1186/s12864-015-1967-5.

Foley C, Chapwanya A, Creevey CJ, Narciandi F, Morris D, Kenny EM, Cormican P, Callanan JJ, O'Farrelly C, Meade KG. 2012. Global endometrial transcriptomic profiling: transient immune activation precedes tissue proliferation and repair in healthy beef cows. BMC Genomics. 13:489. https://doi.org/10.1186/1471-2164-13-489.

Fonzar-Marana RRN, Ferriani RA, Soares SG, Cavalcante-Neto FF, Teixeira JE, Barbosa JE. 2006. Expression of complement system regulatory molecules in the endometrium of normal ovulatory and hyperstimulated women correlate with menstrual cycle phase. Fertil Steril. 86(3):758-61. https://doi.org/10.1016/j.fertnstert.2006.02.095.

Forde N, Carter F, Fair T, Crowe MA, Evans AC, Spencer TE, et al. 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biol Reprod. 81(4):784-94. https://doi.org/10.1095/biolreprod.108.074336.

Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, Okumu LA, McGettigan PA, Mehta JP, McBride R, et al. 2011. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? Biol. Reprod. 85:144–156. https://doi.org/10.1095/biolreprod.110.090019.

Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, Mansouri-Attia N, Sandra O, Loftus BJ, Crowe MA, et al. 2012. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiol. Genomics. 44:799–810. https://doi.org/10.1152/physiolgenomics.00067.2012.

Forde N, Lonergan P. 2012. Transcriptomic analysis of the bovine endometrium: What is required to establish uterine receptivity to implantation in cattle? J Reprod Dev. 58(2):189-95. https://doi.org/10.1262/jrd.2011-021.

Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, Lonergan P. 2012. Effects of Low Progesterone on the Endometrial Transcriptome in Cattle. Biol Reprod. 87(5):124. https://doi.org/10.1095/biolreprod.112.103424.

Foskett AM, Bazhanov N, Ti X, Tiblow A, Bartosh TJ, Prockop DJ. 2014. Phase-directed therapy: TSG-6 targeted to early inflammation improves bleomycin-injured lungs. American journal of physiology. Lung Cell. Mol. Physiol. 306(2):L120–L131. https://doi.org/10.1152/ajplung.00240.2013.

Fourichon C, Seegers H, Malher X. 2000. Effect of disease on reproduction in the dairy cow: a meta-analysis. Theriogenology. 53(9):1729-59. https://doi.org/10.1016/S0093-691X(00)00311-3.

Fox LE, Locke MC, Lenschow DJ. 2020. Context Is Key: Delineating the unique functions of IFNα and IFNβ in disease. Front Immunol. 11:606874. https://doi.org/10.3389/fimmu.2020.606874.

Franco HL, Jeong JW, Tsai SY, Lydon JP, DeMayo FJ. 2008. In vivo analysis of progesterone receptor action in the uterus during embryo implantation. Semin Cell Dev Biol. 19(2):178-86. https://doi.org/10.1016/j.semcdb.2007.12.001.

Friedrich M, Gerbeth L, Gerling M, Rosenthal R, Steiger K, Weidinger C, Keye J, Wu H, Schmidt F, Weichert W, et al. 2019. HDAC inhibitors promote intestinal epithelial regeneration via autocrine TGFβ1 signalling in inflammation. Mucosal Immunol. 12:656-667. https://doi.org/10.1038/s41385-019-0135-7.

Fuentes BM, Quintela Arias LA, Becerra González JJ, del Barrio del Sol L, Mociños Feijóo JE, Guillín Puñal JL, Barrio López M, Prieto Lago A, Díaz Cao JM, Rodríguez GF, et al. 2017. Agreement between postmortem endometrial cytology, biopsy and bacteriology in culled dairy cows. Anim Reprod. 14(4):1024-1033 https://doi.org/10.21451/1984-3143-AR826.

Fuster MM, Wang L. 2010. Endothelial heparan sulfate in angiogenesis. Prog Mol Biol Transl Sci. 93:179–212. https://doi.org/10.1016/S1877-1173(10)93009-3.

Galliou JM, Kiser JN, Oliver KF, Seabury CM, Moraes JGN, Burns GW, et al. 2020. Identification of loci and pathways associated with heifer conception rate in U.S. holsteins. Genes. 11(7):767. https://doi.org/10.3390/genes11070767.

Galvão KN, Bicalho RC, Jeon SJ. 2019. Symposium review: The uterine microbiome associated with the development of uterine disease in dairy cows. J Dairy Sci. 102(12):11786-11797. https://doi.org/10.3168/jds.2019-17106.

Galvão KN, Santos NR, Galvão JS, Gilbert RO. 2011. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. Theriogenology. 76:290-9. https://doi.org/10.1016/j.theriogenology.2011.02.006.

Garg D, Ng S, Baig KM, Driggers P, Segars J. 2017. Progesterone-Mediated Non-Classical Signaling. Trends Endocrinol Metab. 28(9):656–668. https://doi.org/10.1016/j.tem.2017.05.006.

Geisert RD, Morgan GL, Short Jr EC, Zavy MT. 1992. Endocrine events associated with endometrial function and conceptus development in cattle. Reprod Fertil Dev. 4(3):301-5. https://doi.org/10.1071/rd9920301.

Gilbert RO, Shin ST, Guard CL, Erb HN, Frajblat M. 2005. Prevalence of endometritis and its effects on reproductive performance of dairy cows. Theriogenology. 64(9):1879-88. https://doi.org/10.1016/j.theriogenology.2005.04.022.

Gilbert RO. 2012. The effects of endometritis on the establishment of pregnancy in cattle. Reprod Fertil Dev. 24:252-257. https://doi.org/10.1071/RD11915.

Giuliodori MJ, Magnasco RP, Becu-Villalobos D, Lacau-Mengido IM, Risco CA, de la Sota RL. 2013. Metritis in dairy cows: risk factors and reproductive performance. J Dairy Sci. 96(6):3621-31. https://doi.org/10.3168/jds.2012-5922.

Glennon-Alty L, Moots RJ, Edwards SW, Wright HL. 2021. Type I interferon regulates cytokine-delayed neutrophil apoptosis, reactive oxygen species production and chemokine expression. Clin Exp Immunol. 203:151-159. https://doi.org/10.1111/cei.13525.

Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinog. 1999 Jun;25(2):86-91.Decarlo K, Emley A, Dadzie OE, Mahalingam M. Laser capture microdissection: methods and applications. Methods Mol Biol. 2011;755:1-15. https://doi.org/10.1007/978-1-61779-163-5_1.

Gonthier C, Mustafa AF, Ouellet DR, Chouinard PY, Berthiaume R, Petit HV. 2005. Feeding micronized and extruded flaxseed to dairy cows: Effects on blood parameters and milk fatty acid composition. J Dairy Sci. 88:748-756. https://doi.org/10.3168/jds.S0022-0302(05)72738-7.

Greco LF, Neves Neto JT, Pedrico A, Ferrazza RA, Lima FS, Bisinotto RS, Martinez N, Garcia M, Ribeiro ES, Gomes GC, et al. 2015. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on performance and inflammatory responses to a lipopolysaccharide challenge in lactating Holstein cows. J Dairy Sci. 98:602–617. https://doi.org/10.3168/jds.2014-8805.

Greco LF, Neves Neto JT, Pedrico A, Lima FS, Bisinotto RS, Martinez N, Ribeiro ES, Thatcher WW, Staples CR, Santos JEP. 2018. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on spontaneous luteolysis in lactating dairy cows. J Dairy Sci. 101:10536–10556. https://doi.org/10.3168/jds.2018-15065.

Groebner AE, Rubio-Aliaga I, Schulke K, Reichenbach HD, Daniel H, Wolf E, Meyer HH, Ulbrich SE. 2011. Increase of essential amino acids in the bovine uterine lumen during preimplantation development. Reprod. 141(5):685-95. https://doi.org/10.1530/REP-10-0533.

Guillomot M, Betteridge KJ, Harvey D, Goff AK. 1986. Endocytotic activity in the endometrium during conceptus attachment in the cow. J Reprod Fertil. 78(1):27-36. https://doi.org/10.1530/jrf.0.0780027.

Gujral P, Mahajan V, Lissaman AC, Ponnampalam AP. 2020. Histone acetylation and the role of histone deacetylases in normal cyclic endometrium. Reprod Biol Endocrinol. 18:84. https://doi.org/10.1186/s12958-020-00637-5.

Guo Y, Chankeaw W, Chanrot M, Valarcher JF, Chantarapratep P, Bage R, Bongcam-Rudloff E, Andersson G, Charpigny G, Humblot P. 2019. Changes induced by pathogens and metabolic stress on endometrial function in cattle: possible impacts of increased inflammation on fertility. Rev bras reprod anim. 43(2):295-307.

Guo Y, van Schaik T, Jhamat N, Niazi A, Chanrot M, Charpigny G, Valarcher JF, Bongcam-Rudloff E, Andersson G, Humblot P. 2019. Differential gene expression in bovine endometrial epithelial cells after challenge with LPS; specific implications for genes involved in embryo maternal interactions. PLoS One. 14 e0222081. https://doi.org/10.1371/journal.pone.0222081.

Haider S, Gamperl M, Burkard TR, Kunihs V, Kaindl U, Junttila S, Fiala C, Schmidt K, Mendjan S, Knöfler M et al. 2019. Estrogen Signaling Drives Ciliogenesis in Human Endometrial Organoids. Endocrinology. 160(10):2282-2297. https://doi.org/10.1210/en.2019-00314.

Haimerl P, Heuwieser W, Arlt S. 2013. Therapy of bovine endometritis with prostaglandin F2α: a meta-analysis. J Dairy Sci. 96(5):2973-87. https://doi.org/10.3168/jds.2012-6154.

Haimerl P, Heuwieser W, Arlt S. 2018. Short communication: Meta-analysis on therapy of bovine endometritis with prostaglandin F2α-An update. J Dairy Sci. 101(11):10557-10564. https://doi.org/10.3168/jds.2018-14933.

Haimerl P, Heuwieser W. 2014. Invited review: Antibiotic treatment of metritis in dairy cows: a systematic approach. J Dairy Sci. 97(11):6649-61. https://doi.org/10.3168/jds.2014-8462.

Hall JA, Grainger JR, Spencer SP, Belkaid Y. 2011. The role of retinoic acid in tolerance and immunity. Immunity. 35(1):13–22. https://doi.org/10.1016/j.immuni.2011.07.002.

Hall OJ, Klein SL. 2017. Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. Mucosal Immunol. 10(5):1097-1107. https://doi.org/10.1038/mi.2017.35.

Hammon DS, Evjen IM, Dhiman TR, Goff JP, Walters JL. 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet Immunol Immunopathol. 113(1-2):21-9. https://doi.org/10.1016/j.vetimm.2006.03.022.

Hansen TR, Sinedino LDP, Spencer TE. 2017. Paracrine and endocrine actions of interferon tau (IFNT). Reprod. 154:F45-F59. https://doi.org/10.1530/REP-17-0315.

Haubold S, Kröger-Koch C, Tuchscherer A, Kanitz E, Weitzel JM, Hoeflich A, Starke A, Tröscher A, Sauerwein H, Hammon HM. 2020. Effects of a combined essential fatty acid and conjugated linoleic acid abomasal infusion on metabolic and endocrine traits, including the somatotropic axis, in dairy cows. J Dairy Sci. 103:12069-12082. https://doi.org/10.3168/jds.2020-18569.

He M, Wu B, Ye W, Le DD, Sinclair AW, Padovano V, et al. 2020. Chloride channels regulate differentiation and barrier functions of the mammalian airway. Elife. 9:e53085. https://doi.org/10.7554/eLife.53085.

Healy LL, Cronin JG, Sheldon IM. 2014. Endometrial cells sense and react to tissue damage during infection of the bovine endometrium via interleukin 1. Sci Rep. 4:7060. https://doi.org/10.1038/srep07060.

Heinze G, Dunkler D. 2017. Five myths about variable selection. Transpl Int. 30:6-10. https://doi.org/10.1111/tri.12895.

Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE, Sheldon IM. 2006. Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology. 147(1):562-570. https://doi.org/10.1210/en.2005-1113.

Herrera-Camacho J, Soberano-Martinez A, Orozco Duran KE, Aguilar-Perez C, Ku-Vera JC. 2011. Effect of fatty acids on reproductive performance of ruminants. Pages 217–242 in Artificial Insemination in Farm Animals. M. Manafi, ed. Intech, Rijeka, Croatia. https://doi.org/10.5772/16938.

Hjortø GM, Larsen O, Steen A, Daugvilaite V, Berg C, Fares S, Hansen M, Ali S, Rosenkilde MM. 2016. Differential CCR7 Targeting in Dendritic Cells by Three Naturally Occurring CC-Chemokines. Front Immunol. 7:568. https://doi.org/10.3389/fimmu.2016.00568.

Hoelker M, Salilew-Wondim D, Drillich M, Christine GB, Ghanem N, Goetze L, Tesfaye D, Schellander K, Heuwieser W. 2012. Transcriptional response of the bovine endometrium and embryo to endometrial polymorphonuclear neutrophil infiltration as an indicator of subclinical inflammation of the uterine environment. Reprod Fertil Dev. 24:778-93. https://doi.org/10.1071/RD11171.

Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM, Morris DG. 2007. Amino acids in oviduct and uterine fluid and blood plasma during the

estrous cycle in the bovine. Mol Reprod Dev. 74(4):445-54. https://doi.org/10.1002/mrd.20607.

Hutchinson IA, Hennessy AA, Waters SM, Dewhurst RJ, Evans ACO, Lonergan P, Butler ST. 2012. Effect of supplementation with different fat sources on the mechanisms involved in reproductive performance in lactating dairy cattle. Theriogenology. 78(1):12–27. https://doi.org/10.1016/j.theriogenology.2011.12.031.

Hyttel P. 1985. The epithelium of uterine biopsies from cyclic dairy cattle: ultrastructure and endocytotic activity. Acta Anat. 123(2):93-100. https://doi.org/10.1159/000146046.

Inchaisri C, Jorritsma R, Vos PL, van der Weijden GC, Hogeveen H. 2010. Economic consequences of reproductive performance in dairy cattle. Theriogenology. 74(5):835-846. https://doi.org/10.1016/j.theriogenology.2010.04.008.

Iyer S. Characterizing Type I Interferon Mediated Inflammatory Responses During Acute and Chronic Infections. UCLA. 2013. https://escholarship.org/uc/item/769019n3. Accessed 08 Out 2021.

Jain A, Kaczanowska S, Davila E. 2014. IL-1 Receptor-Associated Kinase Signaling and Its Role in Inflammation, Cancer Progression, and Therapy Resistance. Front Immunol. 5:553. https://doi.org/10.3389/fimmu.2014.00553.

Jarry A, Bossard C, Bou-Hanna C, Masson D, Espaze E, Denis MG, Laboisse CL. 2008. Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. J Clin Invest. 118:1132-42. https://doi.org/10.1172/JCI32140.

Johnson H, Torres CG, Carvallo F, Duchens M, Peralta OA. 2015. Endometrial expression of selected transcripts in post-partum of primiparous Holstein cows with clinical and subclinical endometritis. Anim Reprod Sci. 156:34-9. https://doi.org/10.1016/j.anireprosci.2015.02.007.

Ju J, Li L, Xie J, Wu Y, Wu X, Li W. 2014. Toll-like receptor-4 pathway is required for the pathogenesis of human chronic endometritis. Exp Ther Med. 8(6):1896–1900. https://doi.org/10.3892/etm.2014.1990.

Kafi M, Tamadon A, Saeb M. 2015. The relationship between serum adiponectin and postpartum luteal activity in high-producing dairy cows. Theriogenology. 83(8):1264-71. https://doi.org/10.1016/j.theriogenology.2015.01.011.

Kang M, Jeong W, Bae H, Lim W, Bazer FW, Song G. 2018. Bifunctional role of ephrin A1-Eph system in stimulating cell proliferation and protecting cells from cell death through the attenuation of ER stress and inflammatory responses in bovine mammary epithelial cells. J Cell Physiol. 233:2560-2571. https://doi.org/10.1002/jcp.26131.

Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH. 2005a. A comparison of the cytobrush and uterine lavage techniques to evaluate endometrial cytology in clinically normal postpartum dairy cows. Can Vet J. 46(3):255-259.

Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH. 2005b. The effect of a single administration of cephapirin or cloprostenol on the reproductive performance of dairy cows with subclinical endometritis. Theriogenology. 63(3):818-30. https://doi.org/10.1016/j.theriogenology.2004.05.002.

Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH. 2004. Endometrial cytology and ultrasonography for the detection of subclinical

endometritis in postpartum dairy cows. Theriogenology. 62(1-2):9-23. https://doi.org/10.1016/j.theriogenology.2003.03.001.

Kasimanickam R, Kasimanickam V, Kastelic JP. 2014. Mucin 1 and cytokines mRNA in endometrium of dairy cows with post-partum uterine disease or repeat breeding. Theriogenology. 81:952-958.e2. https://doi.org/10.1016/j.theriogenology.2014.01.018.

Kasimanickam RK, Kasimanickam VR, Olsen JR, Jeffress EJ, Moore DA, Kastelic JP. 2013. Associations among serum pro- and anti-inflammatory cytokines, metabolic mediators, body condition, and uterine disease in postpartum dairy cows. Reprod Biol Endocrinol. 11:103. https://doi.org/10.1186/1477-7827-11-103.

Kaulin YA, De Santiago-Castillo JA, Rocha CA, Nadal MS, Rudy B, Covarrubias M. 2009. The dipeptidyl-peptidase-like protein DPP6 determines the unitary conductance of neuronal Kv4.2 channels. J Neurosci. 29(10):3242-51. https://doi.org/10.1523/JNEUROSCI.4767-08.2009.

Kehrli Jr ME, Goff JP, Stevens MG, Boone TC. 1991. Effects of granulocyte colony-stimulating factor administration to periparturient cows on neutrophils and bacterial shedding. J Dairy Sci. 74(8):2448-58. https://doi.org/10.3168/jds.S0022-0302(91)78420-8.

Kershaw EE, Flier JS. 2004. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab. 89(6):2548-56. https://doi.org/10.1210/jc.2004-0395.

Kikuchi K, Yanagawa Y, Onoé K. 2005. CCR7 ligand-enhanced phagocytosis of various antigens in mature dendritic cells-time course and antigen distribution different from phagocytosis in immature dendritic cells. Microbiol Immunol. 49:535-44. https://doi.org/10.1111/j.1348-0421.2005.tb03759.x.

Kim JJ, Kurita T, Bulun SE. 2013. Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. Endocr Rev. 34(1):130-62. https://doi.org/10.1210/er.2012-1043.

Kimura K, Goff JP, Canning P, Wang C, Roth JA. 2014. Effect of recombinant bovine granulocyte colony-stimulating factor covalently bound to polyethylene glycol injection on neutrophil number and function in periparturient dairy cows. J Dairy Sci. 97(8):4842-51. https://doi.org/10.3168/jds.2013-7242.

Klingberg F, Hinz B, White ES. 2013. The myofibroblast matrix: implications for tissue repair and fibrosis. J Pathol. 229:298-309. https://doi.org/10.1002/path.4104.

Kok A, van Knegsel ATM, van Middelaar CE, Engel B, Hogeveen H, Kemp B, de Boer IJM. 2017. Effect of dry period length on milk yield over multiple lactations. J Dairy Sci. 100:739-749. https://doi.org/10.3168/jds.2016-10963.

Kopitar-Jerala N. 2017. The Role of Interferons in Inflammation and Inflammasome Activation. Front Immunol. 8:873. https://doi.org/10.3389/fimmu.2017.00873.

Kovarik P, Castiglia V, Ivin M, Ebner F. 2016. Type I Interferons in Bacterial Infections: A Balancing Act. Front Immunol. 7:652. https://doi.org/10.3389/fimmu.2016.00652.

Krumm CS, Giesy SL, Caixeta LS, Butler WR, Sauerwein H, Kim JW, Boisclair YR. 2017. Effect of hormonal and energy-related factors on plasma adiponectin in transition dairy cows. J Dairy Sci. 100:9418-9427. https://doi.org/10.3168/jds.2017-13274.

Kumar AV, Katakam SK, Urbanowitz AK, Gotte M. 2015. Heparan sulphate as a regulator of leukocyte recruitment in inflammation. Current protein & peptide science. 16(1):77–86. https://doi.org/10.2174/1573402111666150213165054.

Kurita T, Young P, Brody JR, Lydon JP, O'Malley BW, Cunha GR. 1998. Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial cell deoxyribonucleic acid synthesis. Endocrinology. 139(11):4708-13. https://doi.org/10.1210/endo.139.11.6317.

Kurowska P, Mlyczyńska E, Barbe A, Mellouk N, Dupont J, Rak A. 2018. The adipokines in domestic animal reproduction: Expression and role in the regulation of ovarian function. In Payan-Carreira R (ed.) New Insights into Theriogenology. London: IntechOpen. https://doi.org/10.5772/intechopen.81035.

Lamming GE, Darwash AO. 1998. The use of milk progesterone profiles to characterize components of subfertility in milked dairy cows. Anim Reprod Sci. 52:175–190. https://doi.org/10.1016/s0378-4320(98)00099-2.

Lara E, Velásquez A, Cabezas J, Rivera N, Pacha P, Rodríguez-Alvarez L, Saravia F, Castro FO. 2017. Endometritis and In Vitro PGE2 Challenge Modify Properties of Cattle Endometrial Mesenchymal Stem Cells and Their Transcriptomic Profile. Stem Cells Int. 4297639. https://doi.org/10.1155/2017/4297639.

Lawlor L, Yang XB. 2019. Harnessing the HDAC-histone deacetylase enzymes, inhibitors and how these can be utilized in tissue engineering. Int J Oral Sci. 11:20. https://doi.org/10.1038/s41368-019-0053-2.

Lean IJ, Lucy MC, McNamara JP, Bradford BJ, Block E, Thomson JM, Morton JM, Celi P, Rabiee AR, Santos JE. 2016. Invited review: Recommendations for reporting intervention studies on reproductive performance in dairy cattle: Improving design, analysis, and interpretation of research on reproduction. J Dairy Sci. 99:1–17. https://doi.org/10.3168/jds.2015-9445.

LeBlanc SJ. 2008. Postpartum uterine disease and dairy herd reproductive performance: a review. Vet J. 176(1):102-14. https://doi.org/10.1016/j.tvjl.2007.12.019.

LeBlanc SJ. 2012. Interactions of metabolism, inflammation, and reproductive tract health in the postpartum period in dairy cattle. Reprod Domest Anim. 47(5):18-30. https://doi.org/10.1111/j.1439-0531.2012.02109.x.

LeBlanc SJ. 2014. Reproductive tract inflammatory disease in postpartum dairy cows. Animal. 8(1):54-63. https://doi.org/10.1017/S1751731114000524.

LeBlanc SJ. 2020. Review: Relationships between metabolism and neutrophil function in dairy cows in the peripartum period. Animal. 14(S1):s44-s54. https://doi.org/10.1017/S1751731119003227.

Lee MJ, Kim DH, Ryu JS, Ko AY, Ko JH, Kim MK, Wee WR, Khwarg SI, Oh JY. 2015. Topical TSG-6 Administration Protects the Ocular Surface in Two Mouse Models of Inflammation-Related Dry Eye. Investigative ophthalmology & visual science. 56(9):5175–5181. https://doi.org/10.1167/iovs.14-16307.

Lefebvre RC, Stock AE. 2012. Therapeutic efficiency of antibiotics and prostaglandin F2α in postpartum dairy cows with clinical endometritis: an evidence-based evaluation. Vet Clin North Am Food Anim Pract. 28(1):79-96. https://doi.org/10.1016/j.cvfa.2012.01.002.

Lenga Ma Bonda W, Lavergne M, Vasseur V, Brisson L, Roger S, Legras A, et al. 2021. Kallikrein-related peptidase 5 contributes to the remodeling and repair of bronchial epithelium. FASEB J. 35(10):e21838. https://doi.org/10.1096/fj.202002649R.

Leoni G, Nusrat A. 2016. Annexin A1: shifting the balance towards resolution and repair. Biological chemistry. 397(10):971–979. https://doi.org/10.1515/hsz-2016-0180.

Leroy JLMR, Sturmey RG, Van Hoeck V, De Bie J, McKeegan PJ, Bols PEJ. 2014. Dietary fat supplementation and the consequences for oocyte and embryo quality: hype or significant benefit for dairy cow reproduction? Reprod Domest Anim. 49:353-361. https://doi.org/10.1111/rda.12308.

Lewis GS. 2003. Steroidal regulation of uterine resistance to bacterial infection in livestock. Reprod Biol Endocrinol. 1:117. https://doi.org/10.1186/1477-7827-1-117.

Lewis GS. 2004. Steroidal regulation of uterine immune defenses. Anim. Reprod. Sci. 82-83:281–294. https://doi.org/10.1016/j.anireprosci.2004.04.026.

Li MO, Flavell RA. 2008a. Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. Immunity. 28:468-76. https://doi.org/10.1016/j.immuni.2008.03.003.

Li MO, Flavell RA. 2008b. TGF-beta: a master of all T cell trades. Cell. 134:392-404. https://doi.org/10.1016/j.cell.2008.07.025.

Li Q, Kannan A, DeMayo FJ, Lydon JP, Cooke PS, Yamagishi H, Srivastava D, Bagchi MK, Bagchi IC. 2011. The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. Science. 331(6019):912-6. https://doi.org/10.1126/science.1197454.

Li QF, Hao H, Tu WS, Guo N, Zhou XY. 2020. Maresins: anti-inflammatory pro-resolving mediators with therapeutic potential. Eur Rev Med Pharmacol Sci. 24(13):7442–7453. https://doi.org/10.26355/eurrev_202007_21913.

Li R, Tan S, Yu M, Jundt MC, Zhang S, Wu M. 2015. Annexin A2 Regulates Autophagy in Pseudomonas aeruginosa Infection through the Akt1-mTOR-ULK1/2 Signaling Pathway. J Immunol. 195(8):3901-11. https://doi.org/10.4049/jimmunol.1500967.

Lim W, Bae H, Bazer FW, Kim SM, Song G. 2018. C-C motif chemokine ligand 2 regulates lps-induced inflammation and ER stress to enhance proliferation of bovine endometrial epithelial cells. J Cell Physiol. 233(4):3141–3151. https://doi.org/10.1002/jcp.26151.

Lim W, Bae H, Bazer FW, Song G. 2019. Ephrin A1 promotes proliferation of bovine endometrial cells with abundant expression of proliferating cell nuclear antigen and cyclin D1 changing the cell population at each stage of the cell cycle. J Cell Physiol. 234:4864-4873. https://doi.org/10.1002/jcp.27275.

Lin A, Yin J, Cheng C, Yang Z, Yang H. 2017. Decreased expression of FOXA2 promotes eutopic endometrial cell proliferation and migration in patients with endometriosis. Reprod Biomed Online. 36(2):181-187. https://doi.org/10.1016/j.rbmo.2017.11.001.

Liu X, Sun S, Liu D. 2020. IL-17D: A Less Studied Cytokine of IL-17 Family. Int Arch Allergy Immunol. 181:618-623. https://doi.org/10.1159/000508255.

Liu Y, Deng B, Zhao Y, Xie S, Nie R. 2013. Differentiated markers in undifferentiated cells: expression of smooth muscle contractile proteins in multipotent bone marrow mesenchymal stem cells. Dev Growth Differ. 55(5):591-605. https://doi.org/10.1111/dgd.12052.

López-Albors O, Llamas-López PJ, Ortuño JÁ, Latorre R, García-Vázquez FA. 2021. In vivo measurement of pH and CO2 levels in the uterus of sows through the estrous cycle and after insemination. Sci Rep. 11(1):3194. https://doi.org/10.1038/s41598-021-82620-7.

Lopreiato V, Mezzetti M, Cattaneo L, Ferronato G, Minuti A, Trevisi E. 2020. Role of nutraceuticals during the transition period of dairy cows: a review. J Anim Sci Biotechnol. 11:96. https://doi.org/10.1186/s40104-020-00501-x.

Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQ, Hansen PJ. 2009. Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced in vitro. Endocrinology. 150(11):5046-54. https://doi.org/10.1210/en.2009-0481.

Love MI, Anders S, Kim V, Huber W. 2015. RNA-Seq workflow: gene-level exploratory analysis and differential expression. F1000Res. 4:1070. https://doi.org/10.12688/f1000research.7035.1.

Lu H, Ju DD, Yang GD, Zhu LY, Yang XM, Li J, Song WW, Wang JH, Zhang CC, Zhang ZG et al. 2019. Targeting cancer stem cell signature gene SMOC-2 Overcomes chemoresistance and inhibits cell proliferation of endometrial carcinoma. EBioMedicine. 40:276-289. https://doi.org/10.1016/j.ebiom.2018.12.044.

Lukens JR, Barr MJ, Chaplin DD, Chi H, Kanneganti TD. 2012. Inflammasome-derived IL-1β regulates the production of GM-CSF by CD4(+) T cells and γδ T cells. J Immunol. 188:3107-3115. https://doi.org/10.4049/jimmunol.1103308.

Maderna P, Yona S, Perretti M, Godson C. 2005. Modulation of phagocytosis of apoptotic neutrophils by supernatant from dexamethasone-treated macrophages and annexin-derived peptide Ac(2-26). J Immunol. 174(6):3727-33. https://doi.org/10.4049/jimmunol.174.6.3727.

Madoz LV, Giuliodori MJ, Migliorisi AL, Jaureguiberry M, de la Sota RL. 2014. Endometrial cytology, biopsy, and bacteriology for the diagnosis of subclinical endometritis in grazing dairy cows. J Dairy Sci. 97:195-201. https://doi.org/10.3168/jds.2013-6836.

Madoz LV, Rabaglino MB, Migliorisi AL, Jaureguiberry M, Perez Wallace S, Lorenti N, Domínguez G, Giuliodori MJ, de la Sota RL. 2020. Association between progesterone concentration and endometrial gene expression in dairy cows. Domest Anim Endocrinol. 74:106481. https://doi.org/10.1016/j.domaniend.2020.106481.

Maillard V, Uzbekova S, Guignot F, Perreau C, Ramé C, Coyral-Castel S, Dupont J. 2010. Effect of adiponectin on bovine granulosa cell steroidogenesis, oocyte maturation and embryo development. Reprod Biol Endocrinol. 8:23. https://doi.org/10.1186/1477-7827-8-23.

Makrigiannakis A, Coukos G, Mantani A, Prokopakis P, Trew G, Margara R, Winston R, White J. 2001. Expression of Wilms' tumor suppressor gene (WT1) in human endometrium: regulation through decidual differentiation. J Clin Endocrinol Metab. 86(12):5964-72. https://doi.org/10.1210/jcem.86.12.8074.

Mallikarjunappa S, Shandilya UK, Sharma A, Lamers K, Bissonnette N, Karrow NA, Meade KG. 2020. Functional analysis of bovine interleukin-10 receptor alpha in response to Mycobacterium avium subsp. paratuberculosis lysate using CRISPR/Cas9. BMC Genet. 21:121. https://doi.org/10.1186/s12863-020-00925-4.

Mancuso P. 2016. The role of adipokines in chronic inflammation. Immunotargets Ther. 5:47-56. https://doi.org/10.2147/ITT.S73223.

Mann S, Urh C, Sauerwein H, Wakshlag JJ, Leal Yepes FA, Overton TR, Nydam DV. 2017. Short communication: The association of adiponectin and leptin concentrations with prepartum dietary energy supply, parity, body condition, and postpartum hyperketonemia in transition dairy cows. J Dairy Sci. 101:806–811. https://doi.org/10.3168/jds.2017-13752.

Manriquez D, Chen L, Melendez P, Pinedo P. 2019. The effect of an organic rumen-protected fat supplement on performance, metabolic status, and health of dairy cows. BMC Vet Res. 15:450. https://doi.org/10.1186/s12917-019-2199-8.

Mariani F, Roncucci L. 2015. Chemerin/chemR23 axis in inflammation onset and resolution. Inflamm Res. 64(2):85-95. https://doi.org/10.1007/s00011-014-0792-7.

Marsland BJ, Bättig P, Bauer M, Ruedl C, Lässing U, Beerli RR, Dietmeier K, Ivanova L, Pfister T, Vogt L, et al. 2005. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. Immunity. 22:493-505. https://doi.org/10.1016/j.immuni.2005.02.010.

Martin JL, Vonnahme KA, Adams DC, Lardy GP, Funston RN. 2007. Effects of dam nutrition on growth and reproductive performance of heifer calves. J Anim Sci. 85:841-847. https://doi.org/10.2527/jas.2006-337.

Martinez N, Risco CA, Lima FS, Bisinotto RS, Greco LF, Ribeiro ES, Maunsell F, Galvão K, Santos JE. 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. J Dairy Sci. 95:7158-72. https://doi.org/10.3168/jds.2012-5812.

Masterton R, Armstrong EM, More IA. 1975. The cyclical variation in the percentage of ciliated cells in the normal human endometrium. J Reprod Fertil. 42(3):537-40. https://doi.org/10.1530/jrf.0.0420537.

Mateus L, Lopes da Costa L, Bernardo F, Robalo Silva J. 2002. Influence of Puerperal Uterine Infection on Uterine Involution and Postpartum Ovarian Activity in Dairy Cows. Reprod Domest Anim. 37(1):31-5. https://doi.org/10.1046/j.1439-0531.2002.00317.x.

Mattern A, Zellmann T, Beck-Sickinger AG. 2014. Processing, signaling, and physiological function of chemerin. IUBMB Life. 66(1):19-26. https://doi.org/10.1002/iub.1242.

Mayasari N, Chen J, Ferrari A, Bruckmaier RM, Kemp B, Parmentier HK, van Knegsel ATM, Trevisi E. 2017. Effects of dry period length and dietary energy source on inflammatory biomarkers and oxidative stress in dairy cows. J Dairy Sci. 100(6):4961-4975. https://doi.org/10.3168/jds.2016-11857.

McDougall S, Hussein H, Aberdein D, Buckle K, Roche J, Burke C, Mitchell M, Meier S. 2011. Relationships between cytology, bacteriology and vaginal discharge scores and reproductive performance in dairy cattle. Theriogenology. 76(2):229-40. https://doi.org/10.1016/j.theriogenology.2010.12.024.

McDougall S, LeBlanc SJ, Heiser A. 2017. Effect of prepartum energy balance on neutrophil function following pegbovigrastim treatment in periparturient cows. J Dairy Sci. 100(9):7478-7492. https://doi.org/10.3168/jds.2017-12786.

McNamara S, Butler T, Ryan DP, Mee JF, Dillon P, O'Mara PF, Butler ST, Anglesey D, Rath M, Murphy JJ. 2003. Effect of offering rumen-protected fat supplements on fertility and performance in spring-calving Holstein–Friesian cows. Anim Reprod Sci. 79:45–56. https://doi.org/10.1016/s0378-4320(03)00111-8.

Mellouk N, Ramé C, Diot M, Briant E, Touzé JL, Guillaume D, Froment P, Dupont J. 2019. Possible involvement of the RARRES2/CMKLR1-system in metabolic and reproductive parameters in Holstein dairy cows. Reprod Biol Endocrinol. 17(1):25. https://doi.org/10.1186/s12958-019-0467-x.

Mellouk N, Rame C, Naquin D, Jaszczyszyn Y, Touzé JL, Briant E, Guillaume D, Ntallaris T, Humblot P, Dupont J. 2019. Impact of the severity of negative energy balance on

gene expression in the subcutaneous adipose tissue of periparturient primiparous Holstein dairy cows: Identification of potential novel metabolic signals for the reproductive system. PloS one. 14:e0222954. https://doi.org/10.1371/journal.pone.0222954.

Mellouk N, Ramé C, Touzé JL, Briant E, Ma L, Guillaume D, Lomet D, Caraty A, Ntallaris T, Humblot P, Dupont J. 2017. Involvement of plasma adipokines in metabolic and reproductive parameters in Holstein dairy cows fed with diets with differing energy levels. J Dairy Sci. 100:8518–8533. https://doi.org/10.3168/jds.2017-12657.

Miller BA, Brewer A, Nanni P, Lim JJ, Callanan JJ, Grossmann J, Kunz L, de Almeida AM, Meade KG, Chapwanya A. 2019. Characterization of circulating plasma proteins in dairy cows with cytological endometritis. J Proteomics. 205:103421. https://doi.org/10.1016/j.jprot.2019.103421.

Mitchell GB, Albright BN, Caswell JL. 2003. Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. Infect Immun. 71(4):1643-9. https://doi.org/10.1128/IAI.71.4.1643-1649.2003.

Mitko K, Ulbrich SE, Wenigerkind H, Sinowatz F, Blum H, Wolf E, Bauersachs S. 2008. Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. Reprod. 135(2):225-240. https://doi.org/10.1530/REP-07-0415.

Mittal M, Tiruppathi C, Nepal S, Zhao YY, Grzych D, Soni D, Prockop DJ, Malik AB. 2016. TNFα-stimulated gene-6 (TSG6) activates macrophage phenotype transition to prevent inflammatory lung injury. Proc Natl Acad Sci U S A. 113:E8151-E8158. https://doi.org/10.1073/pnas.1614935113.

Moallem U, Lehrer H, Livshits L, Zachut M. 2020. The effects of omega-3 α-linolenic acid from flaxseed oil supplemented to high-yielding dairy cows on production, health, and fertility. Livest Sci. 242 104302. https://doi.org/10.1016/j.livsci.2020.104302.

Moallem U, Shafran A, Zachut M, Dekel I, Arieli A. 2013. Dietary alpha-linolenic acid (ALA) from flaxseed oil improved folliculogenesis and IVF performance in dairy cows, similar to eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids from fish oil. Reprod. 146:603–614. https://doi.org/10.1530/REP-13-0244.

Moallem U. 2009. The effects of extruded flaxseed supplementation to high-yielding dairy cows on milk production and milk fatty acid composition. Anim Feed Sci Technol. 152:232-242. https://doi.org/10.1016/j.anifeedsci.2009.04.018.

Moallem U. 2018. Invited review: Roles of dietary n-3 fatty acids in performance, milk fat composition, and reproductive and immune systems in dairy cattle. J Dairy Sci. 101:8641–8661. https://doi.org/10.3168/jds.2018-14772.

Moore SG, Ericsson AC, Behura SK, Lamberson WR, Evans TJ, McCabe MS, et al. 2019. Concurrent and long-term associations between the endometrial microbiota and endometrial transcriptome in postpartum dairy cows. BMC Genomics. 20(1):405. https://doi.org/10.1186/s12864-019-5797-8.

Moore SG, Ericsson AC, Poock SE, Melendez P, Lucy MC. 2017. Hot topic: 16S rRNA gene sequencing reveals the microbiome of the virgin and pregnant bovine uterus. J Dairy Sci. 100:4953–4960. https://doi.org/10.3168/jds.2017-12592.

Mosley SA, Mosley EE, Hatch B, Szasz JI, Corato A, Zacharias N, Howes D, McGuire MA. 2007. Effect of varying levels of fatty acids from palm oil on feed intake and milk production in Holstein cows. J Dairy Sci. 90:987–993. https://doi.org/10.3168/jds.S0022-0302(07)71583-7.

Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura SL. 2002. The integrin alpha(v)beta8 mediates epithelial

homeostasis through MT1-MMP-dependent activation of TGF-beta1. J Cell Biol. 157:493-507. https://doi.org/10.1083/jcb.200109100.

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D. 1999. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell. 96:319-28. https://doi.org/10.1016/s0092-8674(00)80545-0.

Nagano K. 2019. R-spondin signaling as a pivotal regulator of tissue development and homeostasis. Jpn Dent Sci Rev. 55:80-87. https://doi.org/10.1016/j.jdsr.2019.03.001.

Nakamura M, Gabazza EC, Imoto I, Yano Y, Taguchi O, Horiki N, et al. 2005. Anti-inflammatory effect of activated protein C in gastric epithelial cells. J Thromb Haemost. 3(12):2721-9. https://doi.org/10.1111/j.1538-7836.2005.01635.x.

Nauroy P, Nyström A. Kallikreins: 2019. Essential epidermal messengers for regulation of the skin microenvironment during homeostasis, repair and disease. Matrix Biol Plus. 6-7:100019. https://doi.org/10.1016/j.mbplus.2019.100019.

Neave HW, Lomb J, von Keyserlingk MAG, Behnam-Shabahang A, Weary DM. 2017. Parity differences in the behavior of transition dairy cows. J Dairy Sci. 100:548-561. https://doi.org/10.3168/jds.2016-10987.

Nebert DW, Wikvall K, Miller WL. 2013 Human cytochromes P450 in health and disease. Phil Trans R Soc B. 368:20120431. http://dx.doi.org/10.1098/rstb.2012.0431.

Neupane M, Geary TW, Kiser JN, Burns GW, Hansen PJ, Spencer TE, et al. 2017. Loci and pathways associated with uterine capacity for pregnancy and fertility in beef cattle. PLoS One. 12(12):e0188997. https://doi.org/10.1371/journal.pone.0188997.

Neveu C, Baurhoo B, Mustafa A. 2013. Effect of feeding extruded flaxseed with different forage:concentrate ratios on the performance of dairy cows. J Dairy Sci. 96:3886–3894. https://doi.org/10.3168/jds.2012-6189.

Oguejiofor CF, Cheng Z, Abudureyimu A, Fouladi-Nashta AA, Wathes DC. 2015. Global transcriptomic profiling of bovine endometrial immune response in vitro. I. Effect of lipopolysaccharide on innate immunity. Biol Reprod. 93:100. https://doi.org/10.1095/biolreprod.115.128868.

Okeke EB, Uzonna JE. 2019. The Pivotal Role of Regulatory T Cells in the Regulation of Innate Immune Cells. Front. Immunol. 10:680. https://doi.org/10.3389/fimmu.2019.00680.

Oliveira LM, Teixeira F, Sato MN. 2018. Impact of Retinoic Acid on Immune Cells and Inflammatory Diseases. Mediators Inflamm. 3067126. https://doi.org/10.1155/2018/3067126.

Olson JD, Ball L, Mortimer RG, Farin PW, Adney WS, Huffman EM. 1984. Aspects of bacteriology and endocrinology of cows with pyometra and retained fetal membranes. Am J Vet Res. 45(11):2251-5.

Opgenorth J, Sordillo LM, VandeHaar MJ. 2020. Colostrum supplementation with n-3 fatty acids and α-tocopherol alters plasma polyunsaturated fatty acid profile and decreases an indicator of oxidative stress in newborn calves. J Dairy Sci. 103:3545-3553. https://doi.org/10.3168/jds.2019-17380.

Ørbo A, Moe BT, Grønaas H, Paulssen RH. 2009. Early effects of high concentrations of progesterone and mifepristone A gene expression study of endometrial cancer cells (Ishikawa). J Steroid Biochem Mol Biol. 113(1-2):139-49. https://doi.org/10.1016/j.jsbmb.2008.12.003.

Otto JR, Freeman MJ, Malau-Aduli BS, Nichols PD, Lane PA, Malau-Aduli AEO. 2014. Reproduction and Fertility Parameters of Dairy Cows Supplemented with Omega-3 Fatty Acid-rich Canola Oil. Annu Res Rev Biol. 4:1611-1636. https://doi.org/10.9734/ARRB/2014/7689.

Owusu-Akyaw A, Krishnamoorthy K, Goldsmith LT, Morelli SS. 2019. The role of mesenchymal-epithelial transition in endometrial function. Hum Reprod Update. 25(1):114-133. https://doi.org/10.1093/humupd/dmy035.

Palmquist DL, Jenkins TC. 2017. A 100-Year Review: Fat feeding of dairy cows. J Dairy Sci. 100(12):10061-10077. https://doi.org/10.3168/jds.2017-12924.

Parker-Duffen JL, Nakamura K, Silver M, Zuriaga MA, MacLauchlan S, Aprahamian TR, Walsh K. 2014. Divergent roles for adiponectin receptor 1 (AdipoR1) and AdipoR2 in mediating revascularization and metabolic dysfunction in vivo. J Biol Chem. 289(23):16200-13. https://doi.org/10.1074/jbc.M114.548115.

Pascottini OB, Hostens M, Dini P, Vandepitte J, Ducatelle R, Opsomer G. 2016. Comparison between cytology and histopathology to evaluate subclinical endometritis in dairy cows. Theriogenology. 86(6):1550-1556. https://doi.org/10.1016/j.theriogenology.2016.05.014.

Pascottini OB, Hostens M, Sys P, Vercauteren P, Opsomer G. 2017.Cytological endometritis at artificial insemination in dairy cows: Prevalence and effect on pregnancy outcome. J Dairy Sci. 100(1):588-597. https://doi.org/10.3168/jds.2016-11529.

Pascottini OB, LeBlanc SJ. 2020. Modulation of immune function in the bovine uterus peripartum. Theriogenology. 150:193-200. https://doi.org/10.1016/j.theriogenology.2020.01.042.

Pascottini OB, Van Schyndel SJ, Spricigo JFW, Carvalho MR, Mion B, Ribeiro ES, LeBlanc SJ. 2020. Effect of anti-inflammatory treatment on systemic inflammation, immune function, and endometrial health in postpartum dairy cows. Sci Rep. 10(1):5236. https://doi.org/10.1038/s41598-020-62103-x.

Pascottini OB. 2016. Subclinical endometritis in dairy cattle: A practical approach [PhD Thesis]. Ghent: Ghent University. https://doi.org/10.13140/RG.2.2.13577.85604.

Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martín JD, Flores C, Mantecón AG, Story ME, Pinto FM. 2003. Tachykinins and tachykinin receptors in human uterus. Br J Pharmacol. 139(3):523-32. https://doi.org/10.1038/sj.bjp.0705279.

Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. 2017. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 14(4):417-419. https://doi.org/10.1038/nmeth.4197.

Pavone ME, Dyson M, Reirstad S, Pearson E, Ishikawa H, Cheng YH, Bulun SE. 2011. Endometriosis expresses a molecular pattern consistent with decreased retinoid uptake, metabolism and action. Hum Reprod. 26(8):2157-64. https://doi.org/10.1093/humrep/der172.

Penner GB, Oba M. 2009. Increasing dietary sugar concentration may improve dry matter intake, ruminal fermentation, and productivity of dairy cows in the postpartum phase of the transition period. J Dairy Sci. 92:3341–3353. https://doi.org/10.3168/jds.2008-1977.

Pereira G, Bexiga R, Silva JC, Silva E, Ramé C, Dupont J, Guo Y, Humblot P, Lopes-da-Costa L. 2020. Adipokines as biomarkers of postpartum subclinical endometritis in dairy cows. Reprod. 160:417-430. https://doi.org/10.1530/REP-20-0183.

Pereira G, Guo Y, Silva E, Bevilacqua C, Charpigny G, Lopes-da-Costa L, Humblot P. 2022. Progesterone differentially affects the transcriptomic profiles of cow endometrial cell types. BMC Genomics 23:82. https://doi.org/10.1186/s12864-022-08323-z.

Petit HV, Palin MF, Doepel L. 2007. Hepatic lipid metabolism in transition dairy cows fed flaxseed. J Dairy Sci. 90:4780–4792. https://doi.org/10.3168/jds.2007-0066.

Piantoni P, Lock AL, Allen MS. 2013. Palmitic acid in-creased yields of milk and milk fat and nutrient digestibility across production level of lactating cows. J Dairy Sci. 96:7143–7154. https://doi.org/10.3168/jds.2013-6680.

Piras C, Guo Y, Soggiu A, Chanrot M, Greco V, Urbani A, Charpigny G, Bonizzi L, Roncada P, Humblot P. 2017. Changes in protein expression profiles in bovine endometrial epithelial cells exposed to E. coli LPS challenge. Mol Biosyst. 13(2):392-405. https://doi.org/10.1039/c6mb00723f.

Priest NV, McDougall S, Burke CR, Roche JR, Mitchell M, McLeod KL, Greenwood SL, Meier S. 2013. The responsiveness of subclinical endometritis to a nonsteroidal antiinflammatory drug in pasture-grazed dairy cows. J Dairy Sci. 96(7):4323-32. https://doi.org/10.3168/jds.2012-6266.

Putman AK, Brown JL, Gandy JC, Abuelo A, Sordillo LM. 2019. Oxylipid profiles of dairy cattle vary throughout the transition into early mammary gland involution. J Dairy Sci. 102(3):2481-2491. https://doi.org/10.3168/jds.2018-15158.

Qi X, Lei M, Qin L, Xie M, Zhao D, Wang J. 2016. Endogenous TWEAK is critical for regulating the function of mouse uterine natural killer cells in an immunological model of pregnancy loss. Immunology. 148:70-82. https://doi.org/10.1111/imm.12588.

Qi Y, Jiang D, Sindrilaru A, Stegemann A, Schatz S, Treiber N, Rojewski M, Schrezenmeier H, Vander Beken S, Wlaschek M, et al. 2014. TSG-6 released from intradermally injected mesenchymal stem cells accelerates wound healing and reduces tissue fibrosis in murine full-thickness skin wounds. The Journal of investigative dermatology. 134(2):526–537. https://doi.org/10.1038/jid.2013.328.

Quade BN, Parker MD, Occhipinti R. 2021. The therapeutic importance of acid-base balance. Biochem Pharmacol. 183:114278. https://doi.org/10.1016/j.bcp.2020.114278.

Queckbörner S, Syk Lundberg E, Gemzell-Danielsson K, Davies LC. 2020. Endometrial stromal cells exhibit a distinct phenotypic and immunomodulatory profile. Stem Cell Res Ther. 11(1):15. https://doi.org/10.1186/s13287-019-1496-2.

Queckbörner S, von Grothusen C, Boggavarapu NR, Francis RM, Davies LC, Gemzell-Danielsson K. 2021. Stromal Heterogeneity in the Human Proliferative Endometrium-A Single-Cell RNA Sequencing Study. J Pers Med. 11(6):448. https://doi.org/10.3390/jpm11060448.

Raboisson D, Mounié M, Maigné E. 2014. Diseases, reproductive performance, and changes in milk production associated with subclinical ketosis in dairy cows: A meta-analysis and review. J Dairy Sci. 97:7547–7563. https://doi.org/10.3168/jds.2014-8237.

Rajasagi NK, Reddy PB, Suryawanshi A, Mulik S, Gjorstrup P, Rouse BT. 2011. Controlling herpes simplex virus-induced ocular inflammatory lesions with the lipid-derived mediator resolvin E1. Journal of immunology. 186(3):1735-1746. https://doi.org/10.4049/jimmunol.1003456.

Raliou M, Dembélé D, Düvel A, Bolifraud P, Aubert J, Mary-Huard T, Rocha D, Piumi F, Mockly S, Heppelmann M et al. 2019. Subclinical endometritis in dairy cattle is associated

with distinct mRNA expression patterns in blood and endometrium. PLoS One. 14(8):e0220244. https://doi.org/10.1371/journal.pone.0220244.

Recuero S, Sánchez JM, Mateo-Otero Y, Bagés-Arnal S, McDonald M, Behura SK, et al. 2020. Mating to Intact, but Not Vasectomized, Males Elicits Changes in the Endometrial Transcriptome: Insights From the Bovine Model. Front Cell Dev Biol. 8:547. https://doi.org/10.3389/fcell.2020.00547.

Reverchon M, Bertoldo MJ, Ramé C, Froment P, Dupont J. 2014b. CHEMERIN (RARRES2) decreases in vitro granulosa cell steroidogenesis and blocks oocyte meiotic progression in bovine species. Biol Reprod. 90(5):102. https://doi.org/10.1095/biolreprod.113.117044.

Reverchon M, Ramé C, Bertoldo M, Dupont J. 2014a. Adipokines and the female reproductive tract. Int J Endocrinol. 2014:232454. https://doi.org/10.1155/2014/232454.

Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, Cohen J, Findlay D, Gyssens I, Heuer OE, et al. 2015. The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect. 6:22-9. https://doi.org/10.1016/j.nmni.2015.02.007.

Rockey DC, Weymouth N, Shi Z. 2013. Smooth muscle α actin (Acta2) and myofibroblast function during hepatic wound healing. PLoS One. 8:e77166. https://doi.org/10.1371/journal.pone.0077166.

Rodney RM, Celi P, Scott W, Breinhild K, Lean IJ. 2015. Effects of dietary fat on fertility of dairy cattle: A meta-analysis and meta-regression. J Dairy Sci. 98:5601-5620. https://doi.org/10.3168/jds.2015-9528.

Rourke JL, Dranse HJ, Sinal CJ. 2013. Towards an integrative approach to understanding the role of chemerin in human health and disease. Obes Rev. 14(3):245-62. https://doi.org/10.1111/obr.12009.

Rubel CA, Wu SP, Lin L, Wang T, Lanz RB, Li X, Kommagani R, Franco HL, Camper SA, Tong Q et al. 2016. A Gata2-Dependent Transcription Network Regulates Uterine Progesterone Responsiveness and Endometrial Function. Cell Rep. 17(5):1414-1425. https://doi.org/10.1016/j.celrep.2016.09.093.

Sá Filho MF, Gonella-Diaza AM, Sponchiado M, Mendanha MF, Pugliesi G, Ramos RS, Andrade SCS, Gasparin G, Coutinho LL, Goissis MD et al. 2017. Impact of hormonal modulation at proestrus on ovarian responses and uterine gene expression of suckled anestrous beef cows. J Anim Sci Biotechnol. 8:79. https://doi.org/10.1186/s40104-017-0211-3.

Saini RK, Keum YS. 2018. Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. Life Sci. 203:255-267. https://doi.org/10.1016/j.lfs.2018.04.049.

Saito-Sasaki N, Sawada Y, Mashima E, Yamaguchi T, Ohmori S, Yoshioka H, Haruyama S, Okada E, Nakamura M. 2018. Maresin-1 suppresses imiquimod-induced skin inflammation by regulating IL-23 receptor expression. Sci. Rep. 8(1):5522. https://doi.org/10.1038/s41598-018-23623-9.

Sala E, Genua M, Petti L, Anselmo A, Arena V, Cibella J, Zanotti L, D'Alessio S, Scaldaferri F, Luca G, et al. 2015. Mesenchymal Stem Cells Reduce Colitis in Mice via Release of TSG6, Independently of Their Localization to the Intestine. Gastroenterology. 149(1):163–176.e20. https://doi.org/10.1053/j.gastro.2015.03.013.

Salilew-Wondim D, Ibrahim S, Gebremedhn S, Tesfaye D, Heppelmann M, Bollwein H, Pfarrer C, Tholen E, Neuhoff C, Schellander K, Hoelker M. 2016. Clinical and subclinical

endometritis induced alterations in bovine endometrial transcriptome and miRNome profile. BMC Genomics. 17:218. https://doi.org/10.1186/s12864-016-2513-9.

Sammarco G, Shalaby M, Elangovan S, Petti L, Roda G, Restelli S, Arena V, Ungaro F, Fiorino G, Day AJ, D'Alessio S, Vetrano S. 2019. Hyaluronan Accelerates Intestinal Mucosal Healing through Interaction with TSG-6. Cells. 8(9):1074. https://doi.org/10.3390/cells8091074.

Sánchez MA, García P, Menéndez S, Sánchez B, González M, Flores JM. 2002. Fibroblastic growth factor receptor (FGF-R) expression during uterine involution in goat. Anim Reprod Sci. 69:25-35. https://doi.org/10.1016/s0378-4320(01)00169-5.

Saut JP, Healey GD, Borges AM, Sheldon IM. 2014. Ovarian steroids do not affect bovine endometrial cytokine or chemokine responses to Escherichia coli or LPS in vitro. Reprod. 148(6):593–606. https://doi.org/10.1530/REP-14-0230.

Scaravaggi I, Borel N, Romer R, Imboden I, Ulbrich SE, Zeng S, Bollwein H, Bauersachs S. 2018. Cell type-specific endometrial transcriptome changes during initial recognition of pregnancy in the mare. Reprod Fertil Dev. 31(3):496-508. https://doi.org/10.1071/RD18144.

Schabmeyer ST, Kneidl AM, Schneider JK, Kirsch S, Zablotski Y, Petzl W, Weber F, Zerbe H, Meyerholz MM. 2021. Concentration-Dependent Type 1 Interferon-Induced Regulation of MX1 and FABP3 in Bovine Endometrial Explants. Animals. 11:262. https://doi.org/10.3390/ani11020262.

Schett G, Neurath MF. 2018. Resolution of chronic inflammatory disease: universal and tissue-specific concepts. Nat Commun. 9:3261. https://doi.org/10.1038/s41467-018-05800-6.

Schmitz G, Ecker J. 2008. The opposing effects of n-3 and n-6 fatty acids. Progress in lipid research. 47(2):147–155. https://doi.org/10.1016/j.plipres.2007.12.004.

Senosy WS, Izaike Y, Osawa T. 2012. Influences of metabolic traits on subclinical endometritis at different intervals postpartum in high milking cows. Reprod Domest Anim. 47(4):666-74. https://doi.org/10.1111/j.1439-0531.2011.01941.x.

Seo H, Choi Y, Shim J, Choi Y, Ka H. 2012. Regulatory mechanism for expression of IL1B receptors in the uterine endometrium and effects of IL1B on prostaglandin synthetic enzymes during the implantation period in pigs. Biol Reprod. 87:31. https://doi.org/10.1095/biolreprod.112.099051.

Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, O'Neill LA, Perretti M, Rossi AG, Wallace JL. 2007. Resolution of inflammation: state of the art, definitions and terms. FASEB Journal. 21(2):325-32. https://doi.org/10.1096/fj.06-7227rev.

Serhan CN, Levy BD. 2018. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators. J Clin Invest. 128(7):2657-2669. https://doi.org/10.1172/JCI97943.

Serhan CN, Savill J. 2005. Resolution of inflammation: the beginning programs the end. Nature immunology. 6(12):1191-1197. https://doi.org/10.1038/ni1276.

Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. 2011. Histone deacetylases as regulators of inflammation and immunity. Trends Immunol. 32:335-43. https://doi.org/10.1016/j.it.2011.04.001.

Shaw JL, Petraki C, Watson C, Bocking A, Diamandis EP. 2008. Role of tissue kallikrein-related peptidases in cervical mucus remodeling and host defense. Biol Chem. 389(12):1513-22. https://doi.org/10.1515/BC.2008.171.

Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ. 2009a. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol. Reprod. 81:1025-32. https://doi.org/10.1095/biolreprod.109.077370.

Sheldon IM, Cronin JG, Bromfield JJ. 2019. Tolerance and innate immunity shape the development of postpartum uterine disease and the impact of endometritis in dairy cattle. Annu Rev Anim Biosci. 7:361-384. https://doi.org/10.1146/annurev-animal-020518-115227.

Sheldon IM, Cronin JG, Pospiech M, Turner ML. 2018. Symposium review: Mechanisms linking metabolic stress with innate immunity in the endometrium. J Dairy Sci. 101(4):3655-3664. https://doi.org/10.3168/jds.2017-13135.

Sheldon IM, Lewis GS, LeBlanc S, Gilbert RO. 2006. Defining postpartum uterine disease in cattle. Theriogenology. 65(8):1516-1530. https://doi.org/10.1016/j.theriogenology.2005.08.021.

Sheldon IM, Molinari PCC, Ormsby TJR, Bromfield JJ. 2020. Preventing postpartum uterine disease in dairy cattle depends on avoiding, tolerating and resisting pathogenic bacteria. Theriogenology. 150:158-165. https://doi.org/10.1016/j.theriogenology.2020.01.017.

Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H. 2002. Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reprod. 123:837–845. https://doi.org/10.1530/rep.0.1230837.

Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE. 2009b. Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. Reprod Domest Anim. 44(3):1-9. https://doi.org/10.1111/j.1439-0531.2009.01465.x.

Sheldon IM, Roberts MH. 2010. Toll-like receptor 4 mediates the response of epithelial and stromal cells to lipopolysaccharide in the endometrium. PLoS One. 5(9):e12906. https://doi.org/10.1371/journal.pone.0012906.

Sheldon IM, Williams EJ, Miller AN, Nash DM, Herath S. 2008. Uterine diseases in cattle after parturition. Vet J. 176(1-3):115–121. https://doi.org/10.1016/j.tvjl.2007.12.031.

Sheldon IM. 2004. The post-partum uterus. Vet Clin North Am Food Anim Pract. 20:569-91. https://doi.org/10.1016/j.cvfa.2004.06.008.

Shin HY, Park S, Lee JW. 2017. Positive association between the changes in chemerin and adiponectin levels after weight reduction. Endocr Res. 42(4):287-295. https://doi.org/10.1080/07435800.2017.1300808.

Silva E, Leitão S, Henriques S, Kowalewski MP, Hoffmann B, Ferreira-Dias G, da Costa LL, Mateus L. 2010. Gene transcription of TLR2, TLR4, LPS ligands and prostaglandin synthesis enzymes are up-regulated in canine uteri with cystic endometrial hyperplasia-pyometra complex. J Reprod Immunol. 84(1):66-74. https://doi.org/10.1016/j.jri.2009.10.004.

Silva E, Leitão S, Tenreiro T, Pomba C, Nunes T, Lopes da Costa L, Mateus L. 2009. Genomic and phenotypic characterization of Escherichia coli isolates recovered from the uterus of puerperal dairy cows. J Dairy Sci. 92(12):6000-10. https://doi.org/10.3168/jds.2009-2358.

Singer JW, Fleischman A, Al-Fayoumi S, Mascarenhas JO, Yu Q, Agarwal A. 2018. Inhibition of interleukin-1 receptor-associated kinase 1 (IRAK1) as a therapeutic strategy. Oncotarget. 9:33416-33439. https://doi.org/10.18632/oncotarget.26058.

Singh SP, Häussler S, Gross JJ, Schwarz FJ, Bruckmaier RM, Sauerwein H. 2014. Short communication: Circulating and milk adiponectin change differently during energy deficiency at different stages of lactation in dairy cows. J Dairy Sci. 97:1535-1542. https://doi.org/10.3168/jds.2013-7598.

Singh SP. 2014. Characterization of adiponectin at different physiological states in cattle based on an in-house developed immunological assay for bovine adiponectin [dissertation]. Bonn: University of Bonn. https://bonndoc.ulb.uni-bonn.de/xmlui/bitstream/handle/20.500.11811/5827/3490.pdf?sequence=1&isAllowed=y.

Skarzynski DJ, Jaroszewski JJ, Okuda K. 2001. Luteotropic Mechanisms in the Bovine Corpus Luteum: Role of Oxytocin, Prostaglandin F2α, Progesterone and Noradrenaline. J Reprod Dev. 47(3):125-137. https://doi.org/10.1262/jrd.47.125.

Smolinska N, Dobrzyn K, Maleszka A, Kiezun M, Szeszko K, Kaminski T. 2014. Expression of adiponectin and adiponectin receptors 1 (AdipoR1) and 2 (AdipoR2) in the porcine uterus during the oestrous cycle. Anim Reprod Sci. 146(1-2):42-54. https://doi.org/10.1016/j.anireprosci.2014.02.001.

Soboll G, Shen L, Wira CR. 2006. Expression of Toll-like receptors (TLR) and responsiveness to TLR agonists by polarized mouse uterine epithelial cells in culture. Biol Reprod. 75(1):131-9. https://doi.org/10.1095/biolreprod.106.050690.

Soneson C, Love MI, Robinson MD. 2015. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res. 4:1521. https://doi.org/10.12688/f1000research.7563.2.

Spencer TE, Bazer FW. 2004. Conceptus signals for establishment and maintenance of pregnancy. Reprod Biol Endocrinol. 2 49. https://doi.org/10.1186/1477-7827-2-49.

Spencer TE, Forde N, Lonergan P. 2016. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. J Dairy Sci. 99(7):5941-5950. https://doi.org/10.3168/jds.2015-10070.

Spencer TE, Johnson GA, Burghardt RC, Bazer FW. 2004. Progesterone and Placental Hormone Actions on the Uterus: Insights from Domestic Animals. Biol Reprod. 71(1):2-10. https://doi.org/10.1095/biolreprod.103.024133.

Spencer TE, Kelleher AM, Bartol FF. 2019. Development and Function of Uterine Glands in Domestic Animals. Annu Rev Anim Biosci. 7:125-147. https://doi.org/10.1146/annurev-animal-020518-115321.

Stephensen CB. 2001. Vitamin A, infection, and immune function. Annu Rev Nutr. 21:167-92. https://doi.org/10.1146/annurev.nutr.21.1.167.

Stevison F, Jing J, Tripathy S, Isoherranen N. 2015. Role of Retinoic Acid-Metabolizing Cytochrome P450s, CYP26, in Inflammation and Cancer. Adv Pharmacol. 2015;74:373-412. https://doi.org/10.1016/bs.apha.2015.04.006.

Stone RA, Laties AM, Hemmings HC Jr, Ouimet CC, Greengard P. 1986. DARPP-32 in the ciliary epithelium of the eye: a neurotransmitter-regulated phosphoprotein of brain localizes to secretory cells. J Histochem Cytochem. 34(11):1465-8. https://doi.org/10.1177/34.11.2877023.

Sugimoto MA, Vago JP, Teixeira MM, Sousa LP. 2016. Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance. J Immunol Res. 8239258. https://doi.org/10.1155/2016/8239258.

Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One. 6 e21800. https://doi.org/10.1371/journal.pone.0021800.

Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV. 2019. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 47 D607-D613. https://doi.org/10.1093/nar/gky1131.

Takemura Y, Osuga Y, Yamauchi T, Kobayashi M, Harada M, Hirata T. 2006. Expression of adiponectin receptors and its possible implication in the human endometrium. Endocrinology. 147(7):3203–3210. https://doi.org/10.1210/en.2005-1510.

Taylor TB, Nambiar PR, Raja R, Cheung E, Rosenberg DW, Anderegg B. 2004. Microgenomics: Identification of new expression profiles via small and single-cell sample analyses. Cytometry A. 59(2):254-61. https://doi.org/10.1002/cyto.a.20051.

Tríbulo P, Siqueira LGB, Oliveira LJ, Scheffler T, Hansen PJ. 2018. Identification of potential embryokines in the bovine reproductive tract. J Dairy Sci. 101(1):690-704. https://doi.org/10.3168/jds.2017-13221.

Uken KL, Schäff CT, Vogel L, Gnott M, Dannenberger D, Görs S, Tuchscherer A, Tröscher A, Liermann W, Hammon HM. 2021a. Modulation of colostrum composition and fatty acid status in neonatal calves by maternal supplementation with essential fatty acids and conjugated linoleic acid starting in late lactation. J Dairy Sci. 104:4950-4969. https://doi.org/10.3168/jds.2020-19627.

Uken KL, Vogel L, Gnott M, Görs S, Schäff CT, Tuchscherer A, Hoeflich A, Weitzel JM, Kanitz E, Tröscher A, Sauerwein H, Zitnan R, Bruckmaier RM, Gross JJ, Liermann W, Hammon HM. 2021b. Effect of maternal supplementation with essential fatty acids and conjugated linoleic acid on metabolic and endocrine development in neonatal calves. J Dairy Sci. 104: 7295-7314. https://doi.org/10.3168/jds.2020-20039.

Uz YH, Murk W, Yetkin CE, Kayisli UA, Arici A. 2010. Expression and role of interleukin-23 in human endometrium throughout the menstrual cycle and early pregnancy. J Reprod Immunol. 87(1-2):21-7. https://doi.org/10.1016/j.jri.2010.06.154.

Van Dyke TE. 2017. Pro-resolving mediators in the regulation of periodontal disease. Mol. Aspects Med. 58:21-36. https://doi.org/10.1016/j.mam.2017.04.006.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:research0034.1 https://doi.org/10.1186/gb-2002-3-7-research0034.

Vasudevan S, Kamat MM, Walusimbi SS, Pate JL, Ott TL. 2017. Effects of early pregnancy on uterine lymphocytes and endometrial expression of immune-regulatory molecules in dairy heifers. Biol Reprod. 97:104-118. https://doi.org/10.1093/biolre/iox061.

Vetrano S, Ploplis VA, Sala E, Sandoval-Cooper M, Donahue DL, Correale C, et al. 2011. Unexpected role of anticoagulant protein C in controlling epithelial barrier integrity and intestinal inflammation. Proc Natl Acad Sci U S A. 108(49):19830-5. https://doi.org/10.1073/pnas.1107140108.

Vrtacnik P, Kos S, Bustin SA, Marc J, Ostanek B. 2014. Influence of trypsinization and alternative procedures for cell preparation before RNA extraction on RNA integrity. Anal Biochem. 463 38e44. https://doi.org/10.1016/j.ab.2014.06.017

Wagener K, Gabler C, Drillich M. 2017. A review of the ongoing discussion about definition, diagnosis and pathomechanism of subclinical endometritis in dairy cows. Theriogenology. 94:21-30. https://doi.org/10.1016/j.theriogenology.2017.02.005.

Wall R, Ross RP, Fitzgerald GF, Stanton C. 2010. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev. 68(5):280-9. https://doi.org/10.1111/j.1753-4887.2010.00287.x.

Walsh SW, Williams EJ, Evans ACO. 2011. A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci. 123(3-4):127-138. https://doi.org/10.1016/j.anireprosci.2010.12.001.

Wang CW, Yu SH, Fretwurst T, Larsson L, Sugai JV, Oh J, Lehner K, Jin Q, Giannobile WV. 2020. Maresin 1 Promotes Wound Healing and Socket Bone Regeneration for Alveolar Ridge Preservation. J. Dent. Res. 99(8):930-937. https://doi.org/10.1177/0022034520917903.

Wang X, Li X, Wang T, Wu SP, Jeong JW, Kim TH, Young SL, Lessey BA, Lanz RB, Lydon JP et al. 2018. SOX17 regulates uterine epithelial-stromal cross-talk acting via a distal enhancer upstream of Ihh. Nat Commun. 9(1):4421. https://doi.org/10.1038/s41467-018-06652-w.

Warzecha CC, Sato TK, Nabet B, Hogenesch JB, Carstens RP. 2009. ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. Mol Cell. 33(5):591-601. https://doi.org/10.1016/j.molcel.2009.01.025.

Watanabe R, Sato Y, Ozawa N, Takahashi Y, Koba S, Watanabe T. 2018. Emerging Roles of Tumor Necrosis Factor-Stimulated Gene-6 in the Pathophysiology and Treatment of Atherosclerosis. Int J Mol Sci. 19:465. https://doi.org/10.3390/ijms19020465.

Weigert J, Obermeier F, Neumeier M, Wanninger J, Filarsky M, Bauer S, Aslanidis C, Rogler G, Ott C, Schäffler A et al. 2010. Circulating levels of chemerin and adiponectin are higher in ulcerative colitis and chemerin is elevated in Crohn's disease. Inflamm Bowel Dis. 16(4):630-7. https://doi.org/10.1002/ibd.21091.

Weiss WP, Pinos-Rodríguez JM. 2009. Production responses of dairy cows when fed supplemental fat in low- and high-forage diets. J Dairy Sci. 92:6144–6155. https://doi.org/10.3168/jds.2009-2558.

Westermann S, Drillich M, Kaufmann TB, Madoz LV, Heuwieser W. 2010. A clinical approach to determine false positive findings of clinical endometritis by vaginoscopy by the use of uterine bacteriology and cytology in dairy cows. Theriogenology. 74(7):1248-55. https://doi.org/10.1016/j.theriogenology.2010.05.028.

Whitby S, Zhou W, Dimitriadis E. 2020. Alterations in epithelial cell polarity during endometrial receptivity: A systematic review. Front Endocrinol. 11:596324. https://doi.org/10.3389/fendo.2020.596324.

Williams EJ, Fischer DP, Noakes DE, England GC, Rycroft A, Dobson H, Sheldon IM. 2007. The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology. 68(4):549-59. https://doi.org/10.1016/j.theriogenology.2007.04.056.

Williams EJ, Fischer DP, Pfeiffer DU, England GC, Noakes DE, Dobson H, Sheldon IM. 2005. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology. 63(1):102-117. https://doi.org/10.1016/j.theriogenology.2004.03.017.

Wimmer I, Tröscher AR, Brunner F Rubino SJ, Bien CG, Weiner HL, Lassmann H, Bauer J. 2018. Systematic evaluation of RNA quality, microarray data reliability and pathway analysis in fresh, fresh frozen and formalin-fixed paraffin-embedded tissue samples. Sci Rep. 8 6351. https://doi.org/10.1038/s41598-018-24781-6

Wolf C, Gredig N, Ulbrich SE, Kreuzer M, Berard J, Giller K. 2019. Partitioning of Rumen-Protected n-3 and n-6 Fatty Acids is Organ-Specific in Growing Angus Heifers. Lipids. 54:503-517. https://doi.org/10.1002/lipd.12183.

Wu L, Belyaeva OV, Adams MK, Klyuyeva AV, Lee SA, Goggans KR, Kesterson RA, Popov KM, Kedishvili NY. 2019. Mice lacking the epidermal retinol dehydrogenases SDR16C5 and SDR16C6 display accelerated hair growth and enlarged meibomian glands. J Biol Chem. 294:17060-17074. https://doi.org/10.1074/jbc.RA119.010835.

Xi Y, Ju R, Wang Y. 2020. Roles of Annexin A protein family in autophagy regulation and therapy. Biomed Pharmacother. 130:110591. https://doi.org/10.1016/j.biopha.2020.110591.

Xie Y, Su N, Yang J, Tan Q, Huang S, Jin M, Ni Z, Zhang B, Zhang D, Luo F, et al. 2020. FGF/FGFR signaling in health and disease. Signal Transduct Target Ther. 5:181. https://doi.org/10.1038/s41392-020-00222-7.

Xiong N, Fu Y, Hu S, Xia M, Yang J. 2012. CCR10 and its ligands in regulation of epithelial immunity and diseases. Protein Cell. 3:571-80. https://doi.org/10.1007/s13238-012-2927-3.

Yang Z; Xiao K; Wang W; Tang J; Sun PP; Peng KM, Song H. 2015. The Effect of visfatin on inflammatory reaction in uterus of LPS-induced rats. Int. J. Morphol. 33(1):194-203. https://doi.org/10.4067/S0717-95022015000100031.

Yoshimura T, Oppenheim JJ. 2011. Chemokine-like receptor 1 (CMKLR1) and chemokine (C-C motif) receptor-like 2 (CCRL2); two multifunctional receptors with unusual properties. Exp Cell Res. 317(5):674-84. https://doi.org/10.1016/j.yexcr.2010.10.023.

Zabel BA, Kwitniewski M, Banas M, Zabieglo K, Murzyn K, Cichy J. 2014. Chemerin regulation and role in host defense. Am J Clin Exp Immunol. 3(1):1-19.

Zachut M, Arieli A, Lehrer H, Livshitz L, Yakoby S, Moallem U. 2010. Effects of increased supplementation of n-3 fatty acids to transition dairy cows on performance and fatty acid profile in plasma, adipose tissue, and milk fat. J Dairy Sci. 93:5877–5889. https://doi.org/10.3168/jds.2010-3427.

Zeng B, Lu H, Xiao X, Yu X, Li S, Zhu L, Yu D, Zhao W. 2019. KDF1 is a novel candidate gene of non-syndromic tooth agenesis. Arch Oral Biol. 97:131-136. https://doi.org/10.1016/j.archoralbio.2018.10.0259.

Zeng S, Bick J, Ulbrich SE, Bauersachs S. 2018. Cell type-specific analysis of transcriptome changes in the porcine endometrium on Day 12 of pregnancy. BMC Genomics. 19(1):459. https://doi.org/10.1186/s12864-018-4855-y.

Zhang S, Wang D, Yan Z. 2021. Increasing of matrix metalloproteinase 3 in bovine endometritis. Theriogenology. 175:83-88. https://doi.org/10.1016/j.theriogenology.2021.09.001.

Zhang Y, Huo Y, He W, Liu S, Li H, Li L. 2019. Visfatin is regulated by interleukin-6 and affected by the PPAR-γ pathway in BeWo cells. Mol Med Rep. 19(1):400-406. https://doi.org/10.3892/mmr.2018.9671.

Zhang Z, Broderick C, Nishimoto M, Yamaguchi T, Lee SJ, Zhang H, Chen H, Patel M, Ye J, Ponce A, et al. 2020. Tissue-targeted R-spondin mimetics for liver regeneration. Sci. Rep. 10(1):13951. https://doi.org/10.1038/s41598-020-70912-3.

Zhao YL, Zhu RT, Sun YL. 2016. Epithelial-mesenchymal transition in liver fibrosis. Biomed Rep. 4(3):269-274. https://doi.org/10.3892/br.2016.578.

Zhou JX, Liao D, Zhang S, Cheng N, He HQ, Ye RD. 2014. Chemerin C9 peptide induces receptor internalization through a clathrin-independent pathway. Acta Pharmacol Sin. 35(5):653-63. https://doi.org/10.1038/aps.2013.198.

Zinicola M, Bicalho MLS, Santin T, Marques EC, Bisinotto RS, Bicalho RC. 2019. Effects of recombinant bovine interleukin-8 (rbIL-8) treatment on health, metabolism, and lactation performance in Holstein cattle II: Postpartum uterine health, ketosis, and milk production. J Dairy Sci. 102(11):10316-10328. https://doi.org/10.3168/jds.2019-16335.

Zinicola M, Korzec H, Teixeira AGV, Ganda EK, Bringhenti L, Tomazi ACCH, Gilbert RO, Bicalho RC. 2018. Effects of pegbovigrastim administration on periparturient diseases, milk production, and reproductive performance of Holstein cows. J Dairy Sci. 101(12):11199-11217. https://doi.org/10.3168/jds.2018-14869.

Annex I

REPRODUCTION
RESEARCH

Adipokines as biomarkers of postpartum subclinical endometritis in dairy cows

Gonçalo Pereira[1], Ricardo Bexiga[1], João Chagas e Silva[1], Elisabete Silva[1], Christelle Ramé[2], Joëlle Dupont[2], Yongzhi Guo[3], Patrice Humblot[3] and Luís Lopes-da-Costa[1]

[1]CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Lisboa, Portugal, [2]INRAE, UMR85 Physiologie de la Reproduction et des Comportements, Nouzilly, France and [3]Division of Reproduction, Department of Clinical Sciences, SLU, Uppsala, Sweden

Correspondence should be addressed to L Lopes-da-Costa; Email: lcosta@fmv.ulisboa.pt

Abstract

Adipokines emerged as regulators of metabolism and inflammation in several scenarios. This study evaluated the relationship between adipokines (adiponectin, chemerin and visfatin) and cytological (subclinical) endometritis, by comparing healthy (without), transient (recovered by 45 days postpartum (DPP)) and persistent (until 45 DPP) endometritis cows ($n=49$). Cows with persistent endometritis had higher adiponectin concentrations in plasma (at 21 DPP, $P<0.05$ and at 45 DPP, $P<0.01$) and in uterine fluid (at 45 DPP, $P<0.001$), and higher chemerin concentrations in plasma ($P<0.05$) and uterine fluid ($P<0.01$) at 45 DPP than healthy cows. Cows with persistent endometritis had higher gene transcription in the cellular pellet of uterine fluid and protein expression in the endometrium of these adipokines and their receptors than healthy cows. Adiponectin plasma concentrations allowed to discriminate healthy from persistent endometritis cows, in 87% (21 DPP) and 98% (45 DPP) of cases, and adiponectin and chemerin uterine fluid concentrations at 45 DPP allowed for this discrimination in 100% of cases. Cows with concentrations above the cutoff were a minimum of 3.5 (plasma 21 DPP), 20.4 (plasma 45 DPP), and 33.3 (uterine fluid 45 DPP) times more at risk of evidencing persistent endometritis at 45 DPP than cows with concentrations below the cutoff. Overall, results indicate a relationship between adipokine signalling and the inflammatory status of the postpartum uterus of dairy cows, evidencing that adipokines represent suitable biomarkers of subclinical endometritis, able to predict the risk of persistence of inflammation.

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Introduction

Cow fertility is critical for the sustainable worldwide increasing demand of dairy products and the profitability of the dairy industry (Inchaisri *et al.* 2010, Britt *et al.* 2018). A main factor impairing fertility is the occurrence of postpartum endometritis, which disrupts ovarian and endometrial function leading to a delay in conception and failure in pregnancy establishment (Mateus *et al.* 2002, Sheldon *et al.* 2018). The diagnosis of subclinical endometritis, also termed cytological endometritis, which affects 30–35% of dairy cows between 4 and 9 weeks postpartum (LeBlanc 2008), remains a challenge (Raliou *et al.* 2019). Cytological endometritis is an inflammatory state of the endometrium, detected by histology or cytology, in the absence of purulent vaginal discharge and other clinical signs (Sheldon *et al.* 2006). Due to the invasive nature of the sampling technique, veterinary skills required, time-consuming logistics and cost of the uterine biopsy and swab techniques, the development of reliable, non-invasive biomarkers for the early diagnosis and prognosis of endometritis has been the scope of recent research (Adnane *et al.* 2017, Mayasari *et al.* 2017). The early identification of biomarkers that trigger and/or signal the pathological inflammation of the endometrium would enable to predict uterine health status, administer appropriate prophylactic therapy (Adnane *et al.* 2017) and better manage time of first insemination during the postpartum period.

The adipose tissue serves not only as a depot for lipid storage but also as an endocrine gland that secretes many mediators generally named adipokines (Reverchon *et al.* 2014), including hormone-like mediators as adiponectin, chemerin and visfatin (Kurowska *et al.* 2018). Although adipokines are mainly produced by adipocytes and immune cells found in the stromal vascular fraction of adipose tissue, different cell types outside adipose tissue depots have also been described as primary sources of these mediators (Mancuso 2016, Kurowska *et al.* 2018). Among other functions, adipokines regulate energy metabolism, glucose homeostasis, angiogenesis, reproductive function, immunity and inflammation (Reverchon *et al.* 2014, Mancuso 2016).

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Annex II

Primary antibodies

Antibody name	**Source**	**Dilution**	**Source** (catalogue no.)
ADIPOQ	Rabbit polyclonal	1:100	Homemade as described in Giesy et al. (2012)
ADIPOR1	Rabbit polyclonal	1:50	Antibodies-online GmbH., Aachen, Germany (ABIN2789457)
ADIPOR2	Rabbit polyclonal	1:50	Antibodies-online GmbH., Aachen, Germany (ABIN2788598)
RARRES2	Rabbit polyclonal	1:100	Santa Cruz Biotechnology as described Reverchon et al. (2014b)
CMKLR1	Mouse monoclonal	1:100	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (sc-374570)
Rabbit IgG	Rabbit polyclonal - Isotype Control	1:200	Abcam, Cambridge, UK (ab27478)
Mouse IgG	Mouse polyclonal	1:200	Sigma-Aldrich (Ref PP54)

Annex III

Purity and quality of RNA samples (assessed by NanoDrop Spectrophotometer and Agilent Bioanalyzer 2100) of the cellular pellets from ut*erine flushing used in the qPCR*

Group	*Cow ID*	*260/280 ratio*	*RIN value*
HHP	4463	1.85	8.2
HHP	5645	1.90	7.5
HHP	6450	1.75	7.6
HHP	6480	1.85	8.1
HHP	6460	1.80	7.6
HHP	3030	1.90	7.8
EH	5666	1.75	7.6
EH	5614	1.80	7.8
EH	5724	1.85	8.0
EH	3106	1.90	8.2
EH	5663	1.75	7.9
EH	5626	1.70	7.5
EH	6487	1.80	7.2
EH	6494	1.85	7.6
EH	6492	1.90	8.0
EH	6439	1.70	7.8
EH	271	1.85	8.3
EH	6540	1.90	8.5
EH	6545	1.85	8.0
EH	5707	1.80	7.5
EH	5771	1.90	8.4
EH	2141	1.85	7.9
EH	5780	1.80	8.5
EH	5805	1.75	8.0
EH	6602	1.90	7.8
EE	4454	1.85	7.7
EE	4423	1.75	7.6
EE	5638	1.70	8.0
EE	6523	1.70	7.6
EE	5734	1.85	7.5
EE	5703	1.90	7.6
EE	6575	1.90	7.8
EE	5777	1.85	7.5
EE	6608	1.80	7.4
EE	2297	1.75	7.8
EE	2333	1.80	8.1

HHP - subset of healthy cows pregnant at first AI.
EH - cows with SCE at day 21 postpartum but that recovered by day 45 postpartum.
EE - cows with persistent SCE until day 45 postpartum.
RIN - RNA integrity number.

Annex IV

Oligonucleotide primer sequences

Abbreviation	Name of the gene	Primer sequence 5′–3′	GenBank accession no.	Size (bp)
ACTB	Actin Beta	For: ACGGAACCACAGTTTATCATC Rev: GTCCCAGTCTTCAACTATACC	D12816	188
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	For: TTCAACGGCACAGTCAAGG Rev: ACATACTCAGCACCAGCATCAC	NM_001034034	119
PPIA	Cyclophilin A	For: GCATACAGGTCCTGGCATCT Rev: TGTCCACAGTCAGCAATGGT	NM_178320	217
RARRES2	Chemerin	For: GAGGAGTTCCACAAGCATC Rev: ACCTGAGTCTGTATGGGACA	XM_027538675.1	265
CMKLR1	Chemerin Chemokine-Like Receptor 1	For: CGGCCATGTGCAAGATCAGC Rev: CAGGCTGAAGTTGTTAAAGC	XM_027566482.1	359
GPR1	G Protein-Coupled Receptor 1	For: CTGTCATTTGGTTCACAGGA Rev: AACAACCTGAGGTCCACATC	MK860765.1	629
CCRL2	C-C chemokine receptor-like 2	For: AATTACACGCCAGCACCAGA Rev: AGTCCTTTATATTTTACCAG	NM_001075732	199
ADIPOQ	Adiponectin	For: CACCTTCACAGGCTTCCTTC Rev: AGACTGTCCTGGGAACATGG	NM_174742	219
ADIPOR1	Adiponectin receptor 1	For: GGCTCTACTACTCCTTCTAC Rev: ACACCCCTGCTCTTGTCTG	NM_001034055	144
ADIPOR2	Adiponectin receptor 2	For: GGCAACATCTGGACACATC Rev: CTGGAGACCCCTTCTGAG	NM_001040499	200

Annex V

Data distribution for analysed variables

Variable	n	Shapiro-Wilk test	Distribution
Lactation number	49	< 0.001	Non Normal
Body Weight Loss Calving – 21 DPP	49	0.008	Non Normal
Body Weight Loss Calving – 45 DPP	49	0.286	Normal
Body Weight Loss Calving – 60 DPP	49	0.035	Non Normal
Milk yield by 21 DPP	49	0.461	Normal
Milk yield by 45 DPP	49	0.721	Normal
Milk yield by 60 DPP	49	0.814	Normal
NEFA at 21 DPP	49	< 0.001	Non Normal
NEFA at 45 DPP	49	< 0.001	Non Normal
Metricheck Score at 21 DPP	49	< 0.001	Non Normal
Metricheck Score at 45 DPP	49	< 0.001	Non Normal
PMN% in Cytobrush at 21 DPP	49	< 0.001	Non Normal
PMN% in Cytobrush at 45 DPP	49	< 0.001	Non Normal
Calving – first AI interval	49	< 0.001	Non Normal
Calving – conception interval	40	0.001	Non Normal
AI number/conception	40	0.001	Non Normal
Plasma Adiponectin at 21 DPP	49	< 0.001	Non Normal
Plasma Adiponectin at 45 DPP	49	< 0.001	Non Normal
Uterine fluid Adiponectin at 45 DPP	49	< 0.001	Non Normal
Plasma Chemerin at 21 DPP	49	0.110	Normal
Plasma Chemerin at 45 DPP	49	0.048	Non Normal
Uterine fluid Chemerin at 45 DPP	49	< 0.001	Non Normal
Plasma Visfatin at 21 DPP	49	0.036	Non Normal
Plasma Visfatin at 45 DPP	49	0.079	Normal
Uterine fluid Visfatin at 45 DPP	49	< 0.001	Non Normal
Standardized ADIPOQ mRNA	36	< 0.001	Non Normal
Standardized ADIPOR1 mRNA	36	0.027	Non Normal
Standardized ADIPOR2 mRNA	36	< 0.001	Non Normal
Standardized RARRES2 mRNA	36	< 0.001	Non Normal
Standardized CMKLR1 mRNA	36	< 0.001	Non Normal
Standardized GPR1 mRNA	36	0.008	Non Normal
Standardized CCRL2 mRNA	36	0.418	Normal

DPP – days postpartum.

Annex VI

Agreement between the vaginal discharge Metricheck score and the endometrial cytology PMN percentage at 21 (A) and 45 (B) days postpartum in dairy cows (n = 49)

A		Metricheck			**B**		Metricheck		
	Cytobrush	Positive	Negative	Total		Cytobrush	Positive	Negative	Total
	Positive	25 (51.0%)	5 (10.2%)	30 (61.2%)		Positive	1 (2.0%)	10 (20.4%)	11 (22.4%)
	Negative	7 (14.3%)	12 (24.5%)	19 (38.8%)		Negative	7 (14.3%)	31 (63.3%)	38 (77.6%)
	Total	32 (65.3%)	17 (34.7%)	49 (100%)		Total	8 (16.3%)	41 (83.7%)	49 (100%)

Metricheck score considered positive when vaginal discharge score ≥ 1.

Annex VII

Plasma and uterine fluid concentrations of ADIPOQ, RARRES2 and NAMPT in healthy cows (group HH), cows with SCE at 21 DPP but recovered by 45 DPP (group EH), and cows with persistent SCE until 45 DPP

Parameters	HH (n = 19)	EH (n = 19)	EE (n = 11)
Plasma ADIPOQ at 21 DPP (µg/mL)*	5.46 (4.90 – 6.23) [a]	4.72 (4.70 - 5.21) [a]	6.34 (6.03 - 7.43) [b]
Plasma ADIPOQ at 45 DPP (µg/mL)*	4.72 (4.69 – 5.53) [a]	4.56 (4.44 - 4.64) [b]	7.24 (6.81 - 7.49) [c]
Uterine fluid ADIPOQ at 45 DPP (µg/mL)*	4.71 (4.55 – 4.72) [a]	4.54 (4.32 - 4.57) [a]	10.39 (10.19 - 10.56) [b]
Plasma RARRES2 at 21 DPP (ng/mL)**	2.47 ± 0.05	2.60 ± 0.06	2.48 ± 0.07
Plasma RARRES2 at 45 DPP (ng/mL)*	2.44 (2.36 - 2.56) [a]	2.56 (2.31 - 2.65) [a,b]	2.63 (2.59 - 2.64) [b]
Uterine fluid RARRES2 at 45 DPP (ng/mL)*	1.49 (1.43 - 1.50) [a]	1.42 (1.34 - 1.44) [a]	3.07 (2.91 - 3.38) [b]
Plasma NAMPT at 21 DPP (ng/mL)*	155.93 (129.50 – 166.17) [a,b]	148.07 (140.21 – 155.69) [a]	174.5 (153.31 – 200.93) [b]
Plasma NAMPT at 45 DPP (ng/mL)**	171.35 ± 4.89 [a]	150.84 ± 3.45 [b]	184.98 ± 5.94 [a]
Uterine fluid NAMPT at 45 DPP (ng/mL)*	138.07 (135.21 – 148.07)	153.31 (137.36 - 155.93)	137.36 (121.17 – 155.93)

DPP = days postpartum.
*Values reported as median and (interquartile range) for non-normally distributed data.
**Values reported as mean ± SEM for normally distributed data.
Different letters indicate significant differences between groups (level of significance $P < 0.05$) determined with ANOVA and the non-parametric Kruskal-Wallis-Test with Dunns post-test for normally and non-normally distributed variables, respectively.

Annex VIII

Ability of adipokines to discriminate cows with persistent SCE at 45 DPP

Adipokines	Number of cows under the cutoff value			Number of cows above the cutoff value			OR	OR [CI 95%]	*P* value
	Healthy	Endometritis	Total	Healthy	Endometritis	Total			
Plasma ADIPOQ at 21 DPP (cutoff: 5.9 µg/mL)	31	2	33	7	9	16	19.9	3.5 to 113.3	0.0007
Plasma ADIPOQ at 45 DPP (cutoff: 6.1 µg/mL)	38	1	39	0	10	10	539	20.4 to 14218.7	0.0002
Uterine fluid ADIPOQ at 45 DPP (cutoff: 7.8 µg/mL)	38	0	38	0	11	11	1771	33.3 to 94301.3	0.0002
Plasma RARRES2 at 21 DPP (cutoff: 2.2 ng/mL)	4	0	4	34	11	45	3	0.15 to 60.1	0.4725
Plasma RARRES2 at 45 DPP (cutoff: 2.5 ng/mL)	19	0	19	19	11	30	23	1.3 to 418.1	0.0341
Uterine fluid RARRES2 at 45 DPP (cutoff: 2.2 ng/mL)	38	0	38	0	11	11	1771	33.3 to 94301.3	0.0002

DPP = days postpartum; OR = odds ratio; CI = confidence interval.

Annex IX

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BMC Genomics

RESEARCH Open Access

Progesterone differentially affects the transcriptomic profiles of cow endometrial cell types

Gonçalo Pereira[1†], Yongzhi Guo[2†], Elisabete Silva[1], Claudia Bevilacqua[3], Gilles Charpigny[4], Luís Lopes-da-Costa[1*†] and Patrice Humblot[2†]

Abstract

Background: The endometrium is a heterogeneous tissue composed of luminal epithelial (LE), glandular epithelial (GE), and stromal cells (ST), experiencing progesterone regulated dynamic changes during the estrous cycle. In the cow, this regulation at the transcriptomic level was only evaluated in the whole tissue. This study describes specific gene expression in the three types of cells isolated from endometrial biopsies following laser capture microdissection and the transcriptome changes induced by progesterone in GE and ST cells.

Results: Endometrial LE, GE, and ST cells show specific transcriptomic profiles. Most of the differentially expressed genes (DEGs) in response to progesterone are cell type-specific (96%). Genes involved in cell cycle and nuclear division are under-expressed in the presence of progesterone in GE, highlighting the anti-proliferative action of progesterone in epithelial cells. Elevated progesterone concentrations are also associated with the under-expression of estrogen receptor 1 (*ESR1*) in GE and oxytocin receptor (*OXTR*) in GE and ST cells. In ST cells, transcription factors such as *SOX17* and *FOXA2*, known to regulate uterine epithelial-stromal cross-talk conveying to endometrial receptivity, are over-expressed under progesterone influence.

Conclusions: The results from this study show that progesterone regulates endometrial function in a cell type-specific way, which is independent of the expression of its main receptor PGR. These novel insights into uterine physiology present the cell compartment as the physiological unit rather than the whole tissue.

Keywords: Transcriptome, Progesterone, Endometrium, LCM, RNA-seq

Background

The bovine endometrium is composed of different cell compartments, comprising luminal epithelial (LE), glandular epithelial (GE), and stromal cells (ST), which is submitted to intense tissue remodelling during the estrous cycle, embryo implantation and puerperal involution [1, 2]. Progesterone (P4) released by the corpus luteum (CL) plays a key role regulating endometrial function and remodelling [3, 4]. At the transcriptomic level, this P4 regulation has been evaluated so far, only from the whole endometrial tissue, following luteolysis [5], comparing different oestrus cycle stages [6] or status (diestrus versus anoestrus) and type of ovulation (single versus multiple ovulation) [7]. However, as in other heterogeneous tissues, quantification of gene expression from the whole endometrium may not reflect the specific transcription of the cell compartments. Laser capture microdissection

*Correspondence: lcosta@fmv.ulisboa.pt
†Gonçalo Pereira and Yongzhi Guo shared first authorship.
†Luís Lopes-da-Costa and Patrice Humblot shared senior authors.
[1] CIISA-Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisbon, Portugal
Full list of author information is available at the end of the article

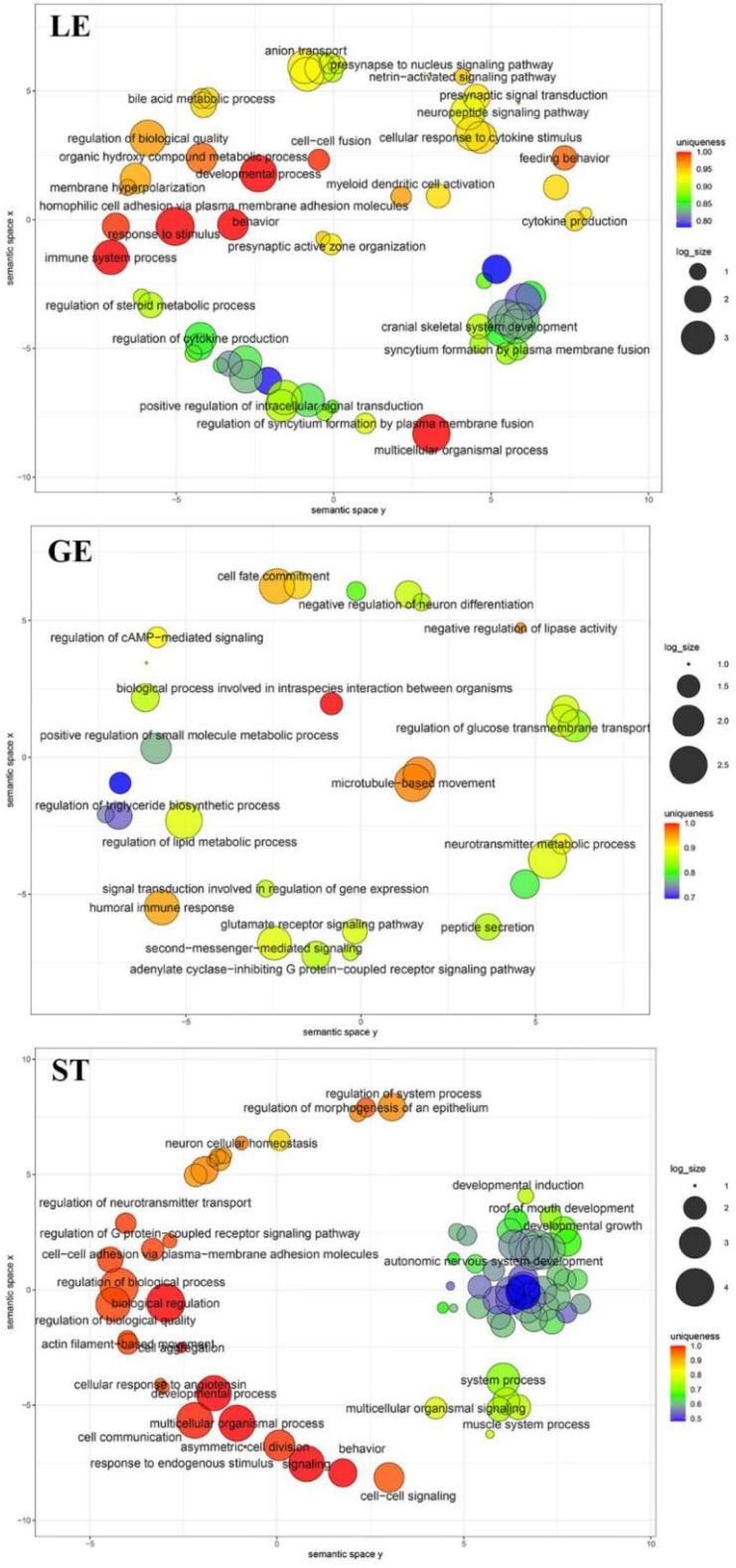
Annex X

Hyperlinks to access Supplementary files 1-9

Supplementary file	Hyperlink
Supplementary file 1	https://data.mendeley.com/datasets/97cznzzcvg/1
Supplementary file 2	https://data.mendeley.com/datasets/bvs2r75sk9/1
Supplementary file 3	https://data.mendeley.com/datasets/b7m2kch89p/1
Supplementary file 4	https://data.mendeley.com/datasets/k6yfhfzg34/1
Supplementary file 5	https://data.mendeley.com/datasets/nxbwdmh99x/1
Supplementary file 6	https://data.mendeley.com/datasets/x5kkf369kn/2
Supplementary file 7	https://data.mendeley.com/datasets/922v7859tg/2
Supplementary file 8	https://data.mendeley.com/datasets/cpb5vznzhn/3
Supplementary file 9	https://data.mendeley.com/datasets/d36t3d438g/3

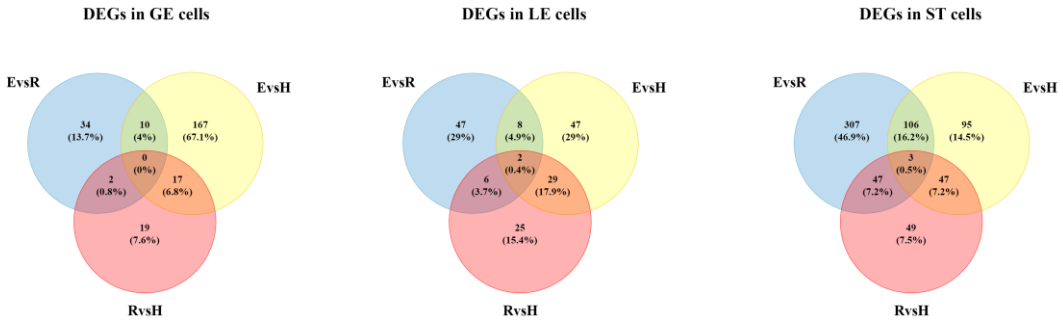
Annex XI

Scatterplot representation of enriched GO terms in semantic space using REVIGO (Supek et al. 2011), from lists of cell-specific genes of luminal epithelial (LE), glandular epithelial (GE) and stromal (ST) cells. Circle size represents the frequency of the GO term in the underlying GOA database (bubbles of more general terms are larger) and colour indicates the uniqueness value



Annex XII

Number of differently expressed genes common between the different comparisons (Persistent subclinical endometritis vs Healthy, Persistent subclinical endometritis vs Recovery, Recovery vs Healthy), in each cell type group (Glandular Epithelial, Luminal Epithelial, Stroma)



Annex XIII

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Effects of feeding rumen-protected linseed fat to postpartum dairy cows on plasma n-3 polyunsaturated fatty acid concentrations and metabolic and reproductive parameters

Gonçalo Pereira, Patrícia Simões, Ricardo Bexiga, Elisabete Silva, Luisa Mateus, Tatiane Fernandes, Susana P. Alves, Rui J. B. Bessa, and Luis Lopes-da-Costa*
CIISA—Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal

ABSTRACT

High-yielding dairy cows experience a negative energy balance and inflammatory status during the transition period. Fat supplementation increases diet energy density, and plasma n-3 polyunsaturated fatty acids (PUFA) have been proposed to improve immune function. This study tested the hypothesis that dietary supplementation with a rumen-protected and n-3 PUFA-enriched fat could ameliorate both the energetic deficit and immune status of postpartum high-yielding dairy cows, improving overall health and reproductive efficiency. At 11 d in milk (DIM), cows were randomly allocated to groups (1) n-3 PUFA (n = 29), supplemented with encapsulated linseed oil supplying additional up to 64 g/d (mean 25 ± 4 g/d) of α-linolenic acid (ALA), or (2) control (n = 31), supplemented with hydrogenated palm oil without ALA content. Fat supplements of the n-3 PUFA and control groups were available through an automated, off-parlor feeding system, and intake depended on the cow's feeding behavior. Plasma ALA concentrations were higher in n-3 PUFA than control cows, following a linear relation with supplement ingestion, resulting in a lower n-6/n-3 ratio in plasma. Metabolic parameters (body condition score and glucose and β-hydroxybutyric acid blood concentrations) were unaffected, but milk yield improved with increased intake of fat supplements. Plasma total adiponectin concentrations were negatively correlated with ingestion of n-3 PUFA-enriched fat supplement, following a linear relation with intake. Conception rate to first AI increased with higher intake of both fats, but a decrease of calving-to-conception interval occurred only in n-3 PUFA cows. Postpartum ovarian activity and endometrial inflammatory status at 45 DIM were unaffected. In conclusion, this study evinced a positive linear relation between rumen-protected linseed fat intake and plasma n-3 PUFA concentrations, which modulated adiponectin expression and improved reproductive parameters.
Key words: n-3 PUFA, adiponectin, linseed, dairy cow

INTRODUCTION

The high-yielding dairy cow transition period is characterized by a state of negative energy balance, which adversely affects cow health, fertility, and milk yield (Raboisson et al., 2014; Abdelli et al., 2017; Sheldon et al., 2019). Fat supplementation has become a common strategy to increase the energy density of diets and minimize the effects of negative energy balance (Palmquist and Jenkins, 2017; Bionaz et al., 2020). However, this may reduce DMI, disturb rumen function, and originate fatty acid (**FA**) isomers that depress milk fat (Chamberlain and DePeters, 2017; de Souza and Lock, 2019; Manriquez et al., 2019). Because FA, namely PUFA, have essential physiologic roles, the beneficial effects of fat supplementation may rely more on FA type than on increased energy intake (Herrera-Camacho et al., 2011). In this context, the PUFA from n-3 (α-linolenic acid, **ALA**; eicosapentaenoic acid, **EPA**; docosapentaenoic acid, **DPA**; and docosahexaenoic acid, **DHA**) and n-6 (linoleic acid, **LA**; and arachidonic acid) families receive special attention due to their roles in reproductive and immune function (Moallem, 2018; Moallem et al., 2020). Increased intake of n-6 PUFA increases the proportion of LA and arachidonic acid in cell membranes, favoring eicosanoid synthesis toward a proinflammatory state (Wolf et al., 2019). In contrast, increased intake of n-3 PUFA increases the proportion of EPA and DHA in cell membranes, which favors eicosanoid synthesis toward an anti-inflammatory state (Greco et al., 2015; Wolf et al., 2019).

Transition dairy cows are good candidates for the modulation of inflammation through nutrition, because they experience a physiologic inflammatory state dur-

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*Corresponding author: lcosta@fmv.ulisboa.pt

Annex XIV

Means (± SE) of milk yield, log-transformed Somatic Cell Counts (lnSCC), and metabolic parameters of cows enrolled in a randomized controlled trial of fat supplementation in postpartum dairy cows. (A) Fat and Protein Corrected Milk (FPCM) yield, (B) lnSCC, (C) Body Condition Score (BCS), (D) blood β-hydroxybutyric acid (BHB), (E) blood glucose, (F) plasma Non-Esterified Fatty Acids (NEFA)