

Inflammation and uterine disease in the postpartum dairy cow



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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, except where otherwise stated:

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Amy Brewer

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Abbreviations

AA	Arachidonic acid
AGP	Alpha-1 acid glycoprotein
AI	Artificial insemination
AMP	Antimicrobial peptide
APP	Acute phase protein
APR	Acute phase response
AZA	5-aza-2'-deoxycytidine
BCA	Bicinchoninic acid assay
BHB	Beta-hydroxybutyric acid
BSA	Bovine serum albumin
CDC	Cytolysin
cDNA	Complementary deoxyribonucleic acid
CRP	C-reactive protein
CYTO	Cytological endometritis
CD	Cluster of differentiation
CL	Corpus luteum
COX	Cyclooxygenase
CRP	C-reactive protein
DAMP	Damage associated molecular pattern
DEG	Differentially expressed genes
DIM	Days in milk
DMI	Dry matter intake
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPP	Days postpartum
DS	Double stranded
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EnPEC	Endometrial pathogenic <i>E.coli</i>
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate

FDR	False discovery rate
GO	Gene ontology
HC	Healthy cow
Hp	Haptoglobin
IG	Immunoglobulin
IL	Interleukin
INF γ	Interferon gamma
INF τ	Interferon tau
IRAKs	IL-1 receptor associated kinases
ITS-X	Insulin Transferrin Selenium X
IU	Intrauterine
KEGG	Kyoto encyclopaedia of genes and genomes
LPS	Lipopolysaccharide
MHC	Major histocompatibility
miRNA	Micro RNA
mRNA	Messenger RNA
MYD88	Myeloid differentiation primary-response protein 88
NAb	Natural antibodies
NEFA	Non-esterified fatty acids
NF κ B	Nuclear factor- kappa B
NGS	Next generation sequencing
NLRP3	NLR family pyrin domain containing 3
PAMP	Pathogen associated molecular pattern
PCA	Principle component analysis
Pen/strep	Penicillin/streptomycin
PG	Prostaglandin
PLA	Phospholipase
PLO	Pyolysin
PMN	Polymorphonuclear leukocytes
PRR	Pathogen recognition receptor
PTX	Pentraxin
PVD	Purulent vaginal discharge
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

RNAseq	RNA sequencing
RPMI	Roswell Park Memorial Institute media
ROS	Reactive oxygen species
RP	Retained placenta
SAA	Serum amyloid A
SAP	Serum amyloid P
SCE	Subclinical endometritis
SNP	Single nucleotide polymorphism
<i>T. pyogenes</i>	<i>Trueperella pyogenes</i>
TEER	Trans-epithelial electrical resistance
TGF	Transforming growth factor
TLR	Toll-like receptor
TMR	Total mixed ration
TNF	Tumour necrosis factor
TP	Total protein
TSA	Trichostatin A

Abstract

Following calving, inflammation of the uterus is common during the first week postpartum and is essential for the reparative process of involution. However, whilst the majority of cows resolve this inflammation a significant proportion fail to do so, resulting in uterine disease and subfertility. Previous work by our group has identified transcriptomic differences in immune genes between cattle that resolve inflammation and those that develop uterine disease as early as 7 days postpartum (DPP), suggesting that the host immune response plays an important role in disease outcome. Here, we hypothesised that even in the context of early postpartum inflammation, cows that go on to develop uterine disease will be identified by a unique inflammatory signature in the circulation and in endometrial epithelial cells 7 DPP.

In this study, we extensively characterised the local and systemic immune response of postpartum dairy cows phenotyped for both clinical and sub-clinical forms of disease. Sampling was carried out at 7 and 21 DPP and involved the collection of blood and endometrial epithelial cells from 112 cattle. Endometrial cells were collected using a cytobrush. Pre-calving bloods (40-60 days prepartum) were also acquired. Cattle were classified as healthy or with uterine disease (either cytological endometritis; CYTO, purulent vaginal discharge; PVD or both) on the basis of vaginal mucus score and >18% polymorphonuclear cell infiltrate into the endometrium at 21 DPP. Blood samples were used to analyse systemic metabolites, immune cells, acute phase proteins (APPs), vitamins and minerals. Endometrial epithelial cells were used for transcriptomic analysis at 7 DPP.

Systemic analysis revealed that increased concentrations of APPs serum amyloid a (SAA) and haptoglobin (Hp) were present 7 DPP in cattle that subsequently developed PVD compared to cows that remained healthy. The same trend was observed for alpha-1-acid glycoprotein (AGP) and in cows with CYTO, though neither reached significance. Analysis of circulating immune cell populations identified a decline in eosinophil numbers in cattle that developed both CYTO and PVD. Interestingly, no difference in neutrophil numbers was detected between groups. Metabolite levels were also assessed, and whilst all cattle were in a state of negative energy balance 7 DPP, concentrations of glucose, urea, non-esterified fatty acids (NEFA) and betahydroxybutyrate (BHB) did not differ between cows that remained healthy and cows that developed disease, which suggests that energy status was not contributing to the pathogenesis of uterine disease in this study.

RNA-seq analysis of endometrial epithelial cells collected using cytobrushes identified differential expression of 294 genes (FDR <0.05) between cows that subsequently resolved

inflammation (n=10) and those that developed disease (n=20). Pathway over-representation analysis of differentially expressed genes (DEG) identified significant changes in immune-related pathways, including the NOD-like receptor signalling pathway, cytokine-cytokine receptor interaction pathway and the Toll-like receptor signalling pathway which were up-regulated in cattle that subsequently developed disease. A major role for the cytokine IL-1 was also highlighted by over-representation of multiple IL-1 signalling pathways. The majority of the DEG were upregulated in cows that developed PVD, and included all genes upregulated in CYTO cows, suggesting a core inflammatory gene signature early post-partum contributes to the onset of uterine disease. This inflammatory signature was validated by qPCR in an independent group of cows (n=56) and included upregulation of pro-inflammatory genes (including *TLR2*, *TLR4*, *NLRP3*, *IL1A*, *IL1B*, *IL8*, and *S100A8*) at day 7 postpartum in cows that failed to resolve inflammation. Despite a large amount of inter-animal heterogeneity, these results suggest that excessive activation and inappropriate regulation of the inflammatory response early postpartum is a key feature of the subsequent development of uterine disease.

In addition to the hyper-inflammatory response observed 7 DPP, cows that developed uterine disease also demonstrated down-regulation of genes associated with cell growth and adhesion when compared to healthy cows. Therefore we hypothesised that excessive inflammation prevents repair and remodelling of the endometrium, contributing to prolonged or unresolved inflammation (endometritis). One of the top differentially expressed genes upregulated in cows that developed both clinical and subclinical endometritis was pentraxin 3 (PTX3). PTX3 is an APP with important roles in the innate immune response and tissue repair. Recently, PTX3 has been found to play a dual role in the regulation of inflammation, either providing protection or amplifying tissue damage. Furthermore, recent evidence suggests PTX3 is capable of affecting epithelial barrier integrity. However, its effect on the endometrial epithelial barrier, particularly in the postpartum cow, has yet to be elucidated. Therefore we decided to investigate the effect of PTX3 on endometrial epithelial barrier repair in an *in vitro* model of the bovine endometrial epithelium designed to mirror the early post-partum uterus of the cow. In addition to confirming the presence of PTX3 in the uterus at protein level, we found that it had a detrimental effect on epithelial barrier repair, following disruption of the epithelium by calcium chelation. Deciphering the mechanisms underlying the effects of PTX3 on epithelial barrier repair could assist in new treatment or prevention strategies for tackling endometritis.

In summary, this study has shown that despite a significant amount of inter-animal heterogeneity, both clinical and subclinical forms of uterine disease are preceded by excessive

activation of the endometrial inflammatory response 7 DPP, which can be detected systemically by an increase in the APPs SAA and Hp and a decline in circulating eosinophils. Furthermore, integrity of the endometrial epithelial barrier is likely to play an important role in preventing disease onset. Not only have we enhanced our understanding of the mechanisms underlying the switch between physiological and pathological inflammation, but we have also paved the way for the development of novel biomarkers for the early detection of uterine disease.

Chapter 1 Introduction

1.1 Bovine reproduction and fertility

Cattle fertility is a major challenge to farmers worldwide. In both beef and dairy systems, a compact calving interval is desirable, however reproductive efficiency is particularly important in seasonal based grazing systems, such as those found in Ireland, New Zealand and Australia, where the aim is to match the peak lactation period of cattle with the abundance of grass growth in spring, enabling farmers to exploit this cheap food source (Figure 1-1). Thus, following a gestation period of around 280 days (9 months), a successful pregnancy is required within a maximum of 12 (seasonal production system) to 16 (non-seasonal production system) weeks after calving (Roche et al., 2018). The goal in an Irish dairy system is to achieve a synchronised annual calving interval across the herd. Cows that calve too late or do not become pregnant within the required time frame are culled from the herd and replaced by heifers, which is economically more expensive for the farmer.

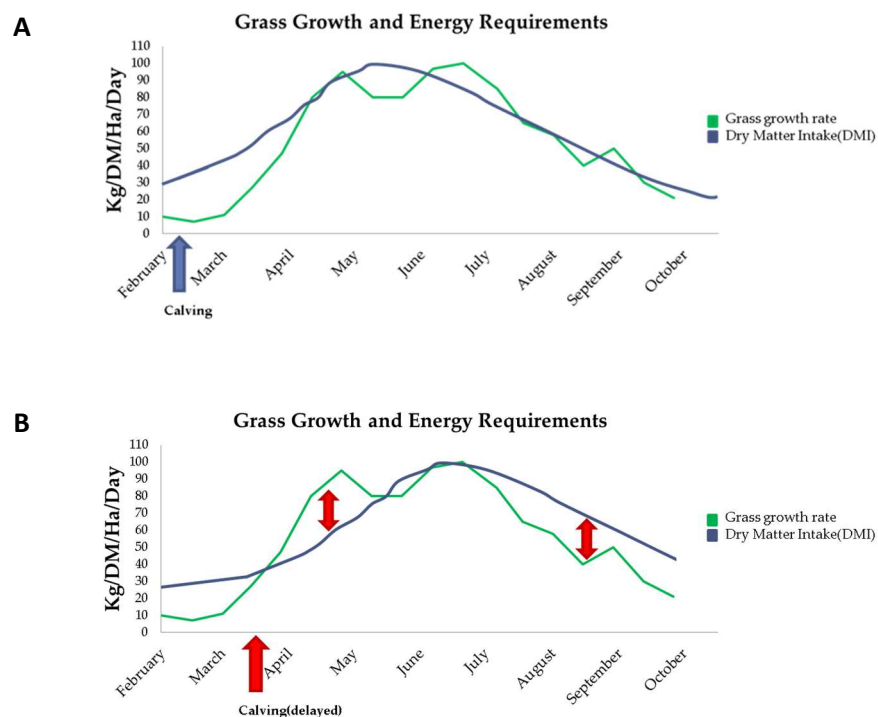


Figure 1-1 The effect of delayed pregnancy in a seasonal based grazing system

A Cows calving in early February in Ireland are able to match their dry matter intake (DMI) requirements with increased grass growth in spring, allowing efficient use of this cheap food source. **B** Uterine disease delays pregnancy, resulting in delayed calving the following year and a misalignment between grass growth and DMI (red arrows).

Over the last 50 years, a decline in dairy cow fertility has been well documented (Walsh et al., 2011). This has been associated with the genetic selection of high yielding cows, particularly of the Holstein-Friesian breed. Milk production has increased substantially since the 1980s, by an average of 46 kg per cow per year in Ireland and up to 131 kg per cow per year in the USA (Dillon et al., 2006). However selection based predominantly on milk yield resulted in a decline in reproductive performance, across a variety of countries with different climates and production systems. More recently, selection indices have shifted from predominantly production related traits to include health and fertility traits, and the decline in fertility has started to reverse (Norman et al., 2009). However, fertility is multi-factorial trait which is not only affected by genetic factors but also environmental and managerial influences, which have changed substantially over time. In addition, the pregnancy rate of multiparous cattle remains at least 10% lower than that of maiden heifers (M Diskin 2017; personal communication), suggesting that fertility is still an issue and is likely to be associated with the negative effects of high milk production on key physiological processes.

During the transition period (3 weeks before to 3 weeks after calving), dairy cows are susceptible to a range of diseases that can negatively impact fertility (Figure 1-2). Each of these diseases extends the calving to pregnancy interval by at least 7, 8, 26, 31 and 40 days in cows with mastitis, retained fetal membranes (RFM), hypocalcaemia, endometritis and lameness respectively (Dobson et al., 2007b).

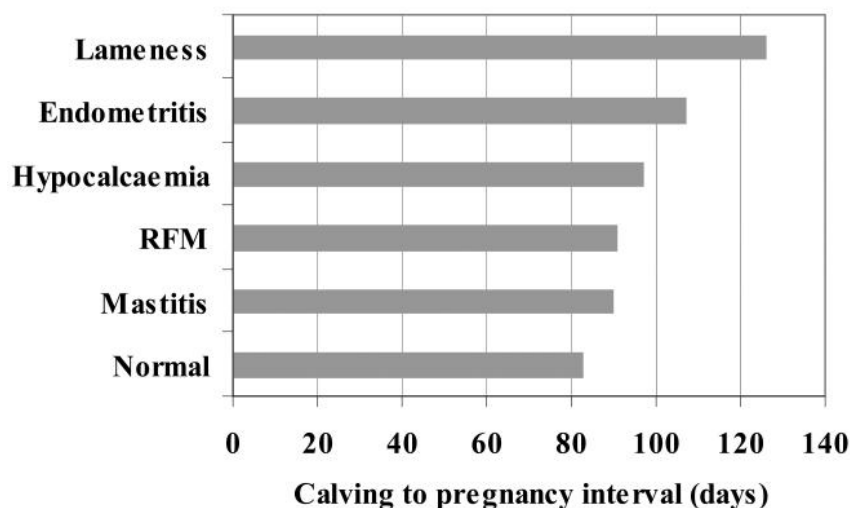


Figure 1-2 Calving to pregnancy intervals for dairy cattle treated for particular clinical conditions

(Dobson et al., 2007a)

Uterine disease is a major contributor to reduced fertility in dairy cattle. It is also very prevalent, with around 40% of cattle suffering from at least one type of uterine disease during the postpartum period (Dubuc et al., 2011b). This includes metritis (inflammation of the uterus before 21 days postpartum which is often associated with systemic inflammation), purulent vaginal discharge (PVD) (detected by the presence of PVD in the cranial vagina at 21 days or more postpartum) and endometritis (inflammation of the endometrium detected by cytology after 21 days postpartum). Conception rates are around 20% lower in cattle suffering from PVD, which also have an average increased calving to conception interval of around 30 days and a reduced pregnancy rate (Fourichon et al., 2000, LeBlanc et al., 2002a). This is also true for cattle with endometritis (Table 1 in (LeBlanc, 2014)). Even after treatment, cows often remain less fertile and a further 3% are culled from the herd for failure to conceive (LeBlanc et al., 2002a, Bosberry and Dobson, 1989). Financially uterine disease is also a big problem for the dairy industry. (Drillich et al., 2001) have estimated the cost of treatment of a single case of metritis to be around €292. As there are currently around 1,343,300 dairy cattle in Ireland (Dec 2017; Central Statistics Office), using the conservative incidence rate of 20% for metritis, uterine disease is estimated to cost the Irish dairy industry over €78 million per year.

Uterine disease may also negatively impact milk production during the early postpartum period. Metritis has been found to decrease milk production by up to 3.7 kg per cow per day (Dubuc et al., 2011a), although other studies have found no effect on milk production. This discrepancy could be due to the effects of parity, as (Østergaard and Gröhn, 1999), (Wittrock et al., 2011) and (Dubuc et al., 2011a) found that multiparous, but not primiparous cows with metritis produced less milk. However it is also likely to be based on the production system in use, as high-yielding North American Holsteins in total mixed ration (TMR) systems are more likely to experience severe negative energy balance in the postpartum period, which decreases available nutrients for mounting an immune response, thus leaving them more susceptible to disease.

The recent abolishment of milk quotas within the EU will intensify the need for good reproductive health in the coming years. By 2020, it is expected that the average herd size in Ireland will have increased by around 24%, whilst annual milk production by the Irish dairy industry is expected to double compared to 2008-9 figures (Teagasc, 2013). Therefore in order to address this issue, it is vital that we gain a better understanding of the bovine immune system and how it functions in postpartum cattle.

1.2 Reproductive physiology of the cow

The uterus exhibits great anatomical variation between species. Cattle and other ruminants possess bicornuate uteri, consisting of a uterine body which splits into two separate uterine horns (Figure 1-3). The uterus is made up of three distinct layers: the outer perimetrium layer of connective tissue, the myometrium which consists of a thick layer of smooth muscle and a thinner outer longitudinal muscle layer joined by connective tissue and blood vessels, and the endometrium.

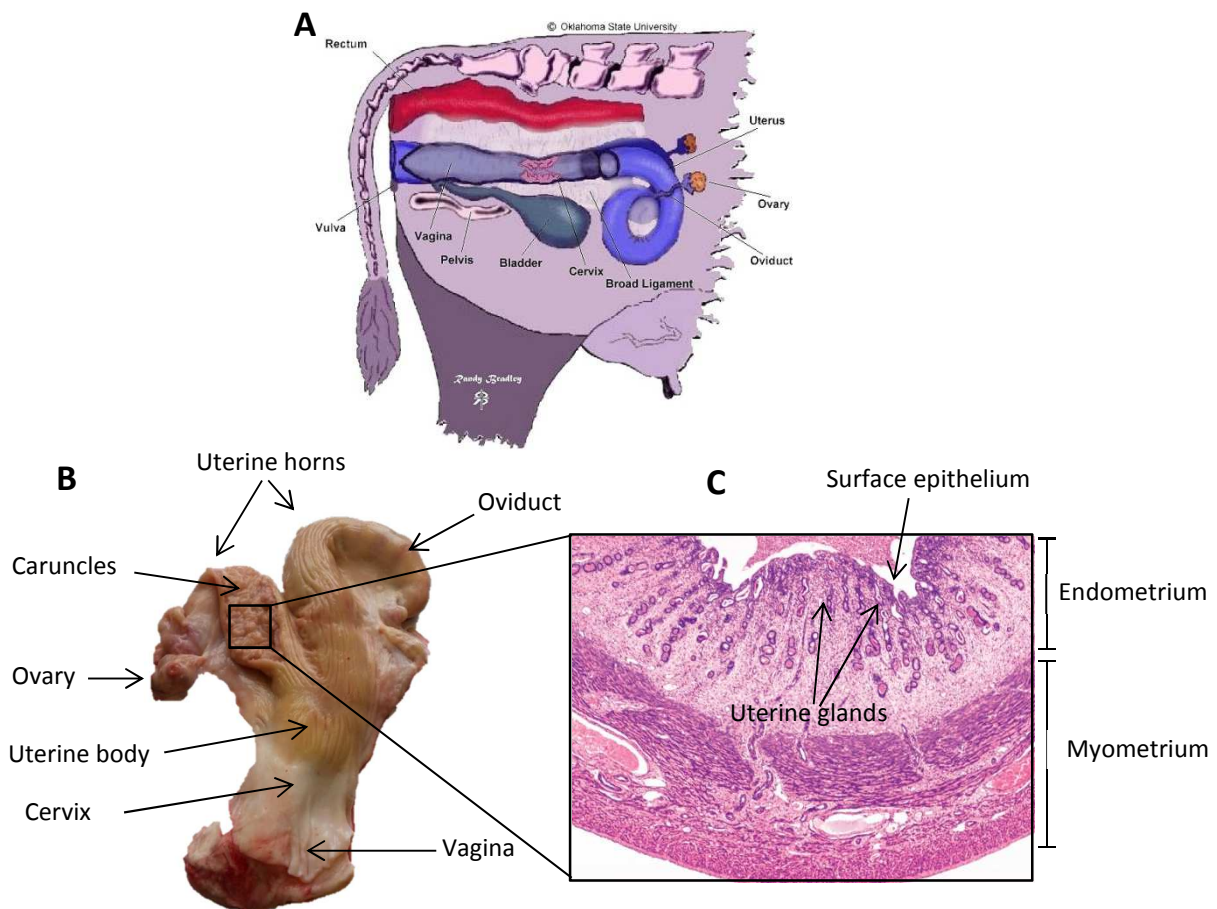


Figure 1-3 Anatomy of the female reproductive tract of the cow

The reproductive tract of the non-pregnant cow is normally situated within the pelvic cavity (A) and consists of the vulva, vagina, cervix, uterus, oviducts and ovaries (A, B). A histological section of the uterus (C) displays the different uterine layers, including the functional epithelial layer and the muscular myometrium. The endometrium consists of a layer of surface epithelial cells and the underlying stroma, along with a number of uterine glands. Images adapted from (A) (Oklahoma State University, 1999) and (C) (Yale University, 2015).

The endometrium is the inner mucosal lining of the uterus, which includes a functional and a basal layer. The functional layer consists of a surface layer of columnar epithelium, which sits on top of connective tissue known as the stroma. The thickness and vasculature of the endometrium is altered in response to hormonal changes (Dawson, 1959). The endometrium also contains glands which are responsible for secreting a fluid known as histotroph, which supports the embryo before and during attachment to the uterus. In ruminants, many button-like projections known as caruncles cover the endometrium, which provide sites of attachment for the placenta. There are usually between 70 and 120 caruncles covering the bovine endometrium, which fuse with fetal cotyledons on the placenta to form placentomes: the site of fetal-maternal interaction (Wooding and Burton, 2008).

1.2.1 Uterine involution

After calving, a number of events must occur before pregnancy can be re-established, including the removal of lochia by uterine contractions, shrinkage of the uterus, necrosis and sloughing of the caruncles and regeneration of the endometrium (Sheldon, 2004). The third stage of labour involves detachment of the fetal membranes, which takes around 6 hours on average. If detachment does not occur within 12 hours, the cow is classed as having retained fetal membranes (RFM) or retained placenta (RP). It can take 3 to 10 days before RFM are completely expelled (Noakes 2001).

Immediately after parturition, the uterus weighs roughly 8-10 kg (Figure 1-4) and measures around 1 metre in length – around 50 times the size of the non-pregnant uterus (Gier and Marion, 1968). The size of the uterus is reduced most rapidly in the first 3 days postpartum (Mori et al., 1974), due to peristaltic contractions which also assist in the removal of lochial discharge. The lochia contains blood and fetal membranes, as well as uterine tissue from the necrotic caruncles which are usually sloughed from the uterine wall at around 12 days postpartum (Sheldon, 2004). Lochia is normally discharged until around 14 to 23 days in milk (DIM) (Lewis, 1997, Mortimer et al., 1997). In normal cows, a large reduction in the size of the uterus occurs between day 10 and 14 after calving, resulting in a uterus less than 50% of the full term size. Cervical involution is considerably slower than that of the uterus (Mortimer et al., 1997), with gross involution of both occurring by around 25 to 47 days postpartum (LeBlanc, 2008). Repair of the endometrium, as observed histologically, takes longer to complete (Morrow et al., 1966).

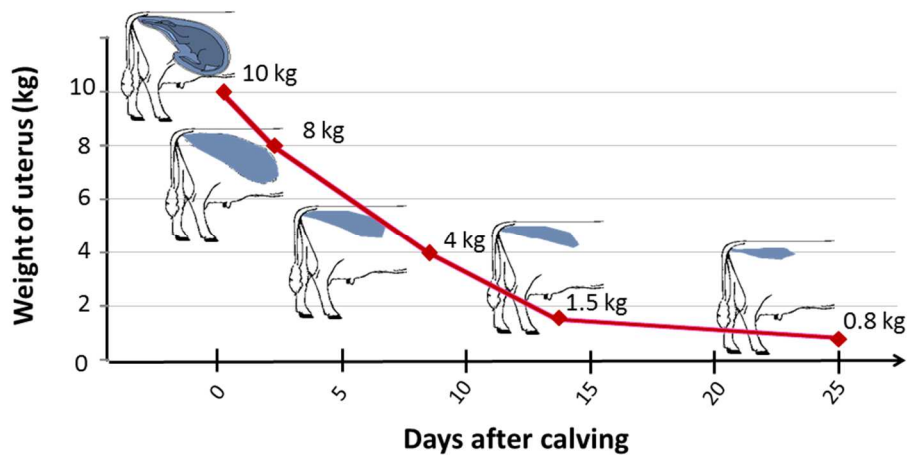


Figure 1-4 Changes in uterine weight during involution in the postpartum cow

It takes around 30 days for the uterus to return to its original size after calving. Image reproduced from (TECHNA, 2018).

1.2.2 Resumption of ovarian cyclicity

The reproductive cycle of the cow (and most other placental mammals) is known as the oestrous cycle. During this cycle, there are periods in which the cow is fertile and sexually receptive (oestrus) and periods when she is not (anoestrus) (Figure 1-5). Cattle are not seasonal breeders, and so this cycle occurs continuously, independent of the time of year. During one oestrous cycle, several waves of follicular maturation occur. During each wave, a group of immature (primordial) follicles begin to mature, yet most are inhibited by the release of progesterone from the corpus luteum (CL) of the previous ovulation. Once the CL degenerates and progesterone concentrations desist, the dominant follicle from a new wave of maturing follicles will go on to ovulate.

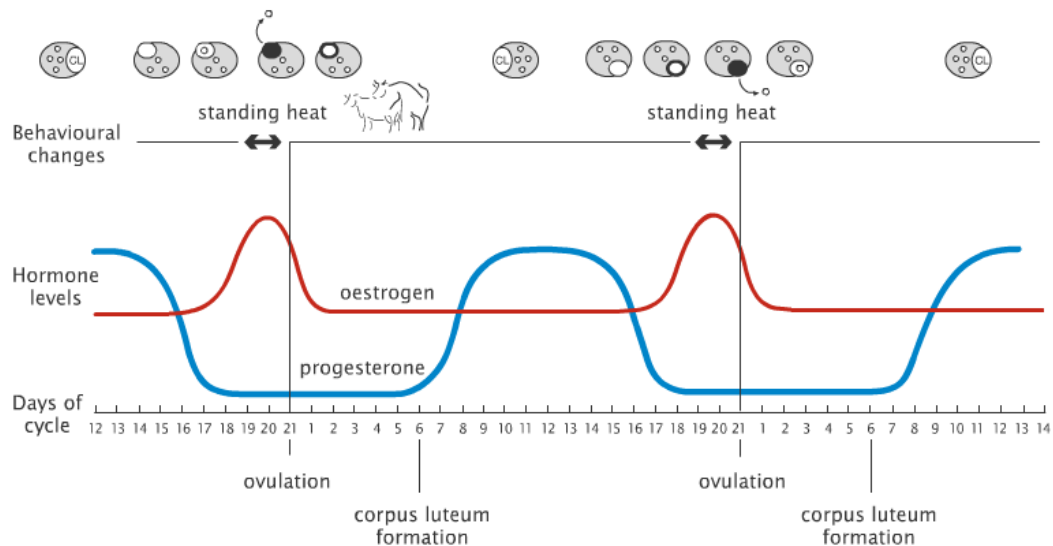


Figure 1-5 The oestrous cycle

The oestrous cycle of cattle has an average duration of 21 days. It consists of a long luteal phase (days 1-17) and a shorter follicular phase (days 18-21). The cycle begins with oestrus, or “standing heat”, which can last from around 4-24 hours and is influenced by the increasing oestrogen concentrations released from maturing follicles in the ovary. Once oestrogen levels reach a threshold level, a surge of luteinising hormone (LH) results in ovulation around 30 hours after the start of oestrus. Following ovulation, the follicular cells originally surrounding the oocyte in the ovary form a corpus luteum (CL), which releases progesterone. If fertilisation does not occur, the CL will regress, removing the inhibitory effect of progesterone on the pituitary and allowing the follicular phase to begin again. Adapted from (Merck Animal Health, 2017).

The length of the postpartum anoestrous period varies in cattle, and is dependent on age, breed, body condition score (BCS) at calving, nutrition and health status. Following parturition, the corpus luteum (CL) begins to degenerate, causing circulating progesterone and oestrogen levels to fall to basal concentrations (Crowe, 2008). This releases the negative feedback on gonadotrophin releasing hormone (GnRH), allowing follicle stimulating hormone (FSH) concentrations to increase, which stimulates the growth and development of new follicles within 3-5 days of parturition (Savio et al., 1990). Once one of the follicles reaches a threshold size (at around 10-14 days postpartum), this follicle becomes dominant, secreting oestradiol and inhibin which suppress FSH secretion and prevent further development of any other follicles (Sheldon et al., 2011). Oestradiol secretion stimulates the release of luteinising hormone (LH) from the pituitary gland, and eventually leads to an LH surge and ovulation at around 15-21 DPP. Following ovulation, the CL is formed, leading to progesterone secretion and the resumption of oestrous cycles. Cattle rarely express signs of oestrus at the first ovulation postpartum, and only around 10% stand to be mounted (Sheldon et al., 2011). The first ovulation is also typically associated with a short luteal phase of around 7-10 days. Most cattle ovulate within 30 days after parturition (McEntee, 1990).

Almost half (45%) of modern dairy cattle suffer from abnormal oestrous cycles after calving (Royal et al., 2000). This typically includes anovulatory anoestrus, where the cow fails to ovulate, or a prolonged luteal phase due to delayed luteolysis. Failure to ovulate is caused by a lack of LH secretion from the pituitary gland, which is often associated with negative energy balance that commonly occurs at the start of lactation. Uterine disease also affects ovarian function. Endotoxins released by bacteria in the uterine lumen have been located in the follicular fluid of cows with uterine disease, and it is thought that they are capable of binding to Toll-like receptors (TLRs) on the granulosa cells surrounding the ovarian follicle, leading to reduced oestradiol secretion and consequently reduced LH secretion (Herath et al., 2007).

1.3 Postpartum uterine disease

Following calving, most cattle experience an influx of bacteria into the uterus (Figure 1-6). Whilst many cows are able to clear the resulting infection, others experience excessive or prolonged inflammation which impairs reproductive performance, leading to infertility during infection and subfertility following resolution of the disease (Sheldon & Dobson 2004). Postpartum uterine diseases include purulent and clinical metritis, endometritis, purulent vaginal discharge, cervicitis and pyometra. Early detection of these diseases is essential, allowing for treatment and recovery before the breeding season begins. This is particularly important in the case of subclinical diseases such as endometritis and pyometra, as a lack of clinical symptoms often means they remain unnoticed by the farmer for an extended period of time.

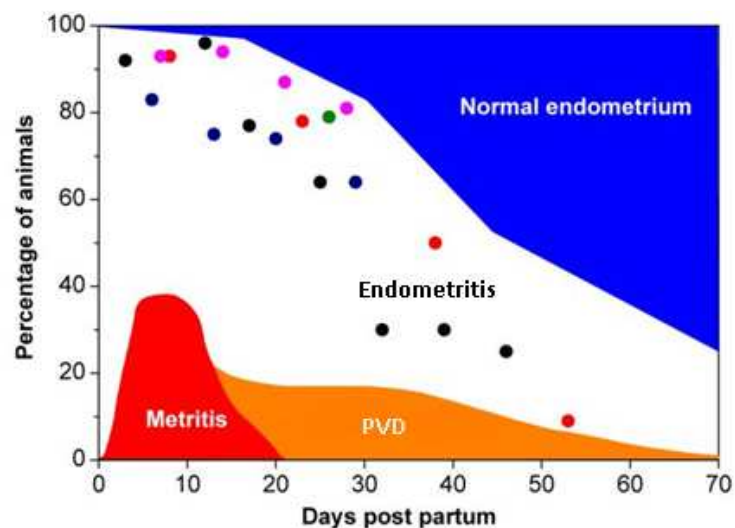


Figure 1-6 Incidence of uterine disease in postpartum cattle

The shaded areas represent the average proportion of cattle with metritis (red), purulent vaginal discharge (orange) or a normal uterus (blue). The rest have subclinical endometritis. Each of the round markers represents the percentage of animals with bacteria isolated from the uterine lumen. Adapted from (Sheldon et al., 2009).

1.3.1 Metritis

The term metritis may refer to either puerperal or clinical metritis. Puerperal metritis is characterised by signs of systemic illness, including fever, reduced milk yield, dullness and reduced appetite, in conjunction with an abnormally large uterus and a fetid watery red-brown uterine discharge. This usually occurs within the first week postpartum. Cattle with an abnormally enlarged uterus and the presence of purulent vaginal mucus without signs of systemic illness within 21 days after calving are classified as having clinical metritis (Sheldon et

al., 2006). Metritis involves inflammation of all layers of the uterus, including the endometrium, submucosa, muscularis and serosa (Bondurant, 1999). Incidence of this disease varies greatly, from around 1% to 50% (Sheldon et al., 2009), which may be partly influenced by the definition of disease used. A meta-analysis of 23 studies revealed a negative effect of metritis on reproductive outcome, which increased time to first service by 7 days and decreased conception rate by 20% (Fourichon et al., 2000).

1.3.2 Purulent vaginal discharge

Traditionally, endometritis was diagnosed by the presence of purulent or muco-purulent vaginal discharge (PVD) at 21 days or more postpartum, and was termed “clinical endometritis”. However, although PVD often accompanies more severe forms of endometritis, it has become apparent that visible discharge can also occur in the absence of endometrial inflammation (Dubuc et al., 2010a) and may instead be indicative of an infection in the lower reproductive tract, such as vaginitis or cervicitis (Deguillaume et al., 2012). Therefore it has been recommended that purulent vaginal discharge (PVD; clinical endometritis) and cytological or subclinical endometritis (CYTO; SCE) be defined as distinct conditions. Diagnosis of PVD requires the examination of vaginal discharge using either a Metrichick device, vaginoscope or simply a gloved hand, which is then scored according to (Williams et al., 2005), based on appearance and often odour as well. Around 15-20 % of cattle are affected by PVD and this is usually associated with impaired reproductive performance (LeBlanc et al., 2002b, McDougall et al., 2007, Dubuc et al., 2010a).

1.3.3 Endometritis

Endometritis (also known as subclinical endometritis (SCE) or cytological endometritis (CYTO)) refers to inflammation of the endometrium, in the absence of any clinical signs of disease. Whilst most cattle experience endometrial inflammation in the first couple of weeks postpartum, some do not resolve, resulting in pathological inflammation of the uterus after 21 DPP, termed endometritis. Endometrial inflammation is determined by assessing the number of polymorphonuclear leukocytes (PMN) in endometrial cytology, which can be obtained using either a uterine cytobrush, lavage or biopsy, or by a more recent adaptation of the cytobrush method, known as cytotape (Pascottini et al., 2015). There is a large amount of discrepancy regarding the criteria for cytological diagnosis of CYTO, due to variations in counting PMN and PMN thresholds for the diagnosis of CYTO. Since no gold standard is available for diagnosing endometritis, PMN thresholds have been based on biological outcomes, such as calving to conception interval. (Kasimanickam et al., 2004) proposed a cut-off point of >18 % PMN at 21-33 DPP, or >10 % PMN at 34-47 DPP, as PMN% above this threshold resulted in a decreased

pregnancy risk. This was based on a receiver operating characteristic (ROC) curve analysis, which assesses sensitivity and specificity over the range of test scores (Gardner and Greiner, 2006). When used alongside prevalence of the disease, a predictive value can be determined to provide interpretive guidance (de Boer et al., 2014). Since then, thresholds of 6.5 to 18 % PMN have been used by different authors depending on time point postpartum, and based on ROC analysis and effects on fertility (Table 1 in (de Boer et al., 2014)). Variation in PMN cut off is likely partly due to the multifactorial nature of fertility, which can be affected by confounders such as poor heat detection, poor semen quality and nutrition. However, (Barański et al., 2012) recently assessed the effect of different PMN thresholds on reproductive performance and found that at 21-28 DPP only a threshold of >18% PMN (as opposed to 8% or 5%) resulted in a significant increase in calving to conception interval, whilst at 35-42 DPP significant differences were found using a threshold of 10% or 8%, but not 5%. It is still unclear as to why these results differ to those that found significant differences using lower thresholds, however it is likely due to differences in farm management that may be influencing postpartum health between herds. The average prevalence of endometritis at 40-60 DPP (immediately before the breeding season) is around 26% (Sheldon et al., 2009), but this varies greatly between herds, from 5% to greater than 50% (Dubuc et al., 2010a). These large differences are due, at least in part, to inconsistencies in timing of diagnosis, diagnostic method and definition of endometritis in addition to real biological differences between populations (de Boer et al., 2014). Endometritis has a negative effect on subsequent fertility, resulting in reduced conception rates and increased pregnancy loss (Cheong et al., 2011).

1.3.4 Physical and environmental risk factors for uterine disease

Several environmental risk factors have been associated with uterine disease. Many are related to trauma and tissue damage, including dystocia and assisted calving, twins, large/male calves, stillbirths and primiparity (Kim and Kang, 2003, Dubuc et al., 2010b, Potter et al., 2010). Retained fetal membranes present the greatest risk factor for uterine disease (Paisley et al., 1986), as the physical barriers between the cervix and uterus are destroyed, and necrotic membranes provide an ideal environment for the growth of pathogenic bacteria. The causes of RFM are largely unknown and are likely to be multi-factorial and possibly genetic (Sheldon, 2014). The hygiene level of the calving environment and cow does not appear to play a significant role in the onset of PVD, suggesting that the cause is more likely due to damage of the physical barriers within the uterus rather than faecal contamination (Potter et al., 2010). However, vulval angle was also a significant risk factor (Potter et al., 2010), which implies that faecal contamination of the vagina could be an issue in certain cows, in addition to drainage of lochia after calving. (Dubuc

et al., 2010b) also reported a difference in risk factors for PVD and endometritis, which included twinning, dystocia, metritis and increased haptoglobin in the first week postpartum for cows with PVD, which contrasted with low body condition score at parturition, hyperketonemia and increased haptoglobin in the first week postpartum for cows with endometritis.

1.3.5 Metabolic risk factors for uterine disease

Metabolic risk factors have also been associated with uterine disease. The transition period (from around 2 weeks prepartum to 3 weeks postpartum) is incredibly challenging for dairy cattle, and tends to be when most metabolic and infectious disease occur, such as mastitis, ketosis, milk fever, fatty liver and uterine disease (Roche et al., 2013). During the postpartum period, dairy cattle are unable to consume enough to meet the energy demands of milk production, so essentially all cows experience a period of negative energy balance in early lactation (LeBlanc, 2014). This is characterised by a decline in dry matter intake (DMI), leading to weight loss and a slight decline in circulating glucose. Glucose is an essential nutrient for many biological processes and so in order to maintain blood glucose levels, the cow begins to mobilise adipose and muscle tissue. The breakdown of adipose tissue results in an accumulation of non-esterified fatty acids (NEFA), which is a good energy source and required for milk fat synthesis. NEFA is released into the bloodstream and therefore commonly measured as a marker of NEB. Excess NEFA is metabolised in the liver and results in the formation of products of incomplete oxidation, such as β -hydroxybutyrate (BHB) and other ketone bodies (Vazquez-Añón et al., 1994) which are also used as markers of NEB. High concentrations of excess NEFA can also be reesterified into triglycerides, which build up in the liver, causing the disorder fatty liver (Strang et al., 1998)

This metabolic imbalance has long been reported to cause a state of immunosuppression during the postpartum period (Machado and Bicalho, 2015). However, (Roche et al., 2013) points out that an increased inflammatory response exists in many tissues of the postpartum cow, and instead refers to a period of immune dysfunction. Recent studies have revealed that adipose tissue is very metabolically active, and fat mobilisation after calving releases pro-inflammatory signals such as TNF and IL-6 (Tilg and Moschen, 2008). These pro-inflammatory factors are capable of blocking insulin signalling (Hotamisligil, 2006), which promotes insulin resistance, and exacerbates further release of NEFA. Fatty acids are also capable of directly stimulating TLR4 in other species, leading to further inflammation (Hotamisligil and Erbay, 2008). Additionally, high concentrations of fatty acids and BHB may directly inhibit PMN function (Ster et al., 2012), which often precedes uterine disease. Impaired chemotaxis of PMN is linked to retained placenta (Kimura et al., 2002), whilst a decline in PMN oxidative burst function has been associated with

metritis and endometritis (Hammon et al., 2006, Cai et al., 1994). PMN impairment may also be caused by the low glucose levels associated with NEB as neutrophils are dependent on glucose uptake and glycolysis for chemotaxis, and glycogen stores for phagocytosis and oxidative burst, even in the presence of glucose (Galvao et al., 2010).

The immune system is also affected by vitamin and mineral deficiencies during the transition period. Milk production requires minerals such as calcium, phosphorus, zinc and copper, resulting in a deficit in the bloodstream (Meglia et al., 2001, Goff et al., 2002). Hypocalcaemia (milk fever) is commonly associated with the occurrence of metritis and clinical endometritis (Martinez de la Torre et al., 2010), which is likely due to reduced neutrophil function caused by compromised cytosolic Ca^{2+} levels needed for phagocytosis (Sayeed, 2000). Calcium is also required for the fusion of secondary granules with the phagosomal membrane during antimicrobial activity (Jaconi et al., 1990), and for neutrophil activation (Bréchar and Tschirhart, 2008). Furthermore, calcium plays a crucial role in smooth muscle function throughout the body, and so hypocalcaemia is often linked to uterine prolapse (Risco et al., 1984), dystocia, RP, slow involution and consequently metritis and endometritis, due to suppressed uterine contractions (Hansen et al., 2003). Hypocalcaemia can cause reduced rumen motility, leading to low DMI and ketosis, and poor teat sphincter closure, resulting in mastitis (Roche et al., 2013). Hypocalcaemia is also affected by magnesium and vitamin D levels, which are required for efficient calcium absorption. An abundance of phosphorus can also have negative effects on vitamin D (and therefore hypocalcaemia) as it inhibits the activity of renal CYP27B1, thereby reducing synthesis of $1,25\text{OHD}_3$, the active metabolite of vitamin D (Silver et al., 1999).

1.3.6 Treatment of endometritis

Antibiotics

The most common treatment for endometritis is the use of intrauterine (IU) antibiotics. IU infusion of cephalixin one month before insemination has been shown to improve the reproductive performance of cattle with PVD, when compared to no treatment (LeBlanc et al., 2002b) (LeBlanc et al., 2002b). This is also true in endometritic cows, where 500 mg IU cephalixin administered at 20-35 DPP increased pregnancy rates compared to untreated animals (Kasimanickam et al., 2005, Denis-Robichaud and Dubuc, 2015). Numerous other antibiotics have been tested, however most have not been approved for IU use and several result in the presence of drug residues in milk, with no published guidelines on withdrawal

times. Some antibiotics such as Lugol's iodine and oxytetracycline also irritate the uterus, causing necrosis of the endometrium (Gilbert and Schwark, 1992).

Prostaglandin $F_{2\alpha}$

Another popular treatment for endometritis is the administration of systemic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), in cases where there is a corpus luteum present. $PGF_{2\alpha}$ induces luteolysis, reducing progesterone levels and initiating oestrus. This is beneficial as it removes the immunosuppressant effects of progesterone and initiates myometrial contractions which help to clear lochia from the uterus. In addition, milk does not have to be discarded following the use of $PGF_{2\alpha}$. One or two injections of $PGF_{2\alpha}$ has been shown to improve reproductive performance in cows with PVD (LeBlanc et al., 2002b, McDougall et al., 2013) but not in those with CYTO (Galvao et al., 2009). A more recent review (Haimerl et al., 2012) and meta-analysis (Haimerl et al., 2013) found that there is limited evidence for a beneficial effect of $PGF_{2\alpha}$ on reproductive performance in PVD cows, and no advantage to using $PGF_{2\alpha}$ for the treatment of CYTO. Other studies have suggested that the timing of treatment may be important, as the time at which cows develop a responsive CL varies considerably, and there is little support for treatment with $PGF_{2\alpha}$ before 4 weeks postpartum (Hendricks et al., 2006). Therefore the outcome of $PGF_{2\alpha}$ treatment for endometritis remains unclear.

Anti-inflammatory drugs

More recent studies have investigated the use of nonsteroidal anti-inflammatory drugs (NSAIDs) for treatment of transition-period disorders in cattle. Flunixin meglumine accelerates uterine involution in cattle with metritis (Amiridis et al., 2001) whilst (Duffield et al., 2009) found that flunixin significantly increased the risk of RFM and metritis. As this drug is a cyclooxygenase inhibitor, it is likely that the incidence of RP and metritis increased due to the suppression of PG synthesis which prevents uterine contractions and expulsion of the placenta. The NSAID carprofen was found to increase pregnancy rate in cattle with CYTO when treated at 21-31 DPP, although there was no effect on PMN% at 42 DPP (Priest et al., 2013). Interestingly, the use of salicylates has been shown to not only improve fertility parameters in postpartum cows, but also enhance long-term milk yield (Bertoni et al., 2004). However, whilst (Trevisi and Bertoni, 2008) found that first service conception rates improved and the incidence of ketosis dropped, they also witnessed an increase in the incidence of metritis. This suggests that decreasing inflammation may result in a trade-off between metabolic and immune function.

Dietary interventions

Supplementing the diet with either antioxidants or n-3 fatty acids may also have a beneficial effect on the immune response of postpartum cattle. Systemic antioxidant concentrations decrease during the postpartum period and are associated with a number of transition cow illnesses (LeBlanc et al., 2004). Supplementing with antioxidants such as vitamin E and selenium improves immune cell function and has been shown to decrease the incidence of RFM (Bourne et al., 2007), which is a well-established risk factor for uterine disease. Supplementing the diet with omega-3 oils results in lower blood concentrations of NEFA and BHB, promoting an anti-inflammatory effect.

In conclusion, IU cephalixin appears to improve reproductive performance in cases of both PVD and CYTO. However, the effect of PGF_{2α} remains unclear. In addition, NSAIDs that reduce the inflammatory response may be beneficial when the correct drug and time of administration are utilised. This can be optimised by increasing our knowledge of the underlying mechanisms of inflammation in the postpartum cow, which may allow development of a treatment that regulates inflammation rather than simply suppressing it. Whilst there are some treatment options available, there still remains a requirement for a rapid “cow-side” test that will allow earlier detection, or even prediction of cattle that develop uterine disease, resulting in earlier treatment and prevention.

1.4 Uterine microbiology

The natural calving process weakens physical barriers against contamination, and involves the production and evacuation of a large amount of necrotic tissue from the uterus. Therefore it is not surprising that the vast majority of cows (80-100 %, (Sheldon and Dobson, 2004)) experience an influx of bacteria into the uterus after calving. These bacteria, which are derived from the environment as well as the animal’s skin and faeces, ascend through the vagina and cervix, which remains dilated during the early postpartum period. Uterine inflammation is thought to be initiated by bacterial infection of the uterus, however the cause of uterine disease remains unknown – it could be due to the pathogenicity or abundance of invading bacteria, or the inflammatory response of the host. Additionally it is important to note that cattle can experience CYTO even in the absence of uterine bacteria, whilst bacteria have also been detected in cattle with PMN% below the threshold for CYTO diagnosis (Ricci et al., 2015). This suggests that bacterial infection is not the sole cause of inflammation within the reproductive tract.

Traditional culture methods have identified *Escherichia coli* (*E. coli*) and *Trueperella pyogenes* (*T. pyogenes*) as the main pathogens causing uterine disease (Doherman et al., 2000b, Williams et al., 2005). The Gram-negative bacterium *E. coli* has been isolated from the uterus within the first few weeks postpartum, which is believed to alter the intra-uterine environment, facilitating subsequent infection with *T. pyogenes* and other opportunistic bacteria (Doherman et al., 2000b, Williams et al., 2007). The detrimental effect of *E.coli* and its endotoxin lipopolysaccharide (LPS) on reproductive performance in cattle has been well documented (Bicalho et al., 2012, Machado et al., 2012). Recent studies have highlighted the importance of specific strains of bacteria and virulence factors that are associated with uterine disease (Sheldon et al., 2010, Bicalho et al., 2010). In 2010, Sheldon *et al.* identified a specific strain of *E. coli* in animals with uterine disease, which was found to be more adherent and invasive than strains from unaffected cows, when applied to endometrial and stromal cells *in vitro*. This strain, which they termed Endometrial Pathogenic *E. coli* (EnPEC), was only found to contain one of seventeen virulence genes commonly associated with adhesion and invasion in other pathogenic *E. coli* strains: the ferric yersiniabactin uptake gene (*fyuA*) which is associated with bacterial iron uptake. However, a larger study by Bicalho *et al.* (2010) identified six virulence factors associated with metritis and endometritis: *fimH*, *hlyA*, *cdt*, *kpsMII*, *ibeA* and *astA*. The *fimH* gene was the most prevalent in cattle with uterine disease, which encodes a type 1 pili adhesive protein that enables bacteria to colonise epithelial surfaces.

The functioning of aerobic microbes and PMN in the uterus during the first few days postpartum results in an anaerobic environment that promotes the growth of anaerobic bacteria such as *T. pyogenes* and *Fusobacterium necrophorum* (*F. necrophorum*). *T. pyogenes* is a Gram-positive opportunistic bacterium, and is regularly associated with the occurrence of metritis and endometritis (Williams et al., 2005, Bicalho et al., 2012, Machado et al., 2012). The primary virulence factor associated with *T. pyogenes* is pyolysin (PLO), a cholesterol-dependent cytolysin (CDC) that is capable of forming large pores in eukaryotic cell membranes (Jost and Billington, 2005). As CDCs require cholesterol to function, they are not normally able to penetrate the intact epithelial lining of the healthy uterus, as epithelial cells contain less cholesterol than the underlying stromal cells. Therefore *T. pyogenes* usually operates as an opportunistic pathogen, taking advantage of damage to the epithelial cell layer caused by parturition or other microbes (Amos et al., 2014). *T. pyogenes* often works in synergy with *F. necrophorum*, a Gram-negative, strictly anaerobic bacterium. *T.pyogenes* assists the invasion of *F.necrophorum* into uterine tissues, whilst *F.necrophorum* produces a leucocidal toxin that protects *T. pyogenes* from phagocytosis, allowing synergistic growth and proliferation of both bacteria (Roberts, 1967).

Although a number of pathogens have been linked to metritis and PVD, the case with CYTO is not so clear. (Prunner et al., 2014a) discovered that the presence of *E. coli* and *T. pyogenes* in the uterus was not linked to the onset of CYTO between 0 and 28 DPP, although it did correlate with a higher bacterial growth density. A study by the same group using Fourier-transform-infrared (FTIR) spectroscopy found no differences between healthy cows and those with CYTO, whilst *T. pyogenes* and *Streptococcus* spp were more commonly isolated from PVD cows (Prunner et al., 2014b). Additionally, (Madoz et al., 2014) were unable to isolate bacteria from CYTO cows. These results suggest that specific pathogens play a minor role in the pathogenicity of CYTO, as opposed to metritis and PVD. However, it is important to note that many of these studies were based on traditional culture-dependent methods, whereas more recent metagenomic studies are also able to identify non-cultivable bacteria. In fact, the uterine microbiome appears to be much more complex and diverse than previously realised, and varies according to postpartum health status and DPP (Santos et al., 2011, Santos and Bicalho, 2012). Interestingly, *E. coli* and *T. pyogenes* were not significantly identified in any cattle using PCR-based methods, which is likely due to the biased selection of fast-growing microorganisms in culture, which may exclude important less abundant or un-culturable bacteria that play a key role in the establishment of uterine disease (Santos and Bicalho, 2012)

Synergistic interactions also exist between endometrial bacteria and bovine herpesvirus 4 (BoHV-4) (Donofrio et al., 2007, Donofrio et al., 2008). BoHV-4 has been identified in a number of reproductive diseases, including metritis, endometritis, vaginitis, mastitis and abortion (Chastant-Maillard, 2015). Primary viral multiplication occurs within the epithelial cells of the endometrium, mammary gland, fetal tissues and vascular endothelia (Lin et al., 1997, Donofrio et al., 2007), followed by replication within lymphocytes and macrophages, where it usually remains latent until activated (Donofrio and van Santen, 2001). However, the pathogenicity of this virus alone appears weak, and symptoms do not usually develop until BoHV-4 is able to co-operate with bacteria in the uterus or udder (Chastant-Maillard, 2015). Indeed, BOHV-4 has also been identified in healthy cattle (Monge et al., 2006), and appears to become reactivated at calving, resulting in seroconversion during the periparturent period in both healthy and metritic cattle (Graham et al., 2005). This is likely due to postpartum stressors such as high cortisol levels. However in metritic animals, isolation of BoHV-4 in uterine fluid usually occurs alongside isolation of bacteria such as *T. pyogenes* and *E. coli* (Monge et al., 2006), suggesting that BoHV-4 predisposes the cow to bacterial invasion. These results were confirmed using an *in vitro* model in which activation and replication of BoHV-4 in endometrial stromal cells was driven by the presence of *E.coli* and LPS, and the resulting pro-inflammatory factor PGE₂ (Donofrio et al.,

2008). This leads to a vicious circle where endometrial tissue damage by BoHV-4 increases the severity of the inflammatory response, again enhancing BoHV-4 replication (Sheldon et al., 2009).

1.5 Uterine immunology

The uterus has a uniquely adapted local immune system, which is capable of tolerating the presence of sperm and the semi-allogenic blastocyst whilst maintaining the ability to fight infectious agents. Back in the 1940s, the phenomenon of immune tolerance by the uterus was first proposed by Sir Peter Medawar, the “father of reproductive immunology”. He claimed that the foetus was able to survive as an allograft due to suppression of the maternal immune system (Medawar, 1953). Twenty years later, Beer and Billingham (1971) built on this idea, hypothesising that immunosuppressive factors were released by the mother and/or the foetus. These initial concepts laid down the foundations for understanding the immune system during pregnancy; however more recent studies have proven some of these ideas incomplete. By 1993, Wegmann and colleagues had proposed that rather than complete suppression of the immune system, pregnancy results in a switch from a type 1 helper lymphocyte (Th1) response to a predominantly Th2 response. Th1 lymphocytes produce mainly pro-inflammatory responses whilst Th2 cytokines have a modulatory or anti-inflammatory response. Although this theory is now generally accepted, it is also considered a rather simplistic explanation as it does not account for the expression of Th1-type cytokines in the endometrium at implantation or parturition (Mor and Cardenas, 2010).

In cattle, immunosuppression during pregnancy begins with the formation of the corpus luteum during the luteal phase of the oestrous cycle. Progesterone released by the CL inhibits immune function in the uterus, which has been demonstrated by the enhanced survival of tissue allografts transplanted into the endometrium of sheep treated with progesterone (Majewski and Hansen, 2002). The bovine conceptus reinforces this immunosuppression by secretion of interferon tau (IFNT) from the trophoblast. As well as inducing maternal recognition of pregnancy, IFNT stimulates expression of immunosuppressive genes in the maternal uterus which both inhibits lymphocyte function and enhances antibacterial and antiviral factors. Prior to blastocyst hatching, the conceptus expresses major histocompatibility (MHC) class I antigens but manages to avoid detection by the maternal immune system due to the presence of the zona pellucida which acts as a physical barrier (Hansen, 2011). Expression of fetal MHC class I antigens on the interplacental trophoblast cells remains low throughout most of pregnancy, which prevents recognition by maternal CD8⁺ T cells. In addition, both classical and non-classical

MHC class I antigens are expressed, the latter of which are known to inhibit leukocyte action (Davies, 2007).

During parturition, a decline in progesterone levels initiates the reversal of these immunosuppressive changes and induces a pro-inflammatory state, essential for delivery of calf and placenta and the postpartum clearance of invading pathogens. Individual variation in the adaption to these immune changes is thought to play a part in the susceptibility of some cattle to uterine infection (Hansen, 2013). Following calving, immune defences in the uterus are already compromised due to the loss of parts of the epithelial layer of the endometrium, which makes up a vital part of the innate immune system. Although we now know that a certain degree of inflammation is beneficial for the involution process (Chapwanya et al., 2012), dysregulation of the inflammatory response (either excessive or inadequate inflammation) in some individuals is believed to be a leading cause of uterine disease and infertility (LeBlanc, 2014).

1.5.1 Inflammation

Inflammation is a protective biological response that is triggered by harmful stimuli such as pathogens, tissue damage and tissue malfunction (Janeway, 2008, Medzhitov, 2008). Regardless of the cause, the main aim of inflammation is to restore tissue homeostasis by removing noxious stimuli and initiating tissue repair (Medzhitov, 2008). The inflammatory response has been best characterised for infection by pathogenic microbes, which are detected by pathogen recognition receptors (PRRs) such as the trans-membrane Toll-like receptors (TLRs) and intracellular NOD (nucleoside-binding oligomerization-domain protein)-like receptors (NLRs) (Janeway, 2008). Pathogen recognition is performed primarily by “classical” immune cells such as tissue-resident macrophages and mast cells, as well as “non-classical” epithelial and stromal cells. This leads to the production of chemokines, cytokines, amines and eicosanoids which promote the extravasation of leukocytes which are usually confined to the bloodstream, into the infected tissue. Endothelial cells lining the blood vessels are stimulated to express adhesion molecules, such as selectins, which bind to integrins and chemokine receptors on leukocytes, encouraging selective migration of leukocytes whilst preventing the movement of erythrocytes (Medzhitov, 2008). Neutrophils are the first immune cell to reach the site of infection, and following activation by pro-inflammatory cytokines or direct pathogen contact, they eliminate pathogens through phagocytosis or degranulation (Nathan, 2006). As neutrophils elicit a non-specific response, inevitably damage to host tissue also occurs (Nathan, 2002). Inflammation was first described by Celsus in AD40 by the observation of ‘rubor, calor, dolor and tumor’; Latin for redness, heat, pain and swelling. These clinical symptoms reflect the increased dilation of local

blood vessels, which assists immune cell extravasation by increasing blood flow at a lower velocity (Janeway, 2008).

A healthy acute inflammatory response involves rapid clearance of noxious stimuli, followed by a resolution phase and tissue repair. Resolution involves the release of anti-inflammatory molecules such as IL-10 and pro-resolving mediators such as lipoxins, resolvins, protectins and maresins (Serhan et al., 2014, Levy et al., 2001). These molecules function to restrict further immune cell infiltration, induce apoptosis in exhausted neutrophils and reprogram macrophages to phagocytose the apoptotic neutrophils (Sugimoto et al., 2016). If the acute inflammatory response is unable to eliminate the pathogen, a progressive shift in cell type occurs, leading to the replacement of neutrophils by macrophages and T cells. If this fails, chronic inflammation can arise.

1.5.2 Defence mechanisms of the bovine uterus

The reproductive tract is a highly conserved system, and thus it makes sense that the primary defence mechanism against pathogens is the innate immune system. The innate immune system is an evolutionary ancient defence system present in animals, plants, fungi and primitive multicellular organisms. The reproductive defence system involves a number of non-specific mechanisms, from the anatomical barriers of the cervix and epithelial lining of the uterus and chemical defences (such as mucus), to more active defence strategies such as pattern recognition receptors and antimicrobial peptides.

1.5.2.1 Mucosal epithelia

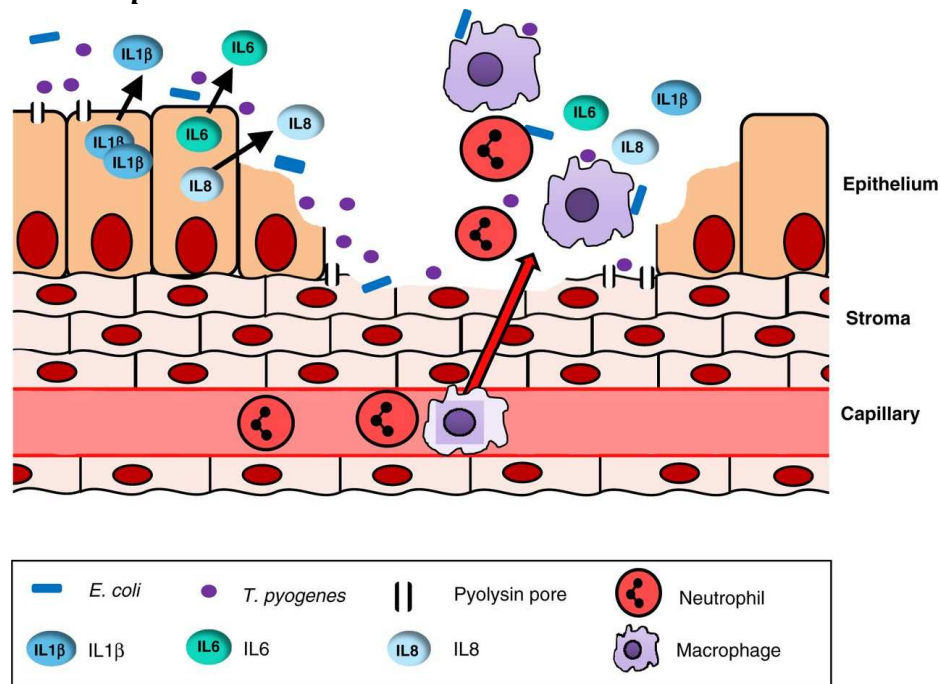


Figure 1-7: Endometrial infection and inflammation.

Following parturition, bacteria enter the uterus and adhere to and invade the endometrium, particularly at areas of tissue damage. Pathogen recognition receptors on endometrial epithelial and stromal cells detect invading PAMPs and release pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 which recruit immune cells from circulation, such as neutrophils and macrophages, to the site of infection. These immune cells destroy invading microbes and help to resolve tissue damage (Sheldon et al., 2014).

The first line of defence in the reproductive tract consists of the physical barriers that normally prevent entry of pathogens in healthy individuals. This includes the vagina and cervix, as well as the layer of columnar epithelium lining the inner wall of the uterus. Recent studies have shown that the epithelium is more than just a barrier, and actively assists in the detection of pathogens. Intact endometrial tissue from the bovine uterus expresses all 10 TLRs, which is not surprising as the endometrium contains immune cells. However, more recent studies have also reported

the expression of TLRs 1-7 and 9 in endometrial epithelial cells cultured *in vitro* (Chapwanya et al., 2013, Davies et al., 2008). Upon TLR activation with LPS, endometrial epithelial cells release pro-inflammatory cytokines such as IL-1 β , IL-6 and IL-8, which attract immune cells to the site of infection (Figure 1-7) (Cronin et al., 2012). They also secrete antimicrobial peptides (AMPs) such as lingual and tracheal antimicrobial peptides (LAP and TAP), which destroy invading bacteria and prevent them from binding to host cell membranes (Davies et al., 2008). Gene expression profiles from endometrial biopsies or cytobrush samples from cows with uterine disease have supported these findings (Herath et al., 2009b, Fischer et al., 2010). Additional defence is provided by goblet cells within the epithelium, which secrete mucus containing enzymes such as mucin 1 (MUC1) and lysozyme. Mucins provide protection by binding to pathogen oligosaccharides, preventing them from reaching the epithelial cell surface. The *MUC1* gene is upregulated both in bovine endometrial epithelial cells stimulated by LPS *in vivo* (Davies et al., 2008) and in cattle suffering from uterine disease (Kasimanickam et al., 2014). Lysozyme expression is also increased in cattle with uterine disease, which breaks down bacterial cell walls (Hoelker et al. 2012). Importantly, activated endometrial cells produce cytokines and chemokines such as TNF α , IL-1 β and IL-8 that attract and activate neutrophils and monocytes, initiating bacterial killing (Roach et al., 2002).

1.5.2.2 Stromal cells

Beneath the surface layer of endometrial epithelium lies the stroma. The stroma consists of stromal cells, blood vessels and uterine glands and provides primarily a structural and connective role. Endometrial stromal cells arise from mesenchymal stem cells (Aghajanova et al., 2010) and have a fibroblast-like appearance and function. However they are referred to as either endometrial stromal cells or endometrial stromal fibroblasts in the literature in order to differentiate them from other fibroblast-like pluripotent precursors that give rise to a range of other cell types (Dunn, 2003). Endometrial stromal cells are well known for their role in reproductive function: the endometrial stroma is hormonally responsive, increasing in thickness during the follicular phase (oestrous) and decreasing during anoestrous. In species with invasive implantation such as the human and mouse, stromal cells also undergo an important transformation known as decidualization, which is required for successful embryo implantation (Dunn, 2003). Following parturition, patches of the epithelial cell barrier are often destroyed, exposing the underlying stromal cells to invading pathogenic bacteria. Therefore stromal cells are also capable of responding to PAMPs such as LPS, which results in the production of IL-6 and IL-8 *in vitro* (Cronin et al., 2012). Pure stromal cell populations express all TLRs apart from TLR5 and TLR8 (Davies et al., 2008).

1.5.2.3 Toll-like receptors

Recognition of invading microbes is carried out by pathogen recognition receptors (PRRs) which are able to detect specific pathogen-associated molecular patterns (PAMPs) present on or released by bacteria, viruses and fungi. Danger signals and damage associated molecular patterns (DAMPs) such as IL1 α , which are released following tissue injury, can also be detected by PRRs. The best characterised family of PRRs are the Toll-like receptors (TLRs). Figure 1-8 outlines the major TLRs and examples of the downward signalling pathways they induce. The first functional mammalian PRR to be discovered was TLR4, which detects LPS from Gram negative bacteria (Poltorak et al., 1998). This occurs with the assistance of two co-receptors, CD14 and MD-2. Since then, a total of 10 TLRs have been discovered in humans and 13 in mice, which primarily bind PAMPs found in prokaryotes, but not eukaryotes. The most important receptors for the recognition of bacterial PAMPs are TLR1, TLR2 and TLR6, which form heterodimers to detect bacterial lipopeptides (Takeuchi et al., 2001, Takeuchi et al., 2002) and TLR4. TLR5 detects the presence of flaggellin, a protein found in bacterial flagella (Hayashi et al., 2001). TLR3, 7, 8 and 9 are localised to intracellular endosomes and are able to detect double-stranded RNA (TLR3), single-stranded RNA (TLR7 and 8), and CpG-rich hypermethylated DNA (TLR9) from viruses and bacteria.

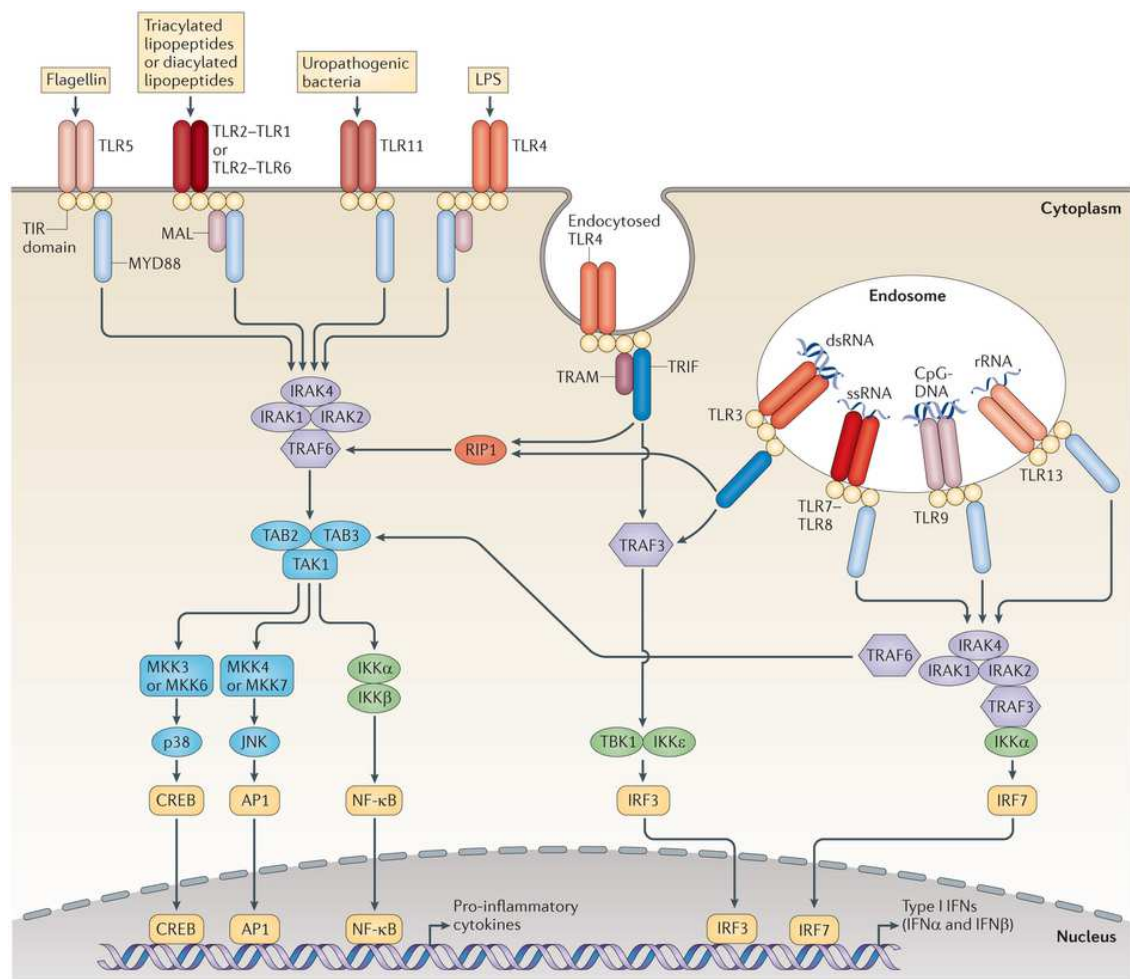


Figure 1-8: TLR signalling pathways

Following infection, PAMPs are detected by TLRs located within the cell membrane at the cell surface, or on endosomes within the cell. Activation of TLRs results in the initiation of downstream signalling pathways, which are initiated through four adaptor proteins: myeloid differentiation primary-response protein 88 (MYD88), MYD88-adaptor-like-protein (MAL), TIR domain-containing adaptor protein inducing IFN β (TRIF) or TRIF-related adaptor molecule (TRAM). Most TLRs (apart from TLR3) predominantly use the MYD88-dependent pathway. MyD88 stimulates interactions between IL-1R-associated kinases (IRAKs) and TNF receptor-associated factor (TRAF) 6 that lead to the activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK)-dependent pathways, resulting in the production of inflammatory mediators such as cytokines, chemokines and prostaglandins (O'Neill et al., 2013).

1.5.2.4 Neutrophils

Neutrophils are the first immune cells on the scene when infection strikes and can tackle a wide range of pathogens. They are the most prevalent white blood cell in mammals, constituting 50-70% of circulating leukocytes in humans. These cells originate from the bone marrow, where signals from cytokines (predominantly granulocyte colony-stimulating factor, G-CSF) invoke maturation. Mature neutrophils travel through the bloodstream to the site of infection, following chemical signals such as IL-8 and IFN γ in a process known as chemotaxis. On arrival neutrophils become activated, initiating a number of pathogen-killing actions. Phagocytosis allows neutrophils to directly ingest pathogens, by internalising and digesting the microbes with ROS and hydrolytic enzymes. The production of ROS is known as oxidative burst. Another key function neutrophils carry out is degranulation – the release of granules with antimicrobial properties. There are several types of granules, which can be grouped into primary (the most toxic), secondary (specific) and tertiary granules, which are released by exocytosis upon stimulation. A hierarchy of granule release occurs in response to increasing intracellular calcium (Ca^{2+}) levels, with high Ca^{2+} concentrations promoting the release of more toxic granules (Sengeløv et al., 1993). A more recent study by (Brinkmann et al., 2004) identified an additional method of pathogen elimination by neutrophils, which involves the generation of neutrophil extracellular traps (NETs). These NETs are composed of chromatin and AMPs which released together are able to effectively trap and kill bacteria. The major advantage of neutrophils is the speed with which they are able to respond, however the production of ROS and AMPs can be very damaging to surrounding cells, which may be why the lifespan of the neutrophil is limited, averaging 8-12 hours in circulation.

Neutrophils play a key role in the postpartum immune response of cattle. The presence of pathogenic bacteria in the uterus after calving results in an influx of neutrophils into the endometrium, followed by rapid clearance during involution in healthy animals (Chapwanya et al., 2012). In animals with uterine disease, neutrophil mobilisation occurs later in lactation and persists longer into the postpartum period, resulting in chronic inflammation and tissue damage (Galvao et al., 2011). Neutrophil function (phagocytic capacity) is also impaired in cattle with uterine disease (Cai et al., 1994, Kim et al., 2005), which is believed to be linked to a greater energy deficit in these animals after calving (Hammon et al., 2006).

1.5.2.5 Monocytes and macrophages

The macrophage is another important phagocyte involved in pathogen clearance. Macrophages are derived from monocytes, which are produced in the bone marrow and released into circulation where they differentiate into either macrophages or dendritic cells. Unlike

neutrophils which are short-lived, macrophages can survive for up to a couple of months. They are attracted to the site of infection by chemotaxis and activated by the inflammatory cytokines IFN γ and TNF, where they ingest aged neutrophils, cellular debris and pathogens (Mosser and Edwards, 2008). Macrophages also act as professional antigen presenting cells to T helper cells, which results in the production of specific antibodies that assist with opsonisation and thereby phagocytosis of pathogens. Additionally macrophages assist with wound healing, removing dead tissue by releasing proteases, and stimulating tissue regeneration with the release of growth factors and other cytokines.

Accumulation of macrophages in the endometrium during pregnancy is common in many species, including the cow (Oliveira and Hansen, 2008). During the peripartum period, CD14⁺ monocytes and macrophages migrate into the caruncular tissue (but not the fetal cotyledons) and are believed to be involved in the release of fetal membranes after birth (Miyoshi and Sawamukai, 2004). In cattle with retained fetal membranes, macrophages isolated from the placentomes of cattle with retained fetal membranes showed reduced activity of the lysosomal enzyme acid phosphatase (Miyoshi et al., 2002). A recent study by (Pomeroy et al., 2017) also found that different subsets of monocytes present in circulation pre-partum were able to predict which cattle later developed postpartum metritis and mastitis, suggesting that these subsets have different functional roles in the inflammatory response.

1.5.2.6 Eosinophils, basophils and mast cells

Eosinophils, basophils and mast cells are all well-known for their roles in fighting multicellular parasites. They are also involved in the dysfunctional immune response associated with allergy and asthma. All three cell types are granulocytes, derived from a common myeloid precursor. Mast cells are located at mucosal sites and in connective tissue, such as the airways and intestines where they are most likely to come into contact with parasites and other pathogens. In contrast, eosinophils and basophils are recruited from circulation to the site of parasite invasion. In addition to these traditional functions, an increasing number of novel roles for these cells are being identified. Eosinophils are involved in immune regulation as well as tissue remodelling and repair. A relevant example is the influx of eosinophils that enter the uterus and cervix during pregnancy, which are thought to be involved in cervical dilation and postpartum remodelling (Timmons et al., 2009). Eosinophil accumulation and degranulation may also play a role in the remodelling and fibrosis that occurs during the inflammatory disease endometriosis (Blumenthal et al., 2000).

1.5.2.7 Antimicrobial peptides

Antimicrobial peptides (AMPs), also known as host defence peptides (HDPs) are oligopeptides found in a wide variety of different species, in both prokaryotes and eukaryotes. In general AMPs are small, consisting of around 12-50 amino acids, and positively charged. AMPs are grouped according to their amino acid composition and structure, of which many different varieties exist. Structural differences also exist between species. AMPs are produced by both professional phagocytic cells (within granules) and epithelial cells, and whilst some are constitutively expressed, others are induced by infection or injury. AMPs are an important component of mucosal immunity, as they non-specifically kill bacteria, fungi and viruses. Most function by binding to the cell membrane of invading microbes and creating pores which causes a loss of essential nutrients and quickly induces cell death. The best studied AMPs are the defensins, which appear to be particularly important in the cow as the bovine genome contains 57 putative β -defensin genes – more than any other mammal (Davies et al., 2008). Expression of lingual antimicrobial peptide (*LAP*), bovine neutrophil β -defensins (*BNBD4*, *BNBD5*) and bovine β -defensins (*BBD19*, *BBD123* and *BBD124*) is present in the endometrium of cattle. Endometrial epithelial cells alone also express tracheal antimicrobial peptide (*TAP*), *LPA*, *BNBD4* and *BNBD5* (Davies et al., 2008). Studies by Wathes and others also found that expression of β -defensin 1 (*DEFB1*) and *TAP*, and the calcium binding AMPs S100A8 and S100A9 were increased in the endometrium of postpartum cattle suffering from severe negative energy balance (Wathes et al., 2009, Swangchan-Uthai et al., 2013).

1.5.2.8 Cytokines & chemokines

Following the detection of invading pathogens by PRRs, pro-inflammatory cytokines are released by both leukocytes and endometrial epithelial and stromal cells. Endometrial cells express TLR4, which detects LPS released by gram negative bacteria such as *E.coli*. Stimulation of TLR4 and its co-receptors cluster of differentiation (CD) 14 and lymphocyte antigen 96 (LY96) induces a signalling cascade, promoting the production of several pro-inflammatory cytokines, such as interleukin (IL)-1 β and IL-6, and the chemokine IL-8 (Sheldon, 2014). Release of pro-inflammatory cytokines initiates an inflammatory response to clear infection, and recruits immune cells to the site of infection.

Interleukin (IL)-1 is one of the most important molecules of the innate immune system. Of the 11 IL-1 family members, it is IL-1 α and IL-1 β that are best studied, due to their potency and the plethora of biological functions documented. Both IL-1 α and β signal through the same receptor and have very similar functions and thus are often referred to simultaneously as IL-1. Typically IL-1 α acts locally, whilst IL-1 β is often secreted into circulation (Sims and Smith, 2010).

Ordinary expression of these cytokines in healthy individuals is very low, and must be induced in response to pathogen or damage induced signals. IL-1 β also requires cleavage from its pro-form to an active form before it is functionally active. This is performed by a multi-protein complex known as the inflammasome, which usually contains the sensor component nucleotide-binding domain and leucine rich repeat pyrin 3 domain (NLRP3), and results in caspase-1 activation which cleaves pro-IL-1 β into its mature form (Schroder and Tschopp, 2010). Serum levels of IL-1 increase at calving, which assists leukocyte transport into the endometrium by inducing vasodilation (Singh et al., 2008). IL-1 also stimulates prostaglandin synthesis and increases circulating calcium concentrations, which induce myometrial contractions to remove lochia from the uterus (Davidson et al., 1995). Cattle that develop retained placenta (RP) and PVD have lower pre-calving serum IL-1 concentrations compared to healthy cows (Islam et al., 2013), which could indicate dysregulation of the immune response before calving. In the same study, circulating IL-1 concentrations decreased in healthy cattle throughout the postpartum period, but increased in those with uterine disease, resulting in a significant difference between the groups by 30 DPP. Local expression of *IL1* is also upregulated in the uterus after calving (Chapwanya et al., 2012), and is increased in cattle with uterine disease (Herath et al., 2009b, Gabler et al., 2010, Fischer et al., 2010), suggesting that excessive inflammation may be contributing to infertility in these animals. It is worth noting that one study produced contradictory results, observing lower expression of *IL1B* at 1 week postpartum in cattle that later developed endometritis (Galvao et al., 2011). Expression of TNF was also lower in the same cattle, leading the authors to conclude that it was an inadequate immune response driving uterine disease in these animals.

Interleukin 6 is also produced during the early stages of the inflammatory response. IL-6 production by immune cells (particularly T cells and macrophages) and stromal cells is stimulated by IL-1 and TNF and as a direct response to PAMPs. IL-6 promotes the maturation of neutrophils, monocytes and natural killer (NK) cells and is a key mediator of the acute phase response (APR). In cattle, peripheral IL-6 concentrations decline after calving. However, IL-6 concentrations remain higher in PVD cows throughout the postpartum period, and lower in those with retained placenta, compared to healthy cattle (Ishikawa et al., 2004). Endometrial *IL6* expression is also upregulated in the early postpartum period (Chapwanya et al., 2009, Chapwanya et al., 2012) and this is higher in cattle with CYTO at 17 DPP (Gabler et al., 2010) and 28-41 DPP (Ghasemi et al., 2012).

The chemokine IL-8 also increases after calving, peaking at 15 DPP in healthy cows, and returning to normal levels by 60 DPP (Chapwanya et al., 2012). IL-8, also known as CXC chemokine ligand 8 (CXCL8), attracts and activates neutrophils, leading to anti-microbial activity (Mitchell et al., 2003). Many cell types are capable of producing IL-8 (such as macrophages and neutrophils), however it is the endometrial epithelial cells that are the dominant producers of IL-8 in the uterus, in response to bacterial stimulation (Chapwanya et al., 2013). Cattle with retained placenta experience lower systemic IL-8 concentrations, which is associated with reduced mobility and myeloperoxidase activity of neutrophils (Kimura et al., 2002). In contrast, *IL8* expression in the uterus increases in accordance with accumulating PMN post-calving, and therefore elevated IL8 expression can result in endometritis, due to an excessive influx of neutrophils into the endometrium, which leads to prolonged inflammation and sub-fertility (Galvao et al., 2011). Excessive *IL8* is also associated with increased PVD (Sheldon, 2014). Interestingly, expression levels of this chemokine are highly variable between individuals, which is at least partly due to genetic polymorphisms in the promoter region of the bovine *IL8* gene (Stojkovic et al., 2016). Indeed, these divergent IL8 haplotypes produce significantly different responses to LPS in bovine endometrial epithelial cells (Stojkovic et al., 2017), suggesting that IL8 haplotype could be an important implication in bacterial infections of the uterus.

Whilst increased expression of IL1, IL6 and IL8 all appear to be linked to the onset of uterine disease, tumour necrosis factor (TNF) expression data is not consistent between studies. TNF is an important regulator of the immune response, and elevated tissue and serum levels are usually described in inflammatory and infectious conditions (Bradley, 2008). Some studies have found that *TNF* is increased in the endometrium of cattle with uterine disease (Gabler et al., 2010, Fischer et al., 2010, Ghasemi et al., 2012, Kasimanickam et al., 2014), whilst others have witnessed reduced expression (Galvao et al., 2011, Chapwanya et al., 2009). Even the systemic results are inconclusive: (Kim et al., 2005) reported increased serum TNF concentrations in endometritic cows at 3 and 4 weeks postpartum, whilst TNF gene expression and protein levels in circulating monocytes were lower in cattle with metritis from calving to 3 weeks postpartum (Galvao et al., 2012). However, these monocytes were stimulated *in vitro* with heat-killed *E.coli* and prior to this basal levels of pro-inflammatory cytokines (including TNF) were higher in monocytes from metritic cows. This suggests that the monocytes may have already been activated *in vivo* (perhaps due to tissue damage at calving), causing a reduced response *in vitro*.

Regulation of the immune response by anti-inflammatory cytokines is important for maintaining immune homeostasis. (Herath et al., 2009b) noted that endometritic cattle had a higher ratio of

IL1 to IL10 in the first week after calving when compared to healthy cows. However, by 3 weeks postpartum *IL10* expression is increased in endometritic cows, suggesting an attempt to regulate inflammation at this time (Foley et al., 2015). Increases in the IL-1 receptor antagonist gene, *IL1RN*, have also been observed at this time point (Gabler et al., 2009).

1.5.2.9 Prostaglandins

Prostaglandins play a key role in the initiation and regulation of the inflammatory response. They are derived from the fatty acid precursor arachidonic acid (AA) which is located in the plasma membrane of cells. Phospholipases (PLAs) release AA, which is then metabolised by cyclooxygenase (COX) enzymes to form PGH₂, a common substrate for production of the four bioactive prostaglandins PGD₂, PGE₂, PGF₂ and PGI₂ by their respective synthase enzymes (Ricciotti and FitzGerald, 2011). Prostaglandin production is normally very low in un-inflamed tissues, but increases rapidly in response to inflammation. The two predominant PGs located in the uterus are PGE₂ and PGF_{2α}.

Infertility in cattle with uterine disease is not only caused by poor uterine health, but also impairment of the oestrous cycle. This is often due to failure of luteolysis, resulting in a prolonged luteal phase and thus delayed conception (Opsomer et al., 2000). In ruminants, luteolysis is induced by pulsatile secretions of prostaglandin (PG) F_{2α} released by the uterus at around day 15-17 of the oestrous cycle (McCracken et al., 1999). But if the animal becomes pregnant, PGE₂ exerts luteoprotective actions, sustaining progesterone secretion to maintain pregnancy (Weems et al., 1997). PGE₂ secretion is stimulated by interferon tau (IFNτ) – a cytokine released by the trophoblast of the developing embryo which also inhibits expression of the oxytocin receptor on endometrial epithelial cells, which is required for PGF_{2α} synthesis (Roberts et al., 1992). This switch from PGF_{2α} to PGE₂ also occurs in cows with PVD and CYTO (Mateus et al., 2003, Baranski et al., 2013) which is thought to be induced by *E.coli* derived LPS (Herath et al., 2009a). The mechanisms behind this increase in PGE₂ were investigated *in vitro*, where stimulation of endometrial epithelial and stromal cells with LPS did not affect the concentration of PGE or PGF synthase enzymes, but levels of PLA2 group IV enzyme (PLA2G6), which liberates AA from the cell membrane, was increased in epithelial cells (Herath et al., 2009a). Prostaglandin D2 (PGD₂) is also believed to play a role in both pregnancy maintenance and inflammation, and expression of the PGD₂ synthase (*PTGDS*) was found to be increased in cattle with CYTO (Gabler et al., 2009, Peter et al., 2015).

1.5.3 The acute phase response

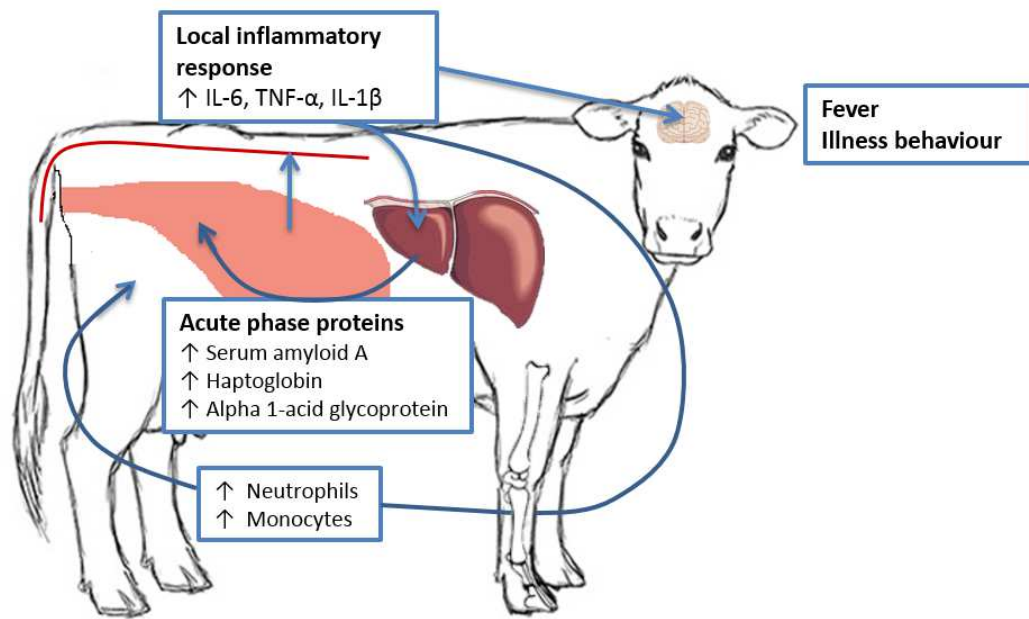


Figure 1-9 The acute phase response in the postpartum cow

Local inflammation in the uterus can induce systemic effects by acting on organs such as the liver, bone marrow and the brain. Pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α activate hepatocytes in the liver, causing the release of acute phase proteins into circulation. They also stimulate the production and release of neutrophils and monocytes from the bone marrow. Additionally, stimulation of the hypothalamus and muscle and fat cells helps to raise the body temperature, inhibiting bacterial and viral replication. Adapted from (Chapwanya, 2009).

When injury or tissue damage become too much for the local defence system, a systemic response is initiated. This affects many organs, and often includes fever, leukocytosis and initiation of the acute phase response (APR) (Ceciliani, 2012). The APR is initiated by TNF- α , IL-1 β and IL-6, which activate hepatocytes in the liver, causing the release of acute phase proteins (APPs) into the plasma (Figure 1-9). Release of these “positive” APPs occurs within just a few hours after infection, which often act as opsonins, assisting in the disposal of pathogens. At the same time a decrease in the secretion of normal blood proteins, or “negative” APPs such as transferrin and albumin occurs, which is believed to increase the number of amino acids available for positive APP synthesis. Positive APPs are often classified by the magnitude of increase during the APR, which varies between species. In cattle, the major APPs (those that increase 10- to 100-fold) are serum amyloid a (SAA) and haptoglobin (Hp) whilst moderate increases (1- to 10-fold) are typically observed in α 1-acid glycoprotein, C-reactive protein and fibrinogen (Cray et al., 2009) Major proteins usually increase rapidly, peaking at 24-48 hours,

whilst moderate APPs elicit a more gradual response, peaking after 2-3 days and declining at a slower rate (Eckersall and Bell, 2010). APPs are very sensitive indicators of inflammation and are often used as biomarkers of disease, although they tend to lack specificity (Ceciliani, 2012).

1.5.3.1 Serum Amyloid A

SAA is a highly conserved protein that is present in most mammalian species. It also functions as a major APP in most mammals, suggesting that it has an important function. The main site of production is in the liver which produces the two circulating isoforms: SAA-1 and SAA-2; however extra-hepatic expression of SAA-3 has been observed in several tissues (Berg et al., 2011), such as the mammary gland during mastitis (Eckersall et al., 2006) and in uterine epithelial cells stimulated with LPS (Chapwanya et al., 2013).

Whilst the biological functions of SAA remain to be elucidated, three major functions have been uncovered. Firstly SAA functions as an apolipoprotein, binding the cholesterol of dying cells and phagocytosed cell membranes of cholesterol-laden macrophages, thereby playing an important role in cholesterol metabolism (van der Westhuyzen et al., 2005). SAA also has immune functions, acting as a chemoattractant for monocytes and neutrophils (Badolato et al., 1994) and as an opsonin for a wide range of Gram-negative bacteria (Hari-Dass et al., 2005).

In cattle, SAA has been assessed as a potential biomarker for uterine disease. SAA concentrations in serum are increased in all cattle within the first week postpartum, but are elevated in cows with metritis or clinical endometritis and persist longer before returning to baseline values (Foley et al., 2015). SAA3 mRNA expression has also been observed in uterine biopsy samples, where it increased in the early postpartum period and expression reflected the severity of inflammation (Chapwanya et al., 2009).

1.5.3.2 Haptoglobin

Haptoglobin (Hp) is an APP expressed mainly in the liver, but also in other tissues such as adipose tissue, ovaries, lungs and placenta (Quaye, 2008). Expression of Hp in hepatocytes is induced primarily by IL-6. The fundamental role of Hp is to bind free plasma haemoglobin (Hb). Haemoglobin is released from red blood cells following haemolysis or cell death, and becomes extremely toxic to cells. The haem group present in Hb is lipophilic which allows it to integrate into and disrupt cell membranes. Haem also contains iron which catalyses the production of reactive oxygen species (ROS) which can cause tissue damage and oxidative stress. Therefore, Hp acts as an antioxidant and indirectly as an anti-inflammatory factor.

Hp also plays a more direct immunomodulatory role. Although Hp is involved in neutrophil recruitment, it also diminishes neutrophil function by inhibiting respiratory burst activity and

lysosomal protease activity. Haptoglobin also induces the release of anti-inflammatory cytokines (IL-10 and heme oxygenase-1, HO-1) from monocytes and macrophages by binding to the CD163 receptor on the surface of these cells (Yang et al., 2018).

Increases in serum Hp after calving have been associated with metritis (Huzzey et al., 2009), purulent vaginal discharge (Williams et al., 2005) and subclinical endometritis (Dubuc et al., 2010b) in postpartum cattle.

1.5.3.3 Alpha-1 acid glycoprotein

Alpha-1 acid glycoprotein (AGP) is part of the immunocalin sub-family, which are a group of binding proteins that also play a role in immunomodulation. In cattle, AGP acts as a potent anti-inflammatory mediator, performing very specific actions on monocytes and granulocytes. High concentrations of plasma AGP released during inflammation acts on monocytes to inhibit migration, by down-regulating the adhesion protein CD-18 (Lecchi et al., 2008). AGP also prevents apoptosis of monocytes, which are involved in the clear up of dead and dying inflammatory cells. In addition, AGP suppresses polymorphonuclear leukocyte (PMN) function by inhibiting the release of ROS and secondary granule exocytosis (Miranda-Ribera et al., 2010). The specific inhibition of secondary granule release is thought to play a role in reducing tissue damage caused by PMN action, as AGP itself is stored within the secondary granules in human and bovine neutrophils, which then takes effect once the cell has reached its site of action. Interestingly, the activity of AGP is heavily regulated by its glycosylation status, which can change based on disease status of the animal. For example, each of the anti-inflammatory activities described above is induced by removal of sialic acid terminal residues from the surface of the AGP molecule (Miranda-Ribera et al., 2010).

Alpha-1 acid glycoprotein has not been as extensively studied as SAA and Hp in postpartum cattle, however high levels of serum AGP have been detected in the serum of postpartum cattle (Williams et al., 2007), which has been related to the growth density of uterine pathogens (Williams et al., 2005), particularly *E.coli* (Sheldon et al., 2001). Increased circulating AGP at 2 weeks postpartum is also linked to delayed involution and increased time to artificial insemination (AI) (Aungier et al., 2014).

1.6 Regulation and resolution of inflammation

In cattle, recent studies have concluded that a rapid and effective inflammatory response within the first week postpartum is a normal and healthy event (Chapwanya et al., 2012). Indeed, a certain degree of inflammation is required during the parturition process, to enable cervical dilation, uterine contractions and rupture and expulsion of fetal membranes (Challis et al., 2009). Furthermore, inadequate expression of a number of inflammatory mediators is associated with retained fetal membranes after calving (Boro et al., 2014). However, factors such as metabolic stress and uterine dysbiosis in the early postpartum period may predispose some cattle to uterine disease by affecting the regulatory mechanisms required for effective resolution of inflammation. Inadequate resolution of inflammation results in chronic inflammation and excessive tissue damage, which leads to dysregulation of tissue healing (Sugimoto et al., 2016). It has been proposed by (LeBlanc, 2014) that cattle experiencing an inadequate or excessive inflammatory response are more likely to suffer from uterine disease and infertility (Figure 1-10). The variables that contribute to this shift from adaptive to pathological inflammation are not well understood, and are the main subject of this thesis.

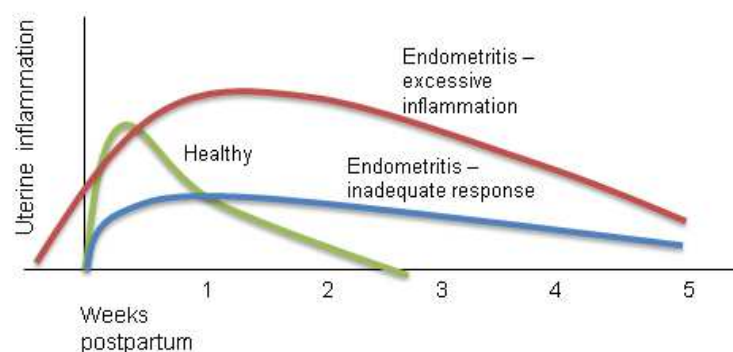


Figure 1-10 Schematic outline of differing immune responses in postpartum cattle

Healthy uterine inflammation occurs within the first week postpartum and is resolved by 3 weeks postpartum. A dysregulated immune response may involve inadequate inflammation, where the innate immune response fails to clear pathogens from the uterus within 3 weeks postpartum, or excessive inflammation, which is severe or prolonged, causing damage which impairs fertility. Adapted from (LeBlanc, 2014).

Inflammation provides protection against infection and tissue damage, but when uncontrolled or unresolved, it can result in tissue damage and disease. Two distinct inflammatory processes have been defined in recent years, referred to as acute and subacute inflammation. Acute inflammation involves a rapid response characterised by the production of inflammatory mediators and increased blood flow to the infected tissue, which encourages the infiltration and

activation of leukocytes. Systemic responses are also initiated, including increased body temperature and heart rate, a decreased appetite and the onset of the acute phase response. A healthy acute inflammatory response is followed by rapid resolution and the restoration of tissue homeostasis. In contrast to this classical inflammatory response, which is initiated by infection and tissue damage, subacute inflammation is associated with tissue malfunction and is often referred to as metabolic inflammation due to its association with metabolic disorders such as type 2 diabetes and obesity (Hotamisligil, 2006). Subacute inflammation is characterised by mild increases in pro-inflammatory mediators and chronic low-grade inflammation. Similar to patients with obesity, cattle with an excessive body condition score at calving are more likely to experience prolonged subacute inflammation. Both types of inflammatory response share the same signalling pathways, and therefore it is usually the magnitude of the inflammatory response that determines whether it is healthy or pathological. However recent studies have also demonstrated the ability of acute and subacute stimuli to produce distinct signals. For example, the concentration of LPS can influence inflammatory response pathways in macrophages. A high dosage activated the classic NF- κ B pathway resulting in a rapid acute response, whilst a low dosage of LPS was incapable of activating NF- κ B and instead resulted in activation of the transcription factor CCAAT/enhancer-binding protein delta which led to mild and prolonged inflammation (Maitra et al., 2011).

The process of resolution begins soon after the onset of inflammation, and involves a number of mechanisms that promote a return to tissue homeostasis (Figure 1-11). This includes restriction of neutrophil tissue infiltration, induction of apoptosis in exhausted neutrophils and efficient clear up by macrophages, downregulation of pro-inflammatory cytokines and chemokines and the reprogramming of macrophages from a classically activated M1 phenotype to a tissue-resident M2 phenotype (Sugimoto et al., 2016). These roles are carried out by anti-inflammatory mediators, such as IL-10, and pro-resolving mediators such as lipoxins, resolvins, protectins and maresins (Serhan et al., 2014, Levy et al., 2001). Pro-resolving effects are different to anti-inflammatory effects, as the latter have an inhibitory or blocking action, whilst the former activate a specific process, such as apoptosis. Inadequate production of anti-inflammatory or pro-resolving factors is likely to result in persistent inflammation. This is evident in patients with ulcerative colitis, who often experience a lack of LXA₄ production (a pro-resolving lipoxin) (Mangino et al., 2006) and Crohn's disease, where decreased expression of annexin A1 in the colon (a pro-resolving peptide) is linked to incomplete resolution of inflammation during therapy (Sena et al., 2013). Additionally, overexpression of pro-resolving inhibitors can also limit resolution of inflammation. For example, the actions of transforming

growth factor (TGF)- β 1, which regulates mucosal inflammation, are blocked by the inhibitor SMAD7 (Monteleone et al., 2001) thereby reducing the ability of TGF β 1 to limit pro-inflammatory cytokine synthesis.

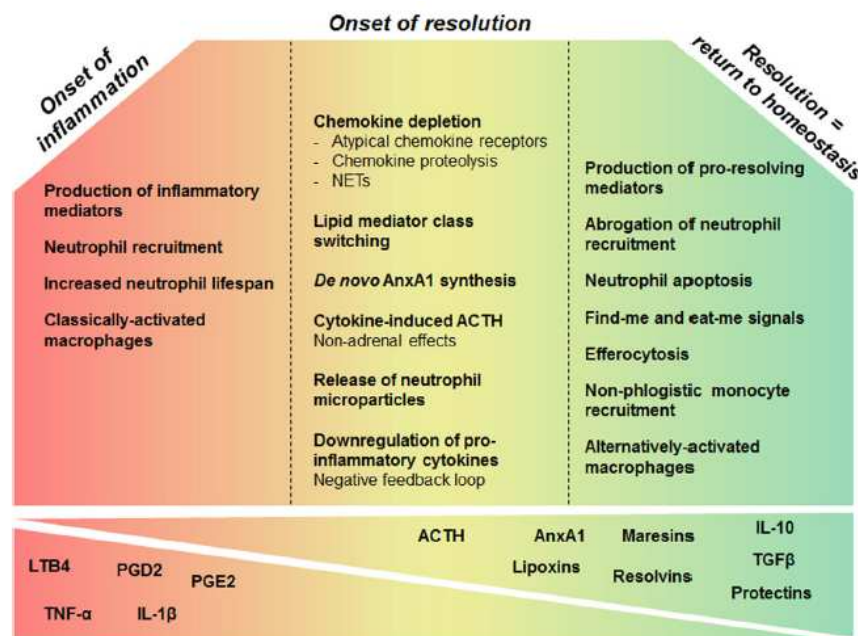


Figure 1-11 Overview of mechanisms involved in the regulation and resolution of inflammation

The onset of inflammation is associated with the production of inflammatory mediators, which recruit immune cells to the site of inflammation and promote leukocyte survival. Once the inflammatory response has been well established, several events occur that promote onset of the resolution phase and a return to homeostasis. This includes chemokine depletion which prevents the recruitment of more immune cells, a switch in production of pro-inflammatory to pro-resolving lipids and the release of neutrophil microparticles which contain pro-resolution mediators such as AnxA1. Many of these resolution mediators activate the apoptosis of granulocytes. Neutrophil apoptosis causes the release of signals that prevent further neutrophil recruitment and induce their own clearance by scavenger cells via “find me and eat me signals”. Efferocytosis of these apoptotic neutrophils also results in a switch in macrophage function, from a pro- to an anti-inflammatory state. Each of these events promotes the return to tissue homeostasis (Sugimoto et al., 2016).

Often mechanisms that are involved in the initiation of inflammation also play a role in the onset of resolution. For instance, NF κ B which plays a central role in the initiation of inflammatory pathways can also assist in the regulation of inflammation. The dual function of this molecule is dimer specific, as the pro-inflammatory active form consists of a p65/p50 heterodimer, whilst the p50/cRel, p65/cRel and p50/p50 dimers typically perform anti-inflammatory actions (Bohuslav et al., 1998). Many pro-inflammatory cytokines are also involved in the initiation of

the resolution process. In rats with intestinal reperfusion injury, IL-1 β promotes the production of IL-10, protecting against local and systemic damage (Souza et al., 2003). Inhibition of IL-1 β resulted in greater tissue damage, increased expression of TNF- α and lethality (Souza et al., 2003).

Neutrophils are well known for their key role against invading pathogens in early inflammation; however they also contribute to the onset of resolution. Proteases released by neutrophils not only degrade the surrounding cells, but are also capable of degrading inflammatory cytokines, such as IL-1 β and TNF- α , but not IL-10 (Gresnigt et al., 2012). Cytokine and chemokine depletion which prevents the recruitment of further neutrophils also occurs through proteolysis by matrix metalloproteinases (MMPs) and the binding of atypical chemokine receptors (ACKR) (Nibbs and Graham, 2013). Additionally, neutrophils undergo a switch in lipid production, from the classic pro-inflammatory eicosanoids (prostaglandins and leukotrienes) to pro-resolving lipoxins and resolvins (Sugimoto et al., 2016). Therefore it is not surprising that neutrophil depletion results in delayed tissue repair (Harty et al., 2010). Macrophages also undergo a phenotypic switch, from a pro-inflammatory to an anti-inflammatory function. Following efferocytosis of apoptotic neutrophils, anti-inflammatory properties are induced in the macrophage, inhibiting production of IL-1 β , IL-8 and TNF- α , whilst enhancing the production of TGF- β 1, PGE and platelet-activating factor (PAF) (Fadok et al., 1998).

It is clear that an effective resolution program is required to achieve tissue homeostasis, and defects in any of the mechanisms that regulate the inflammatory response may result in prolonged and chronic inflammation. A better understanding of the triggers of these pro-resolving effects, which are likely to be tissue and disease specific, will enable us improve treatment for diseases caused by chronic inflammation. Many studies focusing on the regulation of inflammation in postpartum cattle with uterine disease have investigated the role of the transcriptional mechanisms involved, and this is discussed in more detail below.

1.7 Transcriptomics and Next Generation Sequencing

A genome is an organism's complete set of genetic information. In 2009 the bovine genome (*Bos taurus*) was sequenced, and was found to contain approximately 22,000 genes; 14,000 of which are common to all mammals (Elsik et al., 2009). Though the genome remains unchanged throughout an organism's lifetime, gene expression is constantly changing, influencing the physiological phenotype of each individual. Gene expression involves transcription of DNA into messenger (m) RNA. The transcriptome is the absolute number of transcripts present in a tissue or cell type under specific conditions (Wang et al., 2009). This includes protein-coding mRNA, as well as small regulatory RNAs such as microRNAs, which assist in the regulation of mRNA translation (Wang et al., 2009).

1.7.1 Advantages of NGS

Technology for transcriptomic analysis involves hybridization-based techniques (microarrays) and the more recent next generation sequencing (NGS) approach. NGS techniques, such as RNA sequencing (RNA-seq), provide many advantages over existing technologies such as qRT-PCR and microarrays. Firstly RNA-seq does not rely upon existing knowledge of the genome sequence (Wang et al., 2009). It also produces very little background signal, unlike microarrays which produce high background levels due to cross-hybridization (Okoniewski and Miller, 2006). Additionally, RNA-seq methods are more sensitive and have a much larger dynamic range of detection for genes expressed at very low or very high levels (can detect fold changes of >9000; (Nagalakshmi et al., 2008)) compared to microarrays (which detect fold changes of up to a few hundred-fold) (Wang et al., 2009). RNA-seq is also very accurate (assessed by qPCR) and highly reproducible.

1.7.2 Library preparation and sequencing

RNA-seq uses deep-sequencing technologies to measure gene expression. Prior to sequencing, RNA samples must be converted into cDNA libraries. This involves fragmentation of the RNA, followed by reverse transcription to create cDNA. Adapters are ligated to one or both ends of the fragments, which allow the libraries to hybridise to the flow cell during sequencing. The libraries are then amplified by PCR.

Sequencing is commercially available through several NGS platforms, though the sequencing industry has been dominated by Illumina. Illumina (and most other high-throughput sequencing platforms) use an emsembl-based sequencing-by-synthesis method (Bentley et al., 2008). Single stranded DNA library fragments attach to the surface of the glass flow cell at one end by hybridisation of the adaptors (which were ligated during library preparation) to oligonucleotides

that are covalently bound to the flow cell. Each bound fragment is then amplified into a clonal cluster through bridge amplification. In this process, the DNA strand folds over and the adaptor hybridises to a second type of oligo on the flow cell, forming a bridge. These bridges act as templates, allowing the reverse strand to be synthesised by DNA polymerase. Denaturation then occurs which separates the forward and reverse strands of DNA. This process is repeated many times, simultaneously creating millions of clusters of DNA (usually around 1000 copies of the same template) and resulting in clonal amplification of all the fragments. The reverse strands are then washed away, and sequencing of the forward strands occurs. This involves hybridisation of a sequencing primer to the adapter sequence, which is extended using fluorescently tagged nucleotides. Following the addition of each nucleotide, a characteristic fluorescent signal is emitted, based on wavelength and signal intensity. All strands within each cluster are sequenced simultaneously. This process is known as sequencing by synthesis, and the number of cycles determines the length of the read. Short sequence reads can be obtained from one end of a cDNA fragment (single-end sequencing) or both ends (paired sequencing) (Wang et al., 2009).

1.7.3 RNA-seq data analysis workflow

The typical data analysis pipeline for RNA-seq includes generation of sequenced data into FASTQ-format files, mapping of reads to the reference genome and detection of differential gene expression (Kukurba and Montgomery, 2015). Quality control is carried out on the raw sequence data, using software such as FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). This allows for identification of errors in base calling, and enables quality and adaptor trimming of low quality bases from the 3' end of sequence read pairs. Following quality control, reads are either mapped to a reference genome or assembled *de novo*. When mapping RNA-seq reads, it is important to use a “splicing-aware” aligner, that can distinguish between reads aligning over an exon-intron junction and reads with short insertions (Kukurba and Montgomery, 2015). Examples of commonly used RNA-seq aligners include STAR (Dobin et al., 2013) and TopHat (Trapnell et al., 2009). Following read mapping, the number of reads that map to a single gene is counted in a process called summarization (Liao 2014), using software such as featureCounts and htseq-count. Differential expression is carried out in most RNA-seq projects, to detect whether there is a significant difference in the number of reads for a transcript in various conditions. Complex statistical models have been developed for this purpose, which control for technical and biological variability. For example, sequencing read depth can cause artificial differences between

samples. Therefore raw read count data is often transformed into reads per kilobase of exon per million mapped reads (RPKM) values (Mortazavi 2008).

1.7.4 Endometrial transcriptome profiling

The majority of studies investigating the immune response of cattle with uterine disease have focused on targeted gene profiling of specific inflammatory mediators such as cytokines, chemokines and AMPs (Table 1-1). These studies have revealed that an overall increase in the expression of pro-inflammatory factors is detected in the endometrium of cattle with uterine disease, from calving until 7 weeks postpartum. More recently, next generation sequencing (NGS) technologies have begun to shed light on the complexity of the postpartum immune response by characterising the entire endometrial transcriptome. Recent work by our group investigated gene expression in uterine biopsy samples from healthy and cytologically endometritic cattle at 7 and 21 DPP using RNA-sequencing (RNA-seq) (Foley et al., 2015). Whilst all animals were in a pro-inflammatory state at 7 DPP, healthy cattle had resolved by 21 DPP, presenting differential expression of over 4000 genes between the two time points. This included a reduction in inflammatory gene expression and enrichment of calcium signalling and regulation pathways, indicating that these cattle had transitioned to a state of tissue repair and proliferation. The endometritic cattle remained in a pro-inflammatory state at 21 DPP, with differential expression of only 31 genes between 7 and 21 DPP. This prolonged inflammatory response was portrayed by the significant enrichment of immune-related KEGG pathways such as *cytokine-cytokine receptor interaction*, *B cell receptor* and *Toll-like receptor*. Interestingly, though all cows were in a pro-inflammatory state at 7 DPP, there remained differential expression of 73 genes, including the upregulation of a few inflammatory genes in endometritic cows, such as *IL1B* and *IL17A*, which warrant further investigation as potential prognostic biomarkers for uterine disease. A similar study by (Walker et al., 2015) confirmed the presence of a prolonged inflammatory state in CYTO cows, using microarrays to characterise endometrial gene expression at 29 DPP.

One drawback to these studies was the lack of differentiation between PVD and CYTO animals (Foley et al., 2015), or inclusion of PVD cows (Walker et al., 2015), which was amended by a recent study that focused on both (Salilew-Wondim et al., 2016). Microarray analysis of uterine biopsies at 42-60 DPP revealed differential expression of 203 genes in PVD compared to healthy cows, and 28 genes in CYTO compared to healthy, 26 of which were also dysregulated in the PVD cows. This suggests there is a core transcriptome associated with uterine disease, which is directly influenced by the degree of inflammation present. Amongst the top 10 over-represented pathways were *immune system process*, *cell adhesion*, *regulation of apoptotic*

signalling pathway, *G protein coupled receptor signalling pathway* and *chemotaxis*. However, unlike the previous studies mentioned, all but one of the genes involved in *immune system process* and *chemotaxis* pathways were down-regulated in the endometritic cows. In addition, none of the genes classically associated with endometritis (*IL1*, *IL6*, *IL8*, *TNF*) were differentially expressed between healthy and sick cows. The NGS results were reflected *in vitro* within the same study, in endometrial epithelial and stromal cells stimulated with LPS concentrations designed to simulate *in vivo* uterine LPS concentrations of PVD and CYTO cows. Changes in gene expression were more pronounced in epithelial cells than stromal, suggesting that these cells are key regulators of the local immune response. Future *in vivo* investigation into the role of these cells would be beneficial.

Transcriptomic studies have also shown that CYTO affects gene expression during early pregnancy (day 7 of the oestrous cycle, following insemination on day 0) in both the endometrium and resident embryo (Hoelker et al., 2012). Endometritis was found to affect the expression of genes related to immune modulation and cell adhesion in the endometrium, and genes involved in membrane stability, the cell cycle and apoptosis in embryos. Confirmation that the immune response is an important factor in fertility outcome in dairy cattle also arose from a recent paper by (Moran et al., 2015). Focusing on Irish dairy cows of very good or very poor genetic merit for fertility, the authors compared gene expression from uterine biopsies collected on day 7 of the oestrous cycle from non-pregnant cows. Network analysis revealed that the 3 most physiologically relevant networks associated with fertility were *actin and cytoskeletal components*, *immune function* and *ion transportation*. More specifically, down-regulation of inflammatory genes and upregulation of multiple ion transporters and cytoskeletal genes was associated with high fertility.

It is now well established that a prolonged inflammatory state occurs in cattle with uterine disease, however little is known about the factors that regulate this chronic inflammation, and at what point in time this switch from healthy to pathological inflammation occurs. Although some degree of inflammation is beneficial for the normal process of involution and immunity, it would appear that elevated inflammation can impair the fertility and productivity of dairy cattle. Thus further research into the immune response during in the early postpartum period, and improved diagnosis of cows with an enhanced inflammatory status may be able to assist in the development of targeted treatment for uterine disease.

Table 1-1 Timeline of differential gene expression in the endometrium of postpartum cows with uterine disease compared to healthy cows

	<i>Week relative to calving</i>							
	Calving	1	2	3	4	5	6	7+
Immune sensors & signals	↑ <i>TLR4</i> (1)	↑ <i>TLR4</i> (2)	↑ <i>TLR4</i> , 6, 10 (3) ↑ <i>NFKB</i> (3)		↑ <i>NFKB</i> (10)			↑ <i>TLR4</i> (8 ^a)
Cytokines		↓ <i>TNF</i> (1) ↑ <i>IL1A</i> (2) ↑ <i>IL1B</i> (2,9) ↓ <i>IL1B</i> (1) ↑ <i>IL1R2</i> (2) ↑ <i>IL6</i> (1) ↑ <i>IL6</i> (9) ↑ <i>IL17A</i> (9)	↓ <i>TNF</i> (3) ↑ <i>TNF</i> (5) ↑ <i>IL1A</i> (3) ↑ <i>IL1B</i> (5) ↓ <i>IL1B</i> ↑ <i>IL6</i> (3,5) ↑ <i>IL12A</i> (3)	↑ <i>TNF</i> (6) ↑ <i>IL1A</i> (4,9,15) ↑ <i>IL1B</i> (6,9,15) ↑ <i>IL1R2</i> (9) ↑ <i>IL1RN</i> (4) ↑ <i>IL6</i> (9) ↑ <i>IL10</i> (9) ↑ <i>IL17A</i> (9)	↑ <i>TNF</i> (7) ↑ <i>IL1A</i> (10) ↑ <i>IL1B</i> (10) ↑ <i>IL6</i> (7)	↑ <i>TNF</i> (14,15) ↑ <i>IL1A</i> (14,15) ↑ <i>IL1B</i> (1) ↑ <i>IL6</i> (14,15) ↑ <i>IL17A</i> (14)	↑ <i>TNF</i> (15) ↑ <i>IL1A</i> (15) ↑ <i>IL1B</i> (15) ↑ <i>IL6</i> (15)	↑ <i>TNF</i> (8 ^a) ↑ <i>IL1A</i> (13) ↑ <i>IL1B</i> (1,8 ^a ,13 ^c) ↑ <i>IL6</i> (1)
Chemokines			↑ <i>CXCL5</i> (5) ↑ <i>IL8</i> (5)	↑ <i>CXCL5</i> (6,15) ↑ <i>IL8</i> (6,15) ↑ <i>CCL22</i> , 24 (9) ↑ <i>CXCL1/2,3</i> (15) ↑ <i>CXCR2</i> (9,15)	↑ <i>CXCR5</i> (15) ↑ <i>CXCL6</i> (10) ↑ <i>IL8</i> (7, 10) ↑ <i>CCL2</i> (10) ↑ <i>CXCL1/2,3</i> (11) ↑ <i>CXCR2</i> (11)	↑ <i>IL8</i> (15) ↑ <i>CXCR2</i> (15)	↑ <i>CXCR5</i> (15) ↑ <i>IL8</i> (15) ↑ <i>CXCR1/2,3</i> (15) ↑ <i>CXCR2</i> (15)	↓ <i>CXCR4</i> (12 ^b)
Prostaglandins			↑ <i>COX2</i> (5)	↑ <i>PTGDS</i> (4) ↓ <i>PTGES</i> (4)	↑ <i>PTGDS</i> (15)	↑ <i>PTGDS</i> (15) ↑ <i>PTGES</i> (14) ↑ <i>PGHS2</i> (14) ↑ <i>PTGIS</i> (15)	↑ <i>COX1</i> (15) ↑ <i>COX2</i> (15)	↑ <i>PTGDS</i> (15) ↓ <i>PTGDS</i> (12 ^b)
Antimicrobial peptides			↑ <i>TAP</i> (3) ↑ <i>DEFB5</i> (3)	↑ <i>LAP</i> (9) ↑ <i>DEFB5</i> (9) ↑ <i>DEFB7</i> (9) ↑ <i>S100A9</i> (9)	↑ <i>TAP</i> (10) ↑ <i>LAP</i> (10) ↑ <i>DEFB1</i> (10) ↑ <i>DEFB7</i> (10) ↑ <i>DEFB10</i> (10) ↑ <i>DEFB103B</i> (10) ↑ <i>S100A3</i> , 4, 8, 12, 14 (10)			
Acute phase proteins		↑ <i>Hp</i>	↑ <i>Hp</i> (3) ↑ <i>SAA3</i> (3)		↑ <i>SAA3</i> (10)			

References: 1. (Galvao et al., 2011) 2. (Herath et al., 2009b) 3. (Chapwanya et al., 2009) 4. (Gabler et al., 2009) 5. (Gabler et al., 2010) 6. (Fischer et al., 2010) 7. (Ghasemi et al., 2012) 8. (Kasimanickam et al., 2014) 9. (Foley et al., 2015) 10. (Walker et al., 2015) 11. (Gartner et al., 2015) 12. (Salilew-Wondim et al., 2016) 13. (Wagener et al., 2017b) 14. (Johnson et al., 2015) 15. (Peter et al., 2015) ^a 103-196 DPP; ^b 42-60 DPP; ^c 267 ± 110 DPP Table adapted from (LeBlanc, 2012)

Table 1-2 Timeline of systemic inflammatory and metabolic differences in postpartum cows with uterine disease compared to healthy cows

	<i>Week relative to calving</i>								
	-2	-1	Calving	1	2	3	4	5	7+
Haematology		↑neut (2) ↑leuc (2) ↑lymph (2) ↑mono (2)	↑neut (2) ↑leuc (2) ↑lymph (2) ↑mono (2)	↑neut (2) ↑leuc (2) ↑lymph (2) ↑mono (2)	↑neut (2) ↑leuc (2) ↑lymph (2) ↑mono (2)	↑neut (2) ↑leuc (2) ↑lymph (2) ↑mono (2)	↑neut (2) ↑leuc (2) ↑lymph (2) ↑mono (2)		↑neut (7) ↑mono (7)
Immune effectors (gene expression in PBMCs)			↓TNF (10*)	↓IL1B (6, 10*) ↓TNF (10*) ↓IL6 (10*)	↓IL1B (10*) ↓TNF (10*) ↓IL6 (10*)				↑TNF (7) ↑IL12 (7) ↑IL8 (7)
Immune effectors (circulating proteins)	↑IL-6 (1) ↓IL-1 (5) ↑IL-10 (6)	↑IL-6 (1) ↑IL-10 (6)	↑Hp (3) ↓TNF (10*) ↓IL-1 (5) ↑IL-10 (6)	↑Hp (3,8,9) ↑SAA (9) ↑AGP (9) ↓TNF (10*) ↑IL-10 (6)	 ↑IL-10 (6)	↓TNF (10*) ↑TNF (2)	↑TNF (2) ↑IL-1 (5)		↑Hp (7 ^a) ↑SAA (7 ^a) ↑TNF (4,7 ^a) ↑IL-6 (4,7 ^a) ↑IL-1β (4) ↑IL-10 (7 ^a)
Metabolic		↑NEFA (8,12)	↑NEFA (10,11,12) ↑BHB (10,11) ↑Glucagon (11)	↑NEFA (10,12) ↑BHB (8,10,11,12) ↑Glucagon (11)	↑NEFA (12) ↑BHB (10,11,12)	↑NEFA (12) ↑BHB (12) ↑Glucagon (11)		↑NEFA (11) ↑Glucagon (11)	↑Glucagon (11)

References 1. (Ishikawa et al., 2004) 2.(Kim et al., 2005) 3. (Huzzey et al., 2009) 4. (Kasimanickam et al., 2013) 5.(Islam et al., 2013) 6. (Credille et al., 2015) 7.(Brodzki et al., 2015) 7. (Duvel et al., 2014) 8.(Dubuc et al., 2010b) 9.(Williams et al., 2007) 10. (Galvao et al., 2012) 11. (Galvao et al., 2010) 12.(Hammon et al., 2006). Table adapted from (LeBlanc, 2012).

^a60 DPP

*Monocytes stimulated with *E.coli*

1.8 Thesis aims and hypothesis

Hypothesis:

Even in the context of early postpartum inflammation, cows that go on to develop cytological endometritis or purulent vaginal discharge will be identified by a unique inflammatory signature in the circulation and in endometrial epithelial cells at 7 days postpartum.

Specific aims:

1. Assess circulating markers of metabolism and inflammation at 7 DPP in cattle that go on to develop uterine disease, and in those that remain healthy.
2. Examine the endometrial transcriptome at 7 DPP in cattle that go on to develop uterine disease, and in those that remain healthy.
3. Develop an *in vitro* model that reflects the postpartum bovine endometrium in order to investigate some of the mechanisms underlying disease pathogenesis.

Chapter 2 Methods

2.1 List of reagents, materials and equipment used

Table 2-1 List of reagents used with manufacturer details

Reagent	Company	Location
Cell isolation and culture		
Accutase Cell Detachment Solution	Sigma-Aldrich (Merck)	Poole, UK
Amphotericin B solution	Sigma-Aldrich (Merck)	Poole, UK
Bovine Serum Albumin	Sigma-Aldrich (Merck)	Poole, UK
Collagenase II	Sigma-Aldrich (Merck)	Poole, UK
Distilled, sterile, nuclease-free water	Gibco (Thermo Fisher Scientific)	Paisley, UK
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (Merck)	Poole, UK
DNase I	Sigma-Aldrich (Merck)	Poole, UK
Distilled, sterile, nuclease-free water	Sigma-Aldrich (Merck)	Poole, UK
EDTA	Sigma-Aldrich (Merck)	Poole, UK
Ethanol (EMSURE analytical grade)	Sigma-Aldrich (Merck)	Poole, UK
Fetal Bovine Serum	Gibco (Thermo Fisher Scientific)	Paisley, UK
FITC-dextran 4kDa	Sigma-Aldrich (Merck)	Poole, UK
Hank's Balanced Saline Solution	Gibco (Thermo Fisher Scientific)	Paisley, UK
Insulin-Transferrin-Selenium-Ethanolamine (ITS-X)	Sigma-Aldrich (Merck)	Poole, UK
LPS from <i>Escherichia coli</i>	ENZO Life Sciences (Fisher)	Farmingdale, NY, USA
Matrigel basement membrane matrix	Thermo Fisher Scientific	Paisley, UK
Penicillin-streptomycin 100X	Gibco (Thermo Fisher Scientific)	Paisley, UK
Phosphate-Buffered Saline (PBS)	Gibco (Thermo Fisher Scientific)	Paisley, UK
PTX3, human, recombinant	Sino Biological (Thermo Fisher Scientific)	Paisley, UK
RPMI 1640 + GlutaMAX	Gibco (Thermo Fisher Scientific)	Paisley, UK
Trypan blue solution	Sigma-Aldrich (Merck)	Poole, UK
Trypsin 0.25% EDTA	Sigma-Aldrich (Merck)	Poole, UK
Molecular biology		
25OH Vitamin D ELISA	Eagle Bioscience	Santa Clara, CA, USA
Agilent DNA	Agilent Technologies	
Agilent RNA 6000 Nano Kit	Agilent Technologies	Santa Clara, CA, USA
BHB Reagent Set	Randox Laboratories	Crumlin, NI
Calcium Reagent Set	Beckman Coulter	Brea, CA, USA
Chloroform	Sigma-Aldrich (Merck)	Poole, UK
Diff-Quik staining solution	Clin- Tech	Guildford, UK
Ethanol (EMSURE analytical grade)	Sigma-Aldrich (Merck)	Poole, UK
Fast SYBR Green Master Mix	Applied Biosystems (Thermo Fisher Scientific)	Paisley, UK

Glucose Reagent Set	Audit Diagnostics	Cork, Ireland
Haptoglobin bovine ELISA	Tridelta Phase	Maynooth, Ireland
High Capacity cDNA Reverse Transcriptase Kit	Applied Biosystems (Thermo Fisher Scientific)	Paisley, UK
Illumina TruSeq Stranded mRNA Library Prep Kit	Illumina	San Diego, CA, USA
Iron Reagent Set	Beckman Coulter	Brea, CA, USA
Molecular grade H ₂ O	Sigma-Aldrich (Merck)	Poole, UK
NEFA Reagent Set	Randox Laboratories	Crumlin, NI
Pierce BCA Protein Assay Kit	Pierce (Thermo Fisher Scientific)	Paisley, UK
Primers	Sigma-Aldrich (Merck)	Poole, UK
PTX3 ELISA Kit (Bovine)	Cusabio	Houston, TX, USA
Qubit RNA Assay Kit	Life Technologies	Paisley, UK
RNeasy Mini Kit	Qiagen Ltd.	Crawley, UK
RNaseZap	Ambion (Thermo Fisher Scientific)	Paisley, UK
Serum amyloid A bovine ELISA	Tridelta Phase	Maynooth, Ireland
TRIzol	Ambion (Thermo Fisher Scientific)	Paisley, UK
Urea Reagent Set	Audit Diagnostics	Cork, Ireland

Table 2-2 List of materials used with manufacturer details

Material	Company	Location
Cell isolation and culture		
0.2 µm mesh syringe filter	Millipore	Burlington, MA, USA
10/25 ml serological pipettes	Sarstedt	Nümbrecht, Germany
15 & 50 ml centrifuge tubes	Sarstedt	Nümbrecht, Germany
24-well flat bottom tissue culture plates	Cellstar (Greiner Bio-One)	Kremsmünster, Austria
25/50 ml serological pipettes	Sarstedt	Nümbrecht, Germany
3.5 ml transfer pipettes	Sarstedt	Nümbrecht, Germany
40 µm mesh cell strainer	pluriSelect Life Science	Leipzig, Germany
70 µm mesh cell strainer	Falcon	NY, USA
75/175 cm ² tissue culture flasks with vented cap	Sarstedt	Nümbrecht, Germany
Dissection Scissors	Lennox	Dublin, Ireland
Forceps (rat tooth)	Lennox	Dublin, Ireland
Hanging culture transwell inserts 0.4 µm	Millipore	Burlington, MA, USA
Laboratory film	Parafilm	Neenah, WI, USA
Disposable scalpels	Swann-Morton	Sheffield, UK
Petri dishes	Thermo Fisher Scientific	Paisley, UK
Transfer pipettes (3.5 mL)	Sarstedt	Nümbrecht, Germany
Molecular biology		
0.5 mL PCR tube	Eppendorf	Hamburg, Germany
1.5/2.0 mL DNA LoBind tube	Eppendorf	Hamburg, Germany
1.5 mL RNase-free elution tubes	Ambion (Thermo Fisher Scientific)	Paisley, UK

15/50 ml tubes	Sarstedt	Nümbrecht, Germany
Biosphere filter tips P10/20/200/1000 (sterile)	Sarstedt	Nümbrecht, Germany
Cryogenic vials (2 ml; sterile)	Thermo Fisher Scientific	Paisley, UK
Cryotubes (5 ml)	Sarstedt	Nümbrecht, Germany
Cytobrushes	Minitube	Tiefenbach, Germany
DNA Chips	Agilent Technologies	Santa Clara, CA, USA
K ₂ EDTA vacutainer	Vacurette (Greiner Bio-One)	Kremsmünster, Austria
Lithium heparin vacutainer	Vacurette (Greiner Bio-One)	Kremsmünster, Austria
MicroAmp Fast 96-Well Reaction Plate (0.1mL)	Applied Biosystems (Thermo Fisher Scientific)	Paisley, UK
MicroAmp Fast 96-well optical adhesive film	Applied Biosystems (Thermo Fisher Scientific)	Paisley, UK
RNA Nano Chips	Agilent Technologies	Santa Clara, CA, USA
RNase-free elution tubes	Ambion (Thermo Fisher Scientific)	Paisley, UK
Super Premium Microscope Slides	VWR International	Randor, PA, USA

Table 2-3 List of equipment used

Equipment	Company	Location
2100 Agilent Bioanalyzer	Agilent Technologies	Santa Clara, CA, USA
ADVIA 2120	Bayer Healthcare (Siemens Healthineers)	Erlangen, Germany
7500 Fast Real-Time PCR System	Applied Biosystems (Thermo Fisher Scientific)	Paisley, UK
AU400 Clinical Analyzer	Beckman Coulter	Brea, CA, USA
Epithelial Volt-ohm meter	World Precision Instruments	Hertfordshire, UK
Glomax-Multi+ Microplate Reader	Promega	Madison, WI, USA
Mastercycler gradient	Eppendorf	Hamburg, Germany
Mikro 200R centrifuge	Hettich	Tuttlingen, Germany
NanoDrop ND-1000 UV-Vis Spectrophotometer	NanoDrop Technologies Inc.	Wilmington, DE, USA
Qubit™ fluorometer	Thermo Fisher Scientific	Paisley, UK
Thermomixer compact	Eppendorf	Hamburg, Germany

2.2 Experimental design

Sample collection was performed on a commercial, spring-calving dairy farm in Co. Meath, Ireland with an average milk yield of 6417 litres /year. Cattle were mixed-parity Holstein-Friesians and were calved indoors. Approximately 70% of the herd were artificially inseminated. Samples were collected in spring 2015 (February – April) in the morning after milking. Milking frequency during this time was reduced to once daily by the farmer. Cows which experienced difficult deliveries (calving assistance, twins), vulva sores and pneumonia were not included in the study. Samples were collected from 112 cattle at 3 time points per cow: 40-60 days pre-calving and 7 and 21 days postpartum (Figure 2-1). Pre-calving sampling involved collecting blood from the coccygeal vein in EDTA, lithium heparin and Tempus tubes®, whilst sampling at 7 and 21 DPP involved taking blood samples, vaginal swabs and endometrial cytobrushes and recording the vaginal mucus score (VMS). Swab and cytobrush collection was carried out by a veterinary medicine student (Joseph Lim), whilst blood sampling was carried out by qualified farm staff (Eddie Mulligan & Liam McWeeney) and a veterinary nurse (Deirdre King). All animal handling and sample collection was carried out in accordance with the Health Products Regulatory Authority (HPRA). This was in agreement with the European Union (Protection of Animals Used for Scientific Purposes) regulations 2012 (S.I. No. 543 of 2012). Veterinary and health records over the course of sampling were acquired from the farmer. Two cows were removed from the study due to poor health: cow 1822 was suffering from systemic illness (pneumonia) and cow 2640 had severe vulvar tears. Reproductive records (insemination dates) were obtained from the Irish Cattle Breeding Federation (ICBF) and were used to determine the calving to conception interval and number of inseminations per cow for 105 cattle (data unavailable for n=7).

Before blood collection was carried out, the area around the coccygeal vein was wiped clean and sterilised with 70% ethanol. Blood was collected in a 6 mL K₂EDTA vacutainer® for hematology analysis, a 9 mL lithium heparin vacutainer® for metabolite analysis and a 9 mL Tempus tube vacutainer® for analysis of systemic gene expression (data for the latter was part of a Masters project and is not included in this report).

Prior to swab and cytobrush collection, the perineum and external genitalia were wiped cleaned with 70% ethanol. With the aid of veterinary lubricant, a double sheathed swab was inserted into the vagina, rotated clockwise 3 times and returned into the sheath and removed. The swabs were immediately stored in tubes containing 1 x Tris-EDTA (TE) buffer and transferred to dry ice. A double guarded cytobrush was then inserted through the cervix and into the

endometrium. Having a double guarded sheath meant that faecal and vaginal contamination of the brush was avoided. The cytobrush was guided trans-rectally to ensure it was well positioned. Once the cervix was reached, the inner brush was pushed through the outer guard and rotated clockwise along the wall of the uterus three times to collect cells. The cytobrush was then reinserted into the inner guard and removed, and stored in a labelled cryotube on dry ice. All tubes stored on dry ice were transferred to the -80°C freezer on return to the laboratory. Whilst the outer guard was still in place, a second inner cytobrush was inserted which enabled the collection of cells from the same position within the uterus. The second cytobrush was then removed and rolled anticlockwise on a glass microscope slide, which was later stained with Diff-Quik (a modified Wright Giemsa stain) and used to classify the cows cytologically.

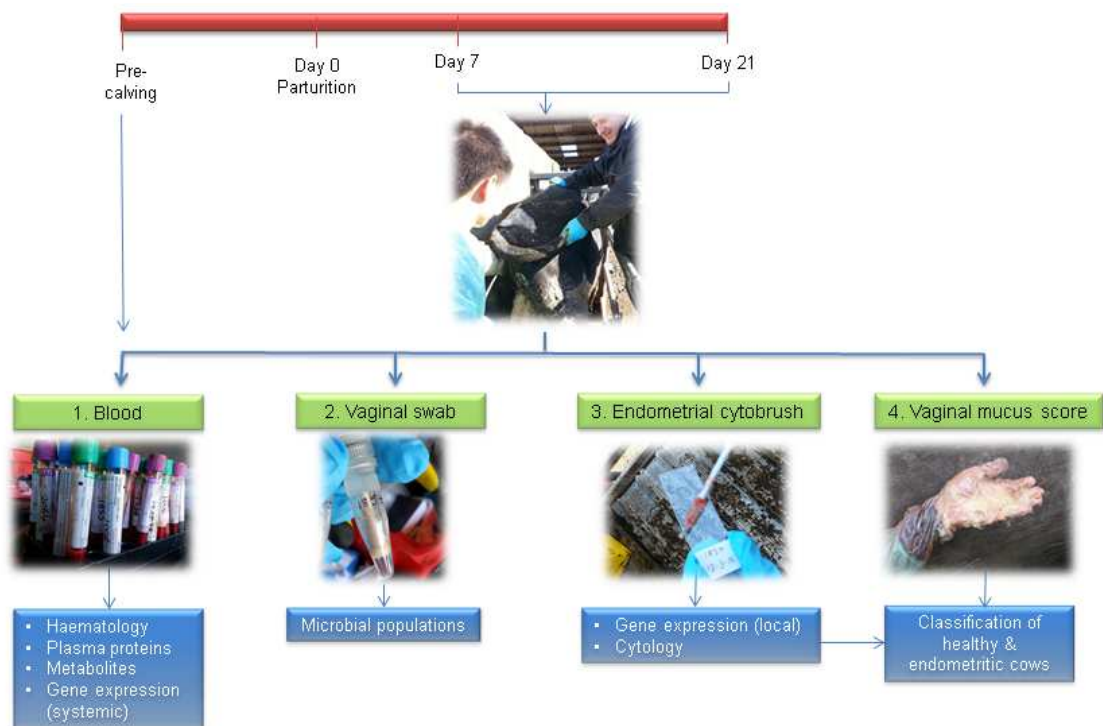


Figure 2-1 Sample collection

Samples were collected from 112 cattle at 40-60 days pre-calving and 7 and 21 days post-calving. Blood samples were collected at each time point. Vaginal swabs, endometrial cytobrushes and a vaginal mucus score were collected at 7 and 21 days postpartum.

2.3 Clinical diagnosis of endometritis

A vaginal mucus score was recorded for each cow at 7 and 21 DPP. Assessment of vaginal mucus was carried out using a gloved hand. A scoring system of 0-3 was used, as previously described by (Williams et al., 2005) (Figure 2-2). Briefly, a score of 0 or 1 was given to vaginal discharge that was clear or contained flecks of pus and the cow was classified as healthy. A score of 2 or 3 was given to samples with over 50% purulent or muco-purulent material present in the vaginal exudate and the cow was diagnosed with purulent vaginal discharge.

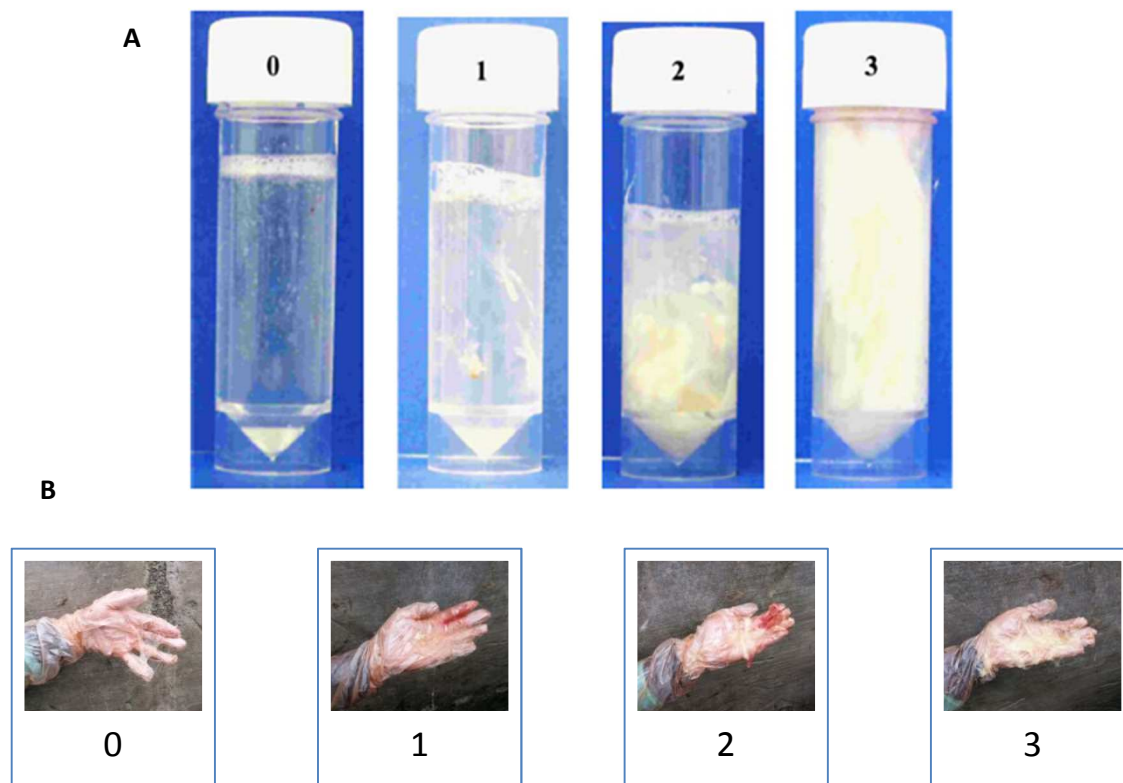


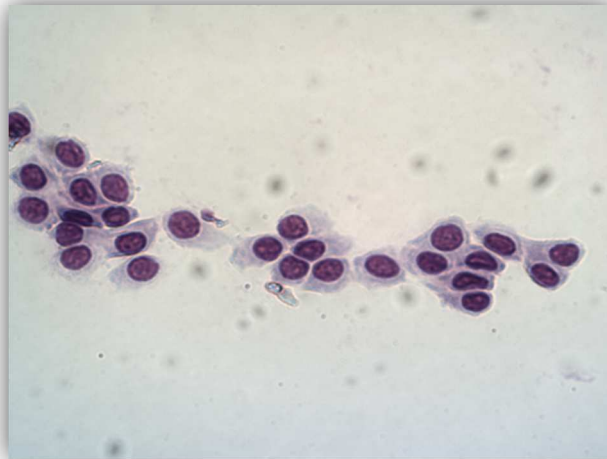
Figure 2-2 The vaginal mucus scoring system used for purulent vaginal mucus diagnosis

The presence of purulent vaginal discharge (PVD) was diagnosed at 21 days postpartum (DPP), using the scoring system developed by (Williams et al., 2005). A score of 0 or 1 was given to vaginal discharge that was clear or contained flecks of pus classified and the cow as clinically healthy. A score of 2 or 3 was given to discharge that contained >50% purulent or muco-purulent material and this diagnosed the cow with purulent vaginal discharge (PVD). **A** Vaginal mucus scoring system - image from (Williams et al., 2005). **B** Demonstration of different vaginal mucus scores using the gloved hand approach.

2.4 Cytological classification

Once the cytology slides had been stained and dried, they were classified by observing the number of PMNs and epithelial cells present (Figure 2-3). A total of 200 cells were counted at 400x magnification. The animals were then classified in line with recent publications, using the recommended cut off of 18% PMN at 21 DPP (Kasimanickam et al., 2004, Barański et al., 2012). Those with $\leq 18\%$ PMN at 21 DPP were classified as healthy, whilst cows with $> 18\%$ PMN at 21 DPP were diagnosed with CYTO.

A



B

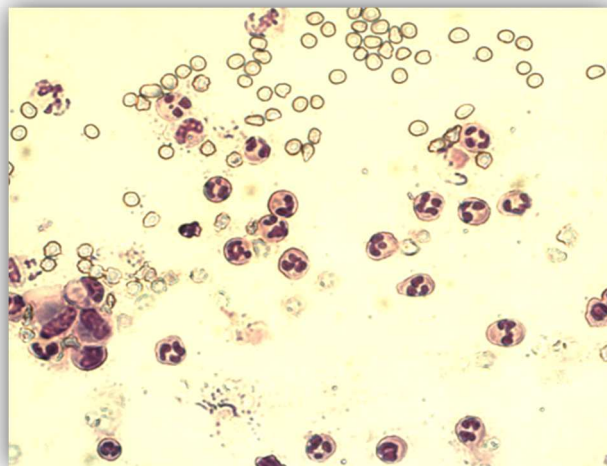


Figure 2-3 Endometrial cytology slides from healthy and endometritic cattle at 21 DPP

Cytological classification was carried out at 21 days postpartum, by counting the number of polymorphonuclear leukocytes (PMNs) and epithelial cells present. Cattle with $\leq 18\%$ PMNs were diagnosed as healthy whilst cattle with $> 18\%$ PMNs were diagnosed with cytological endometritis (CYTO). **A** presents a sheet of epithelial cells in an endometrial cytology image from a healthy cow whilst **B** is a cytology image from a cow diagnosed with CYTO and clearly shows the presence of many PMNs and erythrocytes.

2.5 Whole blood analysis

2.5.1 Haematology

Blood was collected from the coccygeal vein into a spray coated 6 mL K₂EDTA Vacutainer® and inverted several times to ensure mixing of blood with the anticoagulant and prevent clotting. Bloods were transported back to the laboratory within 3 hours of collection, and delivered to the Animal Health Laboratory in Teagasc Grange for analysis. An automated haematology analyser (ADVIA 2120, Bayer Healthcare, Siemens, UK) was used to generate total leukocyte, neutrophil, lymphocyte, monocyte, eosinophil and basophil numbers within each sample.

2.5.2 Metabolites

Blood collected in 9 mL lithium heparinised Vacutainers® was brought back to the lab and centrifuged at 2000 x *g* for 15 minutes at 4 °C. Plasma was collected using disposable Pasteur pipettes and stored in cryotubes at -80°C. Metabolite analysis was performed by the Animal Health Laboratory in Teagasc Grange, where the concentration of glucose, non-esterified fatty acids (NEFA), β -hydroxybutyrate (BHB) and urea were measured. Analysis for each of these metabolites was performed on the Beckman Coulter AU 400 Clinical Analyzer using the protocol supplied by the kit manufacturer. Glucose was measured using the hexokinase method (Neeley, 1972), urea and BHB were measured using the kinetic method (McMurray et al., 1984), whilst NEFA was measured using the colourimetric method (Simonian and Smith, 2006).

2.5.3 Plasma proteins

Blood collected in 9 mL lithium heparinised Vacutainers® was brought back to the lab and centrifuged at 2000 x *g* for 15 minutes at 4 °C. Plasma was stored in cryotubes at -80 °C. Analysis of plasma proteins was performed by the Animal Health Laboratory in Teagasc Grange, where the concentration of serum amyloid A (SAA), haptoglobin (Hp), alpha-1-acid glycoprotein (AGP), vitamin D, calcium and iron were measured. Sample absorbance was read using the GloMax®-Multi Detection System from Promega. The Tridelta Phase™ Serum Amyloid A Assay Kit (a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) kit) was used to measure SAA. The Tridelta Phase™ Haptoglobin Assay Kit (a colorimetric assay) measured Hp concentration. Vitamin D was measured using the Eagle Bioscience 25OH Vitamin D ELISA Kit. Calcium and iron were measured on the Beckman Coulter AU 400 Clinical Analyzer, using Beckman Coulter kits and the colourimetric method (Simonian and Smith, 2006).

2.5.4 Statistical analysis of whole blood data

Whole blood data and reproductive data were analysed using the MIXED procedure in SAS (SAS version 9.4; SAS Institute Inc., Cary, NC, USA). The model included disease as the fixed

independent variable and the parameter of interest as the dependent variable. The interaction of lactation by disease was also tested in the model but was found to be non-significant and was removed. All data were examined for adherence to a normal distribution by plotting residuals against the explanatory variables. Where necessary, the data were log transformed to meet the assumption of normality. Differences between the means were analysed using a Tukey's test at a 95% confidence level.

2.6 RNA extraction

Before RNA extraction began, the fume hood and required instruments were cleaned with 70 % ethanol and treated with Ambion® RNaseZap® (Life Technologies™, Paisley, UK). Endometrial cytobrushes were removed from -80 °C and placed immediately on dry ice until RNA extraction began. The cytobrush was removed from the cryotube using clean forceps, and placed in a 1.5 mL eppendorf tube containing 350 µL of TRIzol reagent. The tube was vortexed vigorously for 1 minute to lyse the cells, and incubated at room temperature for 5 minutes. Chloroform (70 µL per 350 µL Trizol) was added to each sample, shaken vigorously for 15 seconds and centrifuged at 12,000 x g for 15 min at 4 °C. The aqueous phase was carefully removed and transferred to a new tube. Ethanol (70%) was added to the aqueous phase, mixed immediately, and then transferred to an RNeasy spin column. The RNA extraction was completed using the RNeasy kit (Qiagen Ltd., Crawley, UK), following the manufacturer's instructions. This included a number of wash steps using the kit buffers supplied, and finally elution in 30 µL of RNase-free water. The quantity and quality of RNA present in the samples was determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and 2100 Agilent Bioanalyzer respectively.

2.7 Library preparation and sequencing

Thirty samples were chosen for RNA sequencing, based on cow classification at 21 DPP. Cows were classified as either healthy (n=10) or with cytological endometritis (n=10) or purulent vaginal discharge (n=10). Samples from the same cattle were then retrospectively selected at 7 DPP for transcriptomic analysis.

Library preparation was carried out using the Illumina® TruSeq® Stranded mRNA Library Prep Kit, in order to convert the mRNA in a total RNA sample into a library of template molecules of known strand origin, suitable for DNA sequencing. This process began with the isolation and purification of poly-A tailed mRNA from total RNA, using poly-T oligo attached magnetic beads. The isolated mRNA was then fragmented using divalent cations under elevated temperatures. These small fragments of RNA were reverse transcribed to form the first cDNA strand using

reverse transcriptase and random primers. Second strand cDNA synthesis was then carried out using Polymerase I and RNase H, removing the RNA template and synthesising a replacement strand to create double stranded (ds) cDNA. Strand specificity was achieved by replacing dTTP with dUTP in the second strand marking mix, which quenches the second strand during amplification as the polymerase used is not incorporated past this nucleotide. The ends of the double stranded cDNA fragments were then repaired by removing the overhangs at the 5' ends with polymerase activity and removing the overhangs at the 3' end with 3' to 5' exonuclease activity. A single 'A' nucleotide was next added to the 3' end of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction, during which numerous indexing adaptors were ligated to the ds cDNA ends, preparing them for downstream hybridisation to a sequencing flow cell. DNA fragments with adapters attached to both ends were then selectively enriched by performing PCR with primers that anneal to the ends of the adapters. The optimal number of PCR cycles was determined using practice cytochrome RNA samples, which involved minimising the number of cycles to avoid skewing the representation of the library, whilst maintaining a suitable quantity of DNA. The number of PCR cycles was reduced from the 15 cycles recommended in the manufacturer's protocol to 12.

Quality control checks were performed to assess the quality and quantity of the ds cDNA libraries. The Agilent 2100 Bioanalyzer (Agilent Technologies) was used to assess purity of the samples, using the Agilent DNA 1000 kit. Kit components were thawed at room temperature for 30 minutes. The bioanalyzer electrodes were cleaned with 350 μ L of deionised analysis-grade water. The gel was prepared by adding 25 μ L of the dye concentrate into the DNA gel matrix provided, vortexing the solution and transferring it to a spin filter, which was centrifuged at $2240 \times g$ at room temperature for 15 minutes. The flow-through was used in the experiment. A new DNA chip was placed in the chip priming station, and 9 μ L of gel-dye mix was added into the appropriate well. The attached syringe was positioned at 1 mL and the plunger pushed down until it was held by a clip, where it remained for 60 seconds. The clip was released and after 5 seconds the plunger was pulled back to the 1 mL position. The chip priming station was opened and 9 μ L of gel mix was added into the two designated wells on the chip. The DNA marker (5 μ L) was then added into the ladder well and each sample well. Finally the ladder (1 μ L) and samples (1 μ L of each) were added to the appropriate wells and the chip was vortexed for 60 seconds (Agilent Technologies) before running it in the bioanalyzer.

Library quantity was measured using the QubitTM fluorometer. The Qubit working solution was prepared by diluting the dye 1:200 in the buffer supplied. The standards were set up by adding

10 μL of each standard (standard 1: 0 $\text{ng}/\mu\text{L}$; standard 2: 10 $\text{ng}/\mu\text{L}$) to 190 μL buffer. The test samples were prepared by adding 2 μL of each library to 198 μL buffer. The tubes were kept at room temperature for 2 minutes before reading. The standards were used to calibrate the Quibit, before running the rest of the samples, which gave concentration readings in $\mu\text{g}/\text{mL}$.

Five pools of 6 multiplexed samples were constructed, adding equal concentrations of each sample to the pool. Indexes from the Illumina® TruSeq® Stranded mRNA Library Prep Kit were allocated to specific samples following the Illumina® TruSeq® pooling guidelines (Additional Resources: Library Prep Pooling Guide: Index Adapter Sequences). The indexed libraries were sent to Clinical Genomics (Toronto, Canada) where they were sequenced using an Illumina HiSeq 2500, to produce 100 bp single-end reads.

2.8 RNA-seq data analysis

The analysis steps and software used to analyse the transcriptomic data are summarised in Figure 2-4.

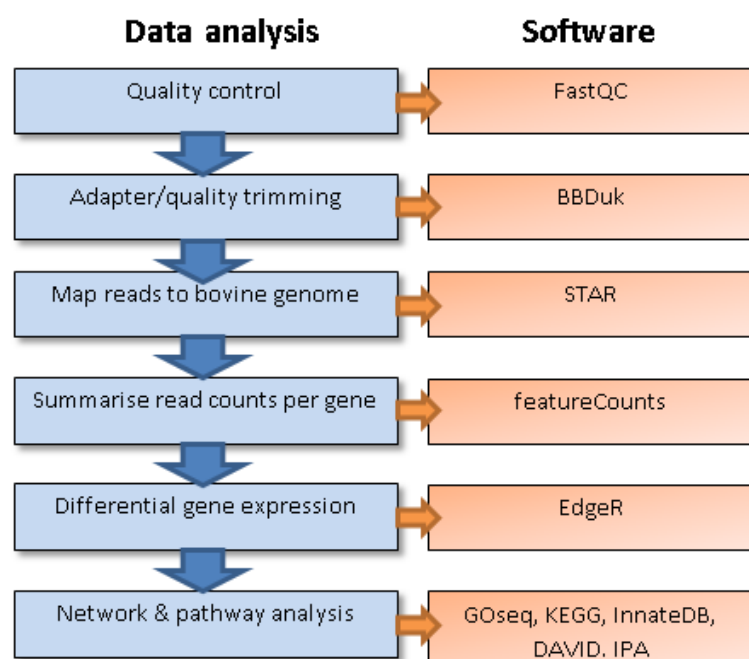


Figure 2-4 Summary of RNA-seq data analysis steps

The data analysis pipeline and software tools used for RNA-seq are summarised here, from quality control through to pathway analysis.

2.8.1 Quality assessment of raw data

Raw sequence reads were downloaded in FASTQ format to a Teagasc server. Quality assessment of the raw sequence data was then carried out using the software FastQC (v 0.1.5; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). This produces a number of different analyses in the form of summary graphs and tables that give the user a quick overview of the data, and any areas in which there may be problems. All samples passed the basic quality statistics. Data was quality and adapter trimmed using the BBDuk java package to trim Illumina adapter sequences and any low quality bases (Phred score <20) from the 3' end of sequence read pairs

2.8.2 Mapping reads to the bovine genome

Reads were aligned to the bovine genome UMD3.1 using the Spliced Transcripts Alignment to a Reference (STAR) aligner (Dobin et al., 2013). A maximum of two mismatches with the reference genome were allowed and only uniquely mapped read pairs were retained for downstream analysis. Read counts overlapping all protein coding genes in the UMD3.1 Ensembl (v.86) annotation were estimated using featureCounts (Liao et al., 2014).

2.8.3 Differential gene expression

Differential gene expression was determined using the R (version 3.3.2) Bioconductor package EdgeR (version 3.18.0) which accounts for biological and technical variation by modelling data as a negative binomial distribution using a generalisation of the Poisson distribution model. To filter out lowly expressed genes, genes with less than one count per million in at least five samples were discarded from the analysis. Remaining gene counts were normalized using the RLE method as implemented in EdgeR (Robinson et al., 2010) to account for varying sequencing depth between samples. Transcript counts are modelled by fitting the data to a negative binomial distribution using moderated tagwise estimates of dispersion and differentially expressed genes are identified with a generalized linear model likelihood ratio test. Differentially expressed genes reported in the present study had a Benjamini Hochberg corrected P value of 0.05. The false discovery rate (FDR) adjusted P-value used to determine genes that were differentially expressed ranged from stringent (FDR<0.05) to less stringent (FDR<0.1) to an unadjusted P-value of 0.05 for subsequent comparisons.

2.8.6 Analysis of biological functions

Pathway analysis was performed on differentially expressed genes using the software Innate DB (Lynn et al., 2008). InnateDB incorporates information from the following publically available databases: PID NCI, KEGG, Reactome, INOH and PID BioCarta. Bovine interactions were

predicted via orthology to human and mouse data. The analysis of cellular and molecular functions was carried out using Ingenuity Pathway Analysis (IPA) Downstream Effects (v. 21249400, Ingenuity Systems, Mountain View, CA; <http://www.ingenuity.com>). The Fisher's exact test corrected by the Benjamini-Hochberg test was used for the identification of over-represented molecular and cellular functions between groups, with a P value of <0.05 and a fold change of ≥ 1.5 . IPA's regulation z-score algorithm was used to predict increases or decreases in functions based on directional changes in the DEGs and expectations derived from the literature.

2.8.7 cDNA synthesis

Endometrial cytobrush RNA from additional animals (n=56) within the same group of cows was used for qPCR validation. Samples with inadequate RNA quality or quantity were not included (n=19). The LH CYTO group were also excluded as it contained only 2 cows and therefore would not have been very representative of the group. Total RNA (1 μ g) from each cytobrush was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions.

2.8.8 Quantitative real-time PCR validation

Following reverse transcription, cDNA were diluted to 5 ng/ μ l with nuclease-free H₂O. qPCR analysis was performed in triplicate using 10 ng of cDNA in 96-well plates using the Fast SYBR Green Master Mix (Life Technologies Inc.). A no-template control and minus reverse transcriptase (RT) were used to check for non-specific amplification. Twelve immune genes were selected for validation of the RNA-seq results, which were chosen due to their involvement in many of the top differentially regulated pathways and functions. Intron-spanning, gene-specific primers were designed using the primer-blast tool on the NCBI website (www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in the appendix). The amplification efficiency for each gene was tested using a serial dilution of pooled cDNA from the tissue of interest. Efficiencies were between 80 and 104% for all genes (data not shown). Four reference genes were tested (*GUSB*, *SDHA*, *HSP90AB1* and *RPS15*), which were selected from the RNA-seq dataset for their stability (i.e. they were not differentially expressed). Genorm analysis software was used to determine the average expression stability (M) value for each gene, and the two genes that were most stable (*HSP90AB1* and *RPS15*) were used for normalisation (Figure 2-5). Relative gene expression levels were calculated using GenEx Software v.5.2.7.44 (2010) (MultiD Analyses AB, Göteborg, Sweden). Adjustments were performed to account for inter-plate variation using the inter-plate calibrator sample included on all plates. The Cq values were adjusted for amplification efficiencies and replicates were averaged. The resulting values were

normalised to the reference genes and relative quantities were calculated, which converted data to a linear scale and compared all samples against the sample with the highest expression within each gene. This resulted in the lowest expressed sample receiving a value of 1, and samples with higher expression >1.

2.8.9 Statistical analysis of qPCR data

Data were analysed using the MIXED procedure in SAS (SAS version 9.4; SAS Institute Inc., Cary, NC, USA). The model included disease as the fixed independent variable and gene as the dependent variable. Lactation, parity (primiparous/multiparous), medication and the interaction of each by disease were also tested in the model but were found to be non-significant and were removed. All data were examined for adherence to a normal distribution by plotting residuals against the explanatory variables. Where necessary, the data were log transformed to meet the assumption of normality. Differences between the means were analysed using a Tukey's test at a 95% confidence level. Relative gene expression qPCR values were all $\log_2$ transformed before statistical analysis, however the corresponding relative quantities are presented to facilitate interpretation of results.

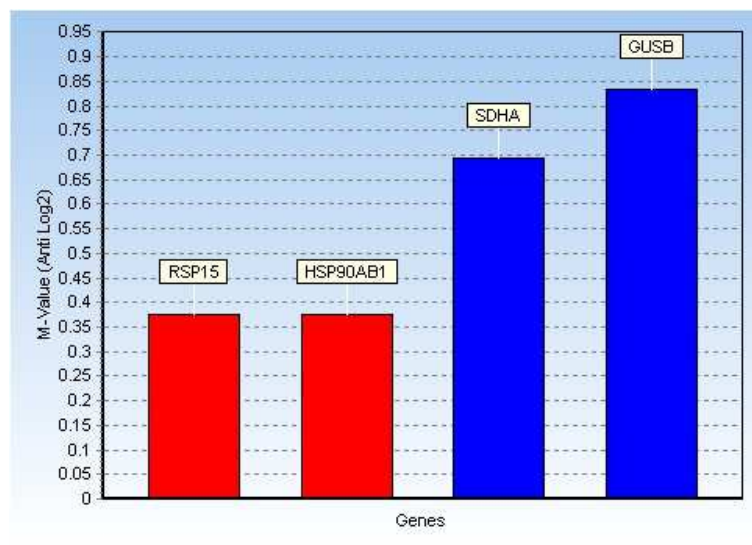


Figure 2-5 GeNorm analysis of reference gene stability

cDNA synthesised from RNA extracted from endometrial cytobrushes (n=56) was used to assess the expression of 4 reference genes by qPCR. The Cq values obtained were imported into Genex, and GeNorm analysis was performed to determine which reference genes were most stable (indicated by a lower M value). *RSP15* and *HSP90AB1* were found to be the most stable (identical M values of 0.376) and therefore the geometric mean of these 2 genes was used for normalisation.

2.9 Cell culture

2.9.1 Isolation and culture of endometrial epithelial cells

Bovine female reproductive tracts were collected from cattle at the local abattoir within 15 minutes of slaughter. Tracts were collected from animals with no evidence of uterine disease. Breed and age were also recorded. Only tracts in the early luteal stage of the oestrous cycle were selected, as progesterone levels should be minimal at this stage and therefore less likely to impact immune function of the cells. The physiological stage of the reproductive cycle was determined by observation of ovarian morphology. Reproductive tracts with a stage I corpus luteum were selected, and only the uterine horn ipsilateral to the corpus luteum was used for tissue dissection. Dissection was carried out at the factory in order to increase the survival rate of the cells harvested. Prior to dissection, the external surface of the tract was washed with 70% ethanol. The uterine horn was opened longitudinally with sterile scissors and the inside surface was rinsed with PBS supplemented with 50 IU/ml penicillin, 50 IU/ml streptomycin and 2.5 g/ml amphotericin B. The uppermost functional layer of the endometrium was then removed in strips using sterile scissors and forceps and placed in 20 ml of RPMI supplemented with antibiotics as above. Samples were transported back to the laboratory at room temperature within 90 minutes of collection. At the laboratory, tissue was washed twice in HBSS supplemented with antibiotics. Tissue was chopped into fine pieces using sterile scissors and forceps and incubated in HBSS with antibiotics for 10 minutes at 37°C. The HBSS was then removed before the addition of 20ml digestive solution, which had been pre-warmed to 37°C. The digestive solution was prepared one day prior to tissue collection, and contained 375 BAEE units of trypsin-EDTA, 50 mg collagenase II, 100 mg bovine serum albumin (BSA) and 10 mg DNase I, made up to 100 ml with HBSS and sterile filtered using a 0.2 micron filter. Samples were incubated in digestive solution at 37°C in a shaking incubator at 150 rpm for 1 hour. Following digestion, the resulting solution was filtered through a 70µm cell strainer in order to remove any cellular debris. The filtrate was collected in 10 ml HBSS with 10% FBS. This solution was then passed through a 40 µm filter (pluriStrainer, pluriSelect, Leipzig, Germany) to isolate stromal cells. As the epithelial cells are larger, they remained on the surface of the filter. This meant we were able to isolate epithelial cells by inverting the filter over a clean 50 ml tube and washing the filter with 10 ml HBSS with 10% FBS. Samples were centrifuged at 700 x g for 10 minutes to pellet the cells. Epithelial cells were then resuspended in 13 ml RPMI supplemented with 10% FBS, 50 IU/ml penicillin, 50 IU/ml streptomycin, 2.5 g/ml amphotericin B and 1 x ITS-X and seeded into a 75 cm² vented tissue culture flask. Cells were stored in an incubator at 37 °C and 5% CO₂ and culture media was changed every 48 hours. Visual examination of cell morphology using a light

microscope was used to assess the purity of the culture. Any contaminating stromal cells were removed using accutase, which lifts stromal cells faster than epithelial cells. Cells were allowed to reach around 80-90% confluency before plating. The establishment and optimisation of a protocol for the isolation, culture and characterisation of bovine endometrial epithelial cells is covered in detail elsewhere (Kelly, 2018).

2.9.2 Polarisation of epithelial cells

Epithelial cells were polarised by culturing them in 0.4 μm semi-permeable trans-well inserts. Inserts were placed in each well of a 24-well plate using forceps. Thawed Matrigel was diluted 1 in 8 in serum-free RPMI and 50 μl was added to each insert. Excess gel was removed from the inserts after 1 hour of room temperature incubation. Epithelial cells were detached from their flasks using 0.25% Trypsin-EDTA and counted using a haemocytometer with Trypan blue staining. Cells were seeded into the inserts at a density of 50,000 cells per well. One well was left blank (without cells). Polarisation was determined by measuring the trans-epithelial electrical resistance (TEER) using an epithelial voltmeter. The two electrodes were rinsed in 70% ethanol. One electrode was placed in the upper compartment and the other in the lower compartment of the well, and the electrical resistance was recorded. To obtain the actual tissue reading, the blank resistance was subtracted. The unit area resistance was then determined by multiplying the tissue reading by the surface area of the filter membrane to give the final TEER in $\Omega\cdot\text{cm}^2$.

2.9.3 Disruption of the epithelial barrier using LPS

Once the polarised epithelial barrier had formed, the aim was to disrupt it using LPS (serotype O55:B5) in order to reflect endometrial damage that occurs following calving. Cells from 3 different cows were challenged with a range of LPS concentrations (2, 10, 20, 100, 200 and 500 $\mu\text{g/ml}$). DMSO (10%) was included as a positive control. Permeability was assessed at 1, 2, 3, 4 and 5 days after challenge by measuring the TEER. A FITC-dextran assay was also performed at 5 days post-challenge as a comparative measure of barrier permeability.

2.9.4 FITC-dextran permeability assay

Paracellular permeability was measured using FITC-dextran. This involved the addition of 200 μg of 4kDa FITC dextran to the apical compartment of each well, including the blank no-cell control. The plate was then incubated at 37 $^{\circ}\text{C}$ with 5% CO_2 for 3 hours. After incubation, 200 μl media from the basolateral compartment of each well was transferred into a black ELISA plate. Fluorescence was measured using a plate reader with filters appropriate for 485 nm and 538

nm excitation and emission respectively. Fluorescence was compared to the no-cell control for analysis.

2.9.5 Calcium switch assay

A calcium switch assay was used to examine the effect of PTX3 treatment on the re-establishment of the epithelial barrier. The assay was performed once endometrial epithelial cells in trans-well inserts had become polarised (TEER $>1000 \Omega \cdot \text{cm}^2$). The TEER was recorded before beginning the assay. Cells were washed in HBSS containing normal Ca^{2+} (1.8 mM), supplemented with 1M HEPES (HBSS+) that had been pre-equilibrated to 37 °C. Cells were incubated in HBSS+ for 15 minutes at 37 °C. The TEER was recorded 15 minutes after incubation and again at 20 minutes to ensure a stable reading (T=0 mins). Extracellular calcium was then chelated by adding Ca^{2+} -free HBSS containing 2nM EDTA and 1M HEPES (Ca^{2+} switch solution) to the apical chamber of each well and incubating for 5 minutes at 37 °C. After 5 minutes, the inserts were washed 3 times in HBSS+ and replaced in HBSS+ with or without the indicated treatment (PTX3 or BSA – see 2.9.9 for details). The TER was recorded straight away (T=5 mins) and again at 15 minute intervals for 3 hours. The results were plotted as percentage of baseline TEER.

2.9.6 Protein extraction from uterine cytobrushes

Protein was extracted from uterine cytobrushes using the TRIzol method. Briefly, this involved immersing each brush in 1 ml TRIzol and vortexing for 30 seconds to lyse the cells. Following 5 minutes of incubation at room temperature, 0.2 ml chloroform was added to each sample and the tube was shaken vigorously for 15 seconds. Samples were incubated at room temperature for 2-3 minutes and then centrifuged at $12,000 \times g$ for 15 minutes at 4 °C. This resulted in separation of the sample into 3 distinct phases. The upper aqueous phase and interphase were removed and 300 μl of 100% ethanol was added to the remaining organic phase to precipitate any remaining DNA. Following 3 minutes of incubation at room temperature, samples were centrifuged at $2000 \times g$ for 5 minutes at 4 °C to pellet the DNA. The resulting supernatant was transferred to a fresh tube and 1.2 ml isopropanol was added to precipitate the protein. This was incubated for 10 minutes at room temperature and then centrifuged at $12,000 \times g$ for 10 minutes at 4 °C. The resulting pellet was resuspended in 2 ml of 0.3M guanidine hydrochloride-95% ethanol and incubated at room temperature for 20 minutes. Samples were then centrifuged at $7500 \times g$ for 5 minutes at 4 °C to pellet the protein. This guanidine hydrochloride-95% ethanol wash was repeated twice more. The protein pellet was then resuspended in 100% ethanol and incubated at room temperature for 20 minutes. Following centrifugation at $7500 \times g$ for 5 minutes at 4 °C, the ethanol supernatant was discarded and the pellet allowed to air-

dry for 5-10 minutes. The pellet was then resuspended in 100 μ l 4X Lamelli Buffer containing protease inhibitors: phenylmethanesulphonyl fluoride (100 μ g/ml), aproptinin (2 μ g/ml), sodium orthovanadate (4 μ g/ml) and leupeptin (2 μ g/ml). This was incubated for 1 hour at 50 °C. Samples were then centrifuged at 10,000 $\times g$ for 10 mins to sediment any insoluble material and the supernatant was transferred to a fresh Eppendorf before being stored at -20°C.

2.9.7 Protein assay

Protein concentration was measured using the BCA assay, following the manufacturer's instructions. BCA reagent B was diluted 1:50 in BCA reagent A and 200 μ l of this solution was used per sample in a 96 well plate. 3 μ l of each sample was added into the reagent mix. The plate was placed in an incubator for 30 mins at 37°C. The concentration of each sample was measured using a spectrophotometer. A standard curve was generated each time with BSA standards and the protein concentration of each sample was calculated from this.

2.9.8 Measurement of PTX3 in uterine cytobrush samples

Measurement of PTX3 protein concentration was performed using the Cusabio bovine pentraxin-related PTX3 ELISA kit. The ELISA was performed following the manufacturer's instructions. A total of 18 cytobrush samples (n=7 healthy and n=11 pathological) were used in the ELISA, and multiple dilutions were included for each sample (50-500 μ g/ml) due to the diverse range in PTX3 concentration and limited window of detection as determined during a practice run. PTX3 concentrations were determined by interpolating values from a sigmoidal standard curve.

Statistical analysis of PTX3 protein concentrations between the 2 groups was performed using Graphpad Prism 7 software. Due to the small sample size tested here, the distribution could not be sensibly assessed, and therefore a non-normal distribution was assumed. The non-parametric Wilcoxon rank sum test (Mann Whitney U test) was carried out. A *P*-value of <0.05 was considered to be statistically significant.

2.9.9 Treatment of epithelial cells with recombinant PTX3

Following disruption of the epithelial barrier using the calcium switch assay, a healthy (5ng/ml) or pathological (500 ng/ml) dose of rPTX3 was administered to the apical or basal compartment of the well for each biological repeat. Each treatment was run in duplicate. An equivalent concentration (500 ng/ml) of bovine serum albumin (BSA) was used to test the effect of adding a peptide on the TEER. Assay media alone was included as a negative control.

Chapter 3 Changes in circulating acute phase proteins and eosinophils in the early postpartum period are associated with the onset of uterine disease in dairy cows

3.1 Introduction

Inflammation of the uterus during the first week postpartum is part of the normal involution process required for tissue repair and return to homeostasis. However, some cows are unable to resolve this inflammation and develop endometritis, which has detrimental effects on subsequent fertility. Current diagnosis of endometritis is carried out 21 DPP or later. Detection of clinical endometritis (PVD) is relatively straightforward as it requires visual assessment of cervico-vaginal mucus which is easily obtainable, and often visible as exudate from the infected cow. However, with the predicted expansion of the Irish dairy herd following the removal of the milk quotas across the European Union in 2015, farmers will have a greater number of cattle to manage, likely leading to lower observation of clinical disease symptoms. In addition, subclinical endometritis (CYTO) also has a negative impact on reproductive performance, but cannot be diagnosed clinically meaning that it often goes unnoticed until the cow does not conceive following insemination. Diagnosis of CYTO involves collection of uterine cells from a biopsy, cytobrush or lavage which is not suitable for routine, large-scale on-farm diagnosis. Therefore the need for a low-cost, non-invasive diagnostic for the detection of uterine disease (particularly subclinical) is critical. Furthermore, prevention is always more economical than treatment of the established disease, and so early diagnosis soon after calving or even prepartum would allow early intervention and a more rapid return to cyclicity.

Local differences in the host immune response and uterine microbiome are evident between healthy and endometritic cows, however systemic differences have been less conclusive. During the transition period, cattle experience extreme metabolic and immunological perturbation which can result in systemic inflammation (reviewed by (Bradford et al., 2015)). The high energy requirements of both parturition and milk production means that most dairy cows cannot eat enough to meet these demands, resulting in a state of negative energy balance during the postpartum period (LeBlanc, 2014). Lactation, rather than calving, appears to have the greatest effect on systemic metabolites when mastectomised and intact cows are compared (Goff et al., 2002). This may explain why high yielding dairy cows often experience greater NEB. During this time, cattle are required to mobilise their fat reserves to produce energy, which increases the supply of non-esterified fatty acids (NEFA) and ketones such as acetoacetate and β -hydroxybutyrate (BHB) (Wathes et al., 2011). Mobilisation of adipose tissue also promotes the production of pro-inflammatory cytokines such as TNF and IL-6 (Tilg and Moschen, 2008), whilst

NEFA itself also initiates inflammation by binding to TLR4 (Hotamisligil and Erbay, 2008). In addition, these cytokines suppress insulin action, resulting in insulin resistance (IR) and reduced circulatory levels of glucose and insulin-like growth factor (IGF) 1. Glucose is the major fuel source of activated leukocytes and therefore depleted circulating glucose levels have been linked to impaired immune function in cows with endometritis. Severe negative energy balance has been associated with uterine disease, however many of these studies are based on total mixed ration (TMR) feed systems, in which cattle are likely to experience different problems to those faced in a pasture-based system.

Whilst the repartitioning of nutrients during the transition period can affect leukocyte function, differences in circulating leukocyte numbers have also been reported. Most studies have focused primarily on neutrophils, as these are the first immune cells to respond to infection and/or damage of the uterus. Cows with retained placenta, metritis and clinical endometritis have an increased number of circulating neutrophils at 15 DPP (Kim et al., 2005, Islam et al., 2014), suggesting that chemotaxis of neutrophils may be impaired, preventing their movement into the uterus and therefore limiting the ability of these cows to fight infection. However, others have found that the opposite is true, with lower systemic neutrophil counts preceding the onset of uterine disease (Cai et al., 1994, Foley et al., 2015). The authors predict that this is due to increased migration of neutrophils into the uterus, which may result in increased tissue damage and prolonged inflammation. Few studies have focused on other immune cell populations in association with endometritis.

Although the mechanisms by which this disease is initiated and sustained remains unclear, dysregulation of the immune response is likely to play an important role (LeBlanc, 2014). The immune system first responds to infection and/or damage of the uterus with an influx of neutrophils into the endometrium and the local production of pro-inflammatory cytokines. Secretion of these cytokines (particularly IL-1, IL-6 and TNF) into circulation initiates the acute phase response, promoting the production of acute phase proteins (APPs) from the liver, which have been explored as potential biomarkers of uterine disease in a variety of species. APPs play an important role in fighting infection. SAA recruits immune cells to the site of infection (Badolato et al., 1994), and acts as an opsonin for a variety of bacteria (Hari-Dass et al., 2005) whilst Hp binds haemoglobin to inhibit iron uptake by microbes, preventing microbial growth. In cattle, SAA and Hp have been recommended as potential biomarkers for metritis (Huzzey et al., 2009) purulent vaginal discharge (Schneider et al., 2013) and cytological endometritis (Dubuc et al., 2010b). AGP is another APP in cattle that has been associated with increased

uterine bacterial counts (Williams et al., 2005) and impaired reproductive performance (Aungier et al., 2014), but hasn't been explored in relation to uterine disease.

The immune system is also affected by vitamin and mineral deficiencies during the transition period. One of the most prevalent transition period disorders is hypocalcaemia (milk fever), which is commonly associated with the occurrence of metritis and PVD (Martinez et al., 2012). Calcium is essential for many biological functions, including uterine contractions (Hansen et al., 2003) and neutrophil function and activation (Jaconi et al., 1990, Bréchar and Tschirhart, 2008). Vitamin D, following conversion to its active form 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), is critical for calcium homeostasis. Vitamin D also plays a role in activated immune cells, stimulating the production of nitric oxide and β -defensin AMPs involved in bacterial elimination (Nelson et al., 2010). Furthermore, *in vivo* studies on mastitis have demonstrated the beneficial role of vitamin D in fighting infection (Lippolis et al., 2011). Another important mineral involved in the immune response is iron. Iron is one of the trace elements involved in the antioxidant defence system (Miller et al., 1993). Systemic concentrations of this mineral have been found to be significantly lower in repeat breeder cows compared to healthy cows (Manickam et al., 1977), however it has not been investigated in relation to uterine disease.

In this study, circulating metabolites, leukocytes, acute phase proteins, vitamins and minerals will be assessed in spring-calving, pasture-based dairy cattle with and without uterine disease. We will assess these parameters at the time of disease diagnosis (21 DPP), as well as during the early postpartum period (7 DPP) and before calving (40-60 days prepartum). In addition we will compare cows with both clinical and subclinical endometritis, in contrast to the mixed phenotype often reported in previous studies.

3.2 Hypothesis and specific aims

We hypothesise that even in the context of prepartum immunosuppression and early postpartum inflammation, cows that go on to develop uterine disease will be identified by a unique inflammatory signature in the circulation at PC and 7 and 21 days postpartum.

Aims:

1. Assess the incidence of uterine disease (both clinical and subclinical) within the herd and the effect on reproductive performance
2. Measure circulating metabolites (glucose, urea, NEFA and BHB), leukocyte populations, acute phase proteins (SAA, Hp and AGP) vitamins (vitamin D) and minerals (calcium and

iron) in blood plasma at all 3 time points and assess differences between healthy and endometritic cows.

3.3 Results

3.3.1 Occurrence and impact of clinical and subclinical disease

The health status of each cow was assessed by vaginal mucus score and endometrial cytology acquired at 21 DPP. Out of the 147 cattle sampled, 35 were excluded from the study as they were either missing a cytobrush sample at one of more time points due to logistical problems on-farm, or there were an insufficient number of cells present on the cytology slide in order to classify the cow. Disease diagnosis at 21 DPP revealed that 76% (n=85) of the herd had one or more forms of uterine disease. Of these animals, 28% (n=31) had cytological endometritis (CYTO), 18% (n=20) had clinical disease (PVD) and 30% (n=34) had both CYTO and PVD (Figure 3-1). The increased calving to conception intervals and greater number of artificial insemination (AI) services required to achieve pregnancy in cattle with uterine disease suggest that uterine disease has a negative effect on reproductive performance, although these results were not significant at the $P<0.05$ level. Additionally, these measurements of reproductive performance include a certain degree of bias, as they do not take into account factors such as oestrus detection, insemination technique, detection of pregnancy and the culling of cows that fail to conceive from the herd.

This study was performed on a commercial farm and therefore a major limitation was the treatment of some cattle with antibiotics and/or anti-inflammatory medication. As a large proportion of the herd received treatment, removal of these animals would have significantly reduced our sample size. Therefore the decision to retain these animals in the analysis was made, and the effect of medication was tested in each of our statistical models. A greater number of cattle that subsequently developed uterine disease received medication (predominantly for mastitis and endometritis) during the sampling period which may have aided a faster return to pregnancy. When medication was included as a factor in our statistical model, disease*medication had a significant effect on the calving to conception interval in healthy cows, which was 98.5 days in healthy cows without medication vs. 174.5 days in healthy cows with medication. The term healthy here refers to the absence of endometritis but not other diseases (such as mastitis) which were not assessed, and therefore it is likely that healthy cows that received treatment had a longer calving to conception interval due to the presence of an additional disease. Whilst medication didn't have an effect on the calving to conception period in cows with uterine disease, it was lower (104 days) in cows that did receive medication than those that didn't (108 days), which suggests that some of these cattle received treatment which may have aided recovery from uterine disease, masking the true effect on fertility.

Primiparous cows had a higher incidence of uterine disease (88%; n=21) than multiparous cows (73%; n=64). Consequently, first parity cows with uterine disease also had a longer calving to conception interval (117 days) and required a greater number of services (2.4) to achieve pregnancy compared to cows (104 days; 1.9 services).

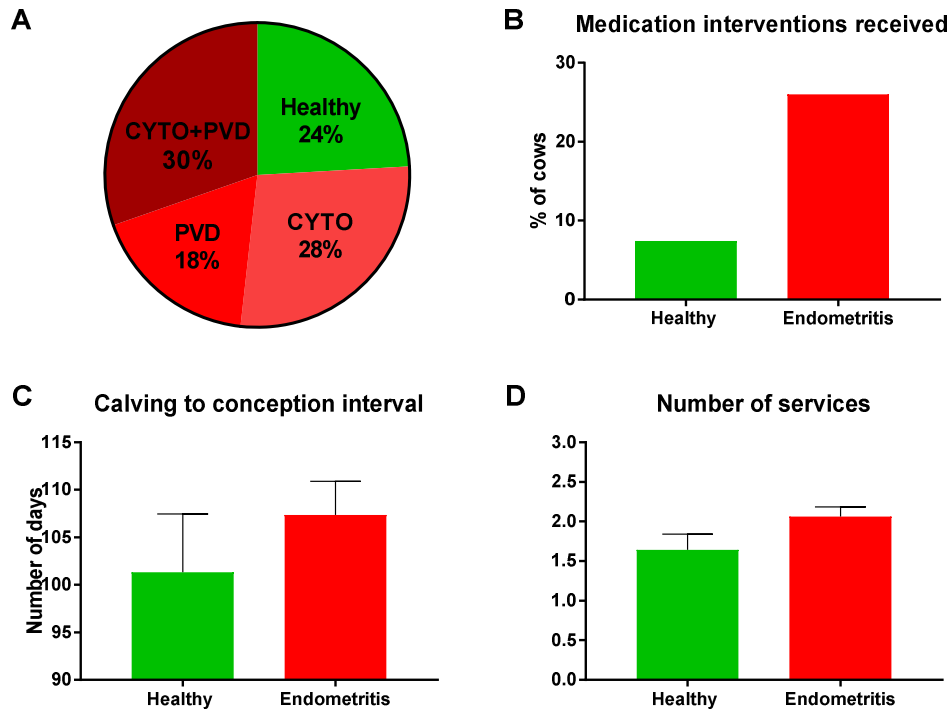


Figure 3-1 Uterine disease had a negative impact on reproductive performance.

A Disease outcome was determined at 21 days postpartum (DPP) (n=112). Cytological endometritis (CYTO) was diagnosed by uterine cytology (presence of >18% polymorphonuclear cells (PMN)) and purulent vaginal discharge (PVD) was assessed according to the vaginal mucus score (VMS). **B** Reproductive performance was assessed based on the calving to conception interval (number of days between calving and final service), the number of artificial insemination services required before pregnancy was achieved and the percentage of cows that received medication during the sampling period (calving to 21 DPP) (n=105).

3.3.2 Metabolic profiles

The metabolic profile of each cow was assessed at pre-calving (PC), and 7 and 21 days postpartum in order to determine whether differences were present between healthy cattle and those that developed uterine disease (Figure 3-2). Glucose concentrations decreased in all cattle during the postpartum period, whilst NEFA and BHB increased. This indicates that all cattle were in a state of negative energy balance after calving. However there were no differences between groups at any time point. As BHB levels were below 1mmol/L 7 DPP, these cows did not have subclinical ketosis (Ingvarsen and Moyes, 2013), which is a risk factor for metritis (Duffield et al. 2009). Plasma urea values were within the same range as previous studies (Cheng et al., 2015), and were not considered “high” by the proposed threshold of 7.5 mmol/L (Wathes et al., 2007). Urea concentrations remained fairly constant between time points, though by 21 DPP urea was slightly lower in cows with PVD.

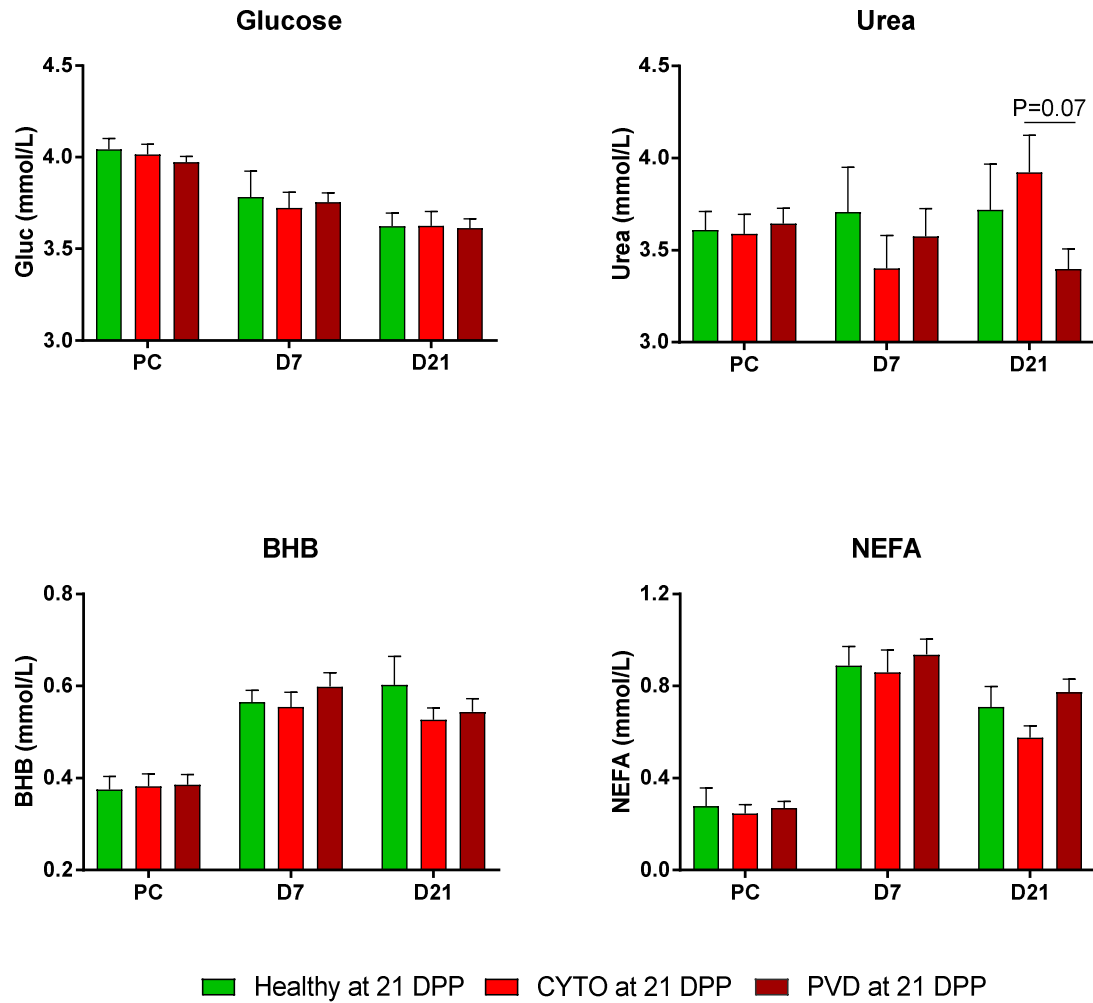


Figure 3-2 Systemic metabolite concentrations were similar in cattle with and without uterine disease

Systemic glucose, urea, betahydroxybutyrate (BHB) and non-esterified fatty acid (NEFA) concentrations were measured in cattle (n=110) at pre-calving (PC) and 7 and 21 days postpartum. Animals were classified by disease status, based on endometrial cytology and vaginal mucus score. A one way ANOVA was used to determine significant differences between groups at each time point (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.3.3 Whole blood leukocyte composition

Whole blood leukocyte counts were analysed in cattle at pre-calving, and 7 and 21 days postpartum (Figure 3-3). Cattle were grouped by disease status 21 DPP. Overall leukocyte numbers increased gradually in all cows during the postpartum period. This appears to reflect the increase in basophil numbers after calving, as neutrophil, monocyte and lymphocyte populations all remained fairly stable. In contrast, eosinophil numbers declined in all animals after calving, and there was a significant drop in eosinophil numbers 7 DPP in cows that later developed uterine disease compared to those that remained healthy. All values were within the hematologic reference ranges provided by the MSD Veterinary Manual (<https://www.msdsvetmanual.com/special-subjects/reference-guides/hematologic-reference-ranges>)

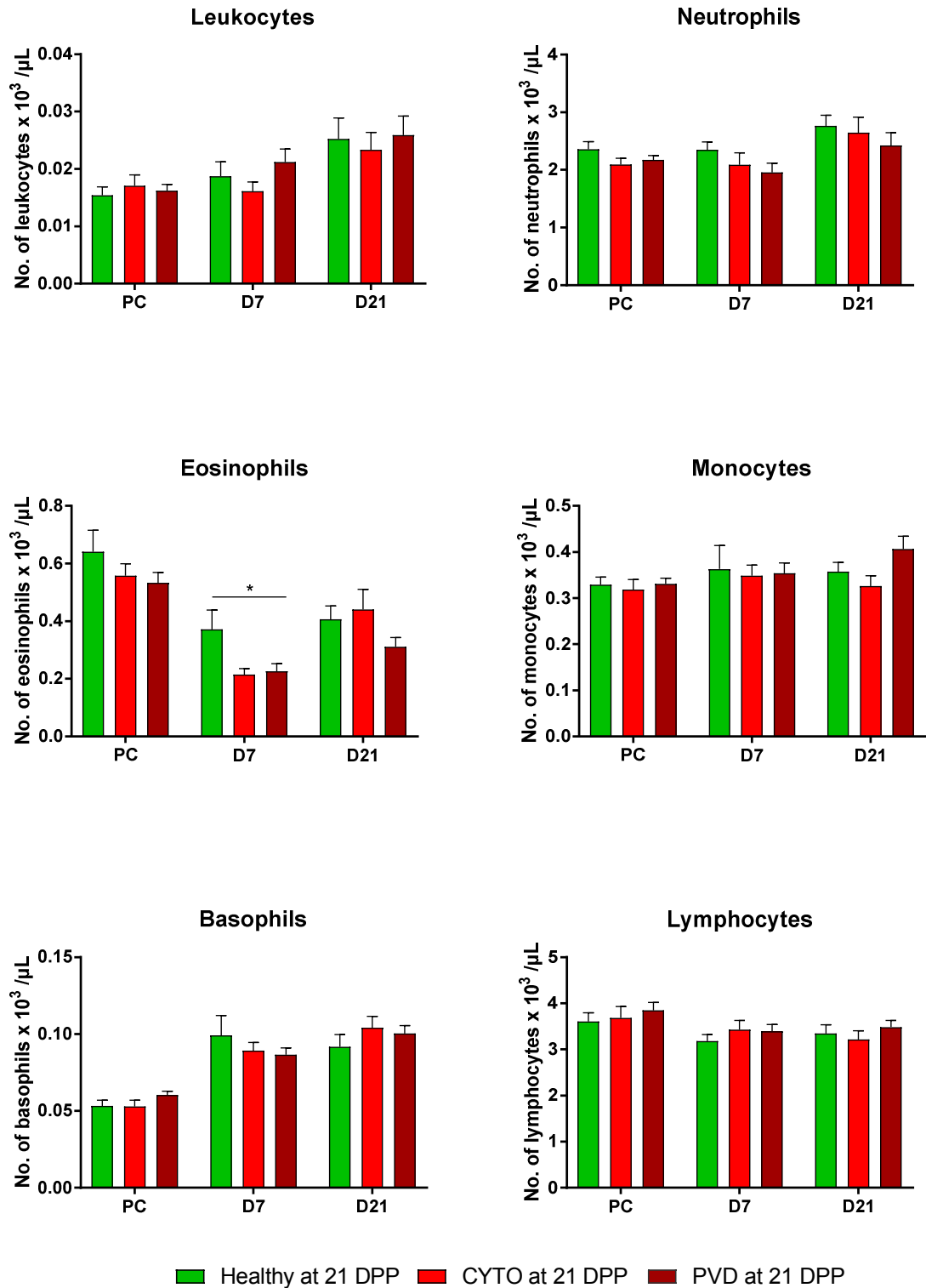


Figure 3-3 Circulating leukocytes were similar in cattle with and without uterine disease

Absolute leukocyte, neutrophil, eosinophil, monocyte, basophil and lymphocyte numbers in whole blood (n=104) were recorded at pre-calving (PC) and 7 and 21 days postpartum (DPP). Animals were retrospectively classified by disease status at 21 DPP. A one way ANOVA was used

to determine significant differences between groups at each time point (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

3.3.4 Acute phase proteins

Serum amyloid A (SAA), haptoglobin (Hp) and alpha-1-acid glycoprotein (AGP) concentrations were analysed in plasma of cattle at pre-calving and 7 and 21 days postpartum (Figure 3-4). All 3 acute phase proteins increased 7 days after calving, which indicates these cattle were in a pro-inflammatory state and is to be expected during the early postpartum period. SAA and Hp levels began to decline by 21 DPP, whereas AGP levels remained high. This reflects their roles as major (SAA and Hp) and moderate (AGP) APPs, the latter of which has a less rapid response and declines at a slower rate. Both SAA and Hp concentrations were higher 7 DPP in cows that developed PVD compared to healthy cows. AGP showed a similar pattern although this did not reach significance. In addition, cows that developed CYTO also had higher levels of Hp 7 DPP, but the response was more subtle and was not significant.

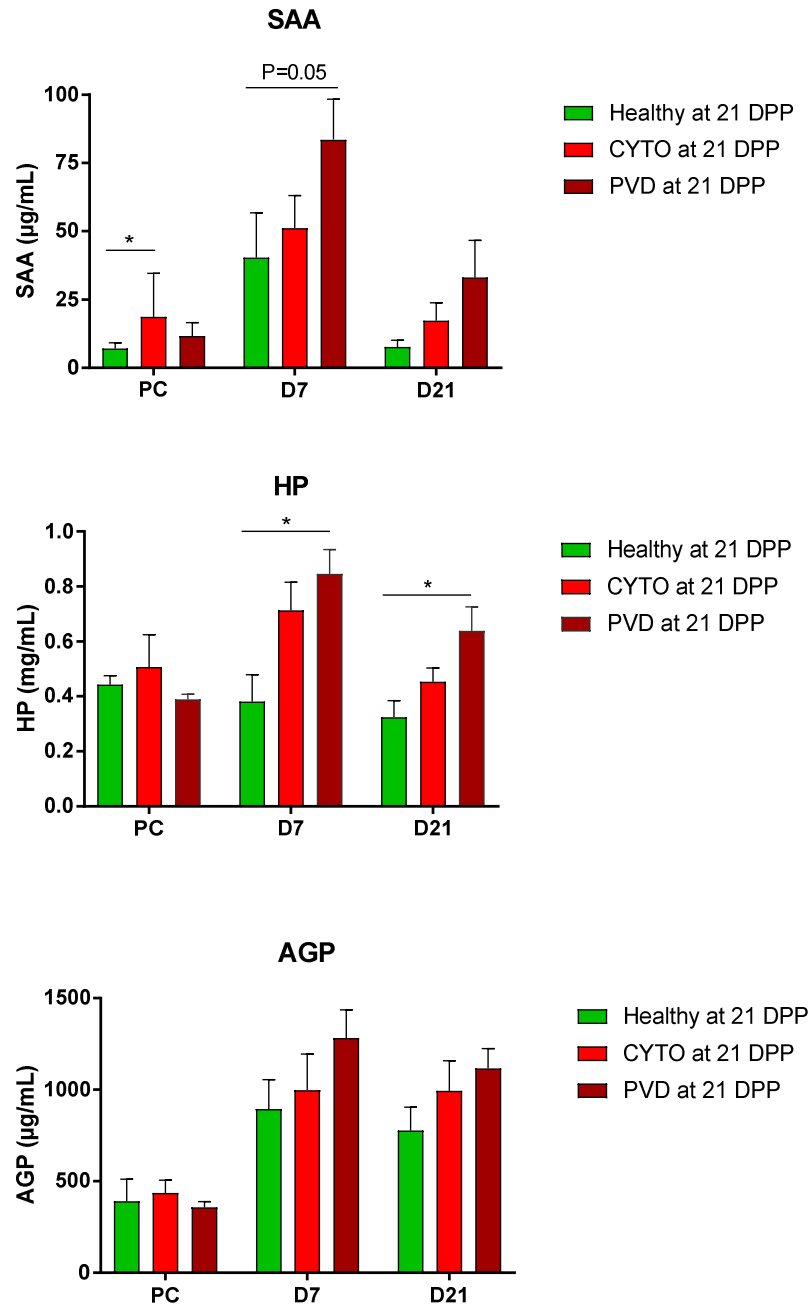


Figure 3-4 Circulating acute phase protein levels were elevated 7 DPP in cattle that subsequently developed uterine disease

Serum amyloid A (SAA) (n=82), haptoglobin (Hp) (n=62) and alpha-1-acid glycoprotein (AGP) (n=62) concentrations were measured in blood plasma of cattle at pre-calving (PC) and 7 and 21 days postpartum. Cattle were retrospectively classified by disease status at 21 DPP. A one way ANOVA was used to determine significant differences between groups at each time point (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

3.3.5 Vitamins and minerals

Vitamin D, calcium and iron concentrations were analysed in the blood plasma of cattle at pre-calving and 7 and 21 days postpartum (Figure 3-5). Cows were grouped by disease status 21 DPP. Vitamin D (25-hydroxyvitamin D; calcifediol) levels were recorded as in addition to its necessity for calcium absorption, vitamin D also plays a vital role in modulation of the immune response, and in a spring-calving herd it is likely that cattle housed all winter will have sub-optimal vitamin D levels. We found that vitamin D concentrations remained fairly stable across groups and time points, although these values were very low by recent recommendations of a minimal threshold of 30ng/ml for optimal immune function in cattle (Nelson et al., 2012). They were also lower in comparison to systemic vitamin D levels measured on other Irish spring-calving dairy farms (data not included). We also measured systemic calcium levels, as calcium is required for a variety of important functions such as uterine contractions and hypocalcaemia (mainly the subclinical form) is a common occurrence in postpartum dairy cows. Blood calcium levels remained stable after calving and did not drop below the most recently proposed subclinical hypocalcaemia threshold of 2.14 mM calcium (Rodríguez et al., 2017), suggesting that all cows had normal levels of blood calcium. Additionally there were no differences between healthy and endometritic cows which suggests that hypocalcaemia did not influence the onset of uterine disease. Systemic iron concentrations were similar in all cows at PC and 7 DPP, but tended towards being lower in cows with PVD 21 DPP.

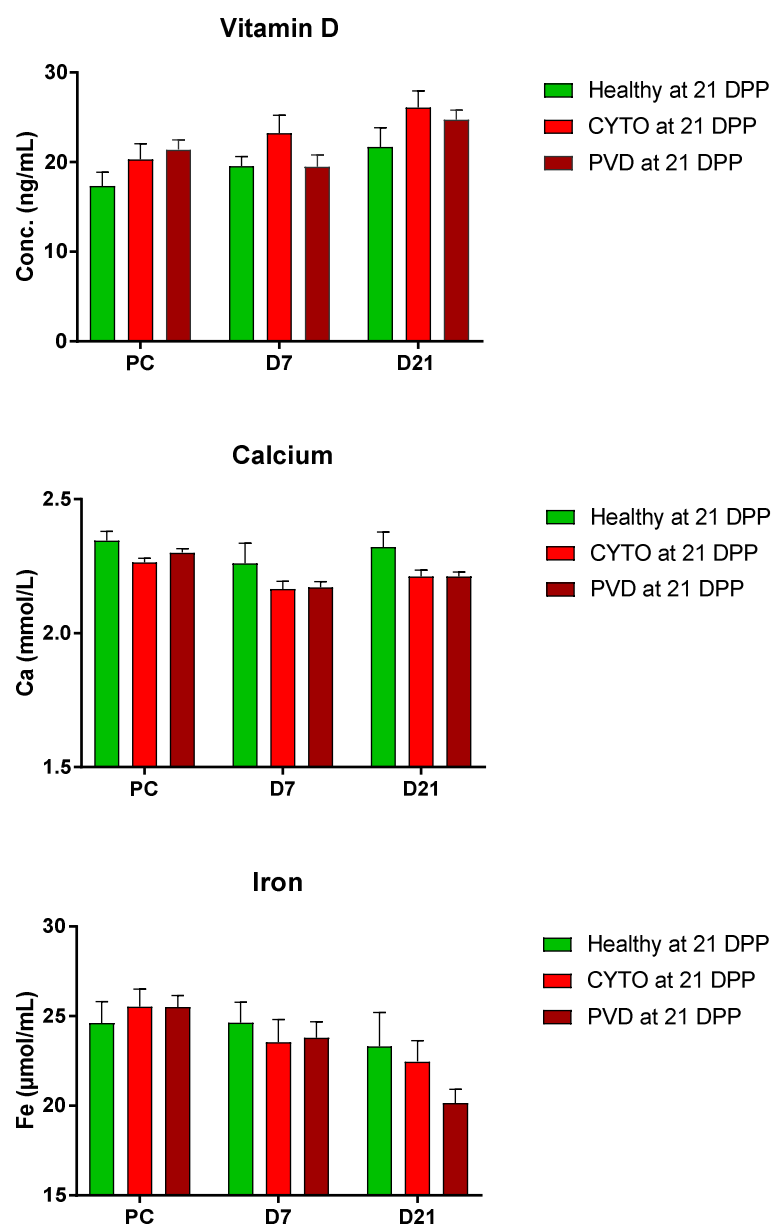


Figure 3-5 Circulating calcium and iron concentrations declined in cattle with uterine disease after calving

Vitamin D (n=74), calcium (n=62) and iron (n=95) concentrations were measured in blood plasma of cattle at pre-calving (PC) and 7 and 21 days postpartum. Cattle were classified retrospectively by disease status 21 DPP. A one way ANOVA was used to determine significant differences between groups at each time point.

3.4 Discussion

Incidence of uterine disease was higher than average in this study, with 76% of cattle experiencing one or more forms of disease 21 DPP. However, this was still within the range of reported incidence rates (Sheldon et al., 2009, Dubuc et al., 2010a). Data on the incidence of uterine disease in extensively pasture-based dairy production systems is limited. Reported incidence rates of PVD in Ireland have ranged from 9 to 73% (Olmos et al., 2009, Crowe and Williams, 2015, Savc et al., 2016), whilst cytological endometritis has been observed in 73% of cattle 21 DPP (Rojas Canadas et al., 2018). Although these studies are not representative of the national picture, it is clear that uterine disease is not only highly prevalent on specific farms (>70% of cows on this farm at 21 DPP), but that the occurrence of disease is associated with reduced fertility and increased veterinary and animal health interventions. Undoubtedly, a proportion of the cows diagnosed in this study would naturally clear infections after 21 DPP but nevertheless in the context of an expanding dairy herd, such associations are unwelcome and require additional investigation. Primiparous cows had a higher rate of disease incidence than multiparous cows, although this did not reach significance. This is in agreement with previous studies (Dubuc et al., 2010b, Potter et al., 2010, Cheong et al., 2011) and may be due to the increased risk of dystocia and therefore uterine damage in heifers due to their smaller size (Meyer et al., 2001, Uematsu et al., 2013). Furthermore, heifers are usually calved at around 24 months of age, when they are not yet physically mature (Coffey et al., 2006) which means they require additional nutrients for their own development as well as that of the calf, which may result in greater negative energy balance.

Energy status of cattle during the transition period is believed to be an important factor in the development of uterine disease. Analysis of metabolites in the current study revealed that all cattle were in a state of NEB post-calving. This was represented by a decline in plasma glucose and an increase in NEFA and BHB. These values were similar to those of other studies, residing in-between those of cattle in mild and severe negative energy balance (Wathes et al., 2009). However, no differences were seen between groups. This was in agreement with previous pasture-based studies that found no association between endometritis and NEFA or BHB at 1 week (Foley et al., 2015) or 6 weeks (Burke et al., 2010) postpartum. In contrast, (Ribeiro et al., 2013) reported increased NEFA in cows with uterine disease in 2 seasonally-based farms in the USA, however it is worth noting that Holstein-Friesians with North American genetics tend to be higher producing animals and therefore more likely to experience negative energy balance postpartum (Lucy et al., 2009). Another metabolite that can fluctuate during the transition period is urea. Urea is a product of protein break-down, and both high and low levels have been

associated with decreased fertility (Wathes et al., 2007). High dietary protein fed to increase milk production results in excessive urea production which can effect the cow's return to cyclicity and embryo survival (Laven and Drew, 1999). It can also decrease uterine pH, impairing sperm motility (Elrod et al., 1993). However in this study reduced urea was present in cows with PVD 21 DPP ($P=0.07$) which could indicate malnutrition or a low protein diet.

Whilst metabolites are the most common measurement of NEB in cattle, a number of studies have suggested that other factors, in particular insulin-like growth factor-1 (IGF1), may be more sensitive markers for the prediction of metabolic disorders (Piechotta et al., 2015). IGF1 is part of the somatotrophic axis, which is the major hormonal pathway involved in the regulation of metabolism. Due to the large energy demands of lactation, the cow begins to repartition nutrients for milk production. Glucose then becomes the nutrient in shortest supply, so the cow employs several key mechanisms to limit the decline in available glucose. This includes down-regulating the expression of growth hormone (GH) receptors in the liver, from around 2 days pre-calving to 2 weeks after parturition. GH usually stimulates IGF1 production in the liver, and so a decrease in hepatic GH receptors leads to decreased circulating IGF1 and increased GH (due to a lack of negative regulation by IGF1). Low IGF1 has been associated with poorer subsequent fertility rates and a higher incidence of cytological endometritis (Valdmann et al., 2018). Levels of IGF1 in pastoral based systems have not yet been reported and is something worth considering in future work. Increased GH stimulates gluconeogenesis and promotes the breakdown of muscle and adipose tissue, producing large amounts of NEFA. Elevated GH and NEFA levels can lead to insulin resistance, as fat mobilisation releases pro-inflammatory cytokines such as IL-1, IL-8 and TNF which block intracellular signalling of the insulin receptor proteins. Increased insulin resistance then consequently leads to reduced glucose uptake.

In addition to metabolic disparities, the abundance and function of circulating leukocytes are also altered during the transition period. Neutrophils are the most prevalent circulating leukocyte and have been the main focus of studies on uterine disease. They are the first immune cell to respond to invading pathogens and are a common hallmark of inflammation. Interestingly, we found no differences in circulating neutrophil number between healthy and endometritic cows or between time points. This is in contrast to previous studies which have reported both increased (Kim et al., 2005, Islam et al., 2014) and decreased (Cai et al., 1994, Foley et al., 2015) neutrophil abundance in cows with uterine disease. Although we saw no difference in neutrophil number, others have emphasised the importance of neutrophil function, which is often impaired in cattle with metritis and endometritis (Hammon et al., 2006).

Decreased neutrophil glycogen stores at calving have also been associated with the onset of uterine disease (Galvao et al., 2011). Additionally, recent studies in humans have characterised distinct sub-populations of neutrophil with divergent functions (Silvestre-Roig et al., 2016). The importance of leukocyte heterogeneity was highlighted by the recent discovery of two distinct monocyte subsets in cattle that were associated with the development of metritis (Pomeroy et al., 2017). One leukocyte population that we did observe differences in was the eosinophil. Eosinophil numbers decreased in all cows 7 DPP compared to pre-calving, but were significantly lower in cows that subsequently developed uterine disease. Eosinophils have traditionally been portrayed as non-specific cells that fight helminthic infections and control allergic and asthmatic reactions. However, more recent studies suggest they also play an important role in immune regulation and tissue remodelling (reviewed by (Jacobsen et al., 2012)). They promote plasma B-cell survival and enhance antibody production (Chu and Berek, 2012). Interestingly, increased eosinophil abundance and degranulation has been identified in fibrotic tissue of women with endometriosis (Blumenthal et al., 2000) and in the gut of patients with inflammatory bowel disease (Lampinen et al., 2008), both of which are chronic inflammatory conditions associated with inappropriate tissue remodelling. Low blood eosinophil numbers in cattle could indicate lack of immune regulation or possibly disruption to epithelial barrier repair in the uterus.

Although the link between haematological cell populations and uterine disease is currently inconclusive, acute phase proteins are very sensitive indicators of inflammation, and are therefore often used as biomarkers of disease in both human and veterinary medicine. Here, we found that SAA, Hp and AGP concentrations increased in all cows 7 DPP, but SAA and Hp were significantly higher in cows that developed PVD. The same pattern was observed for cows that developed CYTO, and for AGP, though neither reached significance. Increased serum Hp has been associated with both CYTO and PVD 21-30 DPP (Heidarpour et al., 2012) and 60 DPP (Brodzki et al., 2015). Furthermore, in agreement with our results, elevated Hp 7 DPP has been associated with the development of PVD (Schneider et al., 2013) and CYTO (Foley et al., 2015), although the latter did not assess the presence of PVD. More specifically, (Dubuc et al., 2010a) reported Hp concentrations of >0.8 g/L during the first week postpartum as a risk factor for CYTO and PVD. Increased Hp levels have also been associated with other uterine diseases including retained placenta (Mordank, 2009) and metritis (Hirvonen et al., 1999, Huzzey et al., 2015). Consequently, the calving to conception interval is longer in cattle with higher serum Hp compared to those with lower serum Hp (Chan et al., 2010) and avoiding the APR has been recommended as an option for improving the fertility of high-yielding dairy cows (Bertoni et al., 2008). However, increased serum Hp is associated with compromised fertility even in the

absence of uterine disease (Burke et al., 2010), which indicates specificity is an issue when assessing APPs as biomarkers for uterine disease.

Fewer studies have investigated the role of SAA and AGP postpartum. Increased SAA has been identified in the serum of endometritic cows at 7 DPP (Foley et al., 2015) and 22 DPP (Brodzki et al., 2015) but not at 5 or 40 DPP (Brodzki et al., 2015). Plasma concentrations of AGP have been found to be associated with a fetid vaginal mucus odour at 21-28 DPP (Williams et al., 2005). AGP and a fetid odour also increased in line with pathogen load in the uterus, particularly numbers of *T.pyogenes* and *Bacillus*, indicating a link between bacteria and the APR. Serum concentrations of APPs are also significantly related to LPS dose (Jacobsen et al., 2004) and uterine pathogen growth density at 7 and 14 DPP (Williams et al., 2007), suggesting that the increased APR of cattle with PVD compared to CYTO in this study could be due to differences in the microbiome between these different manifestations of disease.

Although the sensitivity of APPs (particularly Hp) as indicators of uterine disease has been debated, they are unlikely to be used as biomarkers due to a lack of specificity, as Hp and SAA have also been associated with other postpartum diseases such as mastitis and ketosis (Manimaran et al., 2016). It is worth noting that circulating APP concentrations were much higher in this study compared to those of cows with mastitis (Eckersall et al., 2001) (SAA: mean of 29.9 µg/mL in cows with moderate mastitis vs. mean of 90 µg/mL in cows with uterine disease in our study; AGP: mean of 0.54 mg/mL in cows with moderate mastitis vs. mean of 1.3 mg/mL in cows with uterine disease), although this may well reflect the use of different equipment and reagents between the two studies. Although APPs are primarily produced by the liver, production of SAA3 mRNA 14 DPP has been reported in uterine tissue (Chapwanya et al., 2009) which increased with severity of inflammation. Moreover, endometrial Hp mRNA has also been detected and showed a positive correlation with uterine PMN%. Therefore, local analysis of APP concentrations may provide a more specific biomarker for uterine disease. Indeed, several studies have already investigated the presence of APPs in uterine washings (Brodzki et al., 2015) and cervico-vaginal mucus (CVM) (Adhane et al., 2017). Hp levels were increased in uterine washings of cows with endometritis at 22 and 40 DPP and also in CVM 21 DPP. SAA on the other hand was decreased in cows with uterine disease at 5 DPP in uterine washings, and also in CVM at 21 DPP. This suggests that endometrial SAA may play more of an immunomodulatory role than its circulating counterpart. AGP expression has also been detected in the healthy bovine uterus (Lecchi et al., 2009), but its role in the presence of inflammation has yet to be elucidated and warrants further investigation.

Vitamin and mineral deficiencies during the transition period can also affect immune function. In this study we investigated circulating levels of calcium, vitamin D and iron. Hypocalcaemia may hinder the immune response (Kimura et al., 2006), but its link with uterine disease remains unclear. Clinical hypocalcaemia has been associated with a higher risk of PVD at 23 DPP (Whiteford and Sheldon, 2005), whilst subclinical hypocalcaemia (defined here as <8.59 mg/dL) is associated with greater risk of metritis (Martinez et al., 2012). In line with our own results, other studies reported no differences in serum calcium concentrations at 7 DPP in cows with metritis (Chapinal et al., 2011), or at 6 weeks postpartum in cows with endometritis (Burke et al., 2010). Although cattle in this study did not appear to be calcium deficient, efficient absorption of calcium is also important and relies on vitamin D and magnesium. In addition, there is emerging evidence that vitamin D has important immunomodulatory properties. For example, it regulates AMP expression in bovine mammary epithelial cells (Télez-Pérez et al., 2012). As the primary source from which cattle obtain vitamin D is sunlight or fortified concentrates, limited sun exposure as well as the increased metabolic demands of the periparturient period can result in a large depletion of serum 25(OH)D after calving (Holcombe et al., 2018). Here, we did not see a decline in 25(OH)D postpartum, but this may be due to the fact that levels were already incredibly low (around 20 ng/ml) in comparison to studies performed in the US which saw 25(OH)D levels fall to 82.6 ng/ml 7 DPP (Holcombe et al., 2018). A minimum threshold of 30 ng/ml has been hypothetically proposed based on human epidemiological studies (Cameron et al., 2013) however the concentration for optimal health in dairy cattle remains unknown. Although calcium is the most commonly affected mineral during the transition period, deficiencies of other minerals such as potassium, copper, magnesium and phosphorus have been associated with metabolic and inflammatory diseases postpartum (Roche et al., 2018). Little work has been carried out on the effect of iron deficiency in cattle. Mainly it is thought of as a secondary mineral deficiency which interferes with the absorption of copper (Roche et al., 2018). Additionally, iron deficiency tends to be rare in adult cattle as their requirement is low. However, circulating iron is often diminished during the APR, due to the redistribution of iron from transferrin in the blood into tissue (Kushner, 1982) which is aided by leukocyte lactoferrin. Iron has not been investigated in relation to uterine disease before, however lower concentrations have been reported in repeat breeders (Manickam et al., 1977) suggesting that it may affect fertility. Though we found no significant differences in transferrin-bound iron between healthy and endometritic cows, iron appeared to be subtly lower in PVD cows 21 DPP which could suggest an antimicrobial approach, restricting the amount of iron available for the microbial growth. It also corresponds with increased Hp concentrations 21 DPP

which is known to bind free plasma haemoglobin, which also contains iron, thereby acting as both an antimicrobial agent and an antioxidant by preventing the release of reactive oxygen species.

In summary, the onset of uterine disease in this study was linked to increased Hp and SAA concentrations and lower eosinophil abundance 7 DPP. These differences were greater in cattle that developed PVD, suggesting that CYTO invokes a more subtle inflammatory response. Although APPs are highly sensitive indicators of inflammation, the specificity for uterine disease is low and therefore analysis of APPs and other markers of inflammation in uterine fluid or mucus may result in more relevant biomarkers. New technologies are providing additional non-invasive methods of prognostic biomarker detection, for example liquid chromatography-mass spectrometry (LC-MS) has been used to profile serum metabolic differences in cows that develop metritis (Zhang et al., 2017a) and plasma proteomic differences in cows with endometritis (Zhang et al., 2017b). The proteomic content of circulating exosomes (extracellular vesicles involved in cell-cell communication) in cows with endometritis has also been investigated (Almughlliq et al., 2018). Future work involving the separation of exosomes by their tissue of origin (i.e. acquiring exosomes from the uterus) could prove very useful for increasing the specificity of differential proteins reported. Similarly it may be worth investigating the content of exosomes present in vaginal mucus. As uterine disease is a complex disease, it may be difficult to establish one single biomarker for detection, and so a panel of different biomarkers may be more appropriate.

Chapter 4 Defining the inflammatory gene signature at day 7 postpartum associated with the development of bovine uterine disease

4.1 Introduction

Approximately 40% of cows develop post-partum uterine disease, and the reasons remain unclear (Bromfield et al., 2018). Uterine disease is a significant contributor to reduced fertility in dairy cattle. It results in lower conception rates, increased services per conception and a longer calving interval (Fourichon et al., 2000, LeBlanc et al., 2002a, Dubuc et al., 2010a, LeBlanc, 2014). Moreover, energy diverted into immune and inflammatory processes induced by pathology comes at a cost to production, as well as fertility.

The host immune response plays a principal role in determining disease outcomes, no matter the aetiology or risk factors involved (Machado and Bicalho, 2015). Most cattle experience inflammation of the uterus during the early postpartum period, which is characteristic of the normal involution process (Chapwanya et al., 2012). Dysregulation of inflammation is central to the pathogenesis of endometritis and in his review, (see Figure 1) LeBlanc proposes a model to explain how either excessive or inadequate inflammation can result in endometritis (LeBlanc, 2014). However the molecular networks regulating inflammation and underpinning these divergent responses have not been defined.

Increased expression of pro-inflammatory cytokines, chemokines, antimicrobial peptides (AMPs) and prostaglandins in the uterus of cattle with uterine disease follows a time-dependent sequence (LeBlanc, 2012, Wagener et al., 2017b). However transcriptomic differences within the first week postpartum have not been conclusively demonstrated. Increased expression of *IL1A* and *IL1B* were detected 7 DPP in cattle that subsequently developed endometritis (Herath et al., 2009b), although lower *IL1B* and *TNF* mRNA levels have also been reported in cows that developed uterine disease (Galvao et al., 2011). Upregulation of the pro-inflammatory genes IL-1 and IL-17 were identified 7 DPP using RNA-seq in endometrial biopsies from cows that resolved inflammation and those that developed cytological endometritis (CYTO) (Foley et al., 2015). These findings suggest that despite the fact that most cattle experience uterine inflammation after calving, differences may exist between physiological and pathological inflammation which are able to identify cows at risk of developing subsequent uterine disease very early postpartum. However the sample sizes in these studies were small and the mixed cell population of a biopsy can complicate understanding of the cellular regulatory networks involved. Uterine epithelial cells play an essential role in defence against microbial invasion. Not

only do they maintain a physical barrier, but they are able to recognise pathogen associated molecular patterns (PAMPS) and damage associated molecular patterns (DAMPs) to which they respond through the secretion of pro-inflammatory cytokines and AMPs (Davies et al., 2008, Cronin et al., 2012, Healy et al., 2014). Therefore cytobrushes were used in this study to collect a purer cell population of mainly surface endometrial epithelial cells and immune cells (Cardoso et al., 2017).

Previous transcriptomic studies failed to differentiate between different types of uterine disease (Foley et al., 2015). Traditionally endometritis has been diagnosed by the presence of purulent or muco-purulent vaginal discharge (PVD) at 21 days or more postpartum and was termed “clinical endometritis”. However, although PVD often accompanies more severe forms of endometritis, it can also occur in the absence of endometrial inflammation (Dubuc et al., 2010a) and may instead be indicative of infection or inflammation in the lower reproductive tract, such as the cervix or vagina (Deguillaume et al., 2012). Therefore it has been recommended that PVD and cytological (or subclinical) endometritis (CYTO) be defined as distinct conditions. Endometrial gene expression for both types of uterine disease has been compared to healthy cattle at 42-60 DPP (Salilew-Wondim et al., 2016). A larger number of differentially expressed genes (DEG) were observed in cows with PVD as opposed to cows with CYTO (28 genes; 26 of which were also differentially expressed in cows with PVD), suggesting that similar genes are involved in disease pathogenesis, but PVD elicits a more severe response. However it is not known whether these differences can be detected in the early postpartum period.

In this study, we aimed to characterise the inflammatory gene expression patterns using deep sequencing in a larger panel of post-partum cows with a view to defining early post-partum dysregulated inflammatory patterns that might predict endometritis.

4.2 Hypothesis and specific aims

We hypothesise that even in the context of early postpartum inflammation, cows that go on to develop uterine disease will be identified by a unique inflammatory gene signature in endometrial epithelial cells 7 days postpartum.

Aims:

1. Collect uterine cytobrushes from cattle 7 and 21 days postpartum for RNA and cytological diagnosis
2. Assess the uterine transcriptome 7 DPP of cows that develop uterine disease and those that remain healthy using RNA-seq
3. Validate RNA-seq results in remaining cattle using qPCR

4.3 Results

4.3.1 RNA quality and quantity

Total RNA was extracted from endometrial cytobrushes of 112 cows using a TRIzol-RNeasy hybrid protocol. Optimisation using practice brushes found that this method yielded a greater quantity of RNA than the RNeasy kit alone, likely due to greater potency of TRIzol as a lysis buffer. In addition, use of the RNeasy kit reduced the amount of phenol contamination present in RNA samples compared to using TRIzol alone (data not shown). RNA yield ranged between 0.06 µg/µL and 3.58 µg/µL, with an average of 1.2 µg/µL. RNA quality also varied greatly, ranging from a RIN value of 4.1 to 9, with an average RIN of 7.2. Of the 112 samples extracted, 24 were excluded due to poor quality or quantity of RNA. The variation in RNA quality is likely to reflect the early postpartum intra-uterine environment – a time at which a large number of dying cells are being sloughed from the endometrium. Indeed, RNA quality was generally better at 21 DPP compared to 7 DPP, at which point the endometrium is expected to be almost or fully repaired. Furthermore, RNA quality of healthy cows with little inflammation 7 DPP yielded much higher quality RNA. Thirty samples were chosen for RNA sequencing, based on RNA quality and the inflammatory and disease status of the cow at 7 and 21 DPP. This included 10 healthy cows, 10 with CYTO and 10 with PVD. Cows were diagnosed at 21 days postpartum, and then day 7 samples were retrospectively selected to assess whether differences in gene expression could be detected at this early time point postpartum.

4.3.2 Sequence quality

Sequencing data was generated using 100 base pair single end reads. Quality assessment of the raw reads was carried out using FastQC software, which assigns a Phred score to each nucleotide of the sequenced data. A Phred score 30 means that 99.9% of base calls are correct. The average Phred score here was above 30, which indicates sequence data was of very good quality. Quality and adaptor trimming was subsequently carried out using the BBDuk java package package to trim Illumina adapter sequences and any low quality bases (Phred score <20) from the 3' end of sequence reads.

4.3.3 Alignment of reads to the bovine genome

The splice-aware software STAR (<https://github.com/alexdobin/STAR>) was used to map reads to the bovine genome (UMD3.1). The average sequencing depth (number of input reads) achieved was 32,603,197 (Table 4-1). This was above the recommended “modest” sequencing depth of 30 million reads (of which 20-25 million should map to the genome) required to evaluate the similarity between transcriptional profiles of two polyA+ samples, as recommended by The

Standards, guidelines and best practices for RNA-Seq analysis by the Encyclopedia of DNA Elements (ENCODE) consortium (version 1.0; June 2011). An average of 30,589,318 reads mapped to one location within the bovine genome, whilst an average of 1,782,300 reads mapped to multiple loci. Only uniquely mapped reads were used for downstream analysis. The high percentage of uniquely mapped reads, which was >90% in most samples (85% in one) indicates mapping efficiency was very good.

The full dataset used during the current study is available in the GEO data repository, at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125964>.

Table 4-1 Read alignment results

Cow ID	Number of input reads	Uniquely mapped reads number	Uniquely mapped reads %	Number of reads mapped to multiple loci	% of reads mapped to multiple loci	% of reads unmapped
1066	34261886	32251886	94.13%	1817044	5.30%	0.56%
1183	45977044	43275362	94.12%	2397612	5.21%	0.66%
1194	52329099	49374122	94.35%	2675609	5.11%	0.53%
1239	34873411	33002813	94.64%	1556265	4.46%	0.90%
1340	35705458	33521415	93.88%	1941405	5.44%	0.68%
1378	16066842	14978997	93.23%	979147	6.09%	0.68%
1570	20708122	19133365	92.40%	1377614	6.65%	0.95%
1829	36462522	34243087	93.91%	1978941	5.43%	0.66%
1831	17305303	16223191	93.75%	971136	5.61%	0.64%
1890	39889778	37758135	94.66%	1901244	4.77%	0.58%
1944	28907176	27042116	93.55%	1661922	5.75%	0.70%
2006	19974298	17040881	85.31%	2315470	11.59%	3.10%
2039	28053777	26246542	93.56%	1622011	5.78%	0.66%
2053	24888264	23286366	93.56%	1417367	5.69%	0.74%
2064	39981489	37754942	94.43%	1995420	4.99%	0.58%
2161	35684092	33277429	93.26%	2176305	6.10%	0.64%
2167	40505129	38297150	94.55%	1942770	4.80%	0.65%
2178	38059838	35936511	94.42%	1857495	4.88%	0.70%
2184	21586379	20329324	94.18%	1124489	5.21%	0.62%
2188	44757976	41340413	92.36%	3152490	7.04%	0.60%
2202	24326198	22765949	93.59%	1424721	5.86%	0.56%
2224	32895220	30730943	93.42%	1873948	5.70%	0.88%
2382	32696672	30786517	94.16%	1720668	5.26%	0.58%
2392	27986823	26555280	94.88%	1288262	4.60%	0.51%
2421	42349202	40154490	94.82%	1955888	4.62%	0.57%
2520	24672754	23015295	93.28%	1448876	5.87%	0.85%
2570	34798542	32918076	94.60%	1609709	4.63%	0.78%
2622	58528033	55313486	94.51%	2824785	4.83%	0.66%
2672	20579580	19277806	93.67%	1185249	5.76%	0.57%
2683	23285023	21847657	93.83%	1275154	5.48%	0.70%

4.3.4 Divergence in the endometrial transcriptome 7 DPP between healthy cows and cows that subsequently develop uterine disease

Initial transcriptomic analysis at 7 DPP compared cows that subsequently remained healthy (n=10) to all cows that developed uterine disease, regardless of disease sub-type (n=20). Here we identified 294 genes that were differentially expressed between the two groups (FDR<0.05; FC>1.5). Most of the DEG (n=193) were upregulated in cows that subsequently developed disease and included many immune-related genes. This was reflected in the top 10 DEG, 7 of which showed increased expression in cows with uterine disease (Table 4-2). This included *PTX3* which plays a role in innate immunity, *NLRP3* which is a component of the inflammasome required for IL-1B and IL-18 maturation, *MEFV* which regulates the immune response and L-selectin (*SELL*), an adhesion molecule involved in the migration of leukocytes to sites of inflammation. Pathway analysis performed using InnateDB supported these observations (Figure 4-1). InnateDB incorporates information from the following publically available databases: PID NCI, KEGG, Reactome, INOH and PID BioCarta. When all databases were included in pathway analysis, the most significantly enriched pathway was *IL-1 mediated signalling events*. Over-representation of *Interleukin-1 processing*, *Il-1 signalling pathway* and *IL-1* indicate a strong role for IL-1 pathways in the pathogenesis of uterine disease. Furthermore, over-representation of the *NOD-like receptor signalling pathway* suggests that inflammasome-mediated induction of IL-1 is likely to play an important role. Other pathways highlight the importance of cytokine signalling and NFkB, a transcription factor that plays a key role in regulation of the immune response. When the KEGG database alone was used to generate pathway analysis in InnateDB, over-representation of the Toll-like receptor signalling pathway, chemokine signalling pathway and a number of disease-related pathways also highlighted the importance of differences in the immune response of these two groups of cattle (data not shown).

Table 4-2 Top 10 differentially expressed genes (FDR <0.05) at 7 days postpartum between cattle that remained healthy (n=10) and cattle that developed uterine disease (n=20).

Gene symbol	Ensembl ID	Gene name	Gene functions	FC	FDR
<i>Unknown</i>	ENSBTAG000000046375	Elafin-like	- Elastase-specific protease inhibitor - Antimicrobial peptide	32.62	0.000356
<i>PTX3</i>	ENSBTAG000000009012	Pentraxin 3	- Complement activation & modulation - Regulation of inflammation - Tissue remodelling	-88.25	0.002605
<i>SERPINB4</i>	ENSBTAG000000039037	Serpin Family B Member 4	Serine protease inhibitor	-355.06	0.002605
<i>FAM46A</i>	ENSBTAG000000000995	Family with Sequence Similarity 46 Member A	Probable nucleotidyltransferase that may act as a non-canonical poly(A) RNA polymerase.	4.52	0.002605
<i>NLRP3</i>	ENSBTAG000000001273	NLR Family Pyrin Domain Containing 3	- Sensor component of the NLRP3 inflammasome - Promotes IL-1β/IL-18 maturation	-30.77	0.002605
<i>RUBCNL</i>	ENSBTAG000000013371	RUN and Cysteine Rich Domain Containing Beclin 1 Interacting Protein Like	Possible role in endocytic trafficking and autophagy	-37.34	0.002605
<i>Unknown</i>	ENSBTAG000000023026	Serpin family B member 2-like	Role in complement & coagulation cascades	-182.32	0.003403
<i>MEFV</i>	ENSBTAG000000019123	Pyrin Innate Immunity Regulator	- Regulation of innate immunity/inflammation - Autophagy receptor for degradation of inflammasome components	-131.35	0.003403
<i>MSLN</i>	ENSBTAG000000000177	Mesothelin	- Stimulates colony formation of bone marrow megakaryocytes - Cell adhesion	12.36	0.00347
<i>SELL</i>	ENSBTAG000000011515	L-Selectin	Required for binding & rolling of leukocytes on endothelial cells	-17.44	0.00347

FDR: false discovery rate; FC: fold change (positive values: $\uparrow$ in healthy cows; negative values: $\uparrow$ in cows with uterine disease)

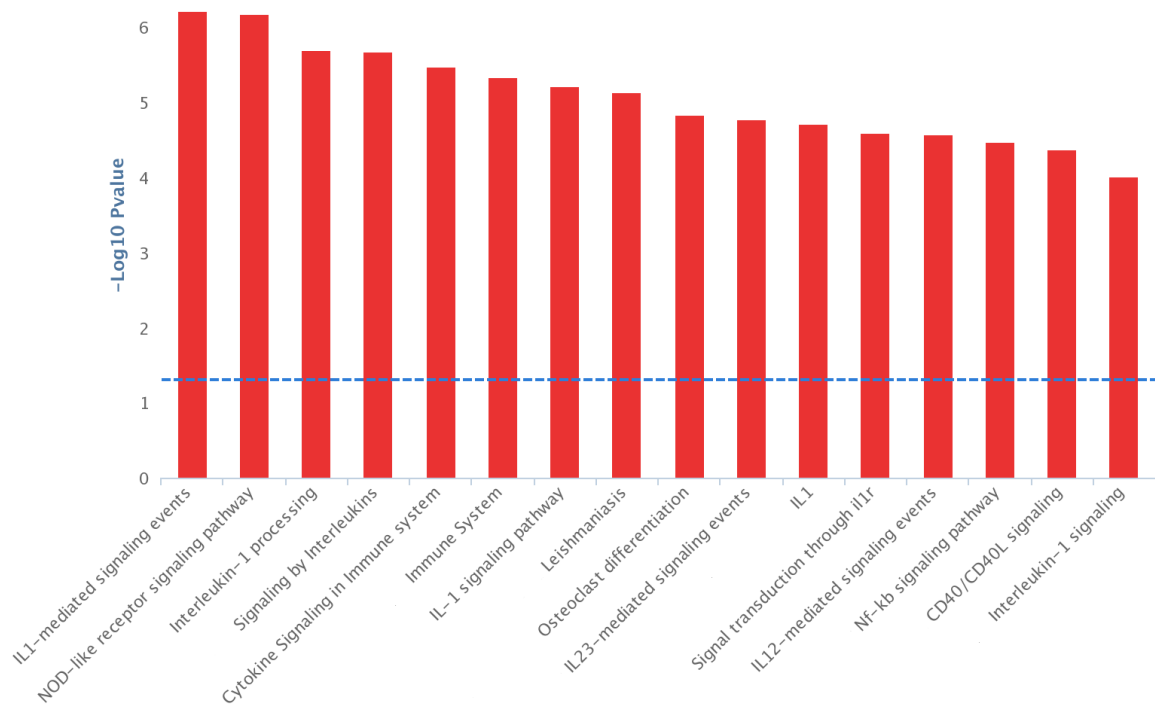


Figure 4-1 Development of uterine disease is preceded by upregulation of immune-related pathways 7 days postpartum.

Pathways over-represented at 7 days postpartum (DPP) between cattle that subsequently remain healthy (n=10) and cattle that developed uterine disease (n=20). Pathway analysis was performed using InnateDB, which incorporates information from the following publically available databases: PID NCI, KEGG, Reactome, INOH and PID Biocarta.

4.3.5 Differences in gene expression 7 DPP associated with clinical or subclinical endometritis

Cattle were next segregated by disease sub-type, to determine whether the uterine transcriptome differed 7 DPP depending on whether they subsequently developed clinical (PVD) or subclinical (CYTO) uterine disease. Cattle that developed PVD had differential expression of 2309 genes compared to healthy 7 DPP, whilst cattle that developed CYTO had only 17 genes that were differentially expressed 7 DPP (Figure 4-2). A less stringent cut-off FDR of <0.1 was used in this comparison as no genes in the healthy vs. CYTO comparison survived the FDR<0.05 cut off. All 17 genes differentially expressed by CYTO cows were also differentially expressed in PVD cows. Most (n=13) of these genes were upregulated in cows that subsequently developed uterine disease and were involved predominantly in activation (*NLRP3*, *RETN*, *SLAMF1*, *CD300LB*) or regulation (*MCEMP1*, *PTX3*, *SERPINB4*, *MEFV*, *IL1R2*) of the immune response.

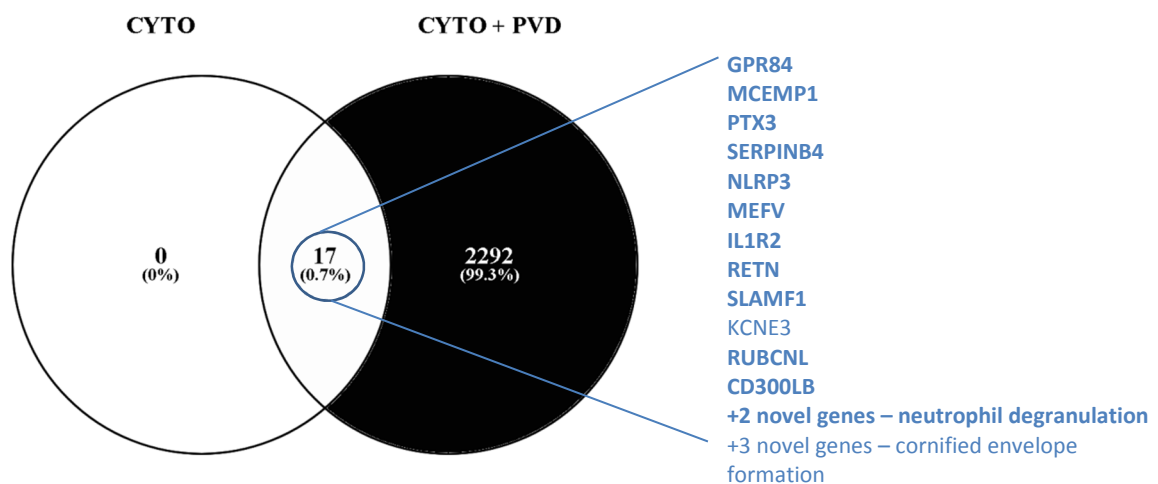


Figure 4-2 Cattle that develop purulent vaginal discharge display a greater number of differentially expressed genes compared to those that develop cytological endometritis at 7 days postpartum.

Each circle represents the number of differentially expressed genes (FDR <0.1; fc >1.5) in each condition (CYTO: cytological endometritis; PVD: purulent vaginal discharge) compared to healthy cows at 7 days postpartum. Genes that were differentially expressed in both conditions are listed on the right (genes in bold were upregulated in cows that developed uterine disease).

4.3.6 Importance of certain immune genes highlighted by large read counts

The importance of many of these differentially expressed immune genes was further highlighted when normalised read counts were considered. Although it is not necessary to correct for gene length when comparing expression of the same gene across samples, it is necessary for ranking gene expression levels within a sample, to account for the fact that longer genes accumulate more reads. Therefore read counts (count per million) were converted to transcripts per million (TPM).

A small number of genes accounted for a large portion of the total transcript number (Table 4-3). Only 16 genes had >1000 TPM. The full list of genes can be viewed in Table 4-4. Interestingly, this included a number of genes involved in the immune response (Figure 4-3). Two isoforms of the SAA gene (*SAA3* and the mammary SAA; *M-SAA3.2*) were among the genes with the greatest read counts, as well as the S100 AMPs *S100A8* and *S100A12*, the IL-1 receptor antagonist *IL1RN* and the chemokine *CXCL8*. The gene *S100A9* was also among those with the greatest read counts with an average of 992 TPM. When the normalised read counts for these genes are presented in graphical format, it becomes obvious that most of these genes (except for SAA) are upregulated in all cows with CYTO compared to healthy, whereas there is greater variation within the PVD group.

Table 4-3 A small number of genes accounted for a large number of reads

Read count (TPM)	Number of genes	% of total transcriptome
<100	24351	98.92
100-500	228	0.93
500-1000	21	0.09
1000-10,000	16	0.06
>10,000	0	0
Total	24617	100

Average (median) transcripts per million (TPM) for healthy cows (n=10) was subtracted from the average (median) TPM for cows with uterine disease (n=20) to determine an overall average read count.

Table 4-4 Genes with the greatest read counts highlights the importance of certain immune genes

Gene symbol	Ensemble ID	Healthy median read count (TPM)	UD median read count (TPM)	UD-Healthy (TPM)
<i>M-SAA3.2</i>	ENSBTAG00000010433	12959.74	6885.41	-6074.33
<i>FTH1</i>	ENSBTAG00000011184	7763.48	12545.02	4781.54
<i>S100A12</i>	ENSBTAG00000012638	862.27	4479.32	3617.05
<i>GAPDH</i>	ENSBTAG00000014731	1756.33	5296.95	3540.63
<i>SPP1</i>	ENSBTAG00000005260	2004.90	5194.05	3189.15
<i>GRP</i>	ENSBTAG00000004796	3398.18	482.11	-2916.07
<i>IL1RN</i>	ENSBTAG00000019665	267.81	3020.09	2752.27
-	ENSBTAG00000037768	571.72	2743.43	2171.70
<i>S100A8</i>	ENSBTAG00000012640	174.33	2251.46	2077.13
<i>SAA3</i>	ENSBTAG00000022396	9012.38	7271.84	-1740.55
<i>TMSB10</i>	ENSBTAG00000005654	4080.79	5758.98	1678.19
<i>CXCL8</i>	ENSBTAG00000019716	109.38	1774.80	1665.42
-	ENSBTAG00000048029	623.84	1962.82	1338.98
<i>CTSK</i>	ENSBTAG00000021035	4081.40	5364.33	1282.93
<i>RPLP1</i>	ENSBTAG00000018320	3207.79	2120.44	-1087.35
<i>SLC2A3</i>	ENSBTAG00000004556	146.42	1147.72	1001.30

Genes with >1000 transcripts per million (TPM) are presented. UD: uterine disease

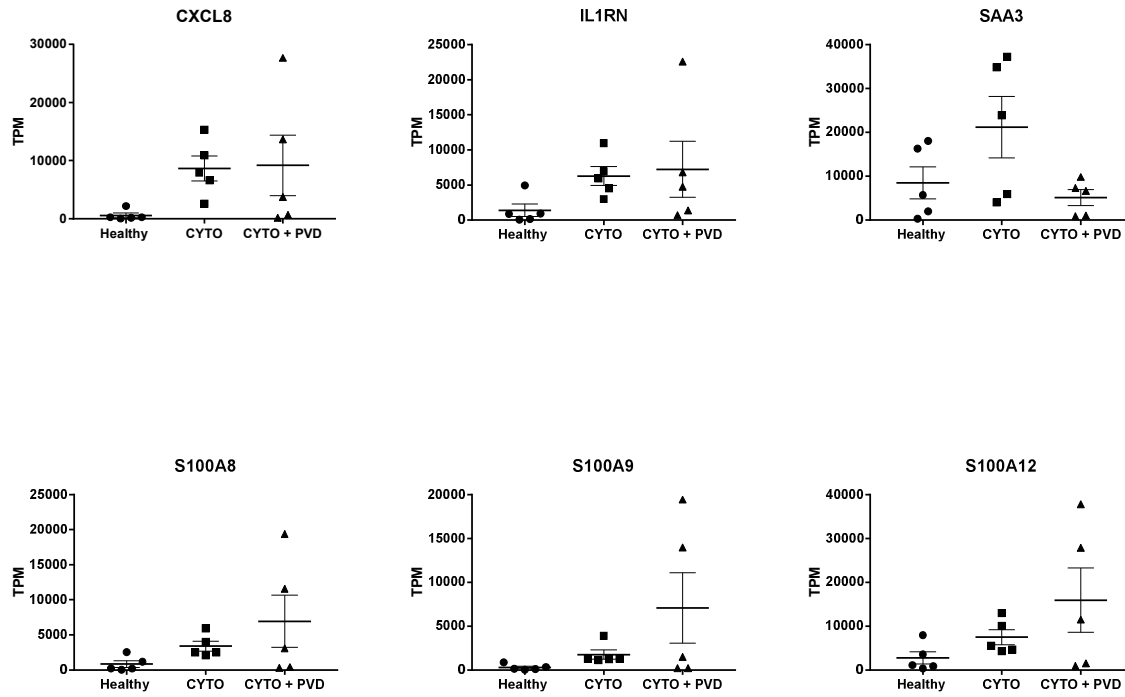


Figure 4-3 Importance of immune gene expression is emphasised by large read counts.

Differentially expressed genes (DEG) were sorted by normalised read count (transcripts per million; TPM) (see table 6). A large number of the genes with the highest read counts had immune functions. Six of these genes are shown here. CYTO: cytological endometritis. PVD: purulent vaginal discharge.

4.3.7 Inflammatory status 7 and 21 DPP

Inflammatory status 7 DPP was assessed at a cellular level by observing the number of uterine PMNs present, as detected by uterine cytology. In this study, the average (median) PMN% at 7 DPP was higher in cows that developed clinical endometritis (53%) or subclinical endometritis (46%) compared to those that remained healthy (21%), although the range in PMN% was similar for all groups (clinical: 2.5-87.5%; subclinical 0-89%; healthy 0-84.5%). The full list of PMN% for each cow can be found in the appendix (Table 8-1).

It has been well established that a certain degree of inflammation after calving is part of the normal involution process. As expected, the majority of the herd (81%) had a high level of inflammation (>18% PMN) 7 DPP (Table 4-5 and Figure 4-4). However, it was interesting to note that a sub-set of cattle (19%) had “low” inflammation 7 DPP (<18% PMN), and this included both cows that remained healthy and those that developed disease. Seeing as inflammatory status of the uterus 7 DPP will naturally influence gene expression due to differences in cell populations present (i.e. epithelial:PMN ratio), we split the cattle into subsets based on their PMN% at day 7 (either “high”: >18% or “low”: <18%).

In addition, cattle with PVD do not always have endometrial inflammation, suggesting that the discharge may be indicative of infection elsewhere in the reproductive tract. Therefore we also split the cows with PVD into two groups: those with (CYTO+PVD) and without (PVD only) uterine inflammation at 21 DPP.

Table 4-5 Animal classification by inflammatory and disease status

	Group	% PMN D7	% PMN D21	VMS D21	N
1	HL Healthy	>18%	≤18%	0-1	14
2	HH CYTO	>18%	>18%	0-1	24
3	HH CYTO + PVD	>18%	>18%	2-3	25
4	HL PVD	>18%	<18%	2-3	22
5	LL Healthy	≤18%	≤18%	0-1	13
6	LH CYTO	≤18%	>18%	0-1	7
	Total				105

Cows were classified by polymorphonuclear leukocyte (PMN) % at 7 and 21 days postpartum (DPP) and vaginal mucus score (VMS) at 21 DPP. Group names indicate the level of inflammation at 7 or 21 dpp (high; H or low; L) using an 18% cut off and the health status of the cow at 21 DPP (healthy, cytological endometritis: CYTO or purulent vaginal discharge: PVD).

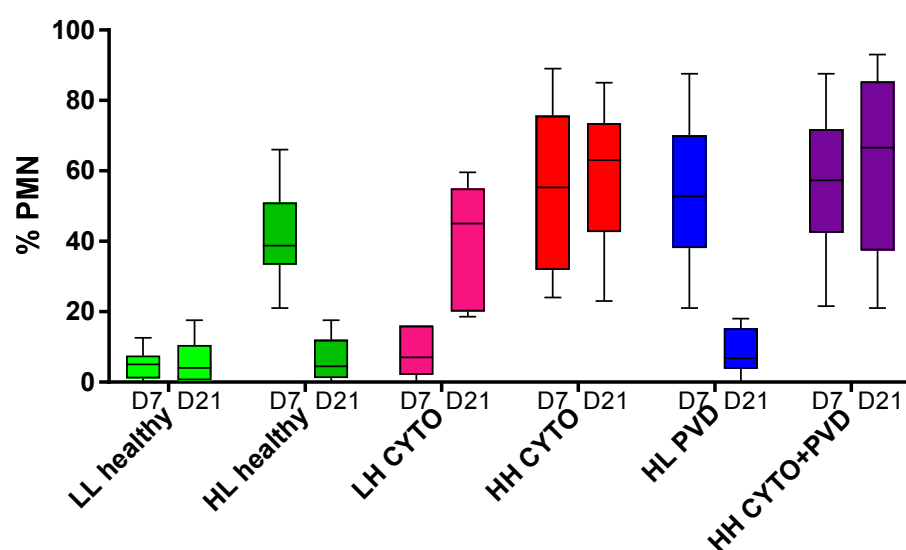


Figure 4-4 Cattle were sub-grouped based on endometrial PMN% at 7 and 21 days postpartum.

Polymorphonuclear (PMN) cell % was determined by counting the number of PMN cells and epithelial cells present on endometrial cytology slides acquired at 7 and 21 days postpartum. A total of 200 cells were counted per slide. An 18% PMN threshold was applied at both time points to separate cows with “high” (H) and “low” (L) inflammation. Group names on the x axis represent the level of inflammation at day 7 and day 21 followed by disease diagnosis at day 21 (CYTO: cytological endometritis; PVD: purulent vaginal discharge). D: days postpartum.

4.3.8 Physiological versus pathological inflammation

As most cattle had “high” inflammation 7 DPP, they became our main focus. Despite the fact that all cows with >18% PMN had uterine inflammation that looked identical between groups at the cellular level (Figure 4-5), transcriptomic differences were detectable between cows that resolved inflammation and cows that developed uterine disease (Figure 4-6). Cows that developed subclinical endometritis (CYTO; n=5) presented more subtle differences in gene expression and so no genes survived the stringent FDR correction. However, large differences in read count of many immune genes between groups indicated important biological differences and thus a less stringent threshold was applied ($P < 0.05$). There were 311 DEG common to both clinical and subclinical disease ($P < 0.05$; Figure 4-6 A). The top 10 DEG differed slightly between the 2 comparisons (Table 4-6 and Table 4-7), but both included upregulation of mast cell expressed membrane protein 1 (*MCEMP1*), G protein coupled receptor 84 (*GPR84*) and pentraxin (*PTX3*) in cows that developed uterine disease. All of the top 10 genes were increased in cows that developed uterine disease. This reflected the overall directional pattern of gene expression as demonstrated by volcano plots for each comparison, which show the majority of genes were upregulated in cows that developed uterine disease (Figure 4-6 B and C). Pathway analysis using IPA indicated that immune signalling pathways played a significant role in disease pathogenesis (Figure 4-6 D and E). In particular, *IL-6 signalling* was upregulated in both CYTO and CYTO + PVD cows, whilst *inflammasome pathway*, *dendritic cell maturation* and *NF- κ B signalling* pathways were increased in CYTO cows and *production of nitric oxide and reactive oxygen species in macrophages*, *tec kinase signalling* and *B cell receptor signalling* were increased in CYTO + PVD cows. This suggests that whilst inflammatory signalling is important for the pathogenesis of both types of disease, clinical disease may be more likely to be associated with bacterial infection and the adaptive immune response. Only one pathway was increased in healthy cows, and this was the *PPAR signalling* pathway. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that are activated by fatty acids. These have roles in lipid clearance, lipid oxidation, cell proliferation and adipocyte differentiation to increase uptake of blood glucose. This suggests they may play a key role in energy homeostasis after calving.

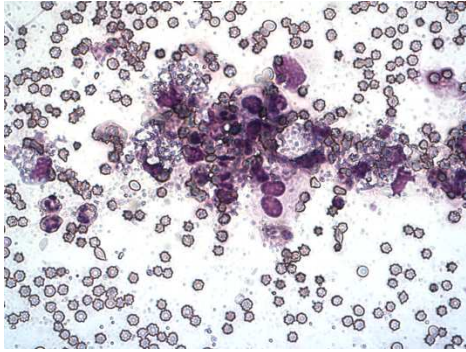
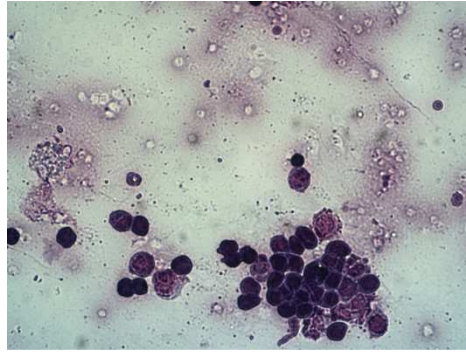
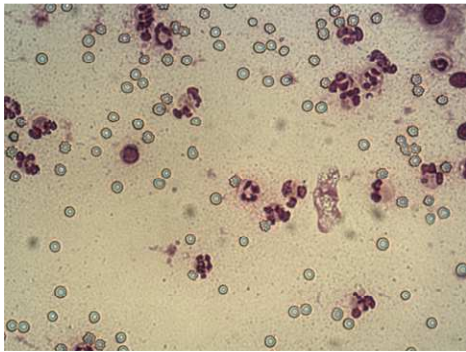
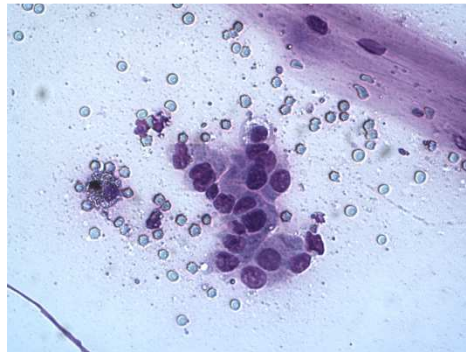
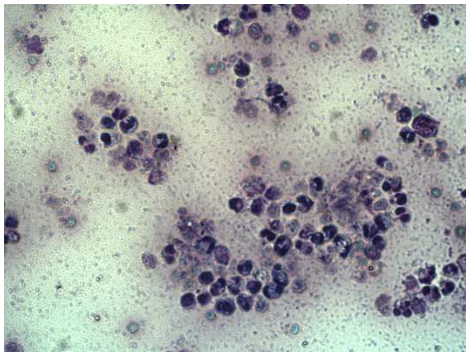
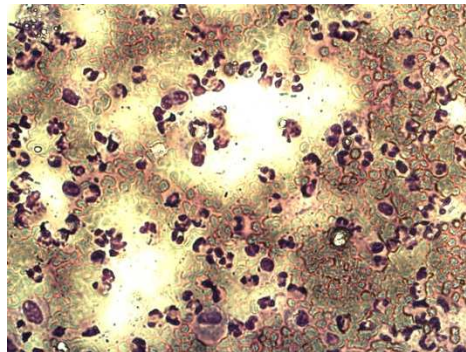
HL Healthy**LL Healthy****HH CYTO****LH CYTO****HH PVD****HL PVD**

Figure 4-5 Cattle were sub-grouped based on endometrial PMN% at 7 and 21 days postpartum.

Polymorphonuclear (PMN) leukocyte % was determined by counting the number of PMN cells and epithelial cells present on endometrial cytology slides acquired at 7 and 21 days postpartum. A total of 200 cells were counted per slide. An 18% PMN threshold was applied at both time points to separate cows with “high” (H) and “low” (L) inflammation. Group names on the x axis represent the level of inflammation at day 7 and day 21 followed by disease diagnosis at day 21 (CYTO: cytological endometritis; PVD: purulent vaginal discharge). D: days postpartum.

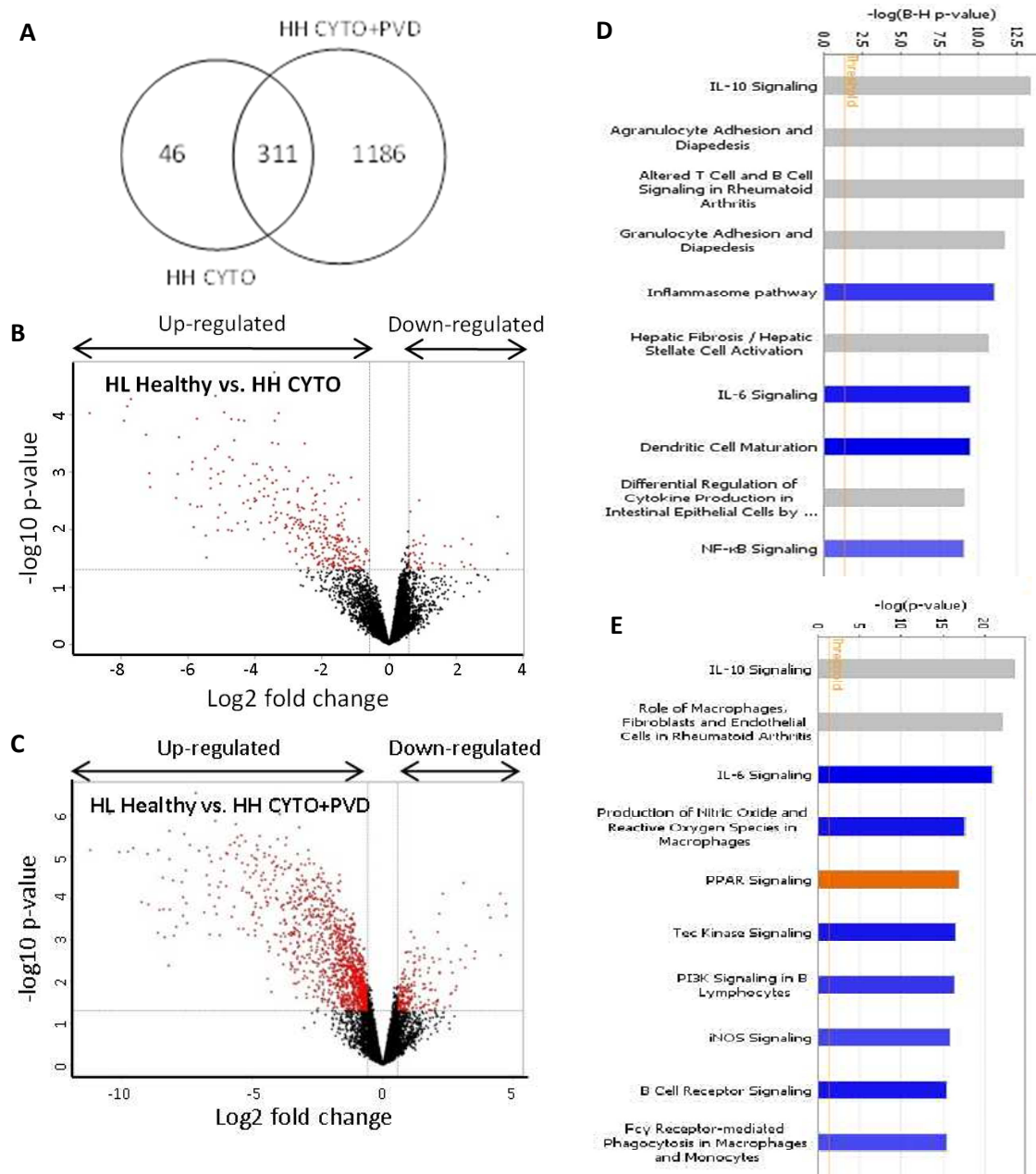


Figure 4-6 The majority of genes differentially expressed at 7 DPP were upregulated in cows that developed uterine disease and had inflammatory-related functions

A Each circle represents the number of differentially expressed genes ($P < 0.05$; $FC > 1.5$) in each condition (CYTO: cytological endometritis; PVD: purulent vaginal discharge) compared to healthy cows at 7 days postpartum (DPP). Only cows with inflammation ($>18\%$ pmn) at 7 DPP were included. **B-C** Gene expression data is presented as volcano plots for each comparison (**B**: healthy vs. HH CYTO, **C**: healthy vs. HH CYTO + PVD) using log values of the fold change and P value. Each data point represents a single gene, with those in red representing genes that survived the cut off thresholds of $P < 0.05$ and $FC > 1.5$. **D-E** Ingenuity pathway analysis (IPA) was used to assess cellular and molecular functions of the DEG between healthy and HH CYTO and healthy and HH CYTO + PVD cows respectively. Over-represented pathways were determined using the Fischer's exact test with a Benjamini-Hochberg correction. A z-score algorithm was used to predict directional changes, whereby pathways significantly increased in cows that developed CYTO were blue, and pathways increased in cows that remained healthy were orange

Table 4-6 Top 10 differentially expressed genes ($P < 0.05$) between HL healthy (n=5) and HH CYTO cows (n=5) at 7 days postpartum.

Gene symbol	Ensembl ID	Gene name	Gene function	FC	P value
<i>AVI1</i>	ENSBTAG00000016913	Advillin	Actin binding	-10.78	1.85E-05
<i>MCEMP1</i>	ENSBTAG00000004714	Mast cell expressed membrane protein 1	- Regulation of mast cell differentiation - Innate immunity	-36.23	4.76E-05
<i>GPR84</i>	ENSBTAG00000015592	G protein-coupled receptor 84	- Fatty acid metabolism - Immune system regulation	-209.49	5.38E-05
<i>Unknown</i>	ENSBTAG00000040586	Serpin peptidase inhibitor, clade B like	Serine protease inhibitor	-225.08	7.16E-05
<i>ALP</i>	ENSBTAG00000014329	Antileukoproteinase	- Antimicrobial peptide - Regulation of inflammation - Wound healing	-30.25	9.07E-05
<i>SCNN1D</i>	ENSBTAG00000017674	Sodium channel epithelial 1 delta subunit	Epithelial sodium ion channel	-9.86	9.43E-05
<i>Unknown</i>	ENSBTAG00000035224	-		-490.58	9.47E-05
<i>PTX3</i>	ENSBTAG00000009012	Pentraxin 3	- Complement activation & modulation - Regulation of inflammation - Tissue remodelling	-53.08	0.000118
<i>RUBCNL</i>	ENSBTAG00000013371	RUN and cysteine rich domain containing Beclin 1 interacting protein like	Possible role in endocytic trafficking and autophagy	-26.09	0.00012
<i>NLRP3</i>	ENSBTAG00000001273	NLR family pyrin domain containing 3	- Sensor component of the NLRP3 inflammasome - Promotes IL-1β/IL-18 maturation	-22.41	0.000123

FC: fold change (positive values: $\uparrow$ in healthy cows; negative values: $\uparrow$ in cows with uterine disease)

Table 4-7 Top 10 differentially expressed genes ($P<0.05$) between HL healthy (n=5) and HH CYTO + PVD (n=5) cows at 7 days postpartum

Gene symbol	Ensembl ID	Gene name	Gene functions	FC	P value
<i>MCEMP1</i>	ENSBTAG00000004714	Mast Cell Expressed Membrane Protein 1	- Regulation of mast cell differentiation - Innate immunity	-141.78	2.92E-07
<i>LPCAT2</i>	ENSBTAG000000019272	Lysophosphatidylcholine Acyltransferase 2	- Platelet-activating factor biosynthesis - Cell membrane biogenesis	-17.53	4.37E-07
<i>GPR84</i>	ENSBTAG000000015592	G Protein-Coupled Receptor 84	- Fatty acid metabolism - Immune system regulation	-1398.57	9.19E-07
<i>ARRDC4</i>	ENSBTAG000000018252	Arrestin Domain Containing 4	- Endocytosis of activated G protein-coupled receptors - Glucose uptake	-15.31	9.36E-07
<i>RUBCNL</i>	ENSBTAG000000013371	RUN And Cysteine Rich Domain Containing Beclin 1 Interacting Protein Like	Possible role in endocytic trafficking and autophagy	-84.66	1.37E-06
<i>TNF</i>	ENSBTAG000000025471	Tumour necrosis factor	- Pyrogen - Cell proliferation & differentiation - Apoptosis	-49.30	1.63E-06
<i>LRK2</i>	ENSBTAG000000016260	Leucine Rich Repeat Kinase 2	Autophagy regulation	-9.15	1.95E-06
<i>BMX</i>	ENSBTAG00000000377	BMX Non-Receptor Tyrosine Kinase	Signalling processes involved in cell migration/proliferation/adhesion/apoptosis	-284.80	2.37E-06
<i>PTX3</i>	ENSBTAG000000009012	Pentraxin 3	- Complement activation & modulation - Regulation of inflammation - Tissue remodelling	-192.58	2.80E-06
<i>ZFP36</i>	ENSBTAG000000008573	ZFP36 Ring Finger Protein	- Regulates protein synthesis - Anti-inflammatory responses	-42.59	3.01E-06

FC: Fold change (positive values: ↑ in healthy cows; negative values: ↓ in cows with uterine disease)

4.3.8.1 Upregulation of tissue remodelling genes in healthy cows 7 days postpartum

Although most genes were upregulated in cattle that developed uterine disease, a small number were also increased in healthy cows at 7 DPP (Table 4-8 and Table 4-9). Most of these genes were involved in cell proliferation (e.g. *ANK3*, *UKP1A* and *PTN*), adhesion (e.g. *CADM1*, *SYNE2* and *PGM5*) and cell structure (e.g. *COLA3*, *COLA4*) which are essential processes required for repair of the endometrium after calving. This suggests that excessive inflammation associated with uterine disease may be preventing tissue regeneration.

Table 4-8 Top 10 differentially expressed genes upregulated in healthy cows compared to CYTO cows at 7 days postpartum

Gene symbol	Ensembl ID	Gene name	Gene functions	FC	P value
<i>SCRN3</i>	ENSBTAG00000012966	Secernin 3	GO annotation: dipeptidase activity	1.86	0.003098
<i>VPS8</i>	ENSBTAG00000005209	VPS8, CORVET Complex Subunit	Involved in vesicle-mediated protein trafficking of the endocytic membrane transport pathway.	1.55	0.004826
<i>SLITRK4</i>	ENSBTAG00000011780	SLIT And NTRK Like Family Member 4	Involved in synaptogenesis.	9.49	0.005967
<i>SYNE2</i>	ENSBTAG00000025450	Spectrin Repeat Containing Nuclear Envelope Protein 2	Forms a link between organelles and the actin cytoskeleton to maintain spatial organisation.	2.04	0.012717
<i>UPK1A</i>	ENSBTAG00000020543	Uroplakin 1A	Regulation of cell development, activation, growth & motility.	4.61	0.014453
<i>GPD1L</i>	ENSBTAG00000009826	Glycerol-3-Phosphate Dehydrogenase 1 Like	Catalyses conversion of sn-glycerol 3-phosphate to glycerine phosphate.	1.67	0.015312
<i>CADM1</i>	ENSBTAG00000000977	Cell Adhesion Molecule 1	Mediates cell-cell adhesion in a Ca²⁺ - independent manner.	1.90	0.017176
<i>LIMCH1</i>	ENSBTAG00000010677	LIM And Calponin Homology Domains 1	Go annotation: actin binding.	2.14	0.018031
<i>MFAP5</i>	ENSBTAG00000000310	Microfibril Associated Protein 5	Component of microfibrils of the extracellular matrix. Promotes attachment of cells to microfibrils.	5.47	0.018169
<i>KALRN</i>	ENSBTAG00000002640	Kalirin RhoGEF Kinase	- Activates specific Rho GTPases. - Regulates neuronal growth via its effects on the actin cytoskeleton. - Induces lamellipodia.	1.75	0.01848

FC: Fold change (positive values: ↑ in healthy cows; negative values: ↓ in cows with uterine disease)

Table 4-9 Top 10 differentially expressed genes upregulated in healthy cows compared to CYTO + PVD cows at 7 DPP

Gene symbol	Ensembl ID	Gene name	Gene functions	FC	P value
<i>TNFRSF19</i>	ENSBTAG00000018300	TNF Receptor Superfamily Member 19	- Activation of JNK and NF-kappa-B. - May promote caspase-independent cell death.	8.52	4.22E-05
<i>COL4A3</i>	ENSBTAG00000021316	Collagen Type IV Alpha 3 Chain	Major structural component of basement membranes	22.69	7.61E-05
<i>SCRN1</i>	ENSBTAG00000016223	Secernin 1	Regulates exocytosis in mast cells.	4.93	7.69E-05
<i>AMPH</i>	ENSBTAG00000031967	Amphiphysin	- Regulates exocytosis in synapses and certain endocrine cell types. - May control the properties of the membrane associated cytoskeleton.	26.34	0.000133
<i>PGM5</i>	ENSBTAG00000033190	Phosphoglucomutase 5	Component of adherens-type cell-cell and cell-matrix junctions.	16.50	0.000144
<i>ANK3</i>	ENSBTAG00000019052	Ankyrin 3	- Links integral membrane proteins to the underlying actin cytoskeleton - Cell motility, proliferation and maintenance of specialized membrane domains.	4.49	0.00023
<i>PTN</i>	ENSBTAG00000002317	Pleiotrophin	- Secreted heparin-binding growth factor - Roles in cell growth, migration and survival	26.58	0.000255
<i>COL4A4</i>	ENSBTAG00000021310	Collagen Type IV Alpha 4 Chain	Major structural component of basement membranes.	17.38	0.00029
<i>SNAPC5</i>	ENSBTAG00000005210	Small Nuclear RNA Activating Complex Polypeptide 5	Subunit of the snRNA-activating protein complex that plays a role in the transcription of snRNA genes.	2.11	0.000619
<i>CPAMD8</i>	ENSBTAG00000009331	C3 And PZP Like, Alpha-2-Macroglobulin Domain Containing 8	Protease inhibitor important in innate and acquired immunity.	4.31	0.000641

FC: fold change (positive values: ↑ in healthy cows; negative values: ↓ in cows with uterine disease)

4.3.8.2 A subset of cattle had low inflammation 7 DPP

Although it is assumed that uterine inflammation is ubiquitous in the first week postpartum, we found that a subset of cattle (19%) had little-to-no inflammation 7 DPP (<18% PMN). Interestingly, healthy cows without inflammation 7 DPP had increased expression of epithelial-specific genes and decreased expression of stromal-specific genes compared to healthy cows with inflammation, which suggests they may have a more intact epithelial barrier (Figure 4-7). Lower expression of the macrophage marker *MSR1* was also observed in 4 out of 5 LL healthy cows. No differences were seen in dendritic cell, B cell or T cell markers between groups. Clear differences in gene expression were apparent when healthy cattle were segregated into subgroups based on inflammatory phenotype 7 DPP (Figure 4-8).

Others within this subset subsequently developed uterine disease; however this was not preceded by the upregulation of inflammatory genes 7 DPP (data not shown). Most of the top 10 DEG between cows with low inflammation that either remained healthy or developed uterine disease were involved in tissue remodelling (*MMP7*), cellular structure (*SPOCK2*, *ACTG2*) and cell-cell communication/adhesion (*GJB1*, *HMCN1*), all of which were upregulated in cows that developed uterine disease. It is likely these genes were downregulated in LL healthy cows as they did not need to perform such extensive repair of the endometrium. It is also important to consider the fact that we were only sampling one small area of the endometrium and it is possible that inflammation was present in other regions of the uterus.

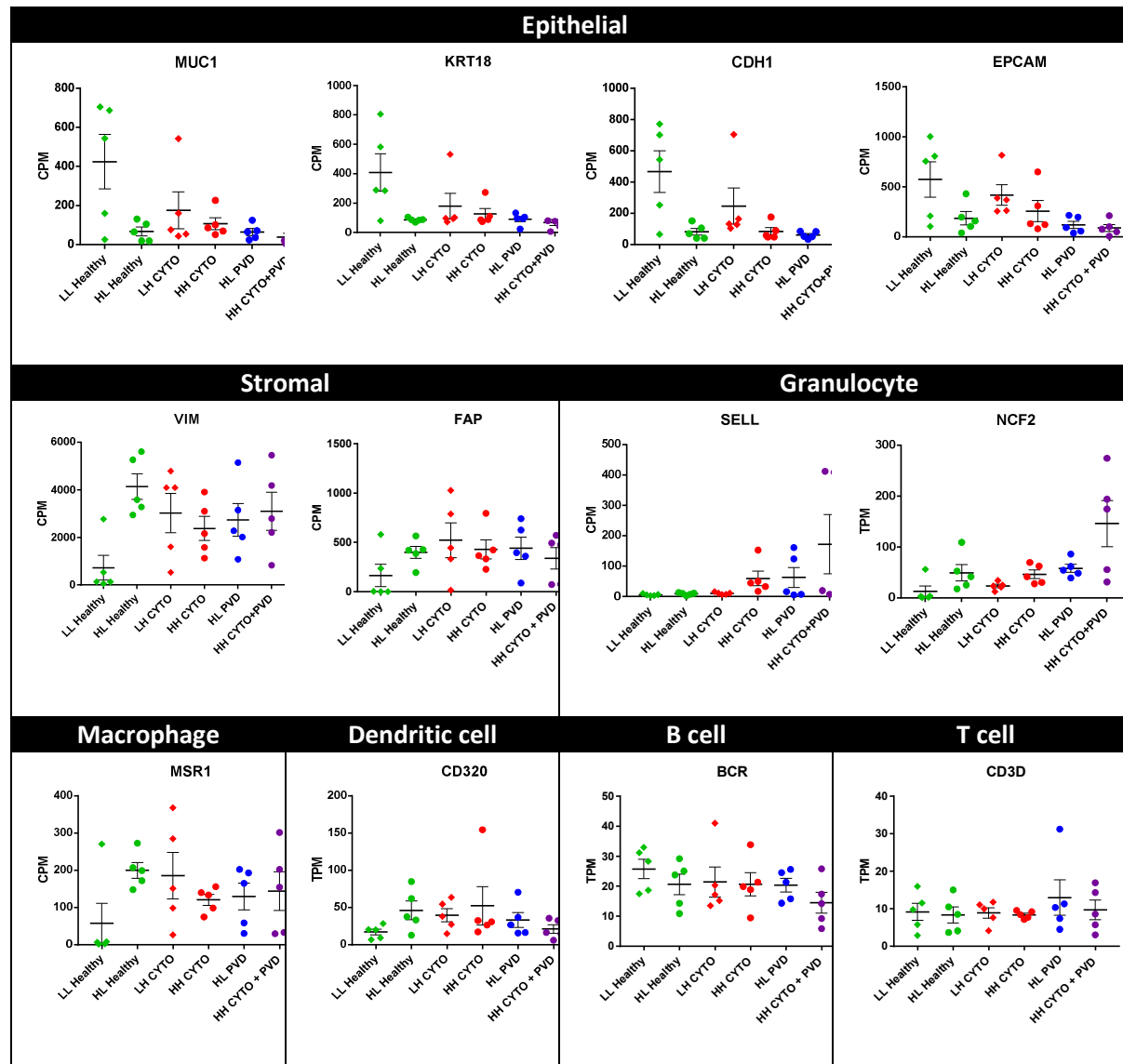


Figure 4-7 Epithelial and granulocyte markers differed between specific sub-groups of cattle.

Expression of genes specific to certain cell types is presented for each group. Data is presented as read counts per million (CPM).

4.3.8.3 PVD sub-groups demonstrate different immune responses 7 DPP

PVD is not always indicative of endometrial inflammation, often representing inflammation in another part of the reproductive tract instead, such as the cervix or vagina. Therefore we segregated the PVD group into cattle with and without inflammation (>18% PMN) at 21 DPP. Cattle that subsequently developed PVD without uterine inflammation displayed significantly lower expression of *TLR2*, *TL4*, *S100A8*, *IL10* and *TGFB2* than those that did have uterine inflammation (Figure 4-8). This suggests that PVD was either indicative of inflammation/infection elsewhere in the reproductive tract, or that it was a sign of resolution.

4.3.8.4 Validation of inflammatory gene signature that precedes the development of uterine disease in additional cattle

Validation of gene expression was performed using qPCR for 12 genes in 56 additional cattle from the same herd (Figure 4-8). Increased expression of pathogen recognition receptors *TLR2*, *TLR4*, inflammasome component *NLRP3*, pro-inflammatory cytokines *IL1A*, *IL1B*, *TNFA*, *IL17*, chemokines *CXCL8*, *CCL20* antimicrobial peptides *S100A8* and anti-inflammatory molecules *IL10* and *TGFB2* were upregulated 7 DPP in cattle that went on to develop uterine disease. Despite a certain degree of inter-animal variation within groups, it is clear that a core inflammatory gene signature 7 DPP is associated with the onset of uterine disease.

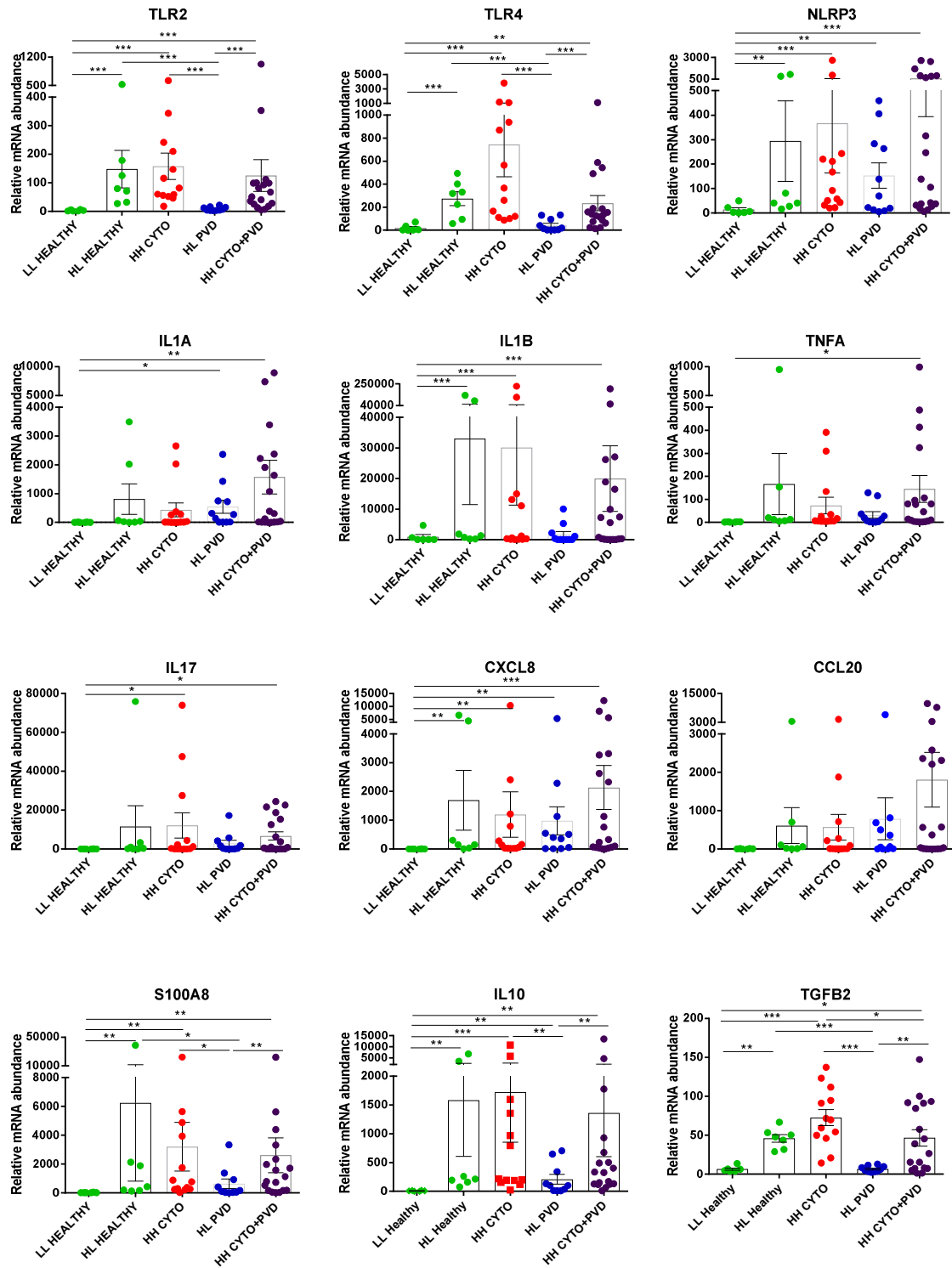


Figure 4-8 Elevated expression of inflammatory genes precedes the onset of uterine disease.

Quantitative PCR (qPCR) was performed on RNA extracted from endometrial cytobrushes (n=56). Gene expression is plotted as proportional to the geometric mean of a reference gene panel composed of *RPS15* and *HSP90A1*. Bars represent mean ± standard error of the mean (SEM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

4.3.8.5 Inflammatory changes were leukocyte independent

Although most cows had high levels of inflammation 7 DPP, variation in the proportion of cells collected from the cytobrush has been identified. The average PMN% was slightly higher in cattle with uterine disease (Figure 4-5). However, gene expression did not show a high correlation with PMN% 7 DPP (Figure 4-9), suggesting that it was the resident endometrial cells (epithelial and stromal) rather than neutrophils driving the differences in gene expression. L-selectin and neutrophil cytosolic factor 2 (NCF2), which is part of the NADPH complex required for oxidative burst function, were increased in cows with CYTO + PVD (Figure 4-7), implying that neutrophil function may differ between groups.

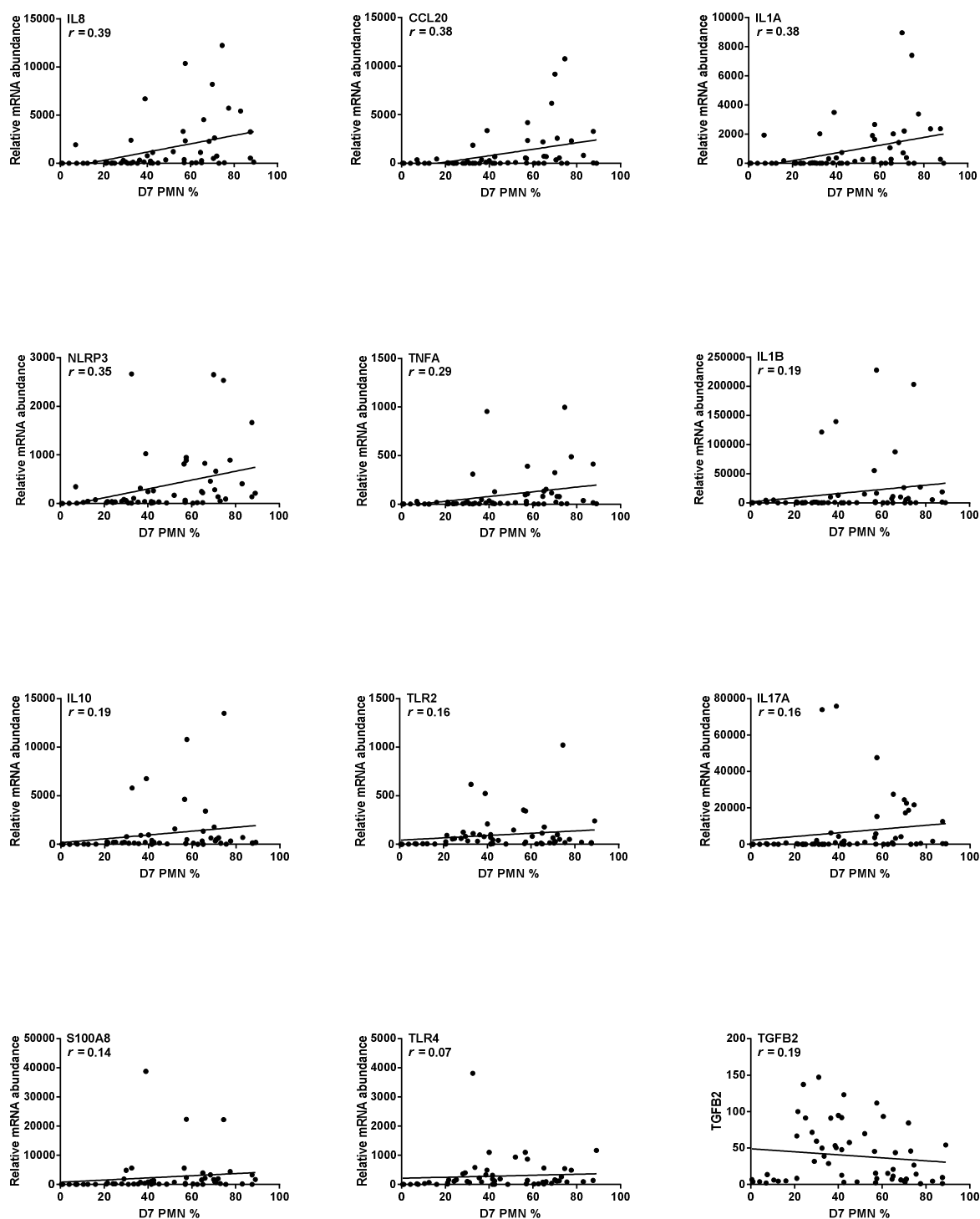


Figure 4-9 Endometrial gene expression is not highly correlated with PMN% 7 days postpartum.

Messenger RNA (mRNA) expression of 12 genes was measured by qPCR (n=56) and plotted against the corresponding endometrial polymorphonuclear leukocyte (PMN) % 7 days postpartum (DPP) as determined by cytological analysis. Correlation r values are indicated for each gene.

4.4 Discussion

Parturition is a metabolically stressful period for the modern dairy cow, and the physiological transition from pregnancy to peak milk production whilst supporting uterine involution requires significant energy reserves (Walsh et al., 2011). While we now know that a certain degree of inflammation is required for normal functioning of the reproductive tract (Chapwanya et al., 2012), the restoration of homeostasis requires the coordination and regulation of multiple interconnected molecular events which serve to regulate those immune process and prevent tissue pathology. Given that context, and the ubiquitous influx of environmental bacteria into the uterus, it is perhaps unsurprising that a significant proportion of cows fail to regulate inflammation and go on to develop clinical and sub-clinical uterine disease. A number of models have been proposed to explain the immune dysregulation that leads to disease (LeBlanc, 2014), but the molecular events underpinning hyper- or hypo-activation of the uterine immune response remains incompletely understood. As a result, appropriate interventions and effective treatments remain elusive.

Building on earlier work by our group (Foley et al., 2015), we hypothesised here that analysis of the early post-partum uterine immune response would identify potentially maladaptive molecular signals that contribute to dysregulated immunity and which leads to the subsequent development of uterine disease. Using RNA-seq, the transcriptomic landscape of cells collected by cytobrush from cows with clinical and sub-clinical uterine disease was compared to healthy counterparts at 7 DPP. Significant heterogeneity was apparent in the magnitude of gene expression differences between individual cows, which is not surprising given natural variation in outbred animal populations combined with the diversity in phenotype on which the original classification was based (clinical or sub-clinical presentation and proportion of PMN present). Clearly the inter-animal variation supports the theory that some cows (in the same environment and under the same management system) adapt to the physiological demands peri-partum without excessive inflammation contributing to disease and understanding these adaptive responses would yield valuable insights into novel targeted approaches. It is likely that some cows with clinical disease for example, are actually clearing infection and the evidence of vaginal discharge may not actually be indicative of active disease. Similarly for cows diagnosed without sub-clinical disease, PMN influx could be present elsewhere in the reproductive tract (e.g. cervix), further complicating accurate disease diagnosis.

All transcriptomic analysis was performed on cells collected by cytobrush at 7 DPP with the ultimate aim of detecting changes in gene expression that precede the development of disease. In order to reduce the impact of the multiple sources of variation, and to tease out subtle

changes associated with each type of disease, cows were classified a number of ways, according to disease classification on day 21 (clinical by VMS or sub-clinical by >18% PMN) and then by degree of cellular infiltration, not only at 21 DPP but also at 7 DPP, again using the 18% threshold. The majority of cows had >18% PMN at 7 DPP, 28% of which subsequently developed cytological endometritis and 55% developed clinical endometritis, alone or in combination. Despite the classifications used, clear differential regulation of immune related genes and pathways were identified. The majority of these differentially expressed genes were significantly upregulated at 7 DPP in cows which subsequently developed uterine disease, including pentraxin 3 which activates the classical pathway of complement activation and facilitates pathogen recognition by immune cells (88 fold increase); Nod-like receptor pathways genes (NLRP3 and MEFV by 30 and 131 fold, respectively); cytokines, such as TNF (49 fold increase) and L-selectin (17 fold increase). An important role for the potent inflammatory cytokine IL-1 was also identified, with a fold increase of 46 and 54 for *IL1A* and *IL1B* respectively in cows that developed uterine disease. This is potentially due to the activation of core differentially regulated pathways of the inflammatory transcription factor NFκB and the NLRP3 inflammasome.

In fact, immune genes accounted for the majority of transcripts being produced by these cells at 7 DPP. Whereas the vast majority (99%) of genes were expressed with a read count of less than 100 reads; 16 genes had read counts between 1,000 and 10,000 TPM. Of these, two isoforms of the acute phase protein *SAA3* were highly expressed and significantly lower in cells from cows which subsequently developed disease. Other immune genes within this class, but which were elevated in the cows which developed disease included *CXCL8*, *S100A8* and *S100A12* and *IL1RN*. The elevated and differential expression of the *IL1RN* gene which encodes the interleukin-1 receptor antagonist is a protein that inhibits the activities of IL1α, thereby highlighting the battle to control inflammation in cows which subsequently develop disease, even at this early time point. Elevation of these genes is very clear at 7 DPP in the cows which develop cytological endometritis. Results suggest that whereas clinical disease, being more advanced, induced a significantly higher proportion of differentially expressed genes but with a higher degree of inter-animal variation. The fact that some of the cows classified with PVD may actually be resolving inflammation is likely to account for a proportion of this variation.

Quantitative PCR was performed on a panel of additional animals to investigate and validate the immune activation profile preceding disease that was identified by the RNA-seq approach. Again, differential expression of a core inflammatory gene subset was apparent with distinct

profiles depending on the degree of PMN cellular infiltrate present at 7 DPP as well as the disease type that was subsequently developed. Clear activation of the Toll-like receptor genes *TLR2* and *TLR4* was detected in most groups, except in the minority of cows that showed no signs of inflammation at 7 DPP. Additionally, cows with PVD only did not have significant TLR gene activation, indicating that they are either resolving disease or disease is associated with another region of the tract – e.g. cervicitis. Similarly, inflammatory cytokine gene expression was elevated in the cows which developed sub-clinical disease with a particular role for *TGFB2*.

Although all cells in the current study for the analysis of gene expression were collected by cytobrush, it is expected that the cell composition will not be homogenous but will in fact reflect a degree of cell damage associated with calving and involution. Whereas 80% of cows exhibited signs of inflammation at 7 DPP (i.e. were classified with >18% PMN), almost 20% were below that threshold, and therefore logically the lack of immune cell infiltrate into the uterus will affect not only gene expression results but also promote the restoration of homeostasis. Therefore, and given the heterogeneity of the gene expression results, we investigated the expression of different cell-type specific markers that may help explain the patterns of gene expression detected by RNA-seq and qPCR. Significant elevation of genes encoding epithelial cell markers in the group of cows which did not display signs of inflammation at 7 DPP is illuminating and suggests that it is the intact epithelial layer that prevents the degree of inflammation detected in the other groups. Conversely, the expression of stromal cell markers was markedly reduced in this class and elevated in all groups that experienced inflammation. Interestingly, it was elevation of genes encoding granulocyte markers that differentiates the healthy group (with inflammation) from the groups that subsequently develop disease. Comparisons between mRNA abundance and the degree of inflammation as measured by % PMN at 7 DPP were moderate for the chemokines IL8 and CCL20 ($r=0.39$ and 0.38 respectively) but overall were weak. Therefore, combined, this data supports the rationale that it is epithelial cell damage and stromal cell exposure to the lumen of the uterus that is primarily responsible for the activation of an inflammatory immune response, and elevation of this response can be detected in cows that subsequently develop uterine disease as early as 7 DPP. In stark contrast to the elevated expression of a number of inflammatory markers in the cows that subsequently develop disease, our analysis identified significant upregulation of genes associated with proliferation and tissue repair in the cows which remain healthy. Promoting expression of these proteins could hold promise to promote tissue regeneration. It has been well established that factors such as dystocia, a large calf or twinning increase the risk of cattle developing PVD (Dubuc et al., 2010b), likely due to excessive damage to the endometrium. Breeding for ease of calving would help to

mitigate this issue. However, in cows that experience a normal calving, a certain degree of tissue damage is to be expected as the placenta becomes detached and therefore it is likely that rapid repair of the endometrium is more important for preventing the development of uterine disease. Work by Wathes and others suggests that delayed endometrial repair occurs in postpartum cattle in severe NEB, which appears to be driven by differential expression of MMPs and IGF binding proteins (IGFBPs) (Wathes et al., 2011). They suggest these molecules may prevent remodelling of the extracellular matrix and the availability of IGF, which promotes healing and proliferation during the wound healing process (Salamonsen, 2003).

So in summary, a number of novel features of the molecular changes that precede both the development of uterine disease as well as the restoration of homeostasis in healthy cows have been identified by the approaches outlined here. Whereas the majority of cows experience some degree of immune activation in the early post-partum period, which is likely associated with improved bacterial clearance, the elevated expression of a core inflammatory gene set signifies a shift from physiological to pathological inflammation. This shift is governed by inflammatory genes in the IL-1 pathway, and is potentially mediated via the NLRP3 inflammasome. This result is reinforced by previous studies performed in different cohorts of cattle that have also identified IL-1 as a key player in the subsequent development of uterine disease (Herath et al., 2009b, Foley et al., 2015). In the minority of cows that do not show PMN influx or inflammatory signs, their cellular transcriptome consists of higher expression of epithelial cell markers, thereby indicating that it is the breach of this cell layer that predisposes the uterus toward exacerbating what already is an inflammatory state. Where an 'inflammatory threshold' is exceeded by the combination of risk factors leading to epithelial cell damage (e.g. dystocia), activation of the stromal cell inflammasome is likely to contribute to immune system dysregulation and tissue pathology.

Uterine inflammation has recently been identified as a key parameter contributing to divergent reproductive outcomes in dairy cows with high or low genetic merit for fertility (Moran et al., 2015). Here, this first analysis of the molecular events preceding the development of disease in dairy cows under a predominantly pasture based system of dairying illustrates the negative impact that uterine disease can have and how minimising damage to the epithelial cell layer of the uterus is the best method for the timely restoration of homeostasis. In agreement with proposed models (LeBlanc, 2014), excessive activation of inflammatory pathways is a key feature preceding disease development and the data from this study will find practical utility in terms of new targets to improve diagnosis of disease. While routine detection of endometrial

epithelial cell transcripts is not currently technically feasible on farm, detection of the inflammatory markers identified here in the mucus of the post-partum cow (Adnane et al., 2017, Adnane et al., 2018) represents a feasible opportunity for the development of more sensitive methods to diagnose disease and improve immune system management of the transition dairy cow.

Chapter 5 Determining the effect of pentraxin 3 on repair of the endometrial epithelial barrier

5.1 Introduction

In chapter 4, we found that the excessive expression of endometrial inflammatory genes during the first week postpartum was associated with subsequent development of uterine disease. Additionally, cows that developed uterine disease experienced down-regulation of genes associated with cell growth and adhesion when compared to healthy cows. Therefore we hypothesised that excessive inflammation prevents repair and remodelling of the endometrium, contributing to prolonged or unresolved inflammation (endometritis). One of the top differentially expressed genes upregulated in cows that developed both clinical and subclinical endometritis was pentraxin 3 (PTX3). PTX3 is part of the pentraxin superfamily, which also includes C-reactive protein (CRP) and serum amyloid P (SAP), all of which function as APPs. PTX3 is an important component of the innate immune response and also contributes to wound healing and tissue repair. In addition, there is growing evidence that it plays a dual role in the regulation of inflammation, either providing protection or amplifying tissue damage (Magrini et al., 2016). Whilst PTX3 has been reported as essential for successful embryo implantation in mice (Tranguch et al., 2007), PTX3 function in the bovine endometrium has not been investigated. Furthermore, whilst recent evidence suggests PTX3 is capable of effecting epithelial barrier integrity (Shindo et al., 2016), its effect on the endometrial epithelial barrier, particularly in the postpartum cow, has yet to be elucidated. Therefore we decided to investigate the effect of PTX3 on endometrial epithelial barrier repair in an *in vitro* model of the bovine endometrial epithelium designed to mirror the early post-partum uterus of the cow.

5.1.1 Postpartum endometrial damage and repair

Unlike the multi-layered epithelium of the vagina, the uterus is lined with a single layer of columnar epithelial cells (Dadarwal et al., 2017). This epithelial barrier is the first line of defence against potentially pathogenic microbes residing in the uterine lumen. Following calving, a key part of uterine involution involves necrosis and sloughing of the caruncles, which is completed by 12-14 DPP (Wagner and Hansel, 1969). It is unclear whether loss of the intercaruncular epithelium also occurs. (Archbald et al., 1972) found that the intercaruncular epithelium remained intact throughout involution, whereas (Gier and Marion, 1968) witnessed regeneration of the intercaruncular areas that began immediately after calving (except in regions of extreme tissue damage) and was completed by 8 DPP. The re-establishment of caruncular epithelium is complete by 19-30 DPP (Archbald et al., 1972). During this time, an incomplete epithelial barrier exposes the underlying stromal cells and increases the cow's

susceptibility to infection and inflammation of the underlying tissue. Therefore rapid repair of the epithelium is important for avoiding the development of uterine disease. The epithelial barrier must also re-establish the junctions that connect individual cells before it regains full functionality. Tight junctions are particularly important as not only do they provide a physical barrier against uterine microbes, but they also separate the apical and basolateral compartments of the cell, allowing it to become polarized. Cell polarity is a key feature of epithelial cells that allows them to direct the secretion of molecules either apically (into the lumen of the uterus) or basolaterally (towards the stroma).

5.1.2 Tight junctions

Neighbouring epithelial cells are joined by three different types of cell junction with unique functions. This includes anchoring junctions which provide mechanical support (adherens junctions, desmosomes and hemidesmosomes), gap junctions which provide channels for cell-cell communication and tight junctions which form strong barriers and regulate the paracellular passage of solutes (O'Connor and Adams, 2010). Tight junctions, adherens junctions and desmosomes together form the apical junctional complex. Dysfunction of tight junction biology is responsible for the “leakiness” of the epithelium and frequently contributes to pathology.

Tight junction biology varies across different tissues. For example, the blood-brain barrier consists of a very “tight” epithelial layer, whilst the gut epithelium is “leaky” to allow greater diffusion of ions and metabolites. This is reflected by differences in the trans-epithelial electrical resistance (TEER), which is around 50-100 $\Omega\cdot\text{cm}^2$ throughout the digestive tract compared to 1,300 $\Omega\cdot\text{cm}^2$ in the brain (Powell, 1981). Polarised bovine endometrial epithelial cells reach a TEER of around 1000-1800 $\Omega\cdot\text{cm}^2$ (MacKintosh et al., 2013, Healy et al., 2015). The TEER reflects the ability of tight junctions to regulate the passage of solutes through the paracellular pathway (Figure 5-1). The electrical current travels through both the transcellular and the paracellular pathway, but since the electrical resistance across the plasma membrane is very high, the paracellular pathway offers the path of least resistance. Measurement of the TEER is commonly performed using a portable voltmeter and chopstick probe (FIGURE 5-1). To accurately assess the TEER of a monolayer, the resistance of a blank well without cells must also be measured, and subtracted from the resistance value of the sample. This value is then multiplied by the area of the insert (e.g. the area of a trans-well insert for a 24-well plate is 0.3 cm^2) to give the TEER value in $\Omega\cdot\text{cm}^2$. The bigger the well is, the more likely the resistance is to vary across the insert. For this reason, Endohm chambers are normally used for 6 and 12 well plates which measure the resistance across the total area covered by cells. Epithelial barrier permeability can also be measured by assessing the movement of molecules that travel

through the paracellular pathway (but not the transcellular) from the apical to the basal side of the trans-well insert. These molecules are often fluorescently (e.g. FITC-dextran) or radioactively (e.g. H^3 -mannitol) labelled.

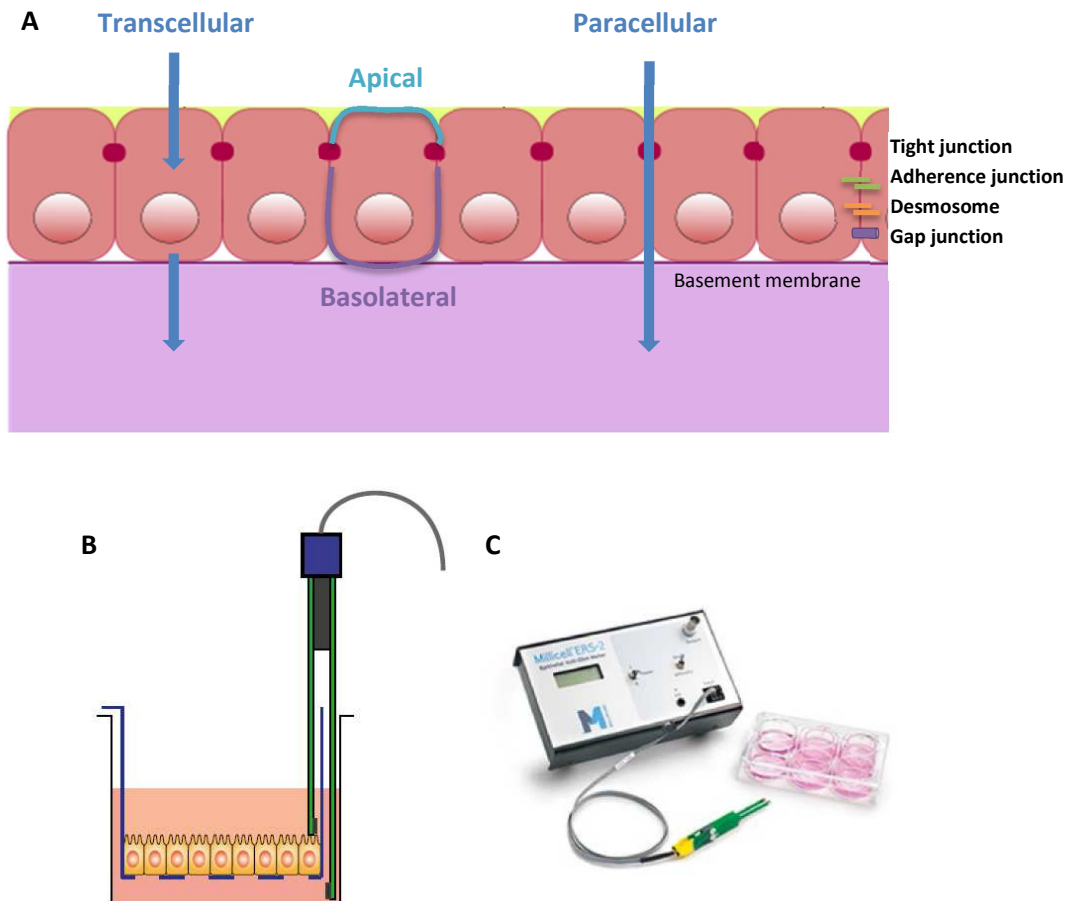


Figure 5-1 Measuring epithelial barrier permeability

A Schematic representation of the different types of cell junctions present between polarised epithelial cells and transport pathways across the monolayer. **B** A drawing of the chopstick electrode used to measure the trans-epithelial electrical resistance (TEER). The probe contains two electrodes, the longer of which is placed in the outer chamber and should just touch the base of the well. The shorter electrode is placed in the insert but should not come into contact with the cell monolayer. Holding the electrode at a 90° angle to the plate, the resistance can then be recorded. **C** An image of the Millicell ERS-2 unit with STX chopstick electrode (Verhoeckx et al., 2015).

Over 40 proteins make up a single tight junction (Gonzalez-Mariscal et al., 2012). These include integral transmembrane proteins that span the gap between cells and peripheral intercellular proteins that link the integral proteins to the actin cytoskeleton (Figure 5-2). Claudins (Latin for “to close”) are the most important of the integral proteins for regulation of paracellular permeability. This has been determined using transgenic mouse models (Turksen and Troy, 2002) and by studying genetic mutations in human diseases (Wilcox et al., 2001). There are 24 members of the claudin family present in mammals, all with slightly different functions. Functional differences arise mainly due to variation in the length of the cytoplasmic tail, which allows post-translational modifications to regulate the tightness and stability of different junctions (Gonzalez-Mariscal et al., 2012). Claudins 1, 2, 3, 4 and 5 are the best studied as they are commonly found in most tissues. Little is known about claudin expression in the uterus. Claudins 1, 5 and 7 have been identified in rat uterine epithelial tight junctions (Mendoza-Rodriguez et al., 2005, Poon et al., 2013). Expression levels of these proteins change during the oestrous cycle and during pregnancy, suggesting that specific tight junction proteins may be important for successful fertilisation. Claudin expression can also change in pathological situations. For example, patients with reflux esophagitis often develop Barrett’s oesophagus, which involves formation of a specialised columnar epithelium that replaces the damaged squamous epithelium. The new columnar epithelium expresses claudin 18 which increases the TEER of the epithelium, thereby protecting against acid damage (Jovov et al., 2007).

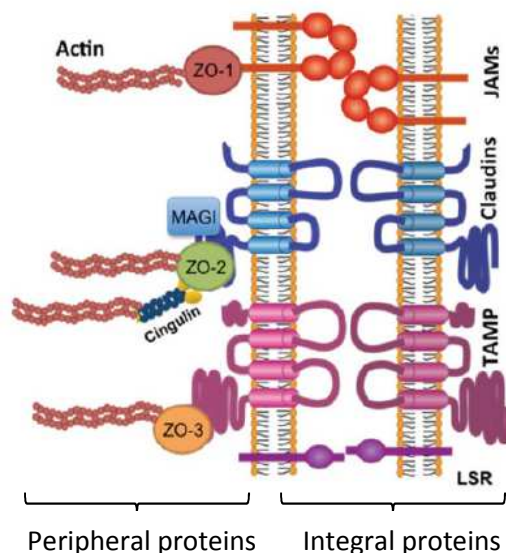


Figure 5-2 Tight junctions are comprised of multiple integral and peripheral proteins
Adapted from (Gonzalez-Mariscal et al., 2012)

Another important integral protein is occludin. Occludin is a tight junction-associated MARVEL protein (TAMP) that plays a key role in adhesion, but also regulates directional re-epithelialisation (Du et al., 2010). Although decreasing occludin expression *in vitro* disrupts epithelial tight junctions, occludin knockout mice are viable and lack of occludin is not obviously detrimental to their epithelial barrier function (Schulzke et al., 2005). This suggests that although occludin is important for cell adhesion, other proteins are somehow able to replace its function. Unlike claudins and occludin, junctional adhesion molecules (JAMs) are not involved in adhesion of cells, but instead act as markers for tight junction assembly (Gonzalez-Mariscal et al., 2012). These proteins are the first to appear during tight junction formation and interact with other proteins such as zona occludens 1 (ZO-1). The correct location of JAMs is therefore essential for achieving epithelial polarity and formation of tight junctions. JAM-A deficient mice have increased gut permeability and susceptibility to colitis (Laukoetter et al., 2007).

The peripheral protein ZO-1 (also known as tight junction protein 1; TJP1) was the first tight junction protein to be discovered. It forms part of the cytoplasmic plaque; a complex network of proteins that connects the integral proteins to the actin cytoskeleton of the cell. ZO-2 and ZO-3 were subsequently discovered and all 3 closely related proteins are directly involved in tight junction formation. ZO proteins interact with claudins and JAMs via their PDZ domains – a common structural domain of 80-90 amino acids that assists in the adhesion of membrane proteins to cytoskeletal proteins. However ZO proteins are not only found in tight junctions, but also adherens junctions and gap junctions. ZO-1 and ZO-2 are essential for the polymerisation of claudins which is required for tight junction strand formation (Umeda et al., 2006). However, only one of the two proteins is required for re-establishment of tight junction strands, suggesting that they have a redundant role in tight junction formation.

Epithelial barrier dysfunction has been associated with the pathogenesis of numerous diseases. For example, a leaky blood brain barrier (BBB) has been linked to stroke, aneurysms, multiple sclerosis, Alzheimer disease and epilepsy. Usually this is due to decreased expression of tight junction proteins. This can lead to the leakage of blood into the central nervous system, resulting in the onset of autoimmune diseases such as multiple sclerosis (Plumb et al., 2002). A weakened epithelial barrier may also be the result of disease. In studies of epilepsy, seizures appear to be both a cause and effect of BBB dysfunction (Marchi et al., 2012). Impaired barrier function is also commonly associated with inflammatory disorders in the gut. Autoimmune conditions such as Crohn's and celiac disease are frequently associated with decreased intestinal TEER and increased expression of pore-forming claudin 2 (Luettig et al., 2015).

Pathogenic organisms can also compromise tight junction function. As the epithelial layer provides a barrier to prevent entry of microorganisms, it is not surprising that pathogens have developed numerous methods of barrier destruction. For example, the hepatitis C virus has developed a mechanism that allows it use occludin and claudins 1, 6 and 9 as receptors to gain entry into host cells (Torres-Flores and Arias, 2015). Other pathogens disrupt the epithelial barrier or destroy tight junctions. The bacterial endotoxin LPS is capable of disassembling tight junctions. LPS commonly causes an increase in epithelial permeability in the gut, suggesting that the microbiome contributes to the pathogenesis of metabolic diseases which are associated with increased serum LPS (Chelakkot et al., 2018).

5.1.3 Pentraxins: PTX3

Pentraxins are an important component of the innate immune response and function as acute phase proteins. These evolutionarily conserved proteins are characterised by a ~200 amino acid domain containing the “pentraxin signature”; His-x-Cys-x-Ser/Thr-Thp-x-Ser; where x represents any amino acid. The classic short pentraxins C-reactive protein (CRP) and serum amyloid P (SAP) are produced by hepatocytes mainly in response to IL-6 (Bottazzi et al., 2016). PTX3 was the first long pentraxin to be discovered (Breviario et al., 1992), and is produced by numerous cell types such as dendritic cells, macrophages, fibroblasts, epithelial cells, endothelial cells, adipocytes, glial cells and granulosa cells in response to inflammatory mediators; in particular IL-1, TNF, bacterial products and DAMPs. PTX3 is also stored in specific granules in neutrophils (although they cannot synthesise the protein themselves), and is released rapidly upon stimulation by invading microbes or at sites of tissue damage (Jaillon et al., 2007). Other members of the long pentraxin subfamily have since been discovered, including guinea pig apelin, neuronal pentraxin 1 (NPTX1) (Schlimgen et al., 1995), neuronal pentraxin 2 (NPTX2, also known as Narp) (Hsu and Perin, 1995), neuronal pentraxin receptor (NPTXR) and PTX4. PTX4 has a different expression pattern to that of the other long pentraxins and does not act as an APP (Martinez de la Torre et al., 2010). In contrast to CRP, PTX3 structure and function is well conserved between humans and mice, which has encouraged the use of genetically modified mice for understanding the role of this protein *in vivo*.

Unlike the pentamer ring structure of CRP and SAP, PTX3 has a quaternary structure, comprising a unique N-terminal domain in addition to the characteristic C-terminal pentraxin domain that is homologous with the short pentraxins. Whilst the human and mouse PTX3 genes are located on chromosome 3, the *Bos taurus* PTX3 gene resides on chromosome 1 and consists of 3 exons which code for the leader region, N- and C- terminal domains of the protein. The

quaternary structure of PTX3 allows it to bind to a wide variety of ligands, meaning that this protein can elicit multiple biological functions.

PTX3 plays a key role in protection against microbial infection. Similar to CRP and SAP, it binds with high affinity to the complement component C1q (Nauta et al., 2003). When PTX3 is surface-bound, this activates the classical complement pathway, whereas the opposite is true for soluble PTX3 which instead inhibits the complement cascade. PTX3 also modulates the complement system by binding to the negative regulator factor H, which activates the alternative pathway and inhibits complement-driven lysis of apoptotic cells (Nauta et al., 2003). Pentraxins also assist in the removal of apoptotic cells from the blood and damaged tissues. PTX3 achieves this by binding to ficolin-1 on dying cells to form a complex that promotes phagocytosis (Ma et al., 2013). PTX3 also acts as an opsonin, binding to specific types of bacteria and fungi that enhance detection and phagocytosis. Indeed, single nucleotide polymorphisms (SNPs) in the PTX3 gene have been associated with impaired clearance of pathogens and greater susceptibility to certain conditions such as tuberculosis (Olesen et al., 2007), aspergillus (Cunha et al., 2014) and urinary tract infections (Jaillon et al., 2014). PTX3 also protects against viral infection. This has been demonstrated by PTX3^{-/-} mice which have increased susceptibility to cytomegalovirus (CMV). In this situation, PTX3 binds to the virus, preventing it from entering host cells and simultaneously activating interferon regulatory factor 3 (IRF3) which leads to production of the anti-viral cytokines IL-12 and interferon gamma (IFN- γ) (Bozza et al., 2006). It is also capable of binding to specific strains of influenza virus, essentially acting as a decoy receptor (Reading et al., 2008).

PTX3 has also been shown to have a role in tissue remodelling, in both physiological and pathological conditions. This function was first discovered in PTX3-deficient female mice with infertility (Scarchilli et al., 2007). Without PTX3, the hyaluronan-rich ECM of the cumulus cells surrounding the oocyte cannot form properly, inhibiting fertilisation. Usually PTX3 produced by cumulus cells interacts with two major components of the ECM: TNF α -induced protein 6 (TNFAIP6) and inter- α -trypsin inhibitor (α 1) which provides the pre-ovulatory oocyte with structural integrity. PTX3 also binds fibroblast growth factors (FGFs) via its N-terminal domain. In endothelial cells the binding of PTX3 to FGF2 prevents angiogenesis (Rusnati 2004) and inhibits smooth muscle proliferation (Camoszi et al., 2005), suggesting that it regulates the process of neovascularisation and repair of damaged blood vessels. More recently, PTX3 was found to play an important role in tissue repair in multiple models of tissue damage in mice (skin wounding, chemically-induced lung and liver damage and arterial thrombosis) (Doni et al.,

2015). PTX3 assisted the process of tissue repair by interacting with fibrin and plasminogen to promote fibrinolysis. Local acidic pH, which usually occurs at sites of tissue damage, is required for this interaction and ensures that it only occurs in the damaged tissue and not in circulating blood. PTX3 is also important for maintaining integrity of the BBB. Downregulation of PTX3 in a mouse model of white matter stroke exacerbated leakage of the BBB, leading to greater damage of white matter tissue (Shindo et al. 2017).

PTX3 also modulates inflammation. It is capable of binding P-selectin, inhibiting leukocyte rolling and recruitment. This allows PTX3 to exert a protective function in LPS-induced lung injury (Han et al., 2011) whilst it also decreases mortality associated with endotoxic shock (Dias et al., 2001). PTX3 deficiency has been linked to detrimental effects in mouse models such as increased tissue damage in cardiac ischemia and reperfusion injury (Salio et al., 2008). However, PTX3 has recently been found to play a dual role in inflammation and is also capable of promoting immunopathology in certain circumstances. Over-expression of PTX3 in mice with *k.pneumoniae* infection resulted in increased lethality due to a decreased influx of neutrophils into the lungs and therefore suppressed bacterial killing when a high bacterial load was present (Soares et al., 2006). However when mice were inoculated with a low bacterial load, over-expressed PTX3 had a protective effect, resulting in increased pro-inflammatory cytokine production and clearance of bacteria by phagocytosis (Soares et al., 2006). Furthermore, administration of recombinant PTX3 enhances tissue damage in mice that have experienced intestinal ischemia and reperfusion (Souza et al., 2003, Souza et al., 2009). This is reflected by increased neutrophil influx, denuded intestinal villi, disintegration of the lamina propria, increased ulceration and the presence of haemorrhage foci. PTX3 also worsens post-ischemic renal injury (Chen et al., 2012). In addition it also enhances tissue damage following mechanical lung damage (Wu et al., 2009, Real et al., 2012).

Due to the detrimental effects of over-expressed PTX3 in certain conditions (e.g. acute tissue injury, increased bacterial load) and its recently characterised role in tissue remodelling, we decided to investigate the actions of this protein on repair of the postpartum bovine endometrium using a 3D *in vitro* model designed to reflect the early postpartum uterus. Whilst all cattle with uterine inflammation at 7 DPP expressed endometrial PTX3, it was significantly elevated in those that developed disease. Therefore we aimed to investigate the effect of both a “healthy” and “pathological” concentration of PTX3 in our model, to determine whether over-expression of this protein has a detrimental effect on endometrial epithelial repair.

5.2 Hypothesis and specific aims

We hypothesise that pathological levels of PTX3 prevents the re-establishment of epithelial barrier integrity in polarised bovine endometrial epithelial cells.

Aims:

1. Establish a model of epithelial barrier disruption in primary polarised bovine endometrial epithelial cells
2. Determine healthy and pathological concentrations of PTX3 present in the bovine uterus 7 DPP
3. Assess the effect of PTX3 on endometrial epithelial barrier repair

5.3 Results

5.3.1 Reduced expression of tight junction proteins in the bovine uterus 7 days postpartum precedes the development of uterine disease

Transcriptomic analysis of the bovine endometrium in Chapter 4 revealed differences in the expression of certain tight junction proteins in cows that subsequently develop uterine disease compared to cows that remain healthy (Table 5-1). All differentially expressed proteins were upregulated in healthy cows. Most of the fold changes associated with protein expression were quite small, except for claudin 10, which was increased 20-fold in healthy cows. As the healthy vs. clinical endometritis comparison includes all healthy cows, it is likely that this difference is driven predominantly by those with “low” inflammation (<18% PMN) at 7 days postpartum. Cows with low inflammation at day 7 had greater expression of epithelial cell markers and lower expression of stromal cell markers, suggesting that they had a more intact epithelial barrier and therefore it is not surprising that they also displayed greater expression of tight junction proteins. Some of these proteins were also differentially expressed between the two healthy groups (*MAGI3* and *MARVELD2*). For these reasons, the most interesting protein here is *TJP1* (*ZO1*) as it is increased in cows with “high” inflammation that subsequently resolve, compared to cows with high inflammation that go on to develop uterine disease.

Table 5-1 Tight junction proteins expressed in the bovine uterus 7 days postpartum

Gene symbol	Gene name	Differentially expressed?	Fold change
<i>CLDN1</i>	Claudin 1	No	-
<i>CLDN4</i>	Claudin 4	No	-
<i>CLDN7</i>	Claudin 7	↑ in healthy vs. clinical endometritis	2.5
<i>CLDN8</i>	Claudin 8	No	-
<i>CLDN10</i>	Claudin 10	↑ in healthy vs. clinical endometritis	20.4
<i>CLDN12</i>	Claudin 12	No	-
<i>OCLN</i>	Occludin	↑ in healthy vs. clinical endometritis	3.1
<i>JAM2</i>	Junctional Adhesion Molecule 2	No	-
<i>JAM3</i>	Junctional Adhesion Molecule 3	No	-
<i>MAGI1</i>	Membrane Associated Guanylate Kinase, WW And PDZ Domain Containing 1	No	-
<i>MAGI3</i>	Membrane Associated Guanylate Kinase, WW And PDZ Domain Containing 1	↑ in healthy vs. clinical endometritis ↑ in LL healthy vs. HL healthy	1.5 1.9
<i>MARVELD2</i>	MARVEL domain containing 2 (<i>Tricellulin</i>)	↑ in healthy vs. clinical endometritis ↑ in LL healthy vs. HL healthy	2.9 3.4
<i>TJP1(ZO-1)</i>	Tight junction protein 1	↑ in healthy vs. clinical endometritis ↑ in HL healthy vs. HH CYTO + PVD	1.8 2.3
<i>TJP2 (ZO-2)</i>	Tight junction protein 2	No	-
<i>TJP3 (ZO-3)</i>	Tight junction protein 3	No	-
<i>PATJ</i>	Crumbs Cell Polarity Complex Component	↑ in healthy vs. uterine disease ↑ in healthy vs. clinical endometritis	2.5 4.4

5.3.2 LPS does not disrupt the intact endometrial epithelial barrier

The establishment and optimisation of a protocol for the isolation, culture and characterisation of bovine endometrial epithelial cells is covered in detail elsewhere (Kelly, 2018). In order to measure the effects of PTX3 on epithelial barrier repair, a model of epithelial barrier disruption needed to be established. Following calving, changes in the uterine microbiome are common due to an influx of bacteria which ascend through the reproductive tract from the environment, as well as metabolic and immunological changes in the local environment which allow certain species to prosper. One of the major pathogens isolated from the uterus during the first few weeks postpartum is *E.coli* (Doherman et al., 2000a, Williams et al., 2005). LPS, the endotoxin found in the cell wall of gram negative bacteria such as *E.coli* is present in large quantities in the bovine uterus early postpartum and plays an important role in disease development through activation of the TLR4 pathway. Up to 98 µg/ml has been detected in uterine washings of heifers 14 DPP (Williams et al., 2007) whilst 2.24×10^4 EU/ml has been reported in lochia of cows with retained placenta (Doherman et al., 2000b) and 7.25×10^4 EU/ml was present in the uterine fluid of endometritic cows (Mateus et al., 2003). LPS has been shown to induce an inflammatory response in polarised bovine endometrial epithelial cells (MacKintosh et al., 2013), however the effect on epithelial barrier integrity has not been investigated. In human intestinal cells, LPS increases epithelial barrier permeability (Guo et al., 2013). This is also true in airway cells (Serikov et al., 2004) and bovine mammary cells (Wellnitz et al., 2016). The concentrations of LPS used in this model ranged from 2 µg/ml (previously validated in endometrial cells (Kelly, 2018)) up to 500 µg/ml. A maximum concentration of 500 µg/ml was used as this was the concentration required to disrupt the epithelial barrier in bovine mammary epithelial cells, whilst 200 µg/ml LPS had no effect (Wellnitz et al., 2016).

Apical stimulation with LPS had no effect on the epithelial barrier at any concentration (Figure 5-3 A). The same batch of LPS had previously been used to successfully invoke an inflammatory response in polarised bovine endometrial epithelial cells in the same laboratory (Paul Kelly; personal communication), confirming that the LPS was biologically active and that the cells expressed the TLR4 receptor. The TEER decreased in all wells 3 hours after LPS treatment, which is not unusual following a media change. The TEER had recovered by 6 hours, and appeared to stabilise over the remaining 18 hours. A decrease in TEER following treatment with 10% DMSO confirmed that TEER measurements were accurate. DMSO was used as a positive control as it is known to be toxic to cells at a concentration of >1% and would therefore induce significant cell damage which decreases the TEER. A previous study in gut epithelial cells found that apical stimulation with LPS had no effect on epithelial barrier integrity even at high concentrations;

however basolateral stimulation with LPS began to disrupt the barrier 4 days later (Guo et al., 2013). The reasoning behind this observation was that the apical membrane surface is in constant contact with high concentrations of LPS and bacteria in the gut lumen and is thus very tolerant to high levels of LPS. This could also be true in the endometrium as the presence of a permanent uterine microbiome has now been well established. Furthermore, disruption of the epithelial barrier after calving would expose the stroma and therefore basolateral side of the epithelium to bacterial endotoxins. Therefore we repeated the experiment, treating basolaterally for up to 120 hours. Again we saw no disruption of the epithelial barrier following LPS treatment. Unexpectedly the TEER was greatest following treatment with the highest concentration of LPS, when treated both apically and basolaterally. To confirm the TEER readings, a FITC-dextran permeability assay was performed at 120 hours (

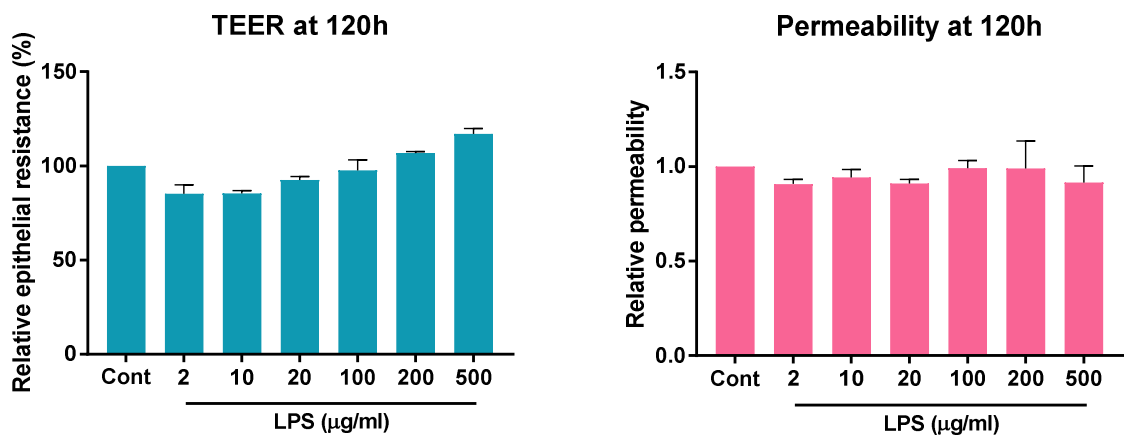


Figure 5-4). This too indicated no difference in epithelial barrier integrity.

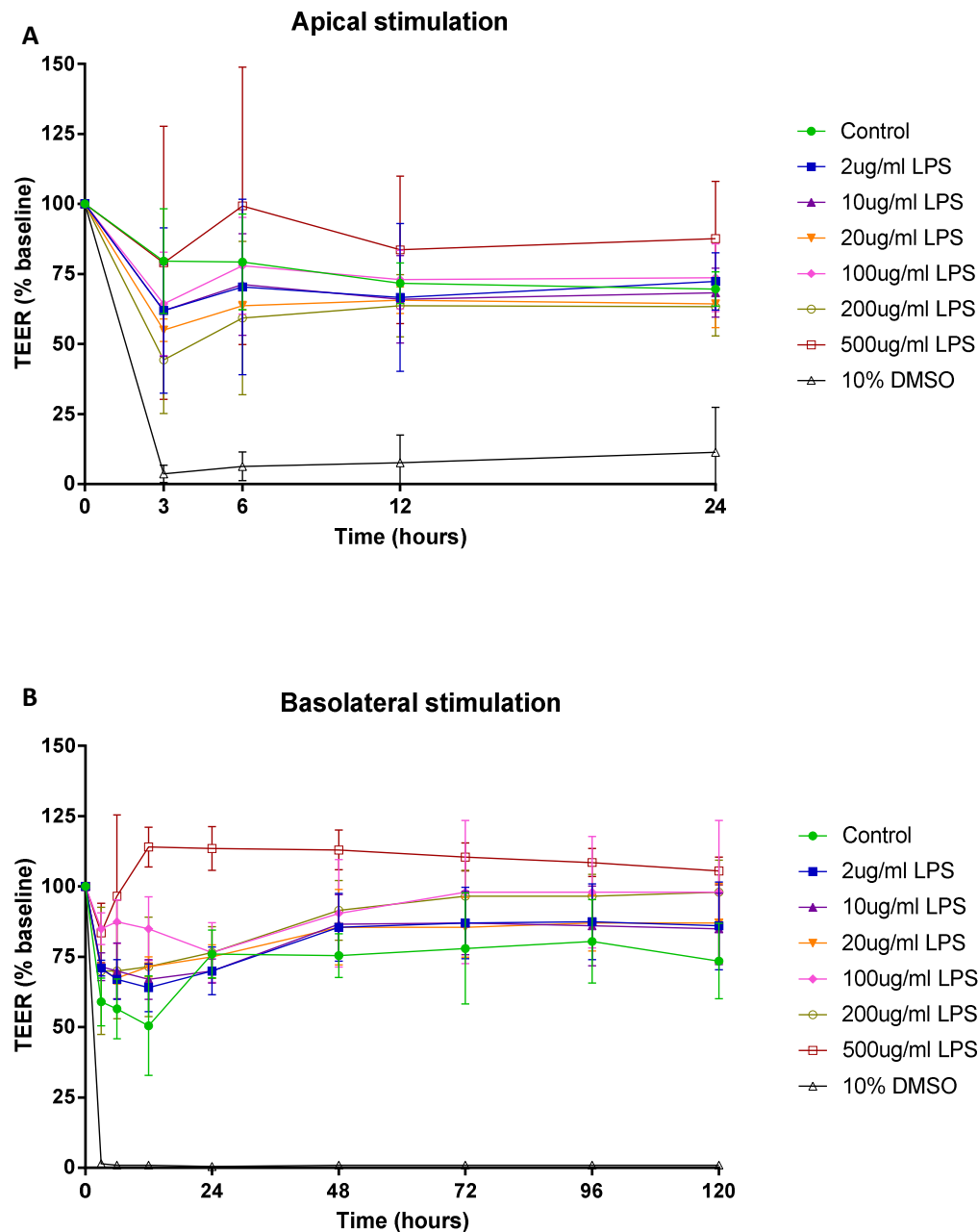


Figure 5-3 Treatment of polarised primary endometrial epithelial cells with LPS did not disrupt the epithelial barrier

Trans-epithelial electrical resistance (TEER) was measured in primary endometrial epithelial cells (n=3) following apical (A) or basolateral (B) stimulation of cells with increasing concentrations of LPS. Dimethyl sulfoxide (DMSO) was included as a positive control. TEER values are presented as a percentage of the baseline measurement for each treatment (time 0h).

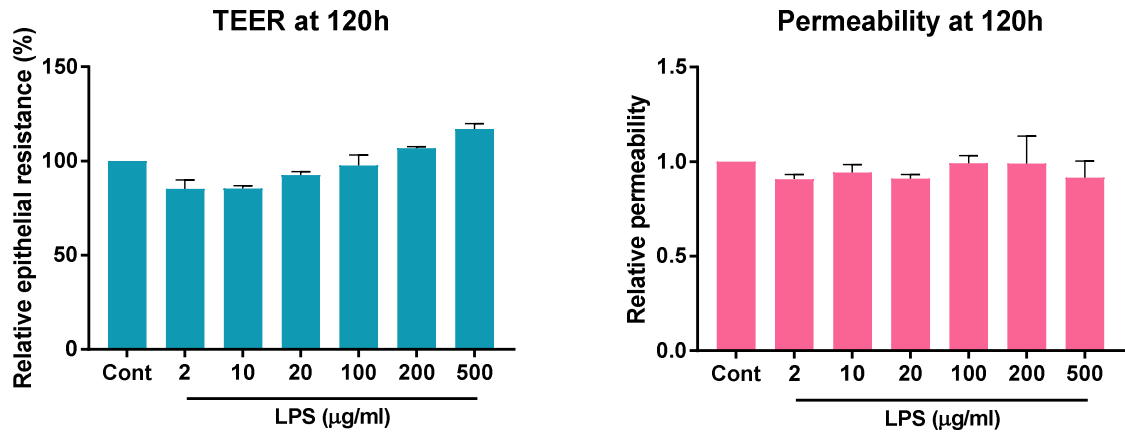


Figure 5-4 Treatment of polarised primary endometrial epithelial cells with LPS for 120 hours did not affect epithelial barrier permeability

Permeability was measured using a FITC-dextran assay. FITC-dextran was added to the apical side of each well and incubated for 3 hours at 37 °C. Fluorescence of the media in the basolateral compartment was measured to determine barrier permeability. Values are plotted as relative to control.

5.3.3 Disruption of the endometrial epithelial barrier using a calcium switch assay

Whilst LPS had no effect on the intact, polarised epithelial barrier, it is unlikely that this reflects the early postpartum uterus, which is characterised by significant loss of the epithelial layer at calving and following sloughing of caruncular tissue. Therefore, a different approach was taken to produce a more physiologically relevant model of barrier disruption. The calcium switch assay has proven effective at disrupting the epithelial barrier in T84 cells, a human colon carcinoma cell line (Glover et al., 2013). However it has not been performed in endometrial epithelial cells before. By depleting calcium using a chelator like EDTA, the junctions between cells are disrupted, but can be re-established over a couple of hours following the addition of calcium-supplemented HBSS. Here, the TEER results confirmed that disruption and subsequent re-establishment of the epithelial barrier occurred (Figure 5-5) and therefore it was concluded that this is a suitable model for testing the effects of PTX3 on endometrial epithelial barrier repair.

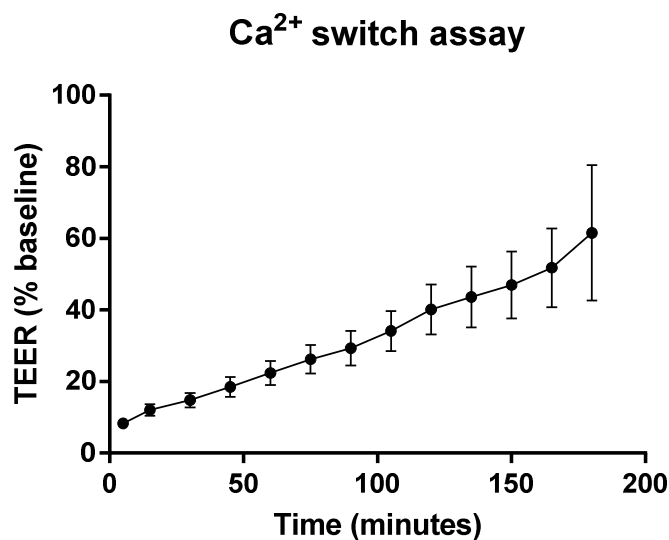


Figure 5-5 Calcium switch assay disrupted the epithelial barrier in primary endometrial epithelial cells

A calcium switch assay was performed on polarised primary endometrial epithelial cells (n=3), which briefly involved depleting calcium from tight junctions by subjecting the cells to the chelator EDTA (2 nM) for 5 minutes before the addition of calcium using supplemented HBSS to observe barrier repair.

5.3.4 Endometrial PTX3 concentration

Before assessing the effect of PTX3 on epithelial barrier repair, it was important to confirm the transcriptomic expression of PTX3 in the endometrium at protein level, and secondly assess the concentration of PTX3 in healthy and endometritic cows so that physiologically relevant concentrations of PTX3 could be used to treat cells *in vitro*. PTX3 mRNA was one of the most significantly differentially expressed genes between healthy and endometritic cows 7 DPP. However, RNA and protein do not always correlate due to the occurrence of post-translational modifications such as methylation and glycosylation. Therefore protein was extracted from endometrial cytobrushes collected 7 DPP and a bovine PTX3 ELISA was performed to quantify the concentration present. An average of 9.7 ng/mg of total protein (TP) (range 0.32-25.02 ng/mg TP) was recorded for healthy cows, whilst an average of 157.8 ng/mg TP (range 0.06-763.73 ng/mg TP) was observed in cattle that developed endometritis. Similarly to the RNAseq results, there was a lot of inter-animal variation, however overall the ELISA confirmed that PTX3 levels are higher in cows that develop endometritis (Figure 5-6 and Table 5-2).

Table 5-2 Endometrial PTX3 concentrations were higher 7 DPP in cows that subsequently developed endometritis

A bovine PTX3 ELISA was performed on protein extracted from endometrial cytobrushes collected 7 days postpartum (DPP). PTX3 mRNA results (from RNAseq data) are also shown for the same cows where available (lack of mRNA results indicates cows that were not sequenced).

	Cow ID	PTX3 mRNA (CPM)	PTX3 protein (ng/mg TP)
Healthy	1066	37.16	0.32
	2053	32.00	16.20
	1831	8.05	3.05
	2392	5.42	25.02
	1761	-	5.23
Endometritis	2064	173.87	0.06
	2184	458.97	1.75
	1378	2868.17	171.58
	2672	3343.80	763.73
	1944	65.25	151.83
	1498	-	4.72
	1984	-	16.48
	2863	-	151.93

CPM: counts per million. TP: total protein.

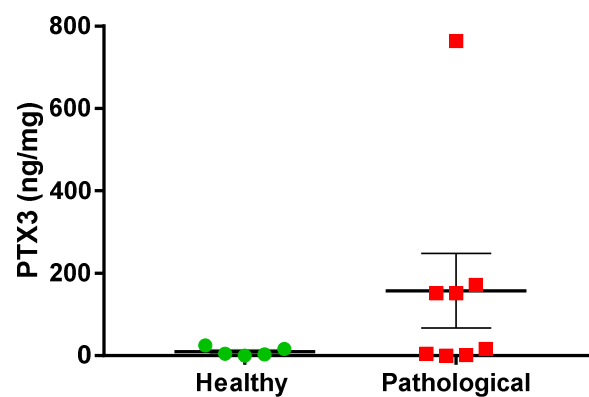


Figure 5-6 Endometrial PTX3 concentrations were higher 7 DPP in some cows that subsequently developed endometritis.

5.3.5 PTX3 protein sequence is well conserved between cows and humans

Although recombinant bovine PTX3 is commercially available, time constraints meant that recombinant human PTX3 was a more viable option for this experiment. In order to make this decision, bovine and human PTX3 protein sequences were first aligned using clustal analysis (Figure 5-7) to investigate homology. There was 82% identity between the two amino acid sequences which confirms that this protein is well conserved between the two species.

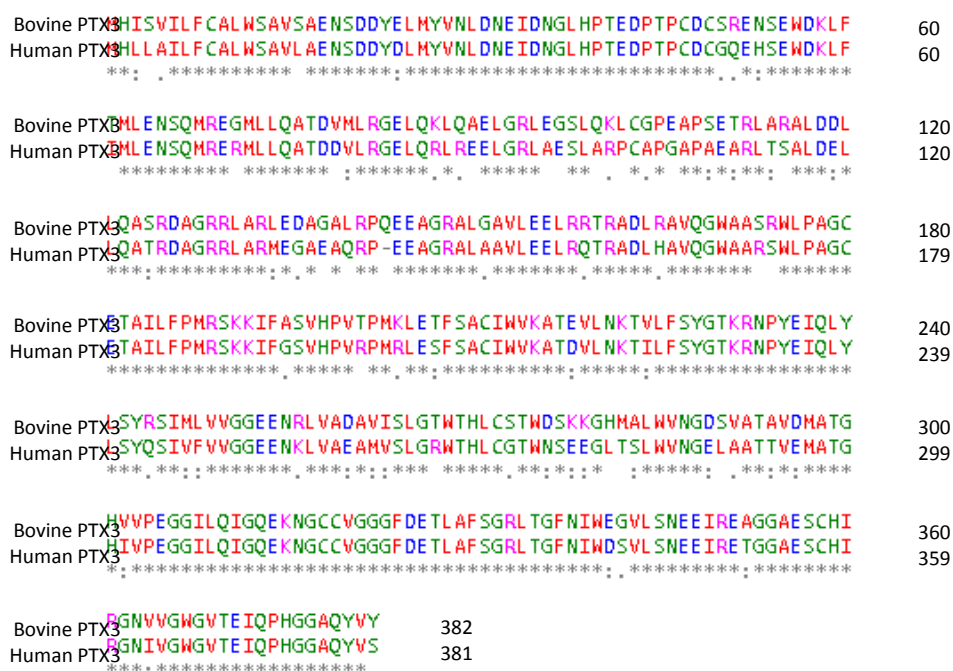


Figure 5-7 Alignment of bovine and human PTX3 protein sequences determined a sequence similarity of 82%

Amino acid sequences for the bovine and human PTX3 proteins were downloaded from GenBank. Sequences were aligned using MUSCLE v. 3.8 (<https://www.ebi.ac.uk/Tools/msa/muscle/>). Amino acid residues are coloured according to their physiochemical properties. Asterisks (*) indicate fully conserved residues; colons (:) indicate strongly biochemically-similar amino acids and periods (.) indicate weakly biochemically-similar amino acids. Numbers indicate position in each individual nucleotide sequence.

5.3.6 Effect of PTX3 on endometrial epithelial barrier repair

In order to determine the effect of PTX3 on endometrial epithelial barrier repair, a calcium switch assay was performed on polarised endometrial epithelial cells (n=4) and a healthy (5ng/ml) or pathological (500ng/ml) dose of PTX3 was added either apically or basolaterally. A high dose of PTX3 was administered for the pathological dose (but still within the range detected by the ELISA) with the aim of seeing a more dramatic effect. In this experiment, the calcium switch assay only worked well in one animal, as the TEER of the control cells of the other 3 animals failed to recover. As the calcium switch assay had previously been validated in the same cell type of 3 separate animals, using the same protocol and under the same conditions, it is likely that the cells used in this experiment were becoming senescent, as they had been cultured for longer which is probably why they did not respond well. The cells in which the calcium switch assay worked showed slower repair of the epithelial barrier following treatment with PTX3 (Figure 5-8). Unexpectedly the healthy dose of PTX3 appeared to have a more detrimental effect on barrier repair than the pathological dose. There was no significant difference observed between apical and basolateral stimulation. Bovine serum albumin (BSA) was included as a negative control to account for the addition of a peptide and because it has previously been shown to have no effect on epithelial barrier integrity in intestinal epithelial carcinoma cell lines (Hurley et al., 2016). However, addition of 500ng/ml BSA in this model prevented epithelial barrier repair and had a greater effect than PTX3. The reasons for this are unclear but could be due to contamination of the BSA aliquot as it was not sterile filtered. A more appropriate negative control for future studies would be a scrambled version of the rPTX3 as this would be of the same molecular weight and polarity.

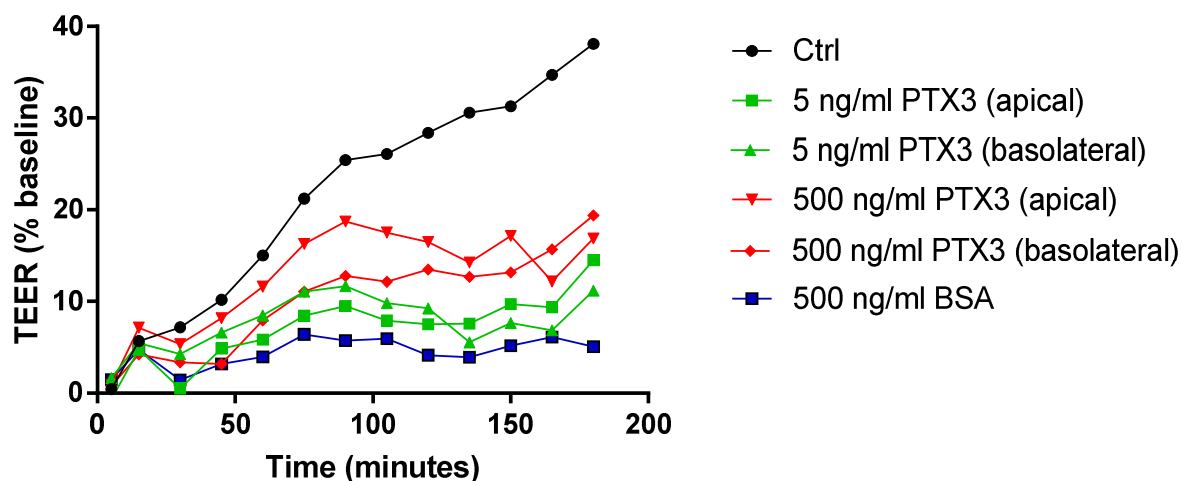


Figure 5-8 PTX3 prevented re-establishment of the endometrial epithelial barrier

A calcium switch assay was performed to disrupt and subsequently re-establish the epithelial barrier in primary endometrial epithelial cells ($n=1$). Cells were treated with a healthy (5ng/ml) or pathological (500ng/ml) dose of recombinant human pentraxin 3 (PTX3), or bovine serum albumin (BSA). Each treatment was performed in duplicate. The trans-epithelial electrical resistance (TEER) was measured every 15 minutes for 3 hours and is presented as a percentage of the baseline value ($T=0h$) for each treatment.

5.4 Discussion

Disruption of the endometrial epithelial barrier is common during calving and the early postpartum period. The process of uterine involution involves regeneration of the intercaruncular epithelium, followed by growth of cells over the caruncles (Sheldon et al., 2008). Risk factors such as dystocia, twinning, parity and calving assistance can result in greater damage of the endometrium which in turn will increase susceptibility to uterine disease (Dubuc et al., 2010b). Interestingly, damage-associated risk factors tend to increase susceptibility to PVD rather than CYTO (Dubuc et al., 2010b). Risk factors specific for CYTO include low body condition score and hyperketonemia. Our RNAseq results indicated that most cattle (80%) had a significant degree of inflammation 7 DPP (>18% PMN) which was associated with a more disrupted epithelium (decreased epithelial cell markers and increased stromal cell markers) in comparison to cows with little-to-no inflammation 7 DPP. When cows with high levels of inflammation were assessed, those that went on to develop uterine disease displayed increased expression of inflammatory genes and decreased expression of genes involved in cell growth and adhesion. This led us to hypothesise that excessive inflammation 7 DPP delays endometrial repair, resulting in prolonged inflammation that leads to endometritis.

An important aspect of re-epithelisation involves the formation of tight junctions between cells. Tight junctions differentiate the apical and basolateral compartments of the cell, allowing the directional secretion of molecules. They are also important for maintaining a strong barrier between cells. Tight junction disruption is evident in many diseases associated with a dysfunctional epithelial barrier and is often due to the deficiency of particular proteins. We identified a number of tight junction proteins that were differentially expressed between cows that developed uterine disease and those that remained healthy. All of these genes were upregulated in healthy cows. The biggest difference was seen in claudin 10 expression (20-fold increase). However, it is likely that these differences were driven predominantly by healthy cows with “low” inflammation (<18% PMN) 7 DPP, with a more intact epithelial barrier (increased expression of epithelial-specific genes). Interestingly, *TJP1* (*ZO1*) was elevated in cows with “high” inflammation that subsequently resolve, compared to cows with high inflammation that go on to develop uterine disease.

Pathogens have developed unique mechanisms that enable them to penetrate the epithelial barrier by hijacking or disrupting tight junction formations. The bacterial endotoxin LPS is capable of disassembling tight junctions and has been reported to cause disruption to the BBB, gut epithelium and mammary mucosa. LPS is also abundant in the postpartum uterus, particularly in cows that develop uterine disease. Using our *in vitro* model, LPS did not affect

barrier integrity. The reasons for this are unclear, but may be related to the “tightness” of the endometrial epithelium when compared to the relatively leaky epithelia of the gut and mammary gland. In addition, although primary cells were used in this model which are more physiologically relevant than cell lines, the presence of mucus-producing glandular epithelial cells has not been confirmed. Primary murine endometrial epithelial cells require the presence of two specific growth factors (EGF and insulin) for the formation of polarised glandular structures (Eritja et al., 2010). The presence of goblet cells, the mucus-producing cells of the intestine, renders intestinal cell lines more permeable as they do not form tight junctions (Srinivasan et al., 2015). Therefore this *in vitro* endometrial epithelial model may possess a tighter and consequently more LPS-resistant epithelial barrier than its *in vivo* counterpart. Furthermore, if loss of at least some of the endometrial epithelium is common during uterine involution, then an intact barrier may not be the best representation of the postpartum uterus. The calcium switch assay, which involved chelating calcium to disrupt the tight junctions and then adding calcium back in to observe barrier reformation worked well in this cell type and better reflects the process of epithelial disruption and subsequent repair in the postpartum uterus.

Once our model of endometrial epithelial disruption had been established, the effects of PTX3 on barrier repair were assessed. PTX3 was the second most significantly DE gene 7 DPP between cows that remained healthy (n=10) and those that developed uterine disease (n=20), with a fold change of 88. It was also upregulated in HH CYTO cows (8th most significantly DE gene; fold change 53) and in HH CYTO + PVD cows (9th most significantly DE gene; fold change 193) when compared to HL healthy. This pattern of endometrial PTX3 overexpression in cows that developed disease was also observed at protein level. Protein results did not always match the mRNA expression, suggesting that post-translational modifications may be involved. However it is also worth noting that PTX3 protein (but not RNA) is also present in neutrophil granules which would also have been present in cytobrush samples. PTX3 performs key roles in defence against pathogens, tissue repair and regulation of inflammatory responses. Therefore PTX3 deficiency often results in increased susceptibility to infection and increased tissue damage. Of particular relevance to this study, PTX3 deficiency was found to compromise integrity of the BBB in hypertensive rats subjected to focal cerebral ischemia, which was associated with decreased expression of claudin 5 and ZO-1 (Shindo et al., 2016). More recently, it has been realised that PTX3 is also capable of promoting immunopathology under certain circumstances. Most of these studies have found that excessive expression of PTX3 worsens tissue injury (Souza et al., 2009, Chen et al., 2012, Wu et al., 2009).

Here, we found that PTX3 had a detrimental effect on epithelial barrier repair. This was observed following both apical and basolateral stimulation. Unexpectedly, a “healthy” concentration of PTX3 (5ng/ml) had a greater effect than the “pathological” dose (500ng/ml). It is interesting to note that a similar result was observed following treatment of bovine endometrial epithelial cells with IL-1 β (Paul Kelly; personal communication). Whilst both apical and basolateral treatment of cells with IL-1 β resulted in impaired barrier repair, higher concentrations of IL-1 β had a more detrimental effect on barrier repair when stimulated apically, whereas the opposite occurred when cells were stimulated basolaterally. Furthermore, treatment of intestinal epithelial cells with IL-1 β also resulted in a dose-dependent outcome, where higher concentrations of IL-1 β had a milder effect on the TEER (Al-Sadi and Ma, 2007). The fact that IL-1 β also impairs re-establishment of the endometrial barrier is very interesting as IL-1 is a key driver of PTX3 expression, and expression of *IL1A*, *IL1B* and the inflammasome component *NLRP3* were also greatly upregulated in cows that developed uterine disease in our study. PTX3 overexpression is also associated with increased IL-1 production in mechanical damage of the lung (Real et al., 2012). Additionally, the importance of the relationship between IL-1 and PTX3 has been reported in a model of cigarette smoke-induced chronic obstructive pulmonary disease (COPD), where PTX3 upregulation was dependent on the IL-1 pathway (Pauwels et al., 2010). The mechanisms by which PTX3 and IL-1 delay repair of the endometrial epithelial barrier remain to be elucidated. Future work involving a larger number of biological repeats and a more appropriate negative control (such as denatured or scrambled rPTX3) in addition to the assessment of relevant tight junction proteins (such as ZO-1) would aid the validation of these findings. Moreover, it would be useful to assess the effects of a PTX3 neutralising antibody on epithelial barrier repair to see if it promotes reestablishment of the barrier.

Expression of PTX3 in other studies of bovine uterine disease has yielded mixed results. Recently, PTX3 was detected in plasma exosomes of cattle with *T.pyogenes*-induced uterine infection (but not healthy cattle) (Almughlliq et al., 2018), supporting our observations. However, (Foley et al., 2015) only observed an increase in PTX3 in healthy cattle 7 DPP compared to 21 DPP, but no difference between healthy and endometritic cows. In addition, no difference in PTX3 expression was detected between cows with and without metritis, but elevated PTX3 was observed in healthy heifers when compared to healthy multiparous cows (Genis et al., 2018). However the same study also reported no differences in *IL6*, *TNFA*, *IL1B* and *CXCL8* between healthy and metritic cows suggesting that metritis was not related to excessive inflammation in this study. Therefore it may be that the role of PTX3 in immunopathology

depends on the intensity of the inflammatory response, in addition to other factors such as the uterine microbiome and degree of uterine damage.

In summary, we found that excessive inflammation and downregulation of cell adhesion and proliferation genes in the early postpartum period are associated with the development of uterine disease. One of top differentially expressed genes, PTX3, was increased at both gene and protein level in the uterus of cows that developed disease. This protein was found to prevent repair of the endometrial epithelial barrier, which may be influenced by excessive endometrial damage or a greater intra-uterine bacterial load. Deciphering the mechanisms underlying the effects of PTX3 on epithelial barrier repair could assist in new treatment or prevention strategies for tackling endometritis.

Chapter 6 General Discussion

The peri-partum period is one of immense stress for the modern day dairy cow. In addition to the metabolic demands of pregnancy, parturition and milk production, she must undergo uterine involution and switch from an immuno-suppressive state during pregnancy to produce an active, regulated inflammatory response in the early postpartum period. Therefore it is no surprise that many cattle experience a range of transition-period diseases during this time. Uterine disease is one of the most prevalent transition period disorders and has a well-documented negative effect on fertility (Fourichon et al., 2000). However, most of these studies have been based on high-yielding Holstein cows housed in TMR systems. Good fertility is particularly important in seasonal pasture-based dairy systems, which predominate in Ireland, as a voluntary waiting period <85 days is required in order to achieve the goal of one cow per calf per year, which is essential for matching peak grass growth in spring to the high energy demands of the lactating postpartum cow. Currently, data on the incidence of uterine disease in cows maintained on grazing systems is limited, but has ranged from 9 to 73% (Olmos et al., 2009, Crowe and Williams, 2015, Savc et al., 2016, Rojas Canadas et al., 2018). Although these studies are not representative of the national picture in Ireland, it is clear that uterine disease is not only highly prevalent on specific farms, but that the occurrence of disease is associated with reduced fertility and increased veterinary and animal health interventions.

Current diagnosis of uterine disease is carried out 21 DPP or later. Detection of clinical endometritis (PVD) at this time point is relatively straightforward as it depends on visual assessment of cervico-vaginal mucus which is easily obtainable, and often visible as exudate from the infected cow. However, with the predicted expansion of the Irish dairy herd following the removal of the milk quotas across the European Union in 2015, farmers will have a greater number of cattle to manage, likely leading to lower observation of clinical disease symptoms in individual cows. In addition, subclinical endometritis (CYTO) also has a negative impact on reproductive performance, but cannot be diagnosed clinically meaning that it often goes unnoticed until the cow fails to conceive following insemination. Diagnosis of CYTO involves collection of uterine cells from a biopsy, cytobrush or lavage which is not suitable for routine, large-scale on-farm diagnosis. Therefore the need for a low-cost, non-invasive diagnostic for the detection of uterine disease (particularly subclinical) is critical. Furthermore, prevention is always more economical than treatment of the established disease, and so early diagnosis soon after calving or even prepartum would allow early intervention and a more rapid return to cyclicity.

The complexity of uterine disease means that it has been linked to a variety of causes and risk factors such as calving difficulties, metabolic imbalance, dysbiosis and an uncontrolled immune response (Dubuc et al., 2010b). High-yielding dairy cows have often been reported to have poorer fertility than those of an average yield (Lucy and Crooker, 2001, Horan et al., 2004), however fertility is a complex trait that is unlikely to be affected by milk yield alone. In fact, several studies have reported higher fertility rates in high yielding cattle when compared to low yielders, or no difference between the two (Grohn and Rajala-Schultz, 2000, Bello et al., 2013). A grass-based dairy cow model was recently established to determine the effects of good vs. poor genetic merit for fertility in cattle of similar milk yield and size (Cummins et al., 2012). Interestingly, cows with poor genetic merit for fertility traits were more susceptible to uterine disease and showed greater up-regulation of inflammation in liver, muscle and endometrium (Moran et al., 2015, Moran et al., 2016). This agrees with our findings, suggesting that despite the fact that most cattle experience inflammation of the uterus after calving, an excessive inflammatory response in the early postpartum period has a negative effect on subsequent uterine health and fertility. The presence of inflammatory differences in organs other than the uterus also provides rationale for investigating systemic biomarkers for uterine disease. A number of models have been proposed to explain the immune dysregulation that leads to disease, which have proposed that it is preceded by either an excessive or inadequate inflammatory response (LeBlanc, 2014, Bradford et al., 2015). However the molecular pathways underpinning these responses are not well understood.

Previous work by our group identified transcriptomic differences in immune gene expression between cattle that resolved inflammation and those that developed uterine disease as early as 7 days postpartum (Foley et al., 2015), suggesting that the host immune response plays an important role in disease outcome. Here, we hypothesised that even in the context of early postpartum inflammation, cows that go on to develop either PVD or CYTO will be identified by a unique inflammatory signature in the circulation and in endometrial epithelial cells 7 DPP, which contributes to a dysregulated inflammatory response. Using RNA-seq, the endometrial transcriptome of cows with clinical and sub-clinical uterine disease was compared to healthy counterparts at 7 DPP. Here, we found that upregulation of IL-1 pathways at 7 DPP plays a prominent role in the pathogenesis of uterine disease. Excessive IL-1 production is a hallmark of many autoinflammatory diseases, as well as some autoimmune diseases such as rheumatoid arthritis and even metabolic disorders like type 2 diabetes (Gabay et al., 2010). In cattle, IL-1 also plays a pathogenic role in mastitis (Xu et al., 2018). An increase in endometrial *IL1A* and *IL1B* production in cows that developed uterine disease has previously been detected in the first

week postpartum (Herath et al., 2009b, Foley et al., 2015), but findings are not conclusive as lower levels of *IL1B* have also been reported in cows that develop disease (Galvao et al., 2011). This suggests cattle in the latter study were unable to evoke a robust enough inflammatory response to clear infection and/or repair endometrial damage after calving. Although it is assumed that uterine inflammation after calving is ubiquitous, our study identified a subset of cattle with very low inflammation 7 DPP (<18% PMN), some of which went on to develop disease. It is possible that as we were only sampling on one day we may have missed or preceded the window of acute inflammation or it may be that a subset of cattle in this study developed endometritis due to an ineffective inflammatory response, as opposed to excessive inflammation. Upregulation of *NLRP3* and the inflammasome pathway suggests that regulation of IL-1 in these cattle may also be inflammasome-mediated. An increase in anti-inflammatory cytokines in the same animals, such as *IL10* and the IL1 receptor antagonist (*IL1RN*) and decoy receptor (*IL1R2*) also implies some attempt to control this hyper-inflammatory response.

Several other pro-inflammatory cytokines and chemokines were also upregulated in most cows that developed uterine disease, including *TNF*, *IL6*, *IL17*, *CCL20* and *CXCL8* which may have been driven by upregulation of the transcription factor NFκB. Interestingly, *CXCL8* was the 11th most abundant transcript expressed in the endometrium at 7 DPP using TPM values. As the primary role of *CXCL8* is to attract and activate neutrophils, it is not surprising that previous studies have reported an increase in endometrial *CXCL8* in accordance with accumulating PMN post-calving (Galvao et al., 2011). Here we found that PMN% was slightly higher on average at 7 DPP in cattle that developed uterine disease, however we only found a mild correlation ($r=0.39$) between *CXCL8* and PMN%. This could be partly explained by genetic differences, as divergent IL-8 haplotypes have been identified in Holstein-Friesian cattle, which produce significantly different responses to LPS in endometrial epithelial cells (Stojkovic et al., 2017). Interestingly, an even weaker correlation exists between PMN% 7 DPP and the expression of cytokines such as *IL1B* ($r=0.18$), which indicates that it is the resident endometrial epithelial cells and the exposed stromal cells at areas of tissue damage that are primarily responsible for generation of an inflammatory immune response. Therefore, cellular inflammation is not always predictive of molecular inflammation, as most cattle appeared very similar when observed cytologically 7 DPP; but demonstrated different molecular signatures. This undermines the suggestion that a threshold of >40% PMNs in the uterus 7 DPP is beneficial for reproductive health (Gilbert and Santos, 2016).

Multiple S100 genes (*S100A8*, *S100A9* and *S100A12*) were also among the most abundant transcripts detected and were all upregulated in cows that developed disease. The S100s are calcium-binding proteins that possess antimicrobial function. The S100A8 and S100A9 proteins form a heterodimer known as calprotectin, which chelates manganese and zinc, thereby limiting the availability of these metals for microbes (Clohessy and Golden, 1995, Loomans et al., 1998). Although each protein alone has some affinity for zinc, formation of the heterodimer in the presence of calcium induces structural changes that improve its affinity for these metals (Striz and Trebichavsky, 2004). Sequestration of zinc also inhibits the action of the zinc-dependent matrix metalloproteinases (MMPs) which are important for processes such as tissue remodelling and inflammation (Isaksen and Fagerhol, 2001). Calprotectin and S100A12 are located mainly in the cytosol of neutrophils and monocytes and are secreted during inflammation (Boussac and Garin, 2000). In fact, faecal calprotectin is often used as a biomarker for inflammatory bowel disease (Summerton et al., 2002). Immunohistochemical staining has revealed the presence of S100A8 and S100A9 in the inflamed bovine endometrium – not only in leukocytes but also in endometrial epithelial and stromal cells (Swangchan-Uthai et al., 2013). This report reinforced the finding of increased *S100A8*, *S100A9* and *S100A12* mRNA production in cultured bovine endometrial cells in response to LPS challenge (Swangchan-Uthai et al., 2012). Both S100A8 and S100A9 are capable of activating TLR4, resulting in the amplification of inflammatory mediator production (Vogl et al., 2007). Additionally, elevated endometrial S100A8 and S100A9 expression has been reported in cows with cytological endometritis at 21 DPP (Foley et al., 2015). Interestingly Foley et al. (2015) also observed upregulation of calcium signalling pathways in healthy cows at 21 DPP. Calcium is important for other functions such as myometrial contractions for the clearance of lochia and endometrial epithelial barrier formation, as seen during our calcium switch assay where chelating calcium resulted in a disrupted epithelium. Whilst we did not detect any differences in circulating calcium concentrations between healthy and endometritic cows, it is possible that the increased requirement of calcium by the S100 proteins could result in local reprioritisation of calcium towards maintenance of uterine defence rather than repair.

The most abundant endometrial transcript detected at 7 DPP was an acute phase protein: the mammary SAA gene, *MSAA3.2*. Additionally, *SAA3* was the 10th most abundant transcript. This suggests that SAA has an important function in the endometrium of the early postpartum cow. The major site of SAA production is in the liver. Increased circulating SAA can be detected in all cattle during the early postpartum period, but has been found to be elevated in cattle that later develop uterine disease, both in this study and others (Brodzki et al., 2015, Foley et al., 2015).

In addition, extra-hepatic expression of the SAA-3 isoform has been observed in a number of tissues, such as the mammary gland and the uterus (Berg et al., 2011). Increased expression of SAA3 has been reported in endometrial biopsies of postpartum cows, where it corresponded with the level of inflammation present (Chapwanya et al., 2009). SAA3 is also highly induced in endometrial epithelial cells challenged with *E. coli* (Chapwanya et al., 2013), highlighting its role as an opsonin for gram-negative bacteria. Additionally, cows with poor genetic merit for fertility, which were also more susceptible to uterine disease, had increased endometrial SAA3 expression at day 7 of the oestrous cycle (Moran et al., 2015). In contrast, analysis of SAA protein in CVM found that SAA was elevated in healthy cows 21 DPP (Adnane et al., 2017). Despite the major role SAA plays in amplification of the inflammatory response, it has also been found to play an immunomodulatory role, encouraging the secretion of the anti-inflammatory cytokine IL10 from neutrophils (De Santo et al., 2010). Therefore the authors suggested that increased SAA in the CVM of healthy cows may indicate a protective measure that prevents tissue damage caused by severe inflammation. It may be that particular SAA isotypes carry out divergent functions in the uterus. In the current study, we found that neither *MSAA3.2* nor *SAA3* was significantly differentially expressed between healthy and endometritic cattle and there was a large amount of inter-animal variation present. Interestingly, however, *SAA2* which has not been reported in the bovine endometrium before was increased 12-fold in cows that developed uterine disease (FDR 0.06), particularly in cows with a heightened inflammatory response. Whilst SAA appears to be an important component of the postpartum immune response, the role it plays is still not completely understood, and warrants further investigation.

Another APP that appears to have an important role in disease pathogenesis in this study is PTX3. PTX3 is a member of the pentraxin superfamily, which makes it 50% homologous to the short pentraxins, CRP and SAP. However, unlike CRP and SAP which are secreted by the liver mainly in response to IL-6, PTX3 is produced by a wide variety of tissues, in response to IL-1, TNF, PAMPs and DAMPs (Bottazzi et al., 2016). Here, PTX3 was one of the top DE genes and was upregulated in cows that developed uterine disease, which was also confirmed at protein level. As healthy cows demonstrated upregulation of genes involved in tissue proliferation and adhesion at 7 DPP, we hypothesised that the hyperinflammatory response present in cows that developed disease was preventing repair of the epithelial barrier during involution. We specifically focused on PTX3 due to recent research that found it is capable of amplifying tissue damage under certain conditions (Souza et al., 2002, Souza et al., 2009). Furthermore, PTX3 not only has immune functions, but also plays an important role in tissue remodelling (Doni et al., 2015) and has been linked to disruption of the blood brain barrier in a mouse model of white

matter stroke (Shindo et al. 2017). Here we found that PTX3 delayed re-establishment of the endometrial epithelial barrier. Further work is required to assess the mechanisms by which this occurs, though it seems likely that the interaction between PTX3 and IL-1 could be important. PTX3 has also been found to have a “ying-yang” effect on inflammation, and only appears to be pathogenic under certain circumstances, usually when additional stressors are present. For example, over-expression of PTX3 in mice with *k.pneumoniae* infection resulted in increased lethality due to a decreased influx of neutrophils into the lungs and therefore suppressed bacterial killing when a high bacterial load was present (Soares et al., 2006). However when mice were inoculated with a low bacterial load, over-expressed PTX3 had a protective effect, resulting in increased pro-inflammatory cytokine production and clearance of bacteria by phagocytosis (Soares et al., 2006). This suggests that bacterial load and/or changes in the microbial populations during the postpartum period could affect PTX3 function. Damage of the endometrial epithelium also allows the entry of microbes into the underlying stroma, leading to the growth of opportunistic bacteria such as *T. pyogenes*, which secretes PLO to form pores in eukaryotic cell membranes in a cholesterol-dependent manner, meaning it cannot usually penetrate the intact epithelium due to a lack of cholesterol (Jost and Billington, 2005). Therefore excessive damage of the endometrium at calving (which is also an established risk factor for PVD) may directly stimulate PTX3 production, in addition to exacerbating the inflammatory response and contributing to uterine dysbiosis, thus resulting in prolonged, chronic inflammation and delaying the return to homeostasis.

The importance of an intact endometrial epithelial barrier has been emphasised by recent histological analysis of uterine samples from cows with metritis, which had lost a large amount of the luminal epithelium in inter-caruncular areas of the endometrium in comparison to healthy cows (Sicsic et al., 2018). Furthermore, many of the risk factors that precede the development of uterine disease cause damage to the uterus at calving, such as dystocia, twinning, primiparity and the gender and size of the calf. Interestingly, we found that a subset of the healthy cows in this study had very low inflammation 7 DPP and this appeared to be associated with a more intact barrier, with greater expression of epithelial cell markers and lower expression of vimentin, a stromal-specific marker. It may be that these cows had a more robust barrier, possibly due to an easier calving, or that less endometrial damage was caused due to a lack of inflammation. Either way, it's likely that a more intact barrier is also more functional, as we saw greater expression of tight junction proteins in these cows. The formation of tight junctions is also essential for directional secretion of molecules either apically into the uterine lumen, or basolaterally into the stroma. Recent studies have shown that IL-6 is predominantly secreted

apically in polarised endometrial epithelial cells in response to bacteria, LPS or IL-1 α , suggesting that an intact epithelium is essential for protecting the underlying stromal cells against noxious stimuli (Healy et al., 2015). Therefore a disrupted epithelium may result in the secretion of IL-6 and other inflammatory into surrounding stroma, resulting in increased tissue damage due to the production of ROS.

Whilst we were able to detect differences in endometrial gene expression between cows that developed uterine disease and those that didn't, heterogeneity remained a key feature of this study. There are several factors that may have influenced the large amount of inter-animal variation observed here. Firstly, it is probably partly due to the current definitions for uterine disease diagnosis in cattle. We attempted to tackle part of this problem by splitting the cows with PVD into two groups: those with and without uterine inflammation at 21 DPP. Following this re-grouping, we found distinct differences in gene expression at 7 DPP between the two groups, which included lower expression of *TLR2*, *TLR4*, *S100A8*, *IL10* and *TGFB2* in cows that subsequently developed PVD only at 21 DPP. This suggests that PVD in the absence of uterine inflammation is more likely to be indicative of infection elsewhere in the reproductive tract, such as the vagina or cervix. There is also a large amount of discrepancy regarding the criteria for cytological diagnosis of endometritis, due to variation in counting PMNs and PMN thresholds (reviewed by (de Boer et al., 2014) and (Wagener et al., 2017a)). Here, we used a threshold of >18% PMN at 21 DPP to diagnose CYTO, which was based on previous findings by (Kasimanickam et al., 2004) and (Barański et al., 2012) who found that PMN% above this threshold resulted in decreased pregnancy risk. Since then, thresholds of 6.5% to 18% have been used to diagnose endometritis, depending on the time point postpartum. These values have been based largely on ROC curve analyses, which assess the sensitivity and specificity over a range of test scores. Whilst it may have been more accurate for us to base our PMN threshold on our own ROC curve analysis rather than a pre-defined threshold, it is important to remember that fertility has a multi-factorial nature and can be affected by a number of confounders, such as poor heat detection, poor semen quality, skill of the AI technician and nutrition. Therefore no PMN threshold will be entirely accurate and it is not surprising that such a large variation in PMN% threshold and incidence of CYTO is detected between farms. In addition, it is likely that a number of cattle diagnosed with uterine disease will spontaneously clear infection and/or resolve inflammation by 21 DPP.

Inter-animal variation in this study could also have been affected by cellular heterogeneity. In this study we collected endometrial cells using cytobrushes, in an attempt to obtain a "purer"

population of predominantly epithelial cells and PMNs as opposed to the mixed cell biopsy (Cardoso et al., 2017). However, due to the lack of epithelial coverage in most cows during the early postpartum period, we also acquired a large number of stromal cells, as assessed by the presence of vimentin expression. Additionally, there was a large variation in the number of PMNs observed at 7 DPP. We therefore attempted to control for some of these differences in cell populations by separating cattle based on their PMN% at 7 DPP, using the same 18% threshold that we used at 21 DPP, to separate cows with “high” and “low” inflammation. By splitting the healthy group of cows in two, we could clearly see differences in gene expression that related to the presence or absence of endometrial inflammation. In addition, healthy cows with low inflammation at 7 DPP had greater expression of epithelial cell markers and lower expression of stromal cell markers suggesting there were differences in the two cell populations which could have influenced gene expression. Although both epithelial and stromal cells can respond to PAMPs and DAMPs, the immune response of each cell type differs slightly (Davies et al., 2008, Cronin et al., 2012). A recent transcriptomics study found that when results were corrected for the average populations of epithelial and stromal cells in endometrial biopsies by computational decomposition, 74% of differentially expressed transcripts became non-significant (Suhorutshenko et al., 2018). Whilst the differences in this study would have been more extreme as different stages of the menstrual cycle (which influences tissue composition) were being compared, future endometrial “omics” studies should take into account cellular composition, where tissue heterogeneity could possibly influence the results. Other options involve sorting cell types by laser microdissection microscopy or fluorescence activated cell sorting (FACS) prior to sequencing, although in addition to being more costly and time consuming, these processes can also affect the viability and activity of isolated cells.

Compelling evidence also indicates that variation in the immune response of cattle is epigenetically regulated. For example, when dermal fibroblasts from cattle that were classed as “high responders” or “low responders” in response to LPS were treated with epigenetic inhibitors, all variability in immune response to LPS was lost (Green and Kerr, 2014). Treatment with the epigenetic inhibitors 5-aza-2'-deoxycytidine (AZA) and trichostatin A (TSA) alone (without LPS challenge) increased expression of *IL8*, *IL6*, *TNF* and *SAA3*, suggesting that these genes are also epigenetically modified under normal conditions (Green and Kerr, 2014). AZA is a de-methylating agent, and therefore as it influences expression of some of the genes that we know are differentially expressed in endometritic cows, it stands to reason that differences in methylation status between healthy and endometritic cattle may influence the expression of certain immune genes. However, (Walker et al., 2015) found no significant associations between

DNA methylation and gene expression between healthy cows and those with CYTO at 29 DPP. However, this could have been influenced by the fact that this analysis was performed on endometrial biopsies, as different cell types also have distinct methylation patterns. Another post-translational modification that influences gene expression is microRNA (miRNA) activity. MiRNAs are small non-coding RNA molecules that silence gene expression by binding to complementary sequences on mRNA molecules. This results in either cleavage of the mRNA strand, destabilisation of the mRNA molecule via the shortening of its mRNA tail, or inefficient translation of mRNA into protein (Fabian et al., 2010). MiRNA analysis of cows with and without CYTO identified 23 differentially expressed miRNAs, which potentially target genes mediating the expression of inflammatory cytokines, in particular NF κ B (Hailemariam et al., 2014). Furthermore, analysis of miRNAs at 7 DPP has identified upregulation of all 5 members of the mir-200 family in cows that developed CYTO (Foley et al., 2015). These miRNAs negatively regulate cell proliferation in the human uterus (Nothnick, 2012) and as miRNAs tend to be well conserved between species, this may also be true in the cow, potentially limiting tissue repair in order to redistribute energy reserves towards mounting an immune response.

In summary, this thesis represents the first study to characterise the endometrial transcriptome of early postpartum dairy cows phenotyped for both clinical and subclinical uterine disease, using next generation sequencing. The additional validation of 12 selected genes in 56 extra cattle makes this the largest transcriptomic study performed in relation to bovine uterine disease to date. We have shown that despite a significant amount of inter-animal heterogeneity, both clinical and subclinical forms of uterine disease are preceded by excessive activation of the endometrial inflammatory response 7 DPP, which can be also detected systemically by an increase in the APPs SAA and Hp and a decline in circulating eosinophils. Furthermore, integrity of the endometrial epithelial barrier is likely to play an important role in preventing disease onset. Not only have we enhanced our understanding of the mechanisms underlying the switch between physiological and pathological inflammation, but we have also paved the way for the development of novel biomarkers for the early detection of uterine disease. It is hoped that this research has highlighted a number of research avenues that can now be explored using hypothesis-driven approaches to continue to improve our understanding of this complex and impactful disease.

Chapter 7 References

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Chapter 8 Appendices

Table 8-1 Animal classification by inflammatory and disease status

Cow ID	Group	Lactation	PMN % D7	PMN % D21	VMS D7	VMS D21	Health status D21
2392	HL Healthy	2	60	1	0	0	Healthy
1570		7	43.5	1	1	0	Healthy
1831		5	36	0	1	1	Healthy
1066		10	53.5	2.5	0	0	Healthy
2053		4	32.5	6.5	0	1	Healthy
1641		6	29	7	0	1	Healthy
1761		6	21	1.5	0	0	Healthy
2361		2	35.5	17.5	1	0	Healthy
2676		1	39	2.5	0	0	Healthy
1733		6	41.5	12	0	0	Healthy
2055		4	66	12	0	1	Healthy
1472		7	38.5	12.5	0	0	Healthy
2478		2	18	5.5	0	0	Healthy
1528		7	47.5	6.5	0	0	Healthy
2223		3	84.5	15.5	0	0	Healthy
1378	HH CYTO	8	85	64	2	1	CYTO
2064		4	57	73	0	1	CYTO
2184		3	76	57.5	0	0	CYTO
2039		4	87	85	0	1	CYTO
1944		5	53.5	62	0	1	CYTO
1498		7	24	76	0	0	CYTO
1984		4	65	33.5	0	0	CYTO
1985		4	25	24.5	1	0	CYTO
2254		3	32.5	68.5	0	1	CYTO
2727		1	40	45	0	0	CYTO
912		11	57.5	35.5	0	1	CYTO
1698		6	30	75	0	1	CYTO
2595		1	28	60	3	1	CYTO
2656		1	73	69	3	0	CYTO
2684		1	42.5	60	1	0	CYTO
2566		1	75.5	67.5	0	1	CYTO
2673		1	52	23	1	1	CYTO
2686		1	89	78.5	0	1	CYTO
2637		1	25.5	90	0	0	CYTO
2671		1	31	83	0	0	CYTO
1571		7	49	60.5	0	0	CYTO
1843		5	58	93	1	1	CYTO

1062		10	66.5	82.5	0	1	CYTO
1204		9	78.5	63	0	1	CYTO
2672	HH CYTO+PVD	1	57	76	3	2	CYTO + PVD
2202		3	53	58	0	3	CYTO + PVD
1239		9	74.5	33	1	3	CYTO + PVD
2161		3	52.5	71.5	1	2	CYTO + PVD
1829		5	86.5	89	0	2	CYTO + PVD
1467		7	60.5	24	0	3	CYTO + PVD
1685		6	56.5	65.5	0	3	CYTO + PVD
1855		5	21.5	62	0	3	CYTO + PVD
2211		3	72	21	0	3	CYTO + PVD
2240		3	31	86	0	2	CYTO + PVD
2245		3	36.5	36.5	0	2	CYTO + PVD
2391		2	23.5	83.5	0	3	CYTO + PVD
2598		1	41.5	29	0	2	CYTO + PVD
2602		1	33.5	40.5	0	3	CYTO + PVD
2629		1	45	69.5	0	3	CYTO + PVD
2639		1	62.5	76	0	3	CYTO + PVD
1871		5	57.5	57	1	3	CYTO + PVD
2213		3	64.5	67.5	0	3	CYTO + PVD
2381		3	57	86	0	2	CYTO + PVD
2443		2	71	40	1	3	CYTO + PVD
1887		5	70	93	0	3	CYTO + PVD
1510		7	77.5	90.5	0	3	CYTO + PVD
2590		1	87.5	92.5	0	3	CYTO + PVD
1854		5	42.5	18	2	2	CYTO + PVD

2094		4	47	95	1	3	CYTO + PVD
1978		4	74.5	93.5	1	3	CYTO + PVD
1194	HL PVD	9	31	6.5	0	2	PVD only
2188		3	62.5	14.5	2	3	PVD only
2622		1	79	3.5	0	3	PVD only
2683		1	39.5	15.5	1	3	PVD only
2520		2	37.5	2	1	2	PVD only
929		11	32.5	1.5	0	2	PVD only
1347		8	68.5	12	1	3	PVD only
1573		7	83	4.5	0	3	PVD only
1676		6	65	4.5	0	3	PVD only
2426		2	48.5	0	0	3	PVD only
2567		1	87.5	7	0	3	PVD only
2604		1	57	6	0	3	PVD only
2024		4	41.5	8.5	0	3	PVD only
1371		8	21	16	1	3	PVD only
1854		5	42.5	18	2	2	PVD only
1814		5	70.5	17	3	3	PVD only
1725		6	27	0.5			
2164		3	31	7.5	1	3	PVD only
2432		2	49.5	11	0	3	PVD only
2410		2	70	9	0	3	PVD only
2580		1	78.5	3	1	2	PVD only
1340	LL Healthy	8	2.5	1	0	1	Healthy
2006		4	6.5	0.5	0	0	Healthy
2570		1	0	1	0	0	Healthy
1890		5	6.5	10.5	0	0	Healthy
2224		3	5	0.5	0	0	Healthy
1487		7	4	7.5	1	0	Healthy
2241		3	0.5	0.5	1	1	Healthy
2409		2	10.5	17.5	1	0	Healthy
2597		2	7.5	10	0	1	Healthy
2636		1	1	4	0	1	Healthy
2210		3	12.5	17	2	1	Healthy
1558		7	2	3	0	1	Healthy
2421	LH CYTO	2	14.5	59.5	0	1	CYTO
1183		9	2	53	0	0	CYTO
2167		3	0	45	0	1	CYTO
2382		2	16	55	0	1	CYTO
2178		4	3.5	28.5	0	0	CYTO
1738		6	7	20	0	1	CYTO
2436		2	16	18.5	0	1	CYTO

Cows (n=112) were classified by polymorphonuclear leukocyte (PMN) % and vaginal mucus score (VMS) at 7 and 21 days postpartum (DPP). Scoring at 21 DPP determined the health status of each cow at this time point. Grey shading indicates cattle that were selected for RNA sequencing

Table 8-2 Oligonucleotide primer sequences used for qPCR validation

Gene symbol	Ensembl ID	Forward primer (5'-3')	Reverse primer (5'-3')
<i>GUSB</i>	ENSBTAG00000000704	ACCATCGCCATCAACAA CAC	TCCGCGTAGTTGAAGAAGT
<i>SDHA</i>	ENSBTAG00000046019	AAGACGTTTCGACAGGGG AAT	ACTCGTCAACCCTCTCCTTG
<i>HSP90AB1</i>	ENSBTAG00000000778	GCATGAAGGAGACGCA GAAG	TCCTTGAGCTGCTGTACACA
<i>RPS15</i>	ENSBTAG00000019718	GCGACATGATCATTCTAC CCG	GGTAGTGGCCGATCATCTCA
<i>NLRP3</i>	ENSBTAG00000001273	CTTTCTGGACTCTGACCG GG	TCTGTCTGACCCCGAGGAAT
<i>IL1A</i>	ENSBTAG00000010349	GCTCGGTTTCAGCAAAGA AGT	GAGGTGGTCAATTCAGAACT GT
<i>IL1B</i>	ENSBTAG00000001321	CCCTGCAGCTGGAGGAA GTA	CTTCGATTTGAGAAGTGCTGA TGT
<i>CXCL8</i>	ENSBTAG00000019716	CATTCCACACCTTTCCAC CC	CCTTCTGCACCCACTTTTCC
<i>CCL20</i>	ENSBTAG00000021326	CAGCAAGTCAGAAGCAA GCA	TTTGGATCTGCACACACAGC
<i>TNFA</i>	ENSBTAG00000025471	CCATCAACAGCCCTCTG GTT	TCACACCGTTGGCCATGA
<i>IL17A</i>	ENSBTAG00000002150	CGTTAACCGGAGCACAA ACT	TCCCAGATCACAGAGGGGTA
<i>TLR2</i>	ENSBTAG00000008008	GGTTTAAAGGCAGAATC GTTTG	AAGGCACTGGGTAAACTGTG T
<i>TLR4</i>	ENSBTAG00000006240	GCTGTTTGACCACTCTG ATTGC	GGGCTGAAGTAACAACAAGA GGAA
<i>S100A8</i>	ENSBTAG00000012640	ATTTTGGGGAGACCTGG TGGG	TGAACCAAGTGTCGCATCC
<i>TGFB2</i>	ENSBTAG00000005359	ACCCTCGGAAAATGCCA TCC	CTGAACTCGGCCTTCACCAA
<i>IL10</i>	ENSBTAG00000006685	GAAGGACCAACTGCACA GCTT	AAAAGTGGATCATTTCCGACA AG