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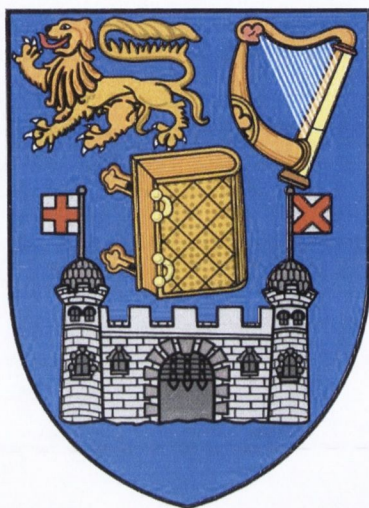
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Systemic and Local Immune Responses Associated with Subclinical Endometritis in the Postpartum Dairy Cow



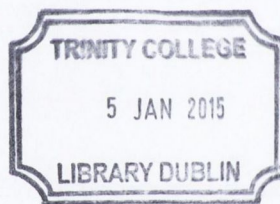
**A thesis submitted to the University of Dublin for the Degree of Doctor
of Philosophy**

2014

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Thesis 10814

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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Uterine swab sample collection

Uterine cytobrush sample collection

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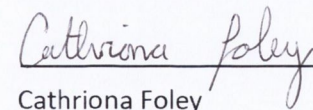
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Publications

Foley, C., C. O. Farrelly, and K. G. Meade. 2011. Technical note : Comparative analyses of the quality and yield of genomic DNA from invasive and noninvasive, automated and manual extraction methods. *Journal of Dairy Science* 94: 3159-3165.

Chapwanya, A., Meade, K. G., Foley, C., Narciandi, F., Evans, A. C., Doherty, M. L., Callanan, J. J., O'Farrelly, C. 2012. The postpartum endometrial inflammatory response: a normal physiological event with potential implications for bovine fertility. *Reproduction Fertility and development* 24(8):1028-39.

Foley, C., Chapwanya, A., Creevey, C., Narciandi, F., O'Farrelly, C., Kenny, E., Cormican, P., Meade, K.G. 2012. Global endometrial transcriptomic profiling: transient immune activation precedes tissue proliferation and repair in healthy beef cows. *BMC Genomics* Sep 18;13:489.

Foley, C., Chapwanya, A., Lynn, D., Callanan, J. J., Narciandi, F., O'Farrelly, C., Meade, K.G. 2013. Divergent innate immune and calcium signalling early postpartum identifies cows with sustained endometrial inflammation associated with endometritis. In preparation.

Abstract

Activation of local innate immune mechanisms by postpartum bacterial contamination of the uterus occurs in all cows and approximately 30% develop endometritis associated with persistent bacterial infection, inflammation and subfertility. The postpartum period is critical for the restoration of homeostasis within the uterus. However, postpartum cows are in the process of an immune transition between an immunosuppressed state during pregnancy to a requirement for uterine immune activation to clear bacterial contamination within a very short timeframe. In this context, early and efficient activation of local innate immune mechanisms is required. However, the regulation of the innate immune response in the postpartum cow remains to be fully understood. Mechanisms that control inflammation and achieve a physiologically functioning endometrium postpartum while avoiding disease have not been identified.

Here, the overall aim was to define local mechanisms that might differentiate cows with healthy and inflamed postpartum endometria locally and systemically by investigating temporal differences between 7 and 21 days postpartum (DPP) within healthy cows and cows with sustained endometrial inflammation, associated with sub-clinical endometritis (SCE) and also between healthy and sub-clinical cows at 7 and 21 DPP, toward the identification of biomarkers that could be used for early diagnosis of uterine disease.

An initial pilot study aimed to characterise uterine involution in healthy cows ($n=3$) at a pan-transcriptomic level in endometrial biopsies using RNA-Seq. 1,107 genes were significantly differentially expressed, 73 and 1,034 were increased 15 days postpartum (DPP) and 30 DPP, respectively (adjusted P -value <0.1). Go-Seq pathway analysis demonstrated that immune pathways were predominantly enriched 15 DPP. In additional animals, real-time qRT-PCR revealed significantly elevated expression of SAA1/2, GATA2, IGF1, SHC2, and SERPINA14 thirty DPP. Validated genes were functionally associated with tissue proliferation and the restoration of uterine homeostasis postpartum.

A follow-on study aimed to define functional differences between cows with a high degree of endometrial inflammation ($n=6$) and healthy cows ($n=9$) displaying relatively low endometrial inflammation 21 DPP. Using histology, neutrophil and leukocyte influx in the epithelium and stroma was graded independently on a scale of 0 to 3 and a cumulative score ≥ 4 categorised cows with a higher degree of endometrial inflammation. Global transcriptomic differences in endometrial biopsies were assessed using RNA-Seq between cows with healthy and inflamed postpartum endometria at both 7 and 21 DPP. Seven DPP a total of 182 genes and 21 DPP a total of 1219 genes were differentially expressed between cows with healthy and inflamed postpartum endometria (FDR <0.1). Go-Seq pathway analysis identified significant perturbation of multiple immune response and calcium signalling pathways between groups 21 DPP. The temporal transcriptomic profile in healthy cows reflects a transient immune response at 7 DPP, preceding more significant calcium signalling 21 DPP which contrasts with a sustained inflammatory response in endometrial samples from inflamed cows. miRNA profiling revealed that all five members of the mir-200 family known to target genes associated with cellular proliferation were significantly elevated 7 days relative to 21 DPP cows that had sustained endometrial inflammation and not in healthy cows.

The culture independent method of Terminal Restriction Fragment Length Polymorphism (T-RFLP) and principal coordinates ordination revealed a significant (p -value <0.05) shift in uterine microbial populations when comparing cows with healthy and inflamed postpartum endometria. Systemically, significantly reduced numbers of circulating neutrophils and eosinophils was observed in inflamed cows 7 DPP ($P<0.05$). Elevated levels of circulating serum proteins haptoglobin (HP) and serum amyloid A (SAA) indicated a significant acute phase response in inflamed cows 7 DPP.

In conclusion inflammation at both a cellular and molecular level was elevated in sub-clinically endometritic cows and the shift in microbial populations may be a contributing factor to the sustained inflammation observed in these cows 21 DPP. Reduced mRNA expression of genes involved in calcium signalling 21 DPP and increased expression of miRNAs that can regulate genes associated with cellular proliferation 7 DPP, appear to be mechanisms influenced by local endometrial inflammation. The results of this study also highlight significant differences in systemic levels of SAA and HP 7 DPP in healthy and SCE cows. Divergent local inflammatory and calcium signalling profiles and systemic acute phase proteins differentiate cows with sustained endometrial inflammation associated with subclinical endometritis from healthy cows and local elevation of *IL-17* gene expression prior to and during subclinical endometritis could become a potential biomarker of subclinical endometritis.

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Abbreviations

AA	Arachidonic Acid
AC	Adenylyl Cyclase
ACTB	β -Actin
AI	Artificial Inseminations
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APP	Acute Phase Protein
ARC	Average Read Count
ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy
BGI	Beijing Genomics Institute
BHB	β -Hydroxybutyrate
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSE	Bovine Spongiform Encephalopathies
CaM	Calmodulin
CAM	Cell Adhesion Molecule
cAMP	3', 5'-Adenosine Monophosphate
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding Sequence
CL	Corpus Luteum
COX	Cyclooxygenase
CPM	Counts Per Million
CREB	cAMP Response Element-Binding Protein
CRP	C Reactive Protein
Ct	Cycle Threshold
DAAI	Days After Artificial Insemination
DAG	Diacylglycerol
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DcR3	Decoy Receptor 3
DE	Differentially Expressed
DNA	Deoxyribonucleic Acid
DPP	Days Postpartum
E2	Oestradiol
ECM	Extra Cellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
ENCODE	Encyclopedia of DNA Elements
ER	Endoplasmic Reticulum
FDR	False Discovery Rate
FSH	Follicle-Stimulating Hormone
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase

GN	Gram Negative
GnRH	Gonadotrophin-Releasing Hormone
GO	Gene Ontology
GPCR	G Protein-Coupled Receptor
GTE _x	Genotype-Tissue Expression
H	Healthy
H&E	Haematoxylin and Eosin
Hb	Haemoglobin
HCM	Hypertrophic Cardiomyopathy
HDL	High Density Lipoprotein
HP	Haptoglobin
hpf	High Power Field
ID	Identification
IGF	Insulin Growth Factor
IKK	IkappaB Kinase
IL	Interleukin
INFT	Interferon- τ
IP ₃	1, 4, 5 Inositol Triphosphate
IP ₃ R	1, 4, 5 Inositol Triphosphate Receptor
IPC	Inter-Plate Calibrator
IRAKs	IL-1 Receptor Associated Kinases
ITIM	Tyrosine-Based Inhibitory Motif
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBP	LPS-Binding Protein
LET _F	Low Expression Tags Filter
LH	Luteinising Hormone
LPS	Lipopolysaccharide
LRC	Library Read Count
MBL	Mannose-Binding Lectin
MDS	Multi-Dimensional Scaling
MHC	Major Histocompatibility
miRNA / microRNA	Micro Ribonucleic Acid
MMP	Bovine Matrix Metallo-Proteinase
MR	Mannose-Binding Receptor
mRNA	Messenger Ribonucleic Acid
MSD	Mating Start Date
mTNF	Membrane Bound Tumour Necrosis Factor
MyD88	Myeloid Differentiation Antigen 88
NCI	National Cancer Institute
NCX	Na ⁺ /Ca ²⁺ Exchanger
NEFA	Non-Esterified Fatty Acid
NET	Neutrophil Extracellular Trap
NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NGS	Next Generation Sequencing
NIH	National Institutes of Health

NK	Natural Killer
NLR	Nucleotide-Binding Domain Leucine-Rich Repeat
NTC	Non-Template Control
P4	Progesterone
PAMP	Pathogen Associated Molecular Pattern
pbUEC	Primary Bovine Uterine Epithelial Cell
PC	Pre-Calving
PCO	Principal Coordinates Ordination
PCR	Polymerase Chain Reaction
PD	Pregnancy Diagnosis
PDG2	Prostaglandin D2
PGE	Prostaglandin E2
PGF	Prostaglandin F2 α
PGFM	Prostaglandin F2 α Metabolite
PGFS	Prostaglandin F2 α Synthase
PGI2	Prostacyclin
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol 4, 5 Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLN	Phospholamban
PMCA	Plasma Membrane Ca ²⁺ -ATPase
PMN	Polymorphonuclear
PPAR	Peroxisome Proliferator-Activated Receptors
PRR	Pattern Recognition Receptor
PWF	Probability Weighting Function
QC	Quality Control
qTR-PCR	Quantitative Real Time Polymerase Chain Reaction
RAGE	Receptor for Advanced Glycation End-products
RIG	RIG-I-Like Receptor
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RP	Ribosomal Protein
rRNA	Ribosomal Ribonucleic Acid
RYR	Ryanodine Receptor
SAA	Serum Amyloid A
SCE / S	Sub-Clinically Endometritic
SERCA pump	Sarcoendoplasmic reticular Ca ²⁺ ATPase
SERPINA14	Serpin Peptidase Inhibitor Clade A Member 14
SH	Src Homology
snoRNA	Small Nucleolar Ribosomal Acid
snRNA	Small Nuclear Ribosomal Acid

SOCE	Store—Operated Ca ²⁺ Entry
SR	Sarcoplasmic Reticulum
sTNF	Soluble Tumour Necrosis Factor
SUZ12	Suppressor of Zeste 12 Homolog
TABs	TAK1-Binding Proteins
TAK1	TGF- β Activated Kinase
TLR	Toll-Like Receptor
TMM	Trimmed Mean of M-Values
TNF α	Tumour Necrosis Factor α
TRAF6	TNF Receptor-Associated Factor 6
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
TSE	Transmissible Spongiform Encephalopathies
UTMP	Uterine Milk Protein
WNT	Wingless Type
ZNF131	Zinc Finger Protein 131
κ B	Kappa B

Chapter 1

General

Introduction

1 General Introduction

1.1 Large Animal Models and Immunology

The field of Immunology has honoured the contribution of animals to its study by immortalising the term *vaccinus*, the Latin for 'of' or 'from cows', within one of the most fundamental immunological concepts; vaccination. In 1798 Edward Jenner established the general principle of vaccination, when he observed that milk maids, who recovered from cowpox, subsequently did not contract the more severe disease known as smallpox. In 1882, Louis Pasteur applied this concept of vaccination to anthrax disease. He created an avirulent form of anthrax, by growing the bacteria at a high temperature and then administered it to sheep to protect them against infection with the virulent form (Tizard 2004).

The value of the bovine species to the study of immunological tolerance, earned Sir Peter Brian Medawar a Noble Prize in 1960. In an effort to distinguish between monozygotic and dizygotic twin calves, Medawar observed that freshly transplanted skin grafts were fully tolerated after birth by each twin calf from the same pregnancy, proving impossible to distinguish between them. He explained for the first time the expression of histocompatible antigens in adult tissues due to prerequisite antigen exposure during fetal development. His concepts, in relation to the fetal allograft and maternal-fetal tolerance, established Medawar as the "Father of reproductive immunology" (Hansen 2010; Billingham *et al.* 1953).

1.2 Bovine Reproductive Immunology

Tolerance and protection of the conceptus, from maternal immune-mediated rejection, is achieved by maternal and fetal anatomical segregation within the placenta, the downregulation of major histocompatibility (MHC) class I antigens by the trophoblast and immunomodulation in the uterus (Davies 2007). Prior to uterine attachment, a bovine conceptus expresses major histocompatibility (MHC) class I antigens on its surface and yet evades recognition by maternal CD8⁺ T cells as it is surrounded by a physical barrier known as the zona pellucida. Blastocyst hatching is associated with low expression of MHC class I antigens, thus avoiding CD8⁺ T cell recognition but increasing its susceptibility towards natural killer (NK) cell lysis (Hansen 2011). However the type of placentation is such in the cow that NK cells are absent at the maternal side of the maternal-fetal interface (Stewart 1998; Moffett & Loke 2006). During placentation most species do not express classical MHC class I antigens in mature trophoblasts, the expression of non-classical MHC class I antigens act as ligands for inhibitory leukocyte receptors such as in the

natural killer cell. In the cow inter-placentomal trophoblast cells express both classical and non-classical MHC I antigens and placental trophoblast cells never express the classical MHC class I antigens (Davies 2007). During the periattachment period of the bovine blastocyst, interferon- τ (IFNT) is secreted from the trophoblast which signals to the uterus inferring maternal recognition of pregnancy and regulation of the maternal immune system (Bauersachs *et al.* 2006, Hansen 2011).

Although the maternal tolerance of a semi-allogenic fetus is essential during pregnancy, all cows must be capable of responding against bacterial contamination (Sheldon & Dobson 2004) and viral infection (Thiry *et al.* 1992; Ackermann 2006). Subsequent to parturition the uterus requires an efficient bacterial and viral defence against invading pathogens, while simultaneously undergoing size reduction, tissue regeneration and remodelling (Gier & Marion 1968). The inflammatory immune response is essential in controlling the level of bacterial contamination in the uterus and also in endometrial tissue remodelling during uterine involution (Chapwanya *et al.* 2010, Gabler *et al.* 2010, Llewellyn *et al.* 2008, Salamonsen 2003).

Uterine involution after parturition is a process the uterus goes through to restore a physiological functioning state, becoming receptive to and supportive of a new conceptus. During involution, the bacterial influx (now regarded as a normal physiological event) is subsequently cleared from the uterus in the majority of cows. However, where this does not occur, prolonged bacterial infection in the uterus is associated with inflammation, histological abrasions in the endometrium and an extended period of involution (Williams *et al.* 2005).

1.3 Reproductive Timeline & Fertility Trends

The optimal reproductive timeline in Irish cattle revolves around springtime calving when there is an abundance of grass growth, one of the cheapest food sources, to provide for the high energy demands of lactating cows. Gestation lasts for ~280 days (9 months) and ideally involves the entire herd beginning to calve in early February, followed by a mating start date (MSD) in mid-April and a breeding season of 12 weeks. First, artificial inseminations (AI) are administered to clinically healthy cows and if pregnancy is not established, repeat 2nd and/or 3rd AIs are performed. Failing pregnancy (usually evident as a cow in heat or Oestrus) natural inseminations with a herd bull are carried out. The goal for Irish farmers is to achieve a synchronised annual calving interval across the herd to achieve the best financial outcome. Cows that calve late or do not become pregnant in the required breeding season are culled (removed) from the herd and are replaced by heifers, increasing economic costs for the farmer.

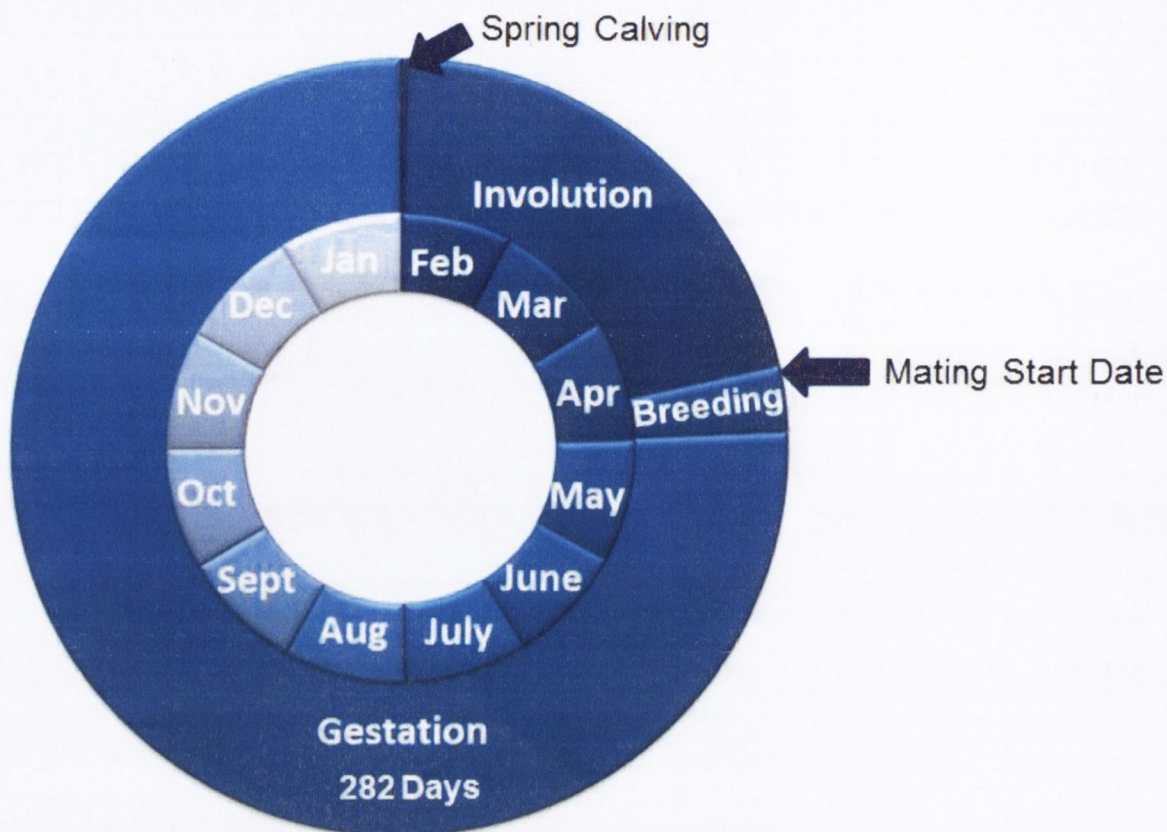


Figure 1.3-1: An optimum reproductive timeline of the cow in a seasonal based grazing system. Spring calving is economically beneficial as there is an abundance of grass growth at this time which is the cheapest food source to provide for the high energy demands of the lactating cow. Subsequent to spring calving, uterine involution must be complete by the Mating Start Date to ensure optimum fertility and cows must breed before May to allow for a 9 month gestation period.

Detection of problem animals prior to the mating start date is important so that these animals can be treated early and inseminated ensuring optimal fertility is achieved (Figure 1.3-1). However, on the farm detection of problem animals relies on visual assessment by the farmer and is usually determined by clinical signs of a disease. There is evidence that in the absence of clinical signs, sub-clinical disease can also cause sub-fertility which may go undetected by the farmer prior to the MSD (LeBlanc *et al.* 2002, LeBlanc 2008, Sheldon 2006, Sheldon *et al.* 2008). Therefore the detection of sub-clinical disease early postpartum would identify animals in need of therapeutic intervention, improving their chances of conceiving to first service within the breeding season, thereby reducing costs and improving fertility.

1.4 Uterine Reproductive Physiology in the Bovine

1.4.1 Physiology and Anatomy

Ruminants possess a uterus comprised of a body, cervix and two horns. The shape of the bovine uterus is known as bipartite as there is incomplete fusion of the uterine body resulting in a division into two parts. The outer layer is the perimetrium and the middle layer is the myometrium, which consists of an inner circular layer of smooth muscle, an intermediate vascular layer and an outer longitudinal layer of smooth muscle. An increase in muscle cell size and number is observed in the uterine wall during pregnancy (Frandsen *et al.* 2003).

The inner mucosal lining of the uterus is the endometrium and consists of glands responsible for secreting a fluid, known as uterine milk or histotroph, to support an embryo before and after it attaches to the uterus. The vasculature and thickness of this tissue is altered due to hormonal changes and pregnancy (Frandsen *et al.* 2003). Projections from the endometrium, known as caruncles, are distributed throughout the ruminant uterus, there are between 70 and 120 are present in the bovine uterus. Caruncles are nonglandular, the maternal side of the fetal-maternal interface during pregnancy which fuse with the fetal cotyledons to form placentomes (Frandsen *et al.* 2003, Wooding & Burton 2008) (Figure 1.4-1).

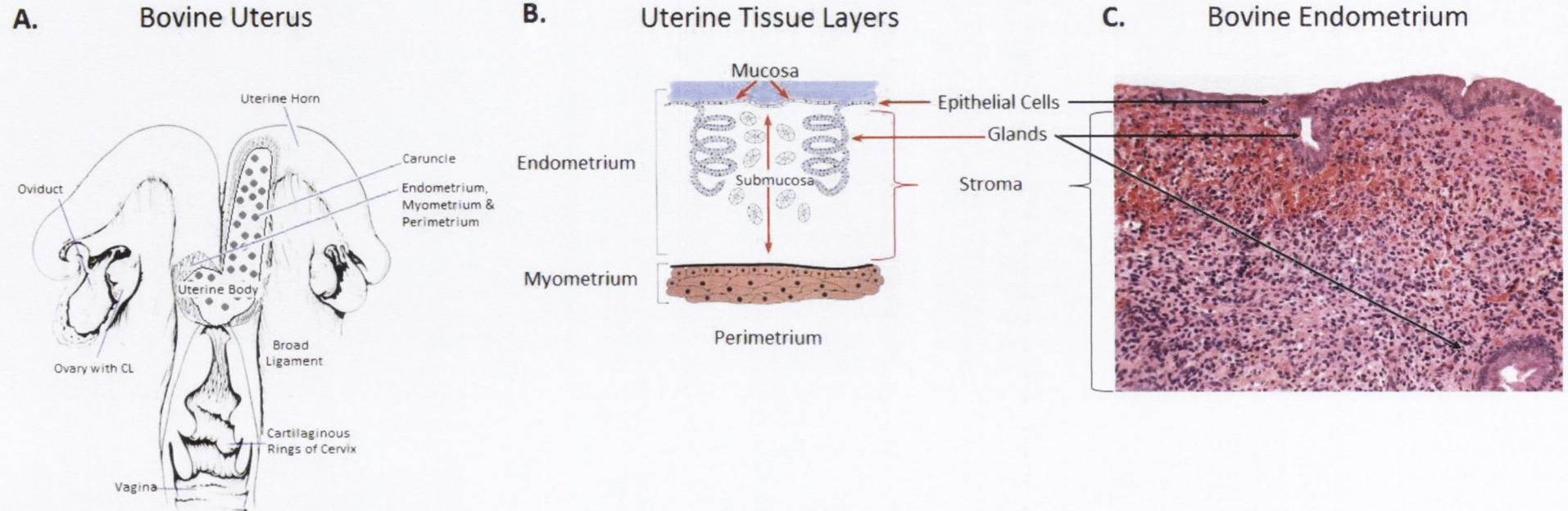


Figure 1.4-1: The bovine uterus and tissue layers. (A) The bovine uterus is bicornuate consisting of two uterine horns, the endometrium is the inner layer exposed to the uterine lumen composed of caruncle and intercaruncle tissue (B) Intercaruncle tissue layers of the uterus contain endometrial glands; both intercaruncle and caruncle endometrial tissues are lined with a mucosal epithelium underneath which is the endometrial stroma (C) A histological section of the bovine endometrium, arrows highlight the presence of endometrial glands in the stroma and epithelial cells lining the endometrium.

Cattle have non-invasive epitheliochorial placentation, which differs from the invasive haemo-monochorial placentation in humans. During bovine implantation, at the fetal-maternal interface, a cellular epithelial layer forms on the maternal side, interdigitating with the outermost fetal membrane, the chorion. Transient binucleate trophoblast cells migrate across the microvillar junction and fuse with uninucleate uterine cells producing a fetomaternal trinucleate cell for the duration of pregnancy (Wooding & Burton 2008). One of the great renaissance artists Leonardo Di Vinci was the first person known to illustrate the correct position of the fetus within the mother's womb. However, he erroneously depicted the human placentation to be the epitheliochorial placentation of a cow, believing that placental structures such as bovine fetal cotyledons, were similar to both species (Figure 1.4-2).

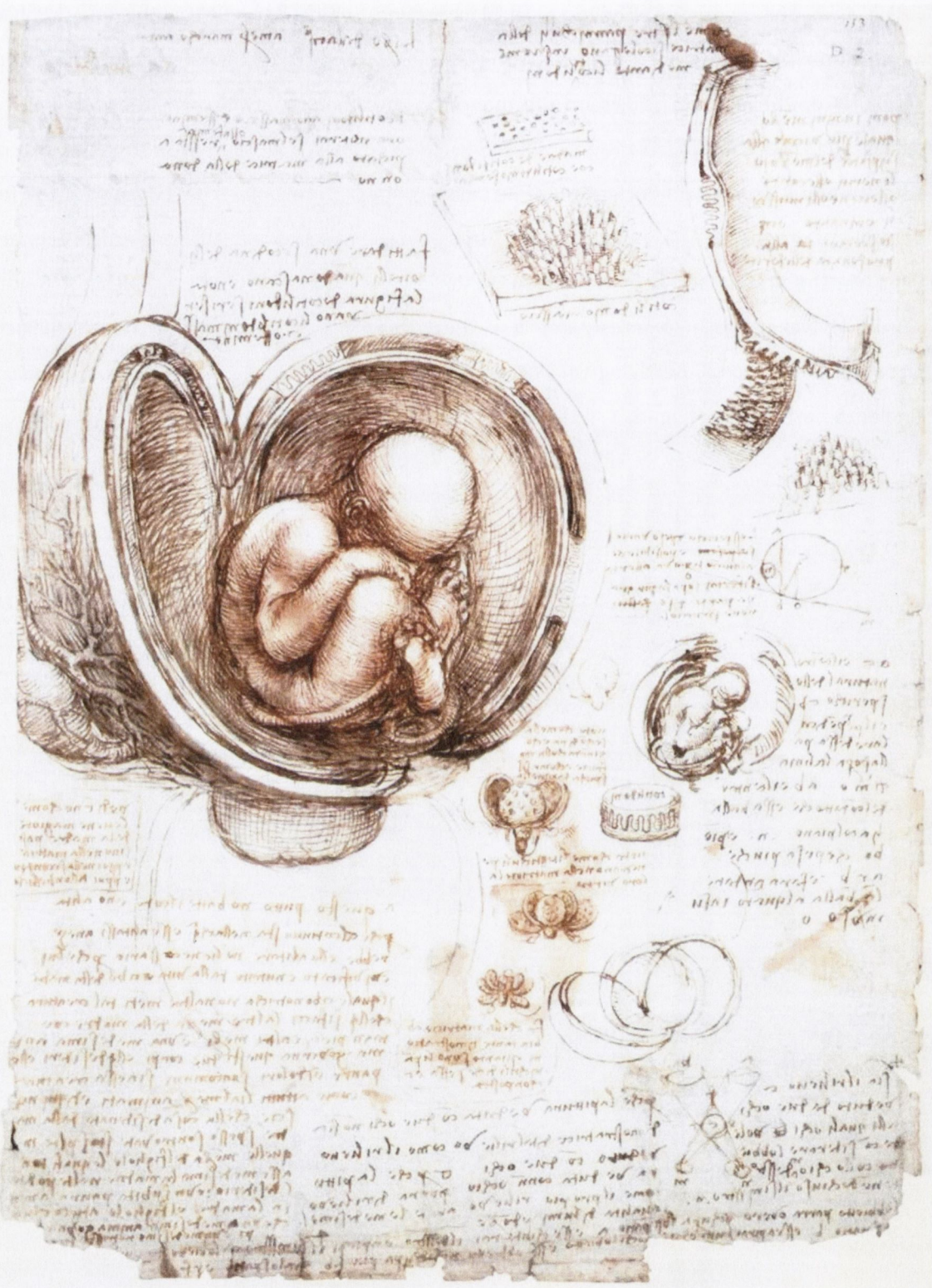


Figure 1.4-2: Leonardo da Vinci's study of the fetus in the womb completed c. 1513 demonstrates a greater understanding of ruminant than human reproductive anatomy at this period in history as human placentation was erroneously depicted the epitheliochorial placentation of the cow. (Presently located in The Royal Collection, Windsor-Castle, Windsor, UK. Source: <http://www.wga.hu/frames-e.html?/bio/l/leonardo/biograph.html>).

1.4.2 Involution

Postpartum uterine involution includes a variety of processes on physiological, histological and molecular levels, involving uterine size reduction, contraction, caruncle sloughing, necrosis, and rejuvenation of endometrial tissue (Gier & Marion 1968). The superficial layer of the caruncle tissue commences sloughing at 6 and 7 days postpartum and subsequently returns to a relatively smooth structure by 15 days postpartum (Archbald *et al.* 1972, Gier & Marion 1968). Regeneration of the caruncle sites involves epithelium coverage by 10 to 20 days postpartum in the non-pregnant horn and by 20 to 30 days in the uterine horn that was previously pregnant (Kiracofe 1980). The intercaruncular regions consist of degenerative and regenerative epithelial cells before 15 days postpartum, after which no degenerative cells are observed (Archbald *et al.* 1972).

The uterus is fully involuted when the previously pregnant uterine horn is the same size as the non-pregnant horn (Zhang *et al.* 2010). The health status of the reproductive organs influences the completion of involution which is extended significantly from 40 days to between 50 and 60 days postpartum due to anatomical damage of the endometrium, degree of infection and mucoidal uterine discharge (Hajurka *et al.* 2005).

Involution of the uterus and the resumption of ovarian cyclicity share a common goal in the provision of conception. However, follicular development may extend the duration of involution (Silvestre *et al.* 2009) and elevated bacterial contamination of the uterus during involution, may delay first postpartum ovulation (Sheldon *et al.* 2002).

1.4.3 Reproductive and Uterine Endocrinology

Cattle are polyoestrous allowing approximately 21 days between each display of oestrous behaviour throughout the year. The oestrous cycle consists of the luteal phase (14 to 18 days) and the follicular phase (for 4 to 6 days). The luteal phase is the period after ovulation during which the corpus luteum (CL) is formed and the follicular phase is the period of CL regression (luteolysis) until ovulation. The homeostatic control of the oestrous cycle is regulated by the hormones of the hypothalamus (gonadotrophin-releasing hormone; GnRH), the anterior pituitary (luteinising hormone; LH and follicle-stimulating hormone; FSH), the ovaries (progesterone; P4, oestradiol; E2 and inhibins) and the uterus (prostaglandin F2 α ; PGF) (Forde *et al.* 2011).

Subsequent to parturition, normal ovarian activity and ovulation in dairy cows is resumed within 15 to 45 days postpartum, while on average beef cows that are allowed to let calves to suckle,

return to normal ovulation within 30 to 130 days postpartum. Progesterone and oestradiol decline to basal levels at parturition, resulting in the resumption of transient increases in FSH concentrations, within 3 to 5 days postpartum, thus stimulating follicular growth and the production of the first dominant follicle by 7 to 10 days postpartum. Dairy and beef cows, undergoing the first estrus cycle display no behavioural signs of oestrus, this silent heat is followed by a short cycle (Crowe 2008, Forde *et al.* 2011). From basal levels at parturition, progesterone concentration begins to increase over 10 to 20 days postpartum in dairy cows and 30 to 40 days postpartum in beef cows (Crowe 2008).

Normally, at basal levels of progesterone, cattle, sheep, and pigs are resistant to uterine infections, however when progesterone levels increase, at the onset of luteal function, they become more susceptible to uterine infections (Lewis 2003). In a study by Seals *et al.* (2002) progesterone remained at basal level from parturition until approximately 12 days postpartum after which levels gradually increased until approximately 21 days postpartum in all cows, regardless of the presence or absence of uterine infection. The study noted that the uterine disease endometritis was associated with an increase in progesterone levels at this time. Progesterone has been shown to be an immunosuppressant, capable of suppressing neutrophil function and lymphocyte proliferation (Par *et al.* 2003, Seals *et al.* 2002, Szekeres-Bartho *et al.* 2001). Down-regulation of uterine eicosanoid synthesis is also associated with the immunosuppressive effects of progesterone, as the majority of eicosanoids have the ability to up-regulate immune cell function *in vitro*. Eicosanoids include prostaglandins and leukotrienes and are synthesised from arachidonic acid through the cyclooxygenase and lipoxygenase pathways (Hoedemaker 1992, Par *et al.* 2003). Chemoattractant eicosanoids include: PGF 2α , LTB 4 , 5-hydroxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid, and lipoxin B 4 attracting neutrophils to the uterus (Hoedemaker *et al.* 1992).

Prostaglandin F 2α (PGF) is produced in the uterus and its metabolite: prostaglandin F 2α metabolite (PGFM), detected in the circulation, is an indicator of uterine PGF production (Kindahl *et al.* 1976). The concentration of circulating PGFM is elevated from basal levels two days prior to parturition and returns to baseline levels 10 to 20 days postpartum, in cows with normal parturition (Edqvist *et al.* 1978). During the first 60 days postpartum Seals *et al.* (2002) observed that levels of PGFM were lower, except from days 15 to 21, in cows diagnosed with endometritis (uterine infection) compared to healthy cows, and suggested that monitoring the levels of levels of PGFM would aid in the early diagnosis of endometritis.

The process of luteolysis in cows involves the regression of the CL, due to a non-pregnant state, allowing the resumption of the next follicular phase of the ovarian cycle. PGF is luteolytic thus promoting luteolysis and PGE is luteotrophic which maintains the CL, especially during pregnancy, therefore extending the luteal phase in the cow (Herath *et al.* 2006, Okuda & Sakumoto 2006). Compared to normal healthy cows, animals with *E. coli* infection in the endometrium have higher LPS and PGE but not PGF in peripheral plasma. The effect of PGE accumulation in the postpartum cow results in a prolonged luteal phase extensively associated with postpartum uterine disease (Herath *et al.* 2009, Herath *et al.* 2009).

1.4.4 Postpartum Microbiology

Within the first two weeks postpartum, consistent bacterial contamination, clearance and recontamination is observed ubiquitously in bovine uteri (Azawi 2008, Sheldon & Dobson, 2004). The spontaneous clearance of bacteria, following a normal parturition, occurs within 10 – 15 days postpartum (Singh *et al.* 2008). During this timeframe postpartum, numerous bacteria have been isolated from the bovine uterus without clinical signs of puerperal metritis including *Streptococcus* spp., *Staphylococcus* spp. and *Bacillus* spp (Sheldon & Dobson 2004).

Elevated bacterial contamination of the uterus, 7 and 21 days postpartum, results in slower growth of the first postpartum dominant follicle and delayed ovulation (Sheldon *et al.* 2002). The predominant bacteria isolated from the uteri of clinically diseased cows were *Arcanobacterium pyogenes* (*A. pyogenes*), *Escherichia coli* (*E. coli*) and different gram negative (GN) obligate anaerobic bacteria: *Fusobacterium necrophrum* (*F. necrophrum*), *Prevotella* spp. and *Bacteroides* spp. (Földi *et al.* 2006, Huszeniczal *et al.* 1999, Zerbe *et al.* 2001). *A. pyogenes* contributes to clinical and reproductive consequences of uterine disease either alone or in combination with other bacteria such as *E. coli* and GN obligate anaerobes. The enhancement of uterine disease severity is achieved by the synergistic action of *A. pyogenes* and; *F. necrophrum*, *Bacteroides* spp. and *Prevotella* spp. (Földi *et al.* 2006, Sheldon & Dobson 2004, Williams *et al.* 2005).

Sheldon *et al.* (2004) categorised cultured aerobic and anaerobic bacteria, known to be associated with pathogenicity within the uterus from numerous studies, into three categories: “(1) bacteria that frequently cause endometritis, (2) bacteria that were an infrequent cause of endometritis, and (3) bacteria not recognised as uterine pathogens” (Table 1.4-1).

Table 1.4-1: Three categories of cultured aerobic and anaerobic bacteria are known to be associated with pathogenicity within the bovine postpartum uterus. (Compiled from a variety of studies and reviewed by Sheldon *et al.* 2004.)

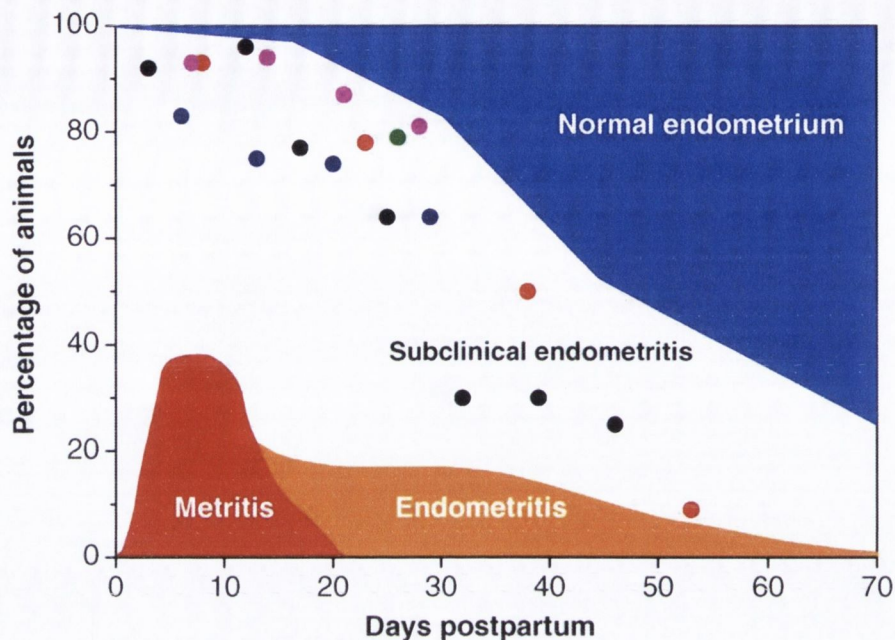
Bacterial category		
Bacteria that frequently cause endometritis	Bacteria that were an infrequent cause of endometritis	Bacteria not recognised as uterine pathogens
<i>Arcanobacterium pyogenes</i>	<i>Acinetobacter spp.</i>	<i>Aerococcus viridans</i>
<i>Prevotella melaninogenicus</i>	<i>Bacillus licheniformis</i>	<i>Clostridium butyricum</i>
<i>Escherichia coli</i>	<i>Enterococcus faecalis</i>	<i>Clostridium perfringens</i>
<i>Fusobacterium necrophorum</i>	<i>Haemophilus somnus</i>	<i>Corynebacterium spp.</i>
	<i>Mannheimia haemolytica</i>	<i>Enterobacter aerogenes</i>
	<i>Pasteurella multocida</i>	<i>Klebsiella pneumonia</i>
	<i>Peptostreptococcus spp.</i>	<i>Micrococcus spp.</i>
	<i>Staphylococcus aureus</i> (coagulase +)	<i>Providencia rettgeri</i>
	<i>Streptococcus uberis</i>	<i>Providencia stuartii</i>
		<i>Proteus spp.</i>
		<i>Propionobacterium granulosa</i>
		<i>Staphylococcus spp. (coagulase –)</i>
		α -haemolytic <i>Streptococci</i>
		<i>Streptococcus acidominimus</i>

Santos *et al.* (2011) performed the only culture-independent evaluation of the uterine microbial populations to date, describing a metagenomic profile of bacteria in the uteri of both healthy and metritic postpartum cows. Five major phyla of bacteria were defined: Gammaproteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, and Tenericutes. In metritic cows Fusobacteria, Bacteroidetes, and Proteobacteria (particularly Gammaproteobacteria) were identified. In the healthy cows the majority identified were *M. varigena* and *U. diversum*. In contrast with culture dependant results, DNA sequences for *A. pyogenes* and *E. coli* were not observed in either healthy or metritic cows.

1.4.5 Postpartum Uterine Disease

Metritis and endometritis are uterine diseases associated with high bacterial load and sustained inflammation, leading to infertility during infection and subfertility after clearance of infection (Sheldon & Dobson 2004). Subfertility in cows is defined as; “any condition leading to failure to establish a pregnancy following involution at 40-50 days postpartum” (Royal *et al.* 2000). A recent study by More *et al.* (2010) has revealed that infertility is the biggest issue for the Irish dairy industry and uterine infection and disease contributes in a major way to this problem.

The incidence of uterine bacterial infection and disease in postpartum dairy cattle.



Sheldon I M et al. Biol Reprod 2009;81:1025-1032



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Figure 1.4-3: The incidence of bacterial infection and disease in dairy cows postpartum. Each circle represents the percentage of cows with bacteria isolated from the uterine lumen and each circle colour represents a different study. The incidence of postpartum uterine disease is represented on the y-axis while the number of days postpartum is on the x-axis (Sheldon *et al.* 2009).

Postpartum uterine disease is classified as either puerperal metritis, clinical metritis, clinical endometritis, subclinical endometritis and pyometra, which cause infertility during and subfertility after resolution of disease. Their detection prior to the breeding season is important to allow early veterinary intervention and recovery. However due to the lack of clinical signs during subclinical endometritis and pyometra, these diseases can go undetected by the farmer prior to breeding, leading to infertility or subfertility (LeBlanc *et al.* 2002, LeBlanc 2008, Sheldon *et al.* 2006, 2008).

Previous studies have observed an incidence of metritis in 40% of dairy cows within one week postpartum. Consequently, clinical endometritis occurs 4 - 6 weeks postpartum in 15 - 20% of dairy cows. Metritis involves a severe inflammatory response in all the layers of the uterus. Within the first 21 days postpartum, puerperal metritis is associated with signs of systemic illness and pyrexia, whereas clinical metritis is not associated with systemic illness. Clinical endometritis has been characterized by the presence of uterine discharge consisting of purulent (>50% pus) detectable in the vagina 21 days or more postpartum and also by the presence of uterine

discharge consisting of mucopurulent (approximately 50% pus, 50% mucus) detectable in the vagina 26 days postpartum (LeBlanc *et al.* 2002, LeBlanc 2008, Sheldon *et al.* 2006, 2008).

A single case of metritis has been estimated to cost €292 on average in Europe (Drillich *et al.* 2001; Martin Sheldon *et al.* 2009). A conservative incidence rate of metritis in dairy cows is 20% and considering there were 1,060,300 dairy cows in Ireland in December 2012 (Central Statistics Office), this uterine disease cost the Irish dairy industry ~€62 million last year (Figure 1.4-3).

Subclinical endometritis is prevalent in 30 - 35% of dairy cows between weeks 4 and 9 postpartum. It is a superficial inflammation of the endometrium without clinical symptoms. Histologically, it is classified by some disruption of surface epithelium, infiltration of inflammatory cells, accumulation of lymphocytes and plasma cells to varying degrees in the superficial layers of the uterus. Subclinical endometritis has also been defined by examination of uterine cytology, using a cytobrush or low-volume uterine lavage (flush) to collect a cytology sample, to detect the presence of >18% neutrophils 21 – 33 days postpartum or >10% neutrophils 34 – 47 days postpartum (Bondurant 1999, Bonnett *et al.* 1991, Sheldon *et al.* 2008). Endometritis has been significantly associated with a lack of pregnancy by 150 days postpartum (Galvão *et al.* 2011). Both sub-clinical and clinical endometritis are conditions in which there is inflammation of the endometrium and only differ in relation to clinical appearance (Fischer *et al.* 2010). The inflamed endometrium of sub-clinically endometritic cows is a tissue phenotype resulting from underlying interactions between inflammatory stimulating molecules and mechanisms of uterine immunology.

1.5 Uterine Immunology

The Nobel Prize laureate, Elie Metchnikoff, known as the “Father of natural immunity”, promoted a comparative approach to understand immunobiology. He is quoted to have once said: “The enormous progress made in the recent years in the field of pathology will surely also fertilize the field of pure zoology and at the same time the evolutionary standpoint of the latter field can provide solutions to medical problems in a comparative pathologic way...” (Kaufmann 2008). In 1883, Metchnikoff carried out an experiment, which contributed to a paradigm shift in the study of pathology, where he used a rose thorn to challenge starfish larvae (Hydra) resulting in the accumulation of phagocytic cells at the site of intrusion (Gordon 2008, Kaufmann 2008). During bacterial infection, opsonisation-dependent phagocytosis functionally links phagocytes, the complement system and acute phase proteins.

Pattern recognition receptors (PRRs) are the primary sensors that activate the innate immune modules (Medzhitov 2007) which are present on the cell surface of innate immune sentinel cells. PRRs detect pathogen associated molecular patterns (PAMPs) on invading micro-organisms (Figure 1.5-1) or damage associated molecular patterns (DAMPs) arising from tissue or cell damage. PRRs are present on “classical” immune migratory cells such as macrophages, neutrophils and dendritic cells or “non-classical” immune/non-migratory cells such as epithelial cells and distinguish between self and non-self (Bauernfeind *et al.* 2011, Hajishengallis & Lambris 2011, Takeuchi & Akira 2010, Weindl *et al.* 2011).

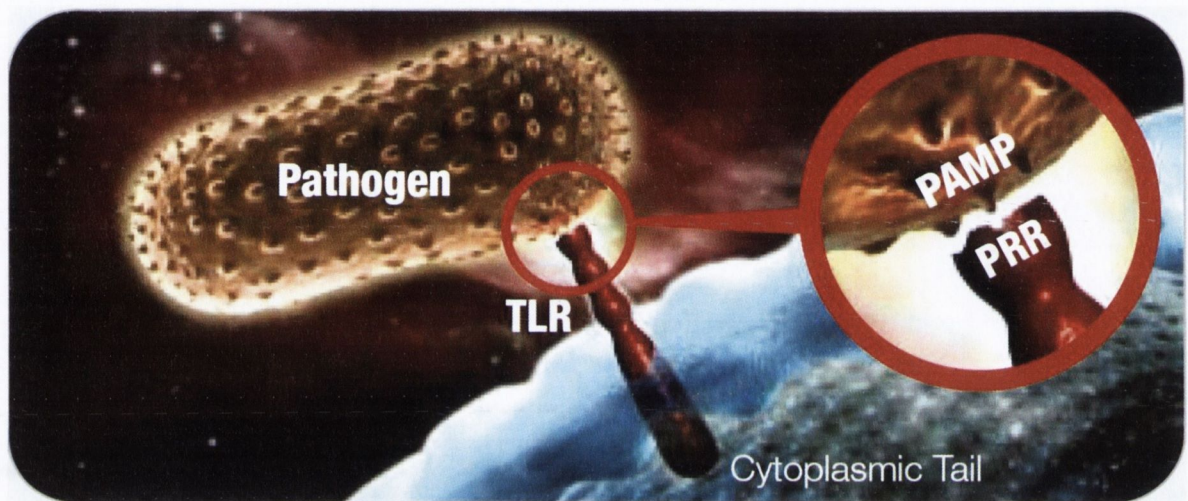


Figure 1.5-1: A pattern recognition receptor (PRR) on the hosts cell membrane recognises and binds to a pathogen associated molecular pattern (PAMP). (Picture reference: http://sabiosciences.com/newsletter/PATHWAYS07_TLR.pdf).

The response triggered, upon recognition of PAMPs and DAMPs by PRRs, resulting from microbial infection and tissue injury, stress or malfunction, is known as inflammation (Medzhitov 2008). Circa AD40 Celcus described the key clinical observations of inflammation with four Latin words: ‘rubor, calor, dolor, tumor’ meaning redness, heat, pain and swelling (Nathan 2002). Heat, redness and swelling are indicative of blood vessel dilation and increased permeability, resulting from the effects of cytokines and other inflammatory mediators, to induce increased local blood flow and leakage of fluid containing plasma proteins (Janeway *et al.* 2001, Medzhitov 2008).

A universal inflammatory ‘pathway’, includes the functional categories: inducers, sensors, mediators and effectors (Medzhitov 2008). Infection elicits various components of the inflammatory immune response with distinct functions, proinflammatory mediators coordinate the immune response, and antimicrobial effectors target pathogens directly (Foster & Medzhitov 2009). Within minutes of detecting an exogenous inducer (eg. microbial or non-microbial pathogen) or endogenous inducer (ie. molecules produced by stressed, damaged or

malfunctioning tissue) host cells capable of innate immune activation, initiate an inflammatory immune response. This occurs through PRRs (sensors) activating an intracellular signalling cascade, culminating in the relocation of transcription factors to the cell nucleus, transcription of genes and translation of pro-inflammatory protein mediators which signal and stimulate host cells (effectors), amplifying the inflammatory response to the exogenous or endogenous inducer (Foster & Medzhitov 2009, Medzhitov 2008, Smale 2010).

PRRs can be classified based on the immune function they initiate upon recognition of PAMPs. Extracellular soluble PRRs, such as mannose binding-lectin (MBL), contribute to the opsonisation of the micro-organism and activation of the complement system. Membrane bound PRRs, such as mannose-binding receptor (MR) or the scavenger receptor, on macrophages and dendritic cells (DCs) induce phagocytosis and processing of microbes for T cell presentation. Membrane bound PRR subgroup such as TLRs, induce the production of proinflammatory cytokines, chemokines and antimicrobial peptides as a result of intracellular signalling (Schenten & Medzhitov 2011) (Figure 1.5-2).

There are at least four cell-associated PRR families that recognise PAMPs and DAMPs (Bauernfeind *et al.* 2011):

- a) Toll-like receptors (TLRs)
- b) RIG-I-like receptors (RIGs)
- c) C-type lectin receptors
- d) Nucleotide-binding domain leucine-rich repeats (NLRs)

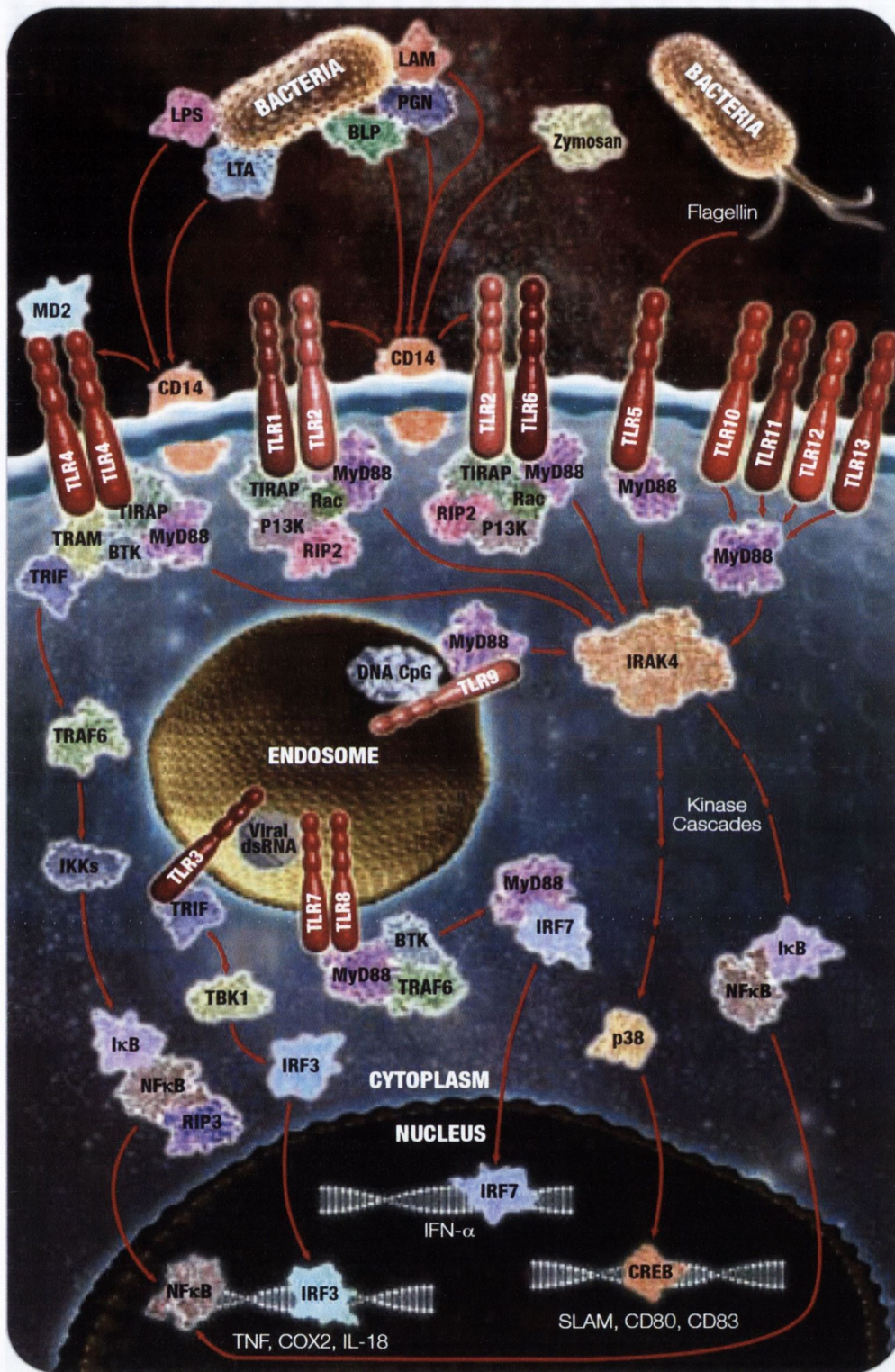


Figure 1.5-2: Toll-Like Receptors (TLRs) are membrane bound pattern recognition receptors (PRRs) that recognise pathogen associated molecular patterns (PAMPs) on the surface of pathogens and transmit a signal from the extracellular environment to inside the cell to activate the cell to produce proinflammatory molecules such as cytokines which are excreted out of the cell to aid in the killing and elimination of the pathogen. (Picture reference: http://sabiosciences.com/newsletter/PATHWAYS07_TLR.pdf).

Inflammation is triggered by infectious stimuli, such as bacterial PAMPs, and sterile stimuli in absence of infection, such as DAMPs released following tissue injury or necrotic cell death. The resulting pro-inflammatory mechanisms triggered in response to sterile tissue injury or necrotic cell death, are similar to those during microbial infection, the reason being that the same PRRs recognise both PAMPs and DAMPs. Additionally there is also a DAMP specific recognition receptor known as Receptor for Advanced Glycation End-products (RAGE), which is expressed on immune cells cardiomyocytes, neurons and endothelial cells. RAGE has the ability to recognise S100/calgranulin family members (Chen & Nuñez 2010; Hofmann *et al.* 1999).

If there is an insufficient or inadequate homeostatic host response to the stimulus or if the stimulus itself is too severe to be dealt with by acute inflammation, chronic sustained inflammation follows. Local responses by tissue-resident cells deal with less severe stimuli, such as macrophages and dendritic cells, whereas the recruitment of leukocytes and plasma proteins from the circulation is required for more intense infection and/or tissue damage. Acute inflammation is characterised by a rapid influx of neutrophils to the site of infection or tissue damage, followed shortly by monocytes that differentiate into macrophages and proliferate in the tissue, affecting the functions of macrophages resident in the tissue. If the acute inflammatory response does not deal with the infection and or tissue damage, sustained acute inflammation and tissue disturbance culminate in chronic inflammation.

During the persistence of inflammation, the host may undergo an adaptive change to promote the return of a homeostatic state, for example the induction of the acute-phase response, a systemic elevation of acute phase proteins (APPs) which helps to clear bacterial infection locally (Medzhitov 2008, Serhan & Savill 2005). Whether the inflammatory stimulant is infectious or sterile the transition from acute to chronic inflammation locally has the potential to elevate systemic molecules of inflammation to assist the host in resolving and/or eliminating the inflammatory stimulant.

1.5.1 Acute Phase Response

The acute phase response is a component of non-specific innate immunity, which is induced by tissue damage, inflammation and infection and is systemic. The release of cytokines, from immune cells and resident cells at the site of infection, into the peripheral blood circulation, target the liver stimulating hepatocytes to produce and secrete acute phase proteins (APPs). Inflammatory processes are either enhanced, or suppressed by APPs, providing antimicrobial action and preventing host damage, respectively. The acute phase response achieves a systemic

state of elevated resistance to infection (Petersen *et al.* 2004, Suffredini *et al.* 1999, Tilg *et al.* 1997) and serves the host by contributing to processes such as preventing infection, clearing pathogens, resolution of inflammation and healing (Cray *et al.* 2009) (Figure 1.5-3). Local expression of APPs in extra hepatic tissues, is either constitutive or in response to infection (Eckersall *et al.* 2006, Lecchi *et al.* 2009, Rahman *et al.* 2010, Uhlar & Whitehead 1999). There is extensive evidence in the literature of APP expression in the uterus of the cow and mare (Berg *et al.* 2011; Gabler *et al.* 2010; Lecchi *et al.* 2012; Wathes *et al.* 2009; Mette *et al.* 2010; Chapwanya *et al.* 2013). The production of APPs by endometrial cells is shown to increase in the presence of LPS (Chapwanya *et al.* 2013) and systemic levels of serum APPs are elevated during uterine disease. Therefore APPs produced by endometrial cells as a result of sustained bacterial infection during uterine disease could elevate levels of systemic APPs (Chan *et al.*, 2010; Huzzey *et al.*, 2009).

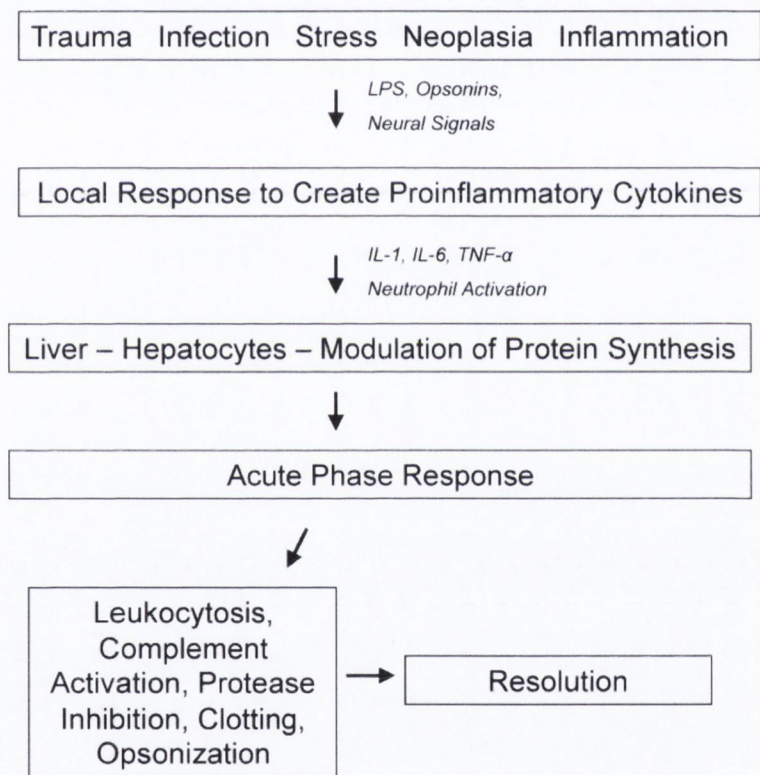


Figure 1.5-3: Initial events that precede the acute phase response begin with a stimulant such as lipopolysaccharide (LPS) on the bacterial cell wall during infection or sterile inflammation such as bruising localised to a specific organ. Either event stimulates the release of cytokines such as IL-1, IL-6 or TNF into the blood which travel to the liver cells known as hepatocytes. This induces the release of acute phase molecules which aid in the resolution of infection or sterile inflammation for example (Cray *et al.* 2009).

IL-1, TNF- α and IL-6 are proinflammatory cytokines produced in response to bacterial infection and tissue damage and they target the liver via the blood circulatory system (Murtaugh *et al.*

1996). Baumann & Gauldie, (1994) classified APPs as either type 1, which are produced by hepatocytes in response to IL-1 type cytokines (including TNF- α), or type 2, which are regulated in response to hepatocytes being stimulated by IL-6 type cytokines. Serum amyloid A (SAA) is a type 1 APP, which is characteristically elevated in serum concentration soon after infection or tissue damage and then quickly reduced to normal levels. Haptoglobin (HP) is an example of a type 2 APP, characterised by a later increase in serum concentration after infection compared to type 1 APPs and remains increased for up to two weeks post infection (Petersen *et al.* 2004).

APPs are classified as either positive or negative, if their concentration is elevated or decreased in the circulation, respectively, in response to an external or internal stimulus. In cattle, positive acute phase proteins include, SAA, HP, and fibrinogen and negative acute phase proteins include, albumin, transferrin and paraoxonase (Guzelbektes *et al.* 2010). All positive APPs have functions which include, modulation of the immune system, transport of proteins and the protection of tissue by damage associated with inflammatory processes (Cray *et al.* 2009).

In the bovine Horadagoda *et al.* (1999) demonstrated that SAA and HP were more sensitive in distinguishing between acute and chronic inflammation than neutrophil and white cell blood cell counts. Another study has shown that the acute phase response precedes clinical appearance of the disease (Huzzey *et al.* 2009). At herd level, the ability to determine subclinical disease by evaluating APP concentration in bovine serum or plasma provides valuable information with respect to prevailing infections. The severity and endurance of the systemic response may, in the future, act as a prognostic tool for certain bovine diseases (Petersen *et al.* 2004).

Parturition is associated with elevation of APP levels and as involution progresses these decrease. Bacterial contamination of the uterus during involution may additionally increase APP concentration postpartum (Sheldon *et al.* 2001). In the absence of infection or disease, parameters influencing some APP concentrations in circulation must be taken into consideration, such as, stress, age and gender. In adult cattle, the stress of transportation was shown to elevate the serum concentrations of SAA and HP in clinically healthy cows and heifers (Lomborg *et al.* 2008).

1.5.1.1 Serum Amyloid A (SAA)

Serum amyloid A (SAA) is produced and released by hepatocytes into peripheral blood circulation in response to stimulation by the IL-1 type cytokines proinflammatory. Although the liver is the predominant source of SAA, it is also produced in extrahepatic tissues, for example in the mammary gland during mastitis (Eckersall *et al.* 2006, Suffredini *et al.* 1999).

Once in circulation, the majority of SAA binds to high density lipoprotein (HDL), it may also exist in a lipid free form, highlighting its role in cholesterol transport to hepatocytes from dying cells. When SAA is bound to HDL, it does not exert its chemotaxic activity allowing HDL to act as an inhibitor of SAA in circulation in this regard (Suffredini *et al.* 1999). Other functions attributed to SAA include, fever inhibition, suppression of neutrophil oxidative burst, chemotaxic influence on monocytes, polymorphonuclear leucocytes and T cells, calcium mobilisation by monocytes and platelet activation (Petersen *et al.* 2004). SAA binds to the gram-negative bacteria ligand OmpA quickly and with high affinity, irrespective of the presence of HDL (Hari-Dass *et al.* 2005). Opsonization by SAA enhances phagocytosis of gram-negative bacteria by innate immune cells (Shah *et al.* 2006).

The potential of SAA, as an early prognostic and diagnostic for endometritis, was assessed measuring its concentration in healthy and infected cows postpartum. Within the first week postpartum, increased SAA concentrations were observed in the clinically healthy cows, after which concentrations decreased to baseline values. Elevated serum SAA levels in cows with uterine infection, persist for 2 months postpartum and then returned to baseline values (Chan *et al.* 2010). It has also been shown that endometrial cells elevate gene expression of SAA in response to *E. coli* (Chapwanya *et al.* 2013).

1.5.1.2 Haptoglobin (HP)

Haptoglobin (HP) is an acute phase protein expressed most abundantly in hepatocytes (Yang *et al.* 1983) and also in extra hepatic tissues such as white and brown adipose tissue, ovary, lung, testis, arteries and placental tissues (Friedrichs *et al.* 1995, Quaye 2008). HP is a type 2 APP, therefore its expression in hepatocytes is stimulated by the cytokine IL-6 (Nakagawa-Tosa *et al.* 1995).

The major role of HP in circulation is to bind free haemoglobin (Hb), released due to haemolysis or red blood cell (RBC) turnover (Quaye 2008). When Hb is not bound to erythrocytes, it is highly toxic, the haem group on Hb disrupts the lipid bilayer of cellular membranes and reactive oxygen species (ROS) production is enhanced by the iron group present in haem (Sadrzadeh *et al.* 1984). The binding of HP to Hb prevents oxidative damage and the release of haem (Quaye 2008).

Additional functions associated with HP include, quenching neutrophil respiratory burst, supporting reverse cholesterol transport, inhibition of COX and LOX enzymes allowing modulation of responses to inflammation or infection to prevent harm to host tissue and regeneration of tissues is promoted by HP (Quaye 2008). In cattle, HP serves as an immunomodulator and regulates lipid metabolism (Petersen *et al.* 2004).

Chan *et al.* (2010) reported an association between a poor prognosis of reproductive performance as a result of the uterine infection metritis and increased HP concentration in postpartum cows. Other studies have also identified a link between elevated HP concentration and reproductive disorders such as metritis in postpartum cows (Chan *et al.* 2004, Huzzey *et al.* 2009). A decrease in HP serum concentration in cows that had toxic puerperal metritis was associated with antimicrobial treatment (Smith *et al.* 1998).

1.5.2 Endometrial Inflammation

In the human reproductive tract, the steroid hormones, tissue injury and/or remodelling associated with physiological processes instigate localised elevation of inflammatory mediators and effectors (Goswami *et al.* 2008, Jabbour *et al.* 2009, Maybin *et al.* 2011). Sex steroids, chemokines, cytokines, growth factors and lipids are inflammatory mediators implicated in reproductive physiological processes such as ovulation, menstruation, implantation, placentation and labour (Jabbour *et al.* 2009). Natural killer (NK) cells, macrophages, B-cells and T-cells, are effectors of endometrial inflammation that accumulate and function during implantation as well as during the menstrual cycle (Maybin *et al.* 2011).

Parturition is a physiological process that imparts substantial tissue injury on the bovine uterus, such as sloughing of the superficial layer of caruncle tissue (Archbald *et al.* 1972, Gier & Marion 1968) and degeneration of the epithelial cells within the intercaruncular regions of the postpartum endometrium (Archbald *et al.* 1972). The tissue damage and disruption, sustained as a result of parturition, to the normal anatomical structure and function of the uterus, is characteristic of an open wound. Although some tissues have specialized mechanisms of healing and repair, there are features of acute wound healing that are common to all tissues, which follow a series sequential processes including: (i) coagulation and haemostasis, (ii) inflammation, (iii) proliferation of new tissue and (iv) tissue remodelling (Hart 2002a, Velnar *et al.* 2009).

Neutrophil infiltration is crucial for acute wound healing during inflammation, the lack of which encourages bacterial contamination causing protracted inflammation, thereby impeding progression to the next stage of wound healing (Hart 2002b). In addition to bacterial elimination, neutrophils possess tissue degrading proteases which assist in the removal of dead and attenuated tissue from a wound, in preparation for proliferation of new tissue (Hart 2002a). In mice devoid of neutrophils, a slower rate of endometrial repair is observed compared to wild type mice (Kaitu'u-Lino *et al.* 2007). Inflammation of the bovine endometrium is instigated early postpartum (Chapwanya *et al.* 2010, Gabler *et al.* 2010, Llewellyn *et al.* 2008, Salamonsen 2003),

evident by a predominant neutrophil infiltrate 15 days postpartum (DPP), which reduced by 30 DPP in healthy animals (Chapwanya *et al.* 2012). Once the bacterial presence is eliminated from the wound site, recruitment of neutrophils is terminated and within a few days neutrophil numbers are progressively reduced (Hart 2002a).

Subsequent to an inflammatory stimulus, neutrophils are the first leucocytes to arrive at the site of inflammation, which are then followed by monocyte-macrophages and T-lymphocytes. The recruitment of immune cells to the site of an inflammatory stimulus follows a co-ordinated process orchestrated by the release of chemoattractants and the cascades of cell chemotaxis they stimulate. Chemoattractants are a diverse group of molecules which include lipids such as LBT₄, tissue proteins such as collagen peptides, proteolytic fragments of complement components such as C3a and C5a and chemokines (Sadik & Luster 2012). Once recruited to wounded tissue, macrophages are centrally involved in continuing the debridement process, by secreting tissue destructive proteinases, and also play a crucial role instigating new-tissue formation, by secreting an array of growth, regulatory and chemotactic factors (Hart 2002a).

Local tissue injury inflicted by biopsying the endometrium preceding IVF, is associated with increased rates of pregnancy in women who previously failed to conceive with IVF or embryo transfer. Doubling their chances of conceiving and giving birth (Barash *et al.* 2003). Furthermore, biopsy-induced tissue injury has been shown to stimulate a local pro-inflammatory immune response, facilitating the preparation of a receptive endometrium for embryo implantation (Gnainsky *et al.* 2010). There are three proposed mechanisms by which endometrial receptivity is increased as a result of biopsy-induced tissue injury (Li & Hao 2009):

- a) Rapid growth of endometrial cells.
- b) Promotion of wound healing.
- c) Suspending endometrial receptivity until its development is synchronised with that of the embryo.

Adult stem/progenitor cells are responsible for cellular turn over in regenerating tissues and three types have been identified in the human endometrium, an endometrial mesenchymal stem cell (eMSC), an epithelial progenitor cell and an endothelial progenitor cell (Gargett & Masuda 2010). MSCs in particular have roles in organ homeostasis, wound healing and successful aging. These adult stem/progenitor cells are emerging as promising therapeutic agents for tissue regeneration because of their ease of preparation and immune privilege due to an immunophenotype lacking MHC class II, B7 and CD40 expression. During inflammation MSCs also play an immunomodulatory

role by suppressing the activity of neutrophils, natural killer cells, dendritic cells, T and B cells (Williams & Hare, 2011). Cells with a mesenchymal phenotype have also been isolated in the bovine endometrium (Donofrio *et al.* 2008). Haematopoietic stem cells (HSCs) have also been shown present in the human uterus throughout the menstrual cycle which are lineage-committed and differ from circulating HSCs (Lynch *et al.* 2007). However, there is no evidence in the literature at present that these cells are present in the bovine endometrium.

1.5.2.1 Cellular Inflammation in the Bovine Endometrium

The endometrium is a mixture of cells comprising of epithelial, stromal, endothelial and immune lineages (Cobb & Watson 1995, Okuda *et al.* 2010). Collectively, these cells function to protect the uterus from bacterial contamination and to repair the uterus after parturition. The approaches taken towards better understanding of endometrial function include a reductionist approach, whereby primary epithelial and stromal cells are isolated and individually challenged with PAMPs, cytokines or growth factors *in vitro* (Cronin *et al.* 2012, Davies *et al.* 2008, Okuda *et al.* 2010, Tasaki *et al.* 2010), or more holistic approaches examining endometrial biopsies or explants *ex vivo* within which the entire complement of endometrial cells are present (Borges *et al.* 2012, Chapwanya *et al.* 2009).

1.5.2.1.1 Endometrial Mixed Cell Populations

Postpartum endometrial cells act in a coordinated manner to elicit an immune response preventing bacterial infection and instigating endometrial proliferation and remodelling (Salamonsen 2003). Lipids, hormones, cytokines, growth factors and numerous other proteins and molecules, are secreted from a multitude of cells in the endometrium into the uterine lumen. The constitution of this milieu, the culmination of specific concentrations of proteins and other molecules, is the product of proactive gene expression, highly regulated to fight bacterial contamination and/or infection and repair endometrial tissue. A variety of studies have identified changes in mRNA expression relating to endometrial function at different stages of the oestrus cycle (Bauersachs *et al.* 2005, Blockeel *et al.* 2011, Fischer *et al.* 2010, Mitko *et al.* 2008), after insemination (Beltman *et al.* 2010), at implantation (Mansouri-Attia *et al.* 2009), during changes in energy balance (Wathes *et al.* 2011) and with endometritis (Fischer *et al.* 2010, Gabler *et al.* 2010, Martins *et al.* 2011).

Transcriptomic studies have demonstrated a role for the bovine endometrial immune response during pregnancy and postpartum. The endometrial immune response differs significantly in

pregnant and cycling/non-pregnant cows (Bauersachs *et al.* 2006, Groebner *et al.* 2011, Mansouri-Attia *et al.* 2009) fertile and sub-fertile cows (Walker *et al.* 2012) and pregnancies with a variety of embryo types (Mansouri-Attia *et al.* 2009). Differences in postpartum endometrial immune responses were observed between fertile and sub- or in-fertile cattle (Herath, Lilly, Santos, *et al.* 2009), cows with mild vs. severe negative energy balance (Wathes *et al.* 2009), endometritic vs. healthy cows (Fischer *et al.* 2010, Gabler *et al.* 2010, Galvão *et al.* 2011) and between early and late postpartum healthy cows (Chapwanya *et al.* 2009).

1.5.2.1.2 Endometrial Epithelial Cells

The mucosal epithelial layer in the endometrium of the uterus provides the initial physical barrier between the host and environmental pathogens. Epithelial cells act as microbial sensors distinguishing commensal from pathogenic PAMPs through membrane bound receptors, such as TLRs. Bovine endometrial epithelial cells express *TLRs 1* to *7* and *9* (Chapwanya *et al.* 2013; Davies *et al.* 2008). The secretion of antimicrobial substances by epithelial cells is either constitutive or in response to PAMPs, which adversely affects microbes through the actions of high reactive chemical molecules, pore-forming molecules and lytic enzymes (Ganz 2003). The expression of antimicrobial peptides Tracheal and Lingual Antimicrobial Peptides (*TAP* and *LAP*) and *MUC-1* are elevated in response to the PAMP, LPS in bovine endometrial epithelial cells (Davies *et al.* 2008). The recognition of PAMPs also induces chemokine secretion by epithelial cells such as *IL-8*, which attract migratory immune cells to the site of inflammation. These actions provide a non-specific response to microbes, shortly after a threat is recognised. In bovine epithelial cells the elevated gene expression of *IL-8* was observed in response to a bacterial challenge (Chapwanya *et al.* 2013).

1.5.2.1.3 Endometrial Stromal Cells

Although endometrial epithelial cells provide an important role in sensing microbes, degeneration and loss of this layer postpartum (Chapwanya *et al.* 2009) exposes stromal cells beneath to uterine bacteria. Endometrial stromal cells are also capable of sensing the bacterial PAMP LPS, through the TLR4 and MYD88-dependent cell signalling pathways, resulting in the elevation of *IL1B*, *IL6* and *IL8* (Cronin *et al.* 2012). LPS also elevates the expression of *TLRs 1* to *4*, *6*, *7*, *9* and *10* and AMPs *TAP* and *LAP* in bovine endometrial stromal cells (Davies *et al.* 2008).

In long-term culture, bovine endometrial stromal cells develop a mesenchymal phenotype and progress along an osteogenic lineage (Donofrio *et al.* 2008). Mesenchymal stem cells (MSCs) are

defined by their ability to differentiate into various mesenchymal lineages such as adipocytes, chondrocytes and osteoblasts. In human and mouse endometrium, a rare population of endometrial mesenchymal stem/stromal cells (eMSC) has also been identified (Gargett 2007, Gargett & Masuda 2010, Meng *et al.* 2007).

1.5.2.1.4 Neutrophils

A predominant neutrophil infiltrate is evident in the bovine endometrium early postpartum, as a result of tissue injury associated with the process of parturition and bacterial contamination (Chapwanya *et al.* 2010, Gabler *et al.* 2010, Llewellyn *et al.* 2008, Salamonsen 2003), which reduces as involution progresses in healthy cows (Chapwanya *et al.* 2012). Neutrophils are the first type of blood leukocytes to enter an inflamed site which phagocytose, kill and digest microbes. In the uterus neutrophils have various functions including: the recognition and phagocytosis of bacteria, intracellular killing of bacteria (Bondurant 1999) as well as a role in endometrial breakdown in mice and repair (Kaitu'u-Lino *et al.* 2007).

The elimination of ingested bacteria by neutrophils involves the generation of potent oxidants (the respiratory burst) or the release of lytic enzymes and anti-microbial peptides (AMPs) from intracellular granules. A significant proportion, 15%, of total protein in neutrophil granules is accredited to defensins. Some of them are chemo-attractants for monocytes, immature dendritic cells, and T-cells (Tizard 2004). Neutrophils also have the ability to mix these toxic compounds with their own DNA and histones and release these structures extracellularly, termed neutrophil extracellular traps (NET), to physically trap and destroy pathogens (Brinkmann *et al.* 2004). The functional capacity of peripheral bovine neutrophils has been shown to be impaired in postpartum cows (Zerbe *et al.* 2000). Polymorphonuclear cells (PMNs) separated from uterine lochial secretions of Holstein Friesian cows, with and without delayed involution and acute endometritis, had reduced killing ability as bacterial contamination of uteri increased. Transmigrated PMNs actively ingesting/functionally active in the uterus, secrete products that have modulatory effects on freshly recruited PMNs, suppressing the generation of ROS and preventing excessive host tissue damage in the bovine uterus (Zerbe *et al.* 2002).

Neutrophils possess several proteinases which help remove dead and attenuated tissue from a wound in preparation for proliferation of new tissue, such as matrix metallo-proteinases (Hart 2002a). Bovine matrix metallo-proteinase 9 (MMP-9) is secreted by neutrophils upon recognition of an opsonised microbe (Higuchi *et al.* 2007) and has been shown to be implicated in tissue remodelling of the bovine endometrium during pregnancy and pre-partum (Kizaki *et al.* 2008).

MMP-9 is expressed in the caruncle tissue of the bovine endometrium during gestation and in the postpartum period (Takagi *et al.* 2007).

1.5.2.1.5 Mononuclear Phagocytes (Monocytes & Macrophages)

Monocytes are incompletely differentiated myeloid cells when they leave the bone marrow and enter the circulation. Once they leave the circulation and enter a tissue, they differentiate into macrophages. Both monocytes and macrophages are effectors of the innate and adaptive immune responses. Macrophages are activated by microbial PAMPs and cytokines, such as INF- γ , and function to phagocytose and kill micro-organisms, secrete cytokines and present antigens to T cells (Abbas *et al.* 2007). Macrophages are capable of wound debridement by directly phagocytosing cellular debris and secreting tissue remodelling molecules into their surroundings. In addition to tissue remodelling, macrophages also secrete molecules that promote tissue growth and proliferation such as Transforming Growth Factor (TGF) alpha and beta (Hart 2002a).

Bovine macrophages are specifically localised in the parenchyma of endometrial caruncles during pregnancy and after parturition. During pregnancy, macrophages accumulate in the bovine endometrium (Oliveira & Hansen 2008), as gestation progresses macrophage numbers increase until 6-7 months. Macrophage tissue remodelling functions maintain the shape and size of caruncles during gestation (Miyoshi & Sawamukai 2004). However, the contribution of these macrophages to uterine immune function postpartum has yet to be defined (Oliveira & Hansen 2008).

1.5.2.1.6 T-Lymphocytes

T-lymphocytes are one of the principal cells of the adaptive immune system which recognise intracellular antigens presented on major histocompatibility complexes (MHCs) on the surface of antigen presenting cells (APCs). The $\alpha\beta$ classes of T-lymphocytes are CD8⁺ cytotoxic T lymphocytes, which function to kill cells infected with microbes or tumour cells, CD4⁺ helper T lymphocytes, involved in B cell differentiation and activating macrophages and regulatory T cells, which induce suppression of other T cells regulating immune responses and maintain self-tolerance. CD8⁺ and CD4⁺ T cells require antigen to be presented by APCs, such as macrophages and dendritic cells. $\gamma\delta$ T lymphocytes are not MHC restricted and are most abundantly found in mucosal tissue (Abbas *et al.* 2007). T-lymphocyte recruitment to the site of inflammation occurs later than neutrophil and macrophages recruitment. Although not essential to tissue repair, they produce regulatory and growth factors modulating the functions of other cells (Hart 2002a).

During early (33-34 days of gestation) and late pregnancy (1-14 days before parturition) CD4⁺ T cells within the peripheral blood are elevated compared to non-pregnant cows (Oliveira & Hansen 2008). A similar trend in women and mice has also been observed. These cells have been shown to express the transcription factor FOXP3, indicative of T regulatory cells which have a role in immune tolerance (Aluvihare *et al.* 2004, Peterson 2012, Somerset *et al.* 2004). During the estrous cycle, CD4⁺ and CD8⁺ T cells are increased in the bovine endometrium in the late luteal and the follicular phases (Cobb & Watson 1995). Periparturient cows (1-14 days before parturition) have more $\gamma\delta$ T cells compared to non-pregnant cows (Oliveira & Hansen 2008). $\gamma\delta$ T cells have six major roles (Vantourout & Hayday 2013) which could be essential for uterine immune function during and after bovine pregnancy:

- 1) Elimination of stressed or infected cells by cell lysis with granzymes
- 2) Production of chemokines and cytokines that regulate immune and non-immune cells
- 3) Provide assistance for B cells promoting the production of IgE
- 4) Presenting antigens for $\alpha\beta$ T cell priming
- 5) Triggering dendritic cell maturation
- 6) Regulation of stromal cell function by producing growth factors

1.5.2.2 Molecular Inflammation in the Bovine Endometrium

Endometrial functions are orchestrated and modulated by local growth factors (Tasaki *et al.* 2010), hormones (Par *et al.* 2003, Seals *et al.* 2002, Szekeres-Bartho *et al.* 2001), cytokines and chemokines (Chapwanya *et al.* 2012, Cronin *et al.* 2012), acute phase proteins and anti-microbial peptides.

1.5.2.2.1 Tumour necrosis factor α (TNF α)

Tumour necrosis factor α (TNF α) is a key mediator of the acute inflammatory response to gram-negative bacteria and is responsible for many of the systemic complications of severe infections. Mononuclear phagocytes are the major source of TNF production, although other cell types, such as T cells stimulated by antigen, natural killer (NK) cells and mast cells, also produce TNF. Neutrophil and monocyte recruitment to the site of infection are the principal biological functions of TNF, mediated by increasing the expression of endothelial adhesion molecules, stimulating endothelial cells and macrophages to secrete chemokines and neutrophils and macrophages to activate their microbicidal functions (Abbas *et al.* 2007).

A linear decrease of serum TNF α levels week 1 prepartum to 4 weeks postpartum, have been documented in disease-free healthy cows. Maintenance of higher serum TNF α levels postpartum is a result of continuing bacterial infection of the uterus in endometritic cows compared to healthy cows where a drop in serum TNF α is associated with elimination of bacterial infection in the uterus postpartum (Kim *et al.* 2005). *TNF* gene expression in the endometrium between 12 hours until 7 weeks postpartum was shown not to be time dependent (Galvão *et al.* 2011) and also not dependent on the stage of the oestrous cycle 21 to 27 DPP (Fischer *et al.* 2010). *TNF* gene expression in the endometrium was decreased one week postpartum (Galvão *et al.* 2011), elevated between 10 and 45 DPP (Gabler *et al.* 2010) and 21 and 27 DPP (Fischer *et al.* 2010) in inflamed or endometritic uteri compared to control cows.

1.5.2.2.2 Interleukin 1 (IL-1)

Another mediator of the host's inflammatory immune response to infections is IL-1 which is produced primarily by mononuclear phagocytes, also by macrophages, epithelial cells and endothelial cells. There are two forms of IL-1, IL-1 α , which is active in either a caspase-1 cleaved or non-cleaved form, and IL-1 β which is active only in its cleaved form. Low concentrations of IL-1 mediate local inflammation by stimulating increased expression of surface molecules associated with leukocyte adhesion. High concentrations of IL-1 influence the systemic inflammatory response to induce fever, APP synthesis in the liver, stimulation of IL-6 and the production of neutrophils and platelets in the bone marrow (Abbas *et al.* 2007).

During parturition, levels of IL-1 in serum are elevated, enhancing vasodilation which allows increased leukocyte migration to the endometrial tissue. Plasma calcium concentration is increased by IL-1 which stimulates contractions in the myometrium thus removing debris from the uterus. IL-1 stimulates synthesis of prostaglandin which improves contraction and evacuation of the uterus (Abbas *et al.* 2007, Kim *et al.* 2005). *IL1B* gene expression in healthy cows decreased over time postpartum, this change over time was not observed in endometritic cows (Galvão *et al.* 2011). *IL1B* gene expression in the endometrium was decreased one week postpartum, elevated 5 to 7 weeks postpartum (Galvão *et al.* 2011), 21 to 27 DPP (Fischer *et al.* 2010) and 10 to 54 DPP (Gabler *et al.* 2010) in inflamed uteri or endometritic cows compared to healthy cows.

1.5.2.2.3 Interleukin 8 (IL-8)

Chemokines stimulate and regulate the migration of leukocytes from the blood into tissues. IL-8 is an important chemokine in the recruitment of neutrophils to the site of infection or tissue

damage (Hoch *et al.* 1996). *IL8* endometrial gene expression decreased in healthy cows over time postpartum, however in endometritic cows this change over time was not observed (Galvão *et al.* 2011). *IL8* gene expression was increased between 10 & 45 DPP (Gabler *et al.* 2010), 21 & 27 DPP (Fischer *et al.* 2010) and 12 hours & 7 weeks postpartum (Galvão *et al.* 2011) in endometritic cows compared to healthy control cows.

A study in primary bovine uterine epithelial cells (pbUEC) has shown that IL-8 protein expression is higher in the supernatant of co-incubated pbUEC and PMNs than in the supernatant of pbUEC or PMN alone. Thus, PMN stimulate pbUEC to produce IL-8 *in vitro*, without the requirement of an inflammatory PAMP or DAMP (Aloé *et al.* 2012). Therefore endometrial epithelial cells further amplify inflammation in the uterus by recruiting neutrophils to eliminate bacteria.

1.5.2.2.4 Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are less than 100 amino acids in length and are secreted from epithelial and phagocytic cells. The hydrophobic regions of AMPs insert into the lipid-rich membranes of bacteria whereas the hydrophilic regions form channel-like pores or simply cover the membrane which culminates in microbial death (Tizard 2004). There are several classes of AMPs of which Cathelicidins and Defensins are the major classes. The defensins are AMPs composing 28 to 43 amino acids including 6 to 8 conserved cysteine residues. The α defensins are found in significant numbers in neutrophils and in paneth cells of the small intestine while β defensins are found in numerous tissues including the epithelial cells that line the airways, skin, salivary gland, and urinary system. Their synthesis is constitutive or is increased in response to inflammatory cytokines such as IL-1 and TNF α (Abbas *et al.* 2007, Tizard 2004).

Upon recognition of LPS, bovine primary endometrial epithelial cells up regulate the gene expression of antimicrobial peptides (AMPs) *TAP*, *LAP*, *BNBD4*, *BNBD5* (Davies *et al.* 2008). In bovine endometrial epithelial and stromal cells, gene expression of *S100A8*, *S100A9* and *S100A12* was elevated in response to LPS (Swangchan-Uthai *et al.* 2012). The S100 family proteins are involved in several cellular processes which include antimicrobial defence and calcium signalling (Abtin *et al.* 2010; Ramirez-Carrozzi *et al.* 2011).

1.5.2.2.5 Acute Phase Proteins

Inflammatory processes are either enhanced, or suppressed by acute phase proteins (APPs), providing humoral antimicrobial activity and preventing host damage (Petersen *et al.* 2004, Suffredini *et al.* 1999, Tilg *et al.* 1997). APPs are extensively expressed in the liver in response to

cytokines, however they are also expressed locally in extra-hepatic tissues including the bovine endometrium (Gabler *et al.* 2010, Wathes *et al.* 2009). APP haptoglobin (*HP*) gene expression was increased between 10 & 17 DPP and declined by 31 DPP regardless of the endometrial inflammatory profile (Gabler *et al.* 2010). In the mare, an increase in the gene expression of the APP serum amyloid A (*SAA*) in the endometrium was associated with experimentally induced endometritis (Mette *et al.* 2010). In addition to the elevated expression of this APP in response to bacteria and during infection, *SAA* has been shown to be constitutively expressed in the bovine uterus (Berg *et al.* 2011, Lecchi *et al.* 2012). Bovine endometrial cells have also been shown to elevate the expression of *SAA* in response to *E. coli* (Chapwanya *et al.* 2013).

1.5.2.2.6 Prostaglandins

Prostaglandins play key roles in the immune response, generating both pro- and anti-inflammatory effects, and have physiological roles in the female reproductive system. There are four major prostaglandins, namely:

- a) Prostaglandin E_2 (PGE_2).
- b) Prostacyclin (PGI_2).
- c) Prostaglandin D_2 (PGD_2).
- d) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

They are generated ubiquitously by all cell types and act as lipid mediators of local homeostasis. However, there is a change in both the level of production and profile of prostaglandins during an inflammatory response. The immediate increase of prostaglandins during an inflammatory response precedes the recruitment of leukocytes and immune cell infiltrate. Prostaglandins are biosynthesised from arachidonic acid (AA) by sequential actions of PGG/H synthase or by cyclooxygenase (COX) to produce PGH_2 , the common substrate for specific synthase and isomerase enzymes which produce PGE_2 , PGI_2 , PGD_2 or $PGF_{2\alpha}$ (Ricciotti & Fitzgerald 2011).

Bovine prostaglandins are secreted by the uterus under physiological conditions in order to regulate female fertility. $PGF_{2\alpha}$ is luteolytic, inducing regression of the corpus luteum, and PGE_2 is luteotrophic, sustaining the corpus luteum (Arosh *et al.* 2004). Locally prostaglandins also have a biological role in the immune response, and in tissue remodelling. PGE_2 is a mediator in the regulation of immune responses, by exerting both pro- and anti-inflammatory responses, through binding to its different receptors on many types of cells (Ricciotti & Fitzgerald 2011). Its pro-inflammatory effects are mediated primarily through dendritic cells and T cells (Yao *et al.* 2009),

whereas its anti-inflammatory effects are mediated through neutrophils, monocytes and natural killer cells (Harris *et al.* 2002). PGE₂ in combination with vascular endothelial growth factor have been implicated in the process of tissue remodelling (Kamei *et al.* 2004). The time of ovulation in cattle is synchronised by the administration of PGF_{2α} in order to induce regression of the corpus luteum, allowing ovulation of the dominant follicle (Pursley *et al.* 1995). Apart from its physiological role in fertility, PGF_{2α} is increased during acute, sub-chronic, and severe chronic inflammation (Basu 2007, Ricciotti & Fitzgerald 2011).

In the bovine endometrial cell population, there is predominant secretion of PGE₂ rather than PGF_{2α} in response to heat-killed *E. coli*, LPS, *A. pyogenes*, pyolysin, BoHV-4, bacterial DNA, and lipids. PGE₂ acts through the prostaglandin receptors 2 and 4 (*PTGER2* and *PTGER4*), which are expressed on bovine endometrial cells and are usually associated with the down regulation of inflammation. This provides a feedback loop that may be important in the regulation of the immune response to *E. coli* in the bovine uterus (Herath *et al.* 2009, Sheldon *et al.* 2009, Sheldon *et al.* 2009).

1.5.3 Regulation and Resolution of Inflammation

John Hunter was a Scottish surgeon, who in 1794 wrote: "Inflammation in itself is not to be considered as a disease but as a salutary operation consequent to some violence or some disease". His observation highlights that the usual inflammatory response outcome results in protection of the host and a phase of resolution and repair, rather than in the establishment of chronic inflammation leading to loss of tissue function and organ scarring. Events instigated early during acute inflammation, initiate this phase of resolution and repair by inducing a switch to catabolism of pro-inflammatory mediators and apoptosis of leukocytes, followed by phagocytosis which clears dying cells and transports them to the lymphatics. This reduction or removal of leukocytes and debris from the site of inflammation is an active biochemical and metabolic process. Pro-inflammatory prostaglandins are produced during acute inflammation, these influence the generation of dual action anti-inflammatory and pro-resolution lipid mediators such as lipoxins, resolvins and protectins, which are biosynthesised locally and assist in reducing the numbers of infiltrating neutrophils during the phase of resolution (Medzhitov 2008, Serhan & Savill 2005, Serhan *et al.* 2007, Serhan *et al.* 2008).

Regulation of inflammation is a requirement to address the balance between adequate inflammation to clear a pathogen and not so much that will limit host tissue damage. The phagocytic effectors of the inflammatory response, neutrophils, monocytes and macrophages, are

crucial in the host defence against pathogens. However, sustained release of their contents from lysosomal granules, such as reactive oxygen species (ROS), to the extracellular space may also result in severe tissue damage (Serhan *et al.* 2008, Steevels & Meyaard 2011, Weissmann *et al.* 1980).

1.5.3.1 Cytokines

Cytokines are anti-inflammatory mediators that inhibit or reduce phagocytic cell activity thereby controlling the innate immune response. IL-10 is an example of an anti-inflammatory cytokine released during an innate immune response that regulates the activities of macrophages and dendritic cells. The production of IL-12 and expression of co-stimulators and MHC Class II molecules by macrophages and dendritic cells are also inhibited by IL-10 (Abbas *et al.* 2007). During pregnancy, IL-10 has been implicated in the maternal tolerance of fetal allograft and its production is downregulated in placental tissue at term compared to preterm in preparation for the inflammatory process of labour (Hanna *et al.* 2000).

1.5.3.2 Pro-resolution mediators

In situations where persistent inflammation and reduced inflammatory resolution are contributing to disease pathophysiology, pro-resolution mediators such as lipoxins, resolvins and protectins, may be possible targets of therapeutic intervention (Serhan *et al.* 2007, 2008). Induction of pro-inflammatory cytokine expression in the human endometrium has been shown to be counteracted by the anti-inflammatory effects of the pro-resolution lipid mediator lipoxin A₄ (Macdonald *et al.* 2011).

1.5.3.3 Membrane bound inhibitory receptors

Inhibitory receptors on phagocytes are activated by ligands to negatively regulate an immune response, in order to allow removal of the pathogen and avoidance of tissue damage. They regulate the production of cytokines, migration of phagocytes, production of ROS, phagocytosis and induce immune cell apoptosis, via intracellular signalling through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in response to a PAMP or DAMP stimuli (Steevels & Meyaard 2011).

1.5.3.4 Non-signalling membrane and non-membrane bound inhibitory receptors

Decoy receptors are incapable of intracellular signalling however they recognise and bind to inflammatory cytokines and chemokines with high specificity and affinity to regulate their actions (Mantovani *et al.* 2001). Decoy receptors can be either membrane bound or soluble such as decoy receptor 3 (DcR3), which has been categorised as an “immunomodulator and biomarker for inflammatory diseases, autoimmune diseases and cancer” (Lin & Hsieh 2011). The maintenance of human pregnancy involves an active role of DcR3, where suppression of its production in endometrial cells, is associated with anembryonic pregnancies. In the endometrium DcR3 expression fluctuates during the menstrual cycle and is regulated by E₂ and progesterone (Chen *et al.* 2009, Lin & Hsieh 2011). Mouse and rat genomes do not contain DcR3 however DcR3-like sequences are present in cattle (*Bos taurus*) (Lin & Hsieh 2011). IL-1 receptor II (*IL-1R2*) binds IL-1 α and IL-1 β however it lacks the TIR domain and does not transmit an intracellular signal also acting as a decoy receptor and therefore buffering the pro-inflammatory effect of IL-1 α and IL-1 β (Contassot *et al.* 2012).

1.5.4 Complexity of Uterine Immunology

The complexity of the bovine uterine immune response during the pre- and post-partum period is clearly evident from what was discussed earlier and in the large number of published reviews of the literature (I Martin Sheldon *et al.* 2009; Singh *et al.* 2008; LeBlanc 2008; Leblanc *et al.* 2011; Laven *et al.* 2012; LeBlanc 2012; Bondurant 1999; Walsh *et al.* 2011). A reoccurring theme throughout these reviews is that sustained inflammation of the postpartum uterus is detrimental to bovine fertility, yet the mechanisms that initiate and sustain harmful inflammation are not yet well defined or quantified (LeBlanc 2012). There are multiple layers that must be considered in order to appreciate the complexity of the uterine immune response. At tissue level, the bovine endometrium is composed of two major layers which consist of variety of cells including epithelial, stromal, endothelial and immune lineages (Cobb & Watson 1995, Okuda *et al.* 2010). All these cells and cellular components are in constant communication, working with and against each other during physiological and sustained inflammation, ultimately to protect the host. Because dysregulated mechanisms initiating and sustaining inflammation are not yet defined to a great degree, a global holistic picture characterising the mechanisms involved during physiological and sustained inflammation must be characterised. Approaches examining endometrial biopsies or explants *ex vivo* will be advantageous for characterising and comparing the mechanisms involved during physiological and sustained inflammation of the endometrium as the entire

complement of endometrial cells and thus cellular components are present (Borges *et al.* 2012; Chapwanya *et al.* 2009).

Genomics is an important approach towards addressing the complexity associated with comparing physiological and pathological states in the bovine endometrium (Forde *et al.* 2012; Foley *et al.* 2012). Genetic code is present within cells and tissues of all living organisms. However, utilisation of the genetic code begins with transcription of specific genes elevated as the result of a particular physiological and pathological state. The full complement of genes that are transcribed within cells and tissues is known as the transcriptome. State of the art technologies such as RNA-Seq, an application of next generation sequencing, aims to reveal the whole transcriptome to characterise, identify and catalogue all genes expressed within cells and/or tissues and to potentially quantify differential expression in particular physiological and pathological states (Hitzemann *et al.* 2013; McGettigan 2013). Based on previous annotated functional studies, RNA-Seq data analysis pipelines can predict the functional inter-relationship between genes, such as pathway analysis and gene network construction which suggest potential mechanisms that differentiate physiological and pathological states (Hitzemann *et al.* 2013). Despite the heterogeneity of cells, molecules, microbial populations, hormones etc. in the postpartum uterus, RNA-Seq has the potential to highlight mechanisms vital to dealing with and avoiding sustained endometrial inflammation.

1.6 Genomics & Transcriptomics

Genomics combines genetics and molecular biology, within which the aims are to generate a complete data set of all the genes in an organism (known as the genome) and the interactions of genes with each other and with the organism's environment. (Green & Guyer 2011, Ziogas & Roukos 2011).

The transcriptome is the entire complement of transcript species in a cell or tissue, inclusive of mRNAs, non-coding RNAs and small RNAs. The aims of transcriptomics are three fold (Wang *et al.* 2009):

- 1) To catalogue the species of transcripts (both coding and non-coding mRNA and non-coding microRNA).
- 2) To uncover the structure of transcribed genes with respect to their 5' and 3' ends, patterns of splicing and other modifications post-transcription.

- 3) Quantifying the altered expression levels of individual transcripts for a physiological condition, developmental stage or disease.

The importance of transcriptomics was highlighted by the launch of the Genotype-Tissue Expression (GTEx) project in 2010, supported by the National Institutes of Health (NIH), which endeavours to assist researchers to comprehend inherited susceptibility to disease by providing a database and tissue bank for gene expression studies. Within individual tissues global RNA expression will be analysed to assess gene expression levels and quantitative traits. Genetic variation is highly correlated with variation in gene expression which is identifiable as expression quantitative trait loci, or eQTLs (<http://commonfund.nih.gov/GTEx/>).

As the study of genomics and transcriptomics has advanced over recent years, the tools with which genomes and transcriptomes are profiled have progressed in parallel, to achieve greater capacities in data collection and analysis. The emergence of the next generation sequencing application mRNA-Seq permits the surveillance of the entire transcriptome and has many advantages over real-time qRT-PCR and microarrays, such as its capacity to achieve superior digital readout (Wang *et al.* 2009).

1.6.1 Next Generation Sequencing

The Illumina Genome Analyzer is a second generation NGS platform that utilises Illumina sequencing technology which relies on the random attachment, of prepared DNA library fragments, onto a flow cell which is an optically transparent surface (Figure 1.6-1). Extension and bridge amplification of the attached DNA fragments occurs, to generate clusters composed of ~1000 copies of the same template. Each template is then sequenced by initially adding four bases differentially labelled with removable uniquely coloured fluorescent dyes and a blocking molecule. Once the complementary base is bound to the DNA template the blocking molecule prevents subsequent bases from attaching to the template strand and unbound bases are removed. This facilitates the high-sensitivity fluorescence detection of the specific base present, as only the attached base will fluoresce when using laser excitation and total internal reflection optics. After detection, the blocking molecule and fluorescent tags are removed and the process is repeated until roughly 40 – 50 base pair (bp) fragments of the template strand are sequenced. (www.illumina.com/technology/sequencing_technology.ilmn).

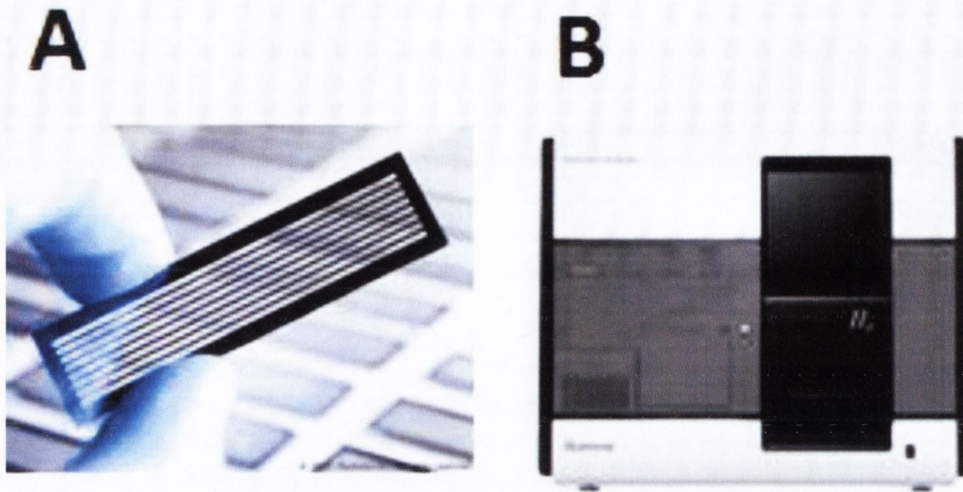


Figure 1.6-1: RNA sequencing involves the preparation of an RNA-Seq library which converts mRNA or microRNA to cDNA fragments preparing it for next generation sequencing. cDNA fragments are attachment to a sequencing flow cell (A) and subsequently high-sensitivity fluorescence detection, in the Illumina Genome Analyzer (B) for example, sequences each base in each cDNA fragment. Sequencing output then requires further data analysis to assess transcriptomic differences between samples of interest.

Next generation sequencing (NGS) has been effective in the application of cell-free nucleic acid for biomarker detection and diagnostics. Fetal trisomy 21 was detected by applying NGS to the free-cell DNA of maternal plasma with high sensitivity and specificity (Chiu *et al.* 2011, Ehrich *et al.* 2011). Snyder *et al.* (2011), through NGS, observed that rejection episodes after heart transplants were associated with elevated concentrations of donor derived DNA in plasma, a characteristic of graft rejection which is in essence a form of tissue damage. Cattle infected with transmissible spongiform encephalopathies (TSE) were shown to have disease-specific patterns of circulating nucleic acids predicted to be associated with the release of DNA into plasma from the neuronal death caused by TSE. Specific patterns of circulating nucleic acids associated with TSE were observed prior to the onset of clinical disease, Bovine spongiform encephalopathies (BSE) (Gordon *et al.* 2009).

1.6.2 mRNA and microRNA Sequencing (RNA-Seq)

mRNA-Seq and microRNA-Seq, or collectively RNA-Seq, are applications of NGS that use a sequence based approach to determine the cDNA sequence of transcripts directly and is not limited to the detection of transcripts corresponding to candidate genomic sequence. This characteristic makes it advantageous to existing technologies such as qRT-PCR and microarray methods, which rely upon existing knowledge of the genome sequence. mRNA-Seq and microRNA-Seq are not limited by the pre-selection of specific candidate genes for absolute gene

expression analysis and achieves superior digital readout of gene sequence compared to relative fluorescence quantification of qRT-PCR and microarrays (Wang *et al.* 2009). Advances in RNA-Seq methodology allow enhanced ability compared to microarrays for, mapping at transcription start sites (TSSs), cataloguing of sense and antisense transcripts, detection of gene fusion transcripts and detection of alternative splicing events. Recent new developments in RNA-Seq technologies include, direct RNA sequencing (DRS) and the reliable profiling of minute RNA quantities (Ozsolak & Milos 2011).

1.6.3 Advantages and Limitations of RNA-Seq

The most notable contributions of short-read sequencing associated with RNA-Seq is the elucidation of the complexity of the transcriptome (Costa *et al.* 2010) encompassing novel data that have uncovered isoform and allelic expression, elongated 3'UTR regions, defined new splice junctions, forms of antisense regulation and intragenic expression. The quantification of gene expression using RNA-Seq is defined by the number of short-reads mapped to a particular transcript, exon or gene which is an estimation of these features expression level (Carninci *et al.* 2005; Graveley *et al.* 2011; Nagalakshmi *et al.* 2008; Trapnell *et al.* 2010). However in the course of estimating differential gene expression there are specific biases that arise during the statistical analysis of the data which are within-sample gene-specific, such as gene length (Oshlack & Wakefield 2009) and GC content (Pickrell *et al.* 2010) or between-sample effects, such as sequencing depth (Dillies *et al.* 2013; Sims *et al.* 2014) and RNA composition (Robinson & Oshlack 2010).

The length of the transcript, exon or gene under investigation has to be accounted for within the analysis of the data, as longer features will ultimately result in more reads than shorter ones (Marioni *et al.* 2008). A normalization method to account for this bias is the RPKM method (reads per kilobase of exon per million mapped reads) (Mortazavi *et al.* 2008) however this method has been shown to be ineffective at normalizing for gene length bias (Bullard *et al.* 2010; Oshlack & Wakefield 2009) for Illumina high-throughput RNA-Seq data in particular (Dillies *et al.* 2013). However statistical analysis packages for high-throughput RNA-Seq data such as TMM (Trimmed Mean of *M*-values) edgeR (Robinson & Oshlack 2010) and DESeq (Anders & Huber 2010) do not need to adjust for gene length bias as each gene is directly compared and therefore the genes length has the same relative influence on the number of read counts in each sample of RNA.

Sequencing depth is defined as “the average number of times that a particular nucleotide is represented in a collection of random raw sequences” and sequencing coverage is achieved by

multiplying the average read length by the number of reads sequenced and dividing by the haploid genome length (Sims *et al.* 2014). The standards, guidelines and best practices for RNA-Seq analysis by the Encyclopaedia of DNA Elements (ENCODE) consortium (version 1.0 June 2011) recommend a modest sequencing depth for the evaluation of similarity between the transcriptional profiles of two polyA+ samples. The example given was 30 million pair-end reads of length greater than 30 nucleotides, of which 20-25 million align to the genome. Seven sequencing platforms have been compared in a recent review by Sims *et al.* (2014) which demonstrated that the Illumina HiSeq 2000 achieved greater than 30x cover of the human genome and produced the highest number of reads per run. Statistical packages available for RNA-Seq data analysis, such as edgeR, account for varying sequencing depths as represented by different library sizes (Robinson *et al.* 2010). An additional feature of edgeR normalises for RNA composition using the TMM method between each pair of samples. The concept behind this normalisation is that highly expressed genes can take up a large proportion of the total library size, at the expense of remaining genes which may be under-sampled which may lead to the false assumption that these genes are down-regulated in that sample (Robinson & Oshlack 2010). However a study by Tarazona *et al.* (2011) suggests that it remains to be seen what the effect of sequencing depth has on the normalization procedures such as TMM. In this study the authors illustrate that by increasing sequencing depth enhances the view on the diversity of the transcriptome however limitations arise at both:

- a. Low sequencing depth – as the identification of positive differential gene expression is more difficult to achieve.
- b. High sequencing depth – as the implication of more reads is the detection of more genes which makes the data noisier making differential gene expression more difficult.

Increasing the sequencing depth with limited biological replicates per treatment for example increases the number of false positive differential expression calls because transcripts that are of low abundance will be detected however their biological relevance can only be definitively modelled through replication. Future improvements for RNA-Seq would include lower prices, allowing researchers to increase the sample size therefore reducing the effect of biological variation (Sims *et al.* 2014).

1.7 Thesis hypothesis, aim and specific objectives

1.7.1 Project Outline

Two distinct studies were performed, an initial pilot study optimised the experimental design, data analysis pipe line and the results informed a more in-depth comprehensive second study. Both studies utilised next generation sequencing technology and RNA-Seq to assess the temporal gene expression in endometrial tissue over two postpartum time points during involution. The pilot study examined physiological endometrial inflammation in healthy animals and the second study evaluated sustained endometrial inflammation in diseased animals during involution. In addition to transcriptomics (mRNA and microRNA) the second study also incorporated haematology, protein expression profiling, metabolite analysis and microbiology.

1.7.2 Hypothesis

Alteration in the early postpartum uterine and systemic immune profile will differentiate healthy cows from cows at risk of developing uterine disease.

1.7.3 Aims

This study aims to firstly define the extent of inflammation in all cows at two time points postpartum during uterine involution and categorise cows as either healthy or sub-clinically endometritic, secondly to characterise the temporal transcriptomic differences within and between groups and thirdly to characterise the systemic profile within and between groups at each time point. This will be carried out in endometrial swabs and biopsies by analysing microbiology, histology and the mRNA and miRNA transcriptome using mRNA-Seq and by assessing whole blood and plasma for cell counts and protein concentration.

1.7.4 Specific Objectives

1. To compare transcriptome profiles between healthy and diseased animals at two early postpartum time points.
2. To compare microbiological populations between groups.
3. To integrate transcriptomic and traditional methods for analysis of physiological and immunological changes in postpartum cows

4. To validate local immune changes at a systemic level in both treatment groups towards the identification of biomarkers for uterine disease.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Experimental Design

In the pilot study endometrial biopsies were collected as part of a previous study at 15 and 30 days postpartum (DPP) from 13 beef cows using the Hauptner equine endometrial biopsy instrument. Biopsies were snap frozen in liquid nitrogen and stored at -80°C. Endometrial tissue inflammation was classified based on histological assessment (Chapwanya *et al.* 2012, 2010). In this study the endometrial transcriptomic profile was assessed by mRNA-Seq (n=3) and candidate gene expression was measured by real-time qRT-PCR (n=5) comparing 15 DPP to 30 DPP samples (Figure 2.1-1). There was one animal common to both analyses. At 15 DPP all samples were classified as grade 3 inflammation and for 30 DPP samples with: mRNA-Seq analysis n=2 grade 1 and n=1 grade 2 inflammation, and real-time qRT-PCR analysis n=4 grade 1 and n=1 grade 2 inflammation.

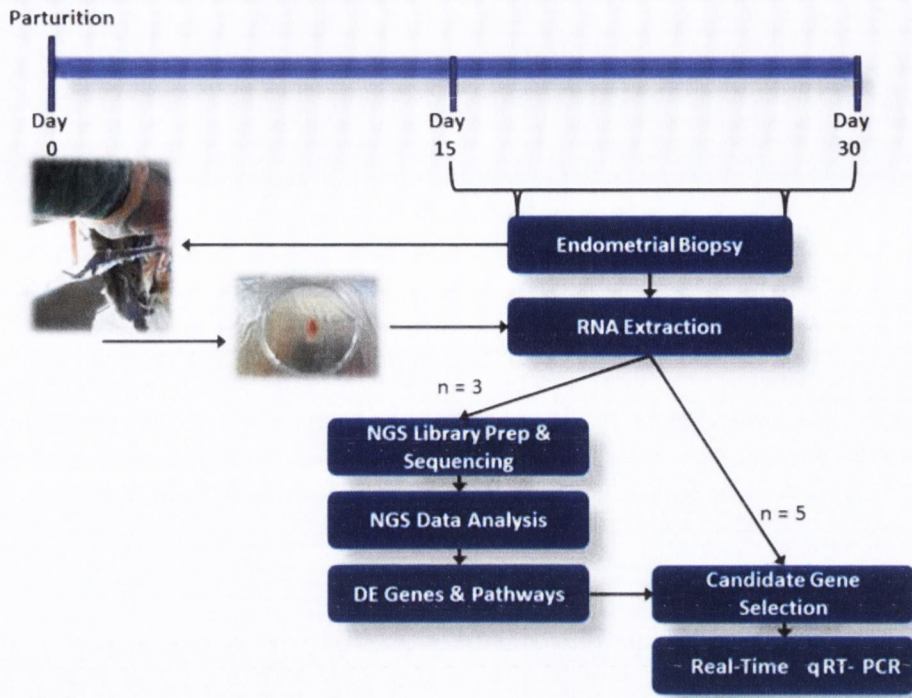


Figure 2.1-1: Flow diagram displaying the experimental design from biopsy collection to gene expression analysis. Endometrial biopsies were collected 15 and 30 days postpartum (DPP) from which RNA was extracted and processed for mRNA-Seq and next generation sequencing (NGS) analysis (n=3). Subsequently candidate genes were selected for validation of gene expression using real-time qRT-PCR.

In the main study twenty two Holstein Friesian cows, of mixed parity, within the same university dairy herd were sampled 7 and 21 days postpartum (DPP) in the morning after milking, over two months. In both the pilot and main study a clinical examination and sample collection was conducted by a veterinarian (Dr. Aspinas Chapwanya), clinical examination involved recording the rectal temperature, body condition score, heart rate and vaginal discharge.

Two cows were removed from the main study due to health complications, cow 3 had a severe womb infection and heart problems and received a selection of drugs for both problems and cow 19 had retained the placenta after birth and was treated with Solvasol for 5 days. Cows 10 and 15 received local administration of Synulox Lactating Cow tubes (Pfizer Animal Health, Ringaskiddy, County Cork, Ireland) to treat mastitis but these cows remained in the study as the withdrawal period of 7 days was surpassed when the biopsies were collected.

The perineum and external genitalia were cleaned using detergent in warm water and 70% ethanol. A sterile disposable speculum (minitube®, Hauptstrasse 41, 84184 Tiefenbach, Germany) was inserted into the vagina and cervix, with the aid of veterinary lubricant, through which a double sheathed uterine culture swab and cytology brush (minitube®, Hauptstrasse 41, 84184 Tiefenbach, Germany) were passed, enhancing accuracy of sampling and avoiding faecal and vaginal contamination of these instruments. The samples were processed once collected which involved placing duplicate uterine culture swabs per cow in 1ml of Tris-EDTA buffer (Sigma Aldrich® Vale Road, Arklow, Wicklow, Ireland). A single cytology brush sample was collected per cow and placed in 1mL of RNA^{later}®, this was not used in downstream analysis as part of this PhD (Sigma Aldrich® Vale Road, Arklow, Wicklow, Ireland). A sterile Hauptner equine endometrial biopsy instrument was covered with a coil sanitary sleeve (IMV Technologies, ZI No. 1, East 61300 L'Aigle, France) and veterinary lubricant was added to the outside of the sleeve. Once the disposable speculum was removed the vet introduced the Hauptner biopsy instrument into the vagina and guided it through the cervix by manipulation *per rectum*. The coil sanitary sleeve was perforated at the external cervical orifice and guided into the post-gravid uterine horn past the bifurcation. Repeat biopsies were taken from the post-gravid horn 7 and 21 DPP in the same cow. Immediately after collection sample processing was performed by placing the biopsy on a sterile petri dish and dividing it in half, one half was snap frozen in liquid nitrogen, for downstream transcriptomics work, and the other half was fixed in formalin solution (Sigma Aldrich® Vale Road, Arklow, Wicklow, Ireland) for downstream histology analysis. Veterinary records over the course of sampling were acquired from the farm manager and a pregnancy diagnosis was carried out on all cows 60 days after artificial insemination, with ultrasound taken by members of Lyons farm

staff. The main study was designed to uncover differences between sub-clinically endometritic and healthy cows, between groups (SCE v Healthy) at either 7 or 21 DPP or within groups over time between 7 and 21 DPP. The independent variables of this experimental design were sub-clinically endometritic cows, healthy cows and day postpartum (7 or 21 DPP). The dependent variables of this experimental design included gene expression (mRNA and microRNA) and microbiology (Figure 2.1-2).

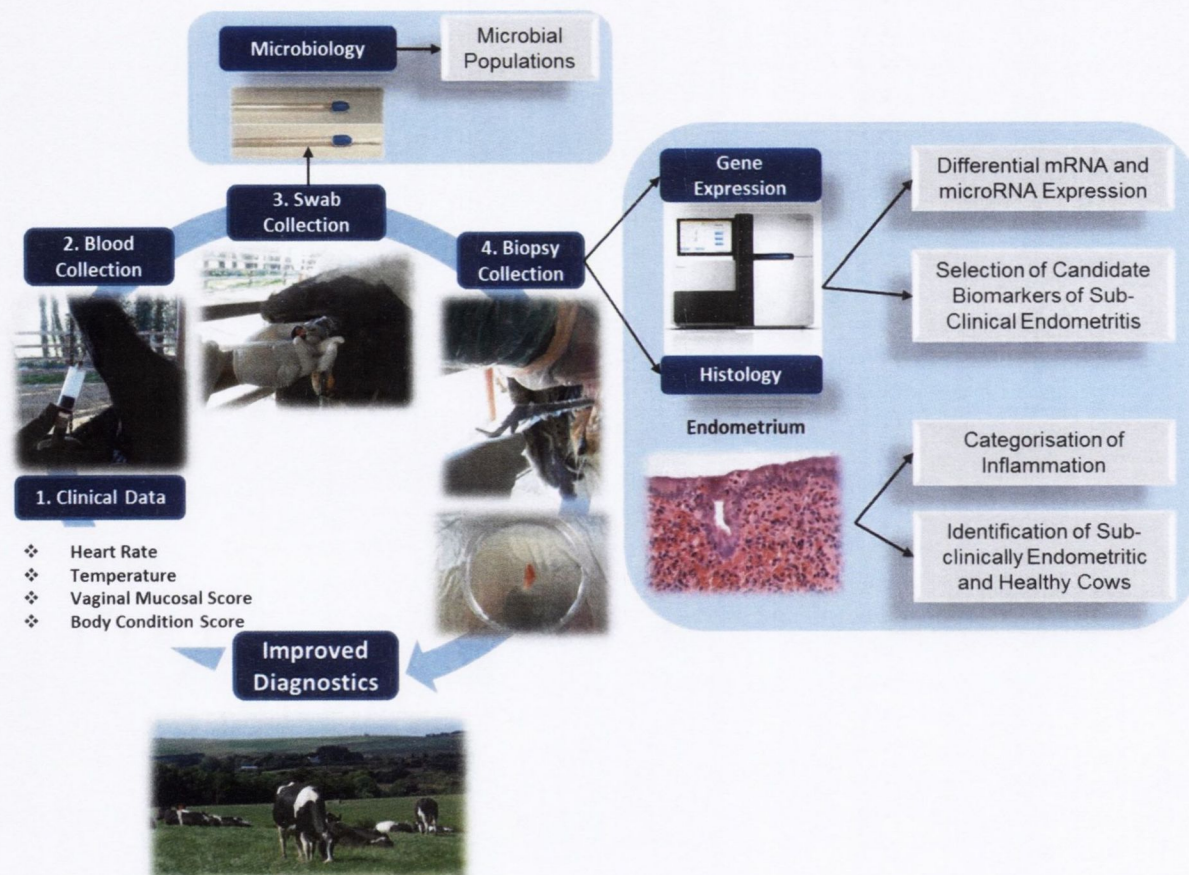


Figure 2.1-2: A flow diagram of experimental design to characterise the regulation of the local endometrial immune response in healthy and sub-clinically endometritic cows 7 and 21 days postpartum. Endometrial swabs were assessed to differentiate microbial populations. Endometrial biopsies were used for differential gene expression and histological assessment of endometrial inflammation. The samples, techniques and apparatus used are highlighted in light blue.

Whole blood collected 7 and 21 days postpartum in 22 postpartum cows 7 DPP, 21 DPP and once pre-partum from 8 animals was analysed. Prior to blood collection the area of skin near the coccygeal vein was cleaned and sterilised using detergent in warm water and 70 % ethanol. 10ml of blood was collected in one spray-coated K₂EDTA Vacutainer®, for haematology and plasma protein analysis, followed by 4ml of blood in one sodium fluoride/Na₂ EDTA Vacutainer®, for blood metabolite analysis. Veterinary records over the course of sampling were acquired and a pregnancy diagnosis was carried out on all cows 60 days after artificial insemination by means of ultrasound.

For quantification of plasma proteins cow 1 was dropped, which was included for the investigation of the local transcriptome, as plasma was not collected 7 DPP. However there was whole blood, for whole blood cell counts, and plasma, for metabolite analysis, collected for cow 1.

This experiment was designed to uncover systemic differences between sub-clinically endometritic and healthy cows, comparing pre-calving, sub-clinically endometritic cows at 7 and 21 DPP and healthy cows 7 and 21 DPP. The independent variables of this experimental design were pre-calving (PC) cows, sub-clinically endometritic (SCE) cows, healthy cows and day postpartum (7 or 21 DPP). The dependent variables of this experimental design included whole blood cell counts, blood plasma metabolites and proteins (Figure 2.1-3). The experimental design was the same for all dependant variables, whereby significance was assessed across all five groups using a non-parametric one way ANOVA. Subsequently for significant dependant variables, comparisons were made between two groups using the combinations in Table 2.1-1.

All experimental procedures were carried out under license from the Irish Department of Health and Children in accordance with the European Community Directive 86-609-EC and were approved by the Animal Research Ethics Committee, University College Dublin.

Table 2.1-1: **Various combinations of independent variables (highlighted in yellow) used to compare dependent variables to profile the systemic immune profile. Independent variables include: Pre-calving (PC) subset of cows, sub-clinically endometritic (SCE) cows, healthy cows, 7 and 21 days postpartum (DPP).**

	PC	SCE 7 DPP	SCE 21 DPP	Healthy 7 DPP	Healthy 21 DPP
PC					
SCE 7 DPP					
SCE 21 DPP					
Healthy 7 DPP					
Healthy 21 DPP					

*Yellow boxes indicate comparisons made between independent variables in rows and columns.

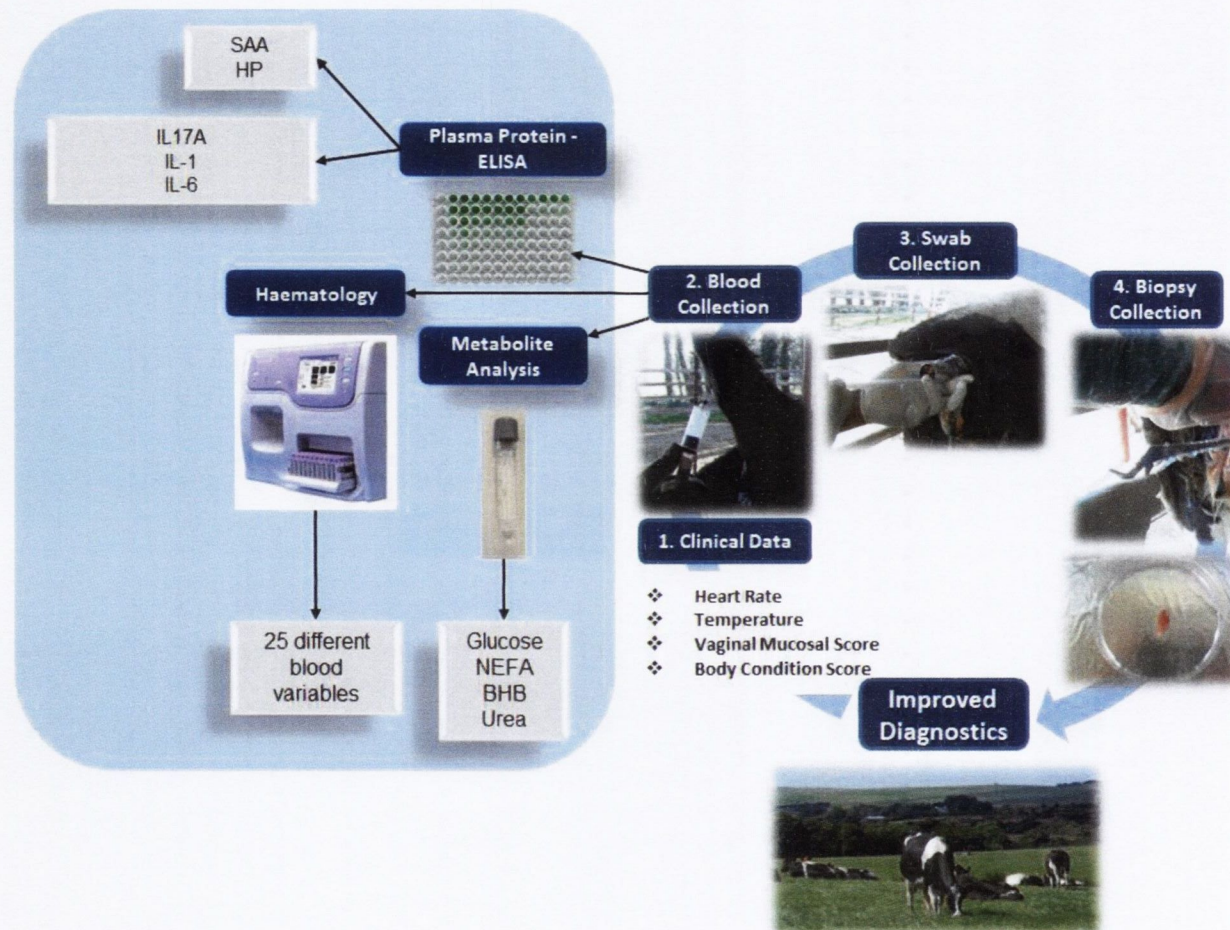


Figure 2.1-3: A flow diagram of experimental design to characterise the systemic haematological, metabolite and immune profile using whole blood from healthy and sub-clinically endometritic 7 and 21 days postpartum. The samples, techniques and apparatus used are highlighted in light blue.

2.2 Total RNA Isolation, Quantification and Quality Assessment

2.2.1 RNA isolation

Method 1a outlined below was implemented for RNA isolation and in chapter 3 initially two different protocols, method 1 and method 2, were analysed to optimise the isolation of RNA using bovine liver and endometrial non-test biopsies prior to using test samples. The first method is the “Method 1” below which was the optimal protocol and subsequently used for test biopsies.

Method 1a: Prior to RNA extraction from biopsies the fume hood and instruments were cleaned with 70 % ethanol and treated with Ambion® RNaseZap® (Life Technologies™, Paisley, UK). To accurately record the weight of each biopsy a 2 ml microcentrifuge tube with 600 µl of TRIzol® (Life Technologies™, Paisley, UK) was weighed on a laboratory precision balance. Immediately after removal from -80 °C the biopsy was placed into the same microcentrifuge tube with TRIzol® and weighed once more. A Pro Scientific homogeniser model PRO200 (PRO Scientific Inc., Oxford, CT, USA) was used to homogenise the biopsy in TRIzol® with 3 sec pulses 10 times. Once all biopsies were homogenised a further 400 µl of TRIzol® was added to achieve a final volume of 1ml of TRIzol®, tubes were inverted and incubated at room temperature for 5 min. 200 µl of chloroform was then added, tubes were vigorously shaken for 15 sec and samples were incubated at room temperature for 3 min. Samples were then centrifuged at 12,000 x g for 15 min at 4 °C. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube; 500 µl isopropanol was added, mixed by inverting the tubes, incubated at room temperature for 10 min and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was removed and the RNA pellet retained, to which 1 ml of 75 % ethanol was added, tubes were pulse vortexed at low speed and centrifuged at 12,000 x g for 5 min at 4 °C. The supernatant was removed and the RNA pellet was air dried for 5-10 min and then reconstituted in 30 µl RNase-free water (Sigma-Aldrich).

Method 1b: Total RNA was then cleaned up using the Qiagen RNeasy® Plus Mini kit (Qigen Ltd) by adding 320 µl RLT buffer and adding the final volume of 350 µl to a gDNA eliminator column and centrifuging at 8000 x g for 30 sec. 350 µl 70 % ethanol was added to the flow through and mixed well by pipetting up and down. 700 µl of sample was added to an RNeasy spin column and centrifuged at 8,000 x g for 15 sec. The flow through contained RNA fragments less than 200 nucleotides and immediately frozen at -80 °C for downstream miRNA analysis. RNA fragments greater than 200 nucleotides remained on the RNeasy column to which 700 µl of RW1 buffer was added then centrifuged at 8,000 x g for 15sec and flow through was discarded. 500 µl RPE was added to the column and centrifuged at 8,000 x g for 15 sec which was repeated except

centrifuged for 2 min the second time. The column was centrifuged once more at 10,000 x g to remove excess liquid and then placed in a new 1.5 ml microcentrifuge tube. 30 µl of nuclease-free H₂O was added to the column which was centrifuging at 8,000 x g for 1 min, the flow through was retained as it contained total RNA and it was immediately placed at -80 °C.

Method 2: This method differed from “Method 1” in the following ways (a) the upper aqueous layer was transferred directly to the gDNA eliminator column of the Qiagen RNeasy® Plus Mini kit (Qigen Ltd) (b) RNA was not precipitated prior to addition to the gDNA eliminator column.

2.2.2 RNA quantity and quality assessment

The NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) calculated RNA concentration based on absorbance readings, the accuracy of these readings vary depending on extraction methods and residual molecules eg. proteins. The instrument was initialised by adding 1.5 µl dH₂O to the lower optic surface. The appropriate nucleic acid and constant 40-RNA was selected and the absorbance was set at A₂₆₀. The instrument was blanked with 1.5 µl dH₂O, 1.5µl of sample was loaded and the RNA concentration was recorded.

The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the quality of RNA extracted. RNA concentrations from the NanoDrop spectrophotometer reading were diluted to achieve 3-5 µl at 100 ng/µl concentration. Reagents were equilibrated to room temperature for 30min pre-use. 350 µl RNase Zap and RNase-free water were used to clean the electrodes in the bioanalyser. The gel was prepared by the addition of 550 µl Nanogel matrix into a spin column and centrifugation for 10 min at 13,000 x g. To 65 µl of the filtered gel 1 µl of dye was added. The gel-dye mixture was vortexed and centrifuged at 1,500 x g for 10 min. Sample and ladder were denatured for 2 min at 70 °C. The chip was placed in the chip priming station and 9 µl of the gel-dye mixture was added into appropriate well. The attached syringe was adjusted to 1 ml and the plunger was pressed down until held by a catch. After 30sec the catch was released and after 5 sec the plunger was slowly released to the 1 ml mark. The chip priming station was opened and 9 µl of the gel mix into two appropriate wells on chip. 5 µl of RNA 6000 Nano marker was added into the ladder well and each of 12 sample wells. 1 µl of sample and ladder were added to the appropriate wells. The chip was vortexed for 60 sec in the vortex mixer (Agilent Technologies), after which the chip was run in the Bioanalyser.

Additionally the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), as described above, calculated the 260/230 ratio values which are usually

between 2.0 and 2.2. If the ratio was lower than 2 it may have indicated the presence of contaminants which absorb at 230 nm.

2.3 mRNA Seq Library Preparation & Sequencing

2.3.1 mRNA-Seq library preparation

mRNA-Seq library preparations were carried out to convert total mRNA into a cDNA library specific for next generation sequencing. Two different kits used and protocols were followed as per manufactures instructions. In the pilot study the Illumina® mRNA Sequencing Sample Preparation Kit (Protocol: September 2009) was used and for the main study the Illumina® TruSeq® RNA sample preparation kit v2 was used (Chesterford Research Park, Little Chesterford, Nr Saffron Walden, Essex, CB10 1XL, UK). The general outline of each protocol included the isolation and purification of poly-A tailed mRNA from total RNA with poly-T oligo-attached magnetic beads and then isolated RNA was fragmented with divalent cations at an elevated temperature. These fragments were then reverse transcribed using random hexamer primers to form the first cDNA strand, followed by the removal of the RNA template and synthesis of a second strand to generate double stand (ds) cDNA using DNA polymerase I and RNase H. The ends of these ds cDNA fragments were then repaired by filling in the overhangs at the 5' ends with polymerase activity and removing the overhangs at the 3' end with 3' to 5' exonuclease activity. To the 3' ends of the ds cDNA fragments a single 'A' nucleotide was added in order to avoid ligation to other ds cDNA fragments during the adapter ligation reaction which ligated numerous indexing adapters to the ds cDNA ends which allowed downstream hybridation to a sequencing flow cell. cDNA template purification, size selected fragments which were subjected to template enrichment and library validation. The resulting cDNA was quantified using a Qubit® Quantitation Platform and HS dsDNA kit (Invitrogen, Paisley, UK).

The quantity of cDNA in the final sequencing cDNA library was assessed using the Quant-iT™ dsDNA HS assay kit and the Qubit™ fluorometer (Life Technologies™, Paisley, UK). A Quant-iT™ working solution was made by combining 1 µl Quant-iT™ reagent and 199 µl Quant-iT™ buffer x n (n= the number of standards plus samples). To 0.5 µl Quant-iT™ assay tubes 199 µl of Quant-iT™ working solution was added to 1 µl of sample and 190 µl of Quant-iT™ working solution was added to 10 µl of standards. All tubes were mixed for 3 sec and incubated at room temperature for 2 min. Standards were used to calibrate the Qubit™ fluorometer, samples were measured and sample concentration was calculated.

As a result of the pilot study in chapter three it was decided to include additional steps to optimise the Illumina® TruSeq® RNA sample preparation kit procedure in the main study in chapter 4. An mRNA-Seq library was not prepared for two samples due to low total RNA quality. The sample size for mRNA-Seq library preparation was therefore 30 samples from 15 animals 7 and 21 days postpartum. The added steps involved in the Illumina® TruSeq® RNA sample preparation kit protocol in regards to optimisation, library preparation, assessment of library purity and pooling are detailed below.

Optimisation: The mRNA-Seq library preparation protocol below was optimised with non-test total RNA extracted with the optimal RNA isolation method from bovine liver and endometrial samples prior to using test samples by varying (a) the concentration of input RNA and (b) the number of PCR cycles at the final PCR enrichment stage.

mRNA-Seq library preparation: The Illumina® TruSeq® RNA sample preparation kit v2 (Chesterford Research Park, Little Chesterford, Nr Saffron Walden, Essex, CB10 1XL, UK) was used to convert mRNA into a cDNA libraries for DNA sequencing. Input total RNA was uniformly 4 µg in a volume of 50 µl nuclease-free H₂O for each sample (Appendices: Table A-2). Indexes from Illumina® TruSeq® v2 A and B kits were allocated to specific samples prior to library construction so that at least two unique and compatible bar codes were in each pool which made provision for a pooling strategy following the Illumina® TruSeq® pooling guidelines. After adapter ligation the ds cDNA fragments were enriched by PCR which created the final sequencing cDNA library. In the PCR enrichment stage of the protocol 10 PCR cycles were used altered from 15 PCR cycles in the manufacture's protocol.

The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the purity of the ds cDNA libraries. Reagents were equilibrated to room temperature for 30 min pre-use. 350 µl RNase Zap and RNase-free water were used to clean the electrodes in the bioanalyser. The gel was prepared by the addition of 25 µl DNA dye concentrate into the DNA gel matrix, vortexed for 10 sec and added to spin filter tube. This was centrifuged at 2240 x g at room temperature for 15 min, the flow through was used for the experiment. A DNA chip was placed in the chip priming station and 9 µl of the gel-dye mixture was added into appropriate well. The attached syringe was adjusted to 1 ml and the plunger was pressed down until held by a catch. After 60 sec the catch was released and after 5 sec the plunger was slowly released to the 1 ml mark. The chip priming station was opened and 9 µl of the gel mix into two appropriate wells on chip. 5 µl of DNA marker was added into the ladder well and each of 12 sample wells. 1 µl of sample and DNA ladder were added to

the appropriate wells. The chip was vortexed for 60 sec in the vortex mixer (Agilent Technologies), after which the chip was run in the Bioanalyser.

Sample pooling: Seven equimolar pools of 5 multiplexed samples were constructed. Indexes from Illumina® TruSeq® v2 kits A and B were allocated to specific samples following the Illumina® TruSeq® pooling guidelines (Appendices: Table A-3).

10nM stocks for each sample were created and were calculated by firstly calculating the molarity of each sample. The Qubit™ fluorometer concentration (ng/μl) was multiplied by 1000 to convert it to g/L, divided by the molecular weight of 1 double stranded base pair, divided by the average length of fragments in a cDNA library and multiplied by 1000 to convert from M to nM. Secondly the volume of cDNA library and resuspension buffer (Illumina® TruSeq® RNA sample preparation kit v2, Chesterford Research Park, Little Chesterford, Nr Saffron Walden, Essex, CB10 1XL, UK) required to create 10nM stocks was then calculated with the $M1V1=M2V2$ formula (Appendices: Table A-4).

Example: $(22.40 \text{ ng}/\mu\text{l}) \times (10^3) \times (1/649 \text{ g}) \times (1/339 \text{ bp}) \times (10^3) = 101.88 \text{ nM}$

$101.88 \text{ nM} \times V1 = 10 \text{ nM} \times 25 \mu\text{l}$

$V1 = \underline{2.45 \mu\text{l cDNA}}$

$25 \mu\text{l} - 2.45 \mu\text{l} = \underline{22.6 \mu\text{l resuspension buffer}}$

2.3.2 Next generation sequencing

The next generation sequencing of libraries prepared for both the pilot and main studies in chapter 3 and 4 were outsourced. Prior to sequencing the sample libraries from the pilot study only were validated and samples were loaded into individual lanes on a flow cell, cluster amplified and sequenced using the Illumina® Genome Analyzer II (GAII) at TrinSeq, the Trinity Genome Sequencing Laboratory, in the Molecular Medicine Institute, Trinity College Dublin (<http://www.medicine.tcd.ie/sequencing>). One sample was single-end and the remainder were paired-end sequenced. The image files created during sequencing of fragment clusters were converted into sequence using Illumina Software to achieve 40 base pair (bp) paired end reads. These reads were then subjected to filtering which removed low-quality sequence and primer contamination. The quality of the reads was assessed using the FastQC software (version 0.4.3) (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). The assessments used included per base sequence quality, per sequence quality, over represented sequences and per base N count.

The sequencing of cDNA libraries in chapter four was outsourced to the Beijing Genomics Institute (BGI) (BGI Hong Kong Co., Limited, 16 Dai Fu Street, Tai Po Industrial Estate, Tai Po, New Territories, Hong Kong). Initially BGI carried out quality control analysis on the sample pools with the Agilent 2000 Bioanalyser (Agilent Technologies) and the Illumina® sequencing library qPCR quantification protocol. The cDNA libraries were sequenced on an Illumina® HiSeq™ 2000 and produced 50 bp reads paired end. The Off Line Basecaller (OLB)-1.9.4 was used to call bases and to convert a .bcl file to a qseq file. Software developed by BGI was then used to convert qseq files to demultiplexed fastq files.

2.4 Next Generation Sequencing Data Analysis

2.4.1 Sequence files download, integrity check and unzipping

In chapter four the method of attaining the output from the BGI sequencing facility required the following protocol. Seventy sequence files, MD5 checksums files and sequencing statistics files were downloaded via the internet from a BGI server (cdts.genomics.hk) to a Teagasc server (hcux323.teagasc.net) by a file transfer protocol with the *mget* command allowing the download of multiple files. Once downloaded MD5 checksums were created with the software *md5sum* (version 6.12) for each sequence file, these were compared to the MD5 checksums created by BGI for the same files and if they were identical the integrity of the downloaded sequence file was maintained. The downloaded sequence files were unzipped using the software *gzip* (version 1.3.12) with the command *gunzip*.

2.4.2 Quality assessment of sequence files

In both chapters 3 and 4 quality control checks of the raw sequence data were carried out with FastQC software (version 0.10.0). The FastQC report produced a list of 11 different modules which were used to evaluate if there were any problems which should be addressed before continuing with further analysis. FastQC imported the unzipped FastQ files received from BGI and produced a quality control summary represented by graphs and tables for each quality control module.

2.4.3 Outline of the next generation sequencing data analysis pipeline

The general outline of the next generation sequencing pipeline was the same for chapter 3 and 4 mRNA-Seq data. Each analytical step involved the use of a software package to implement a specific method to achieve a particular result/output which was then used as input for the next step in the analysis pipeline. The initial step involved mapping the raw reads to the bovine genome using TopHat, followed by summarising the number or read counts per gene with HtSeq Count, trimmed mean of M-values (TMM) normalization method

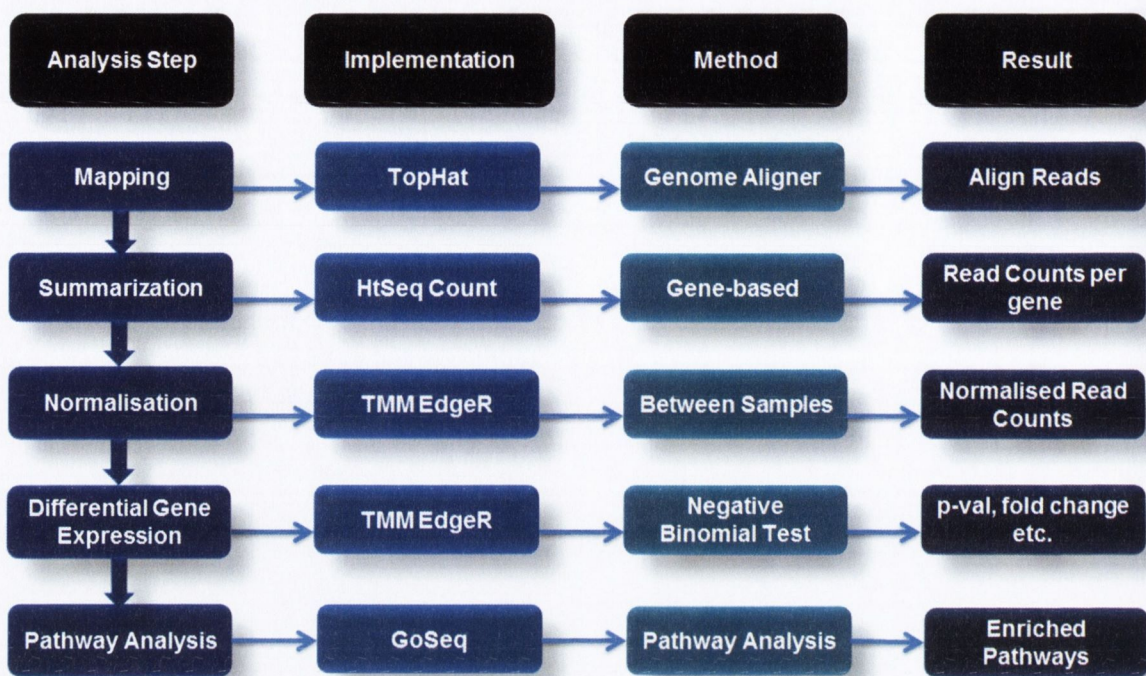


Figure 2.4-1: Flow diagram presenting the steps involved in mRNA-Seq data analysis. Each analytical step involved the use of a software package to implement a specific method to achieve a particular result/output which was then used as input for the next step in the analysis pipeline.

2.4.4 Mapping Reads to the bovine genome

Chapter 3 – Reads were mapped to the bovine genome (version Btau_4.0.62) using the genome aligner TopHat (version 1.3.1). The TopHat script assumed an inner mate distance (-r) of 200 bp, reads were not strand specific (fr-unstranded), a segment length of 20 bp (each read cut into 2 x 20 bp segments and mapped independently) and segment mismatches of 1 were allowed in each segment alignment for independently mapped read segments. Output files were converted from BAM to SAM files. The SAM files were then sorted by read name rather than chromosomal location.

Chapter 4 – In chapter four only, prior to aligning sequences to the genome with TopHat was used to estimate the inner mate distance between paired reads was required for each library. To estimate the inner mate distance mate pairs were aligned to the bovine genome with Bowtie2 (version 2.0.5) using a maximum fragment length (-X) of 500 and minimum fragment length (-l) of 0 for valid paired-end alignments. The output files created by this alignment were sorted using the picard-tools (version 1.60) SortSam option and the sort order was “coordinate”. Picard-tools

(version 1.60) CollectInsertSizeMetrics option was then used to estimate the inner mate distance between mate pairs for each library.

Tophat version 2.0.6 was used to align sequences for each library to the bovine genome (UMD3.1.69) by specifying the mean inner distance between mate pairs (-r), the standard deviation for the distribution on inner distances between mate pairs (--mate-std-dev), the library type (--library-type fr-unstranded), the sequencing pipeline output file type (--solexa1.3-quals) and the sensitivity of the Bowtie2 (version 2.0.5) search for the best alignment of each read (--b2-very-sensitive). Additional options used that differed from default specifications were; 12 threads used to align reads (-p 12), up to 1 alignment to the reference genome for a given read was allowed (-g 1), the removal of paired reads that mapped discordantly, reporting concordantly mapped reads only (--no-discordant), reporting only reads where both reads in a pair matched (--no-mixed) and setting the segment length to at least 24 bases as read lengths were 49 base pairs for this project (--segment-length 24).

2.4.5 Summarizing read counts per gene

Htseq-count (version 0.5.3 in chapter 2 and version 0.5.3p9 in chapter 3, <http://www-huber.embl.de/users/anders.HTSeq>) was used to summarize the number of aligned reads per exon (-t exon) using the union mode (-m union) and the Ensembl (version 62 in chapter 2 and version 69 in chapter 3) annotation of the bovine genome. Ensembl gene ID's (-i gene_id) were used in the output file to identify the number of read counts per gene. The data analysed was not strand specific therefore the option "-s" was selected as "no".

2.4.6 Normalisation and differential gene expression

Bioconductor package EdgeR (chapter 3 - version 1.6.12 and chapter 4 - version 3.0.8) (Robinson *et al.* 2010) was employed in R software (version 2.11.0). To account for biological and technical variation data was modelled as a negative binomial distribution using a generalisation of the Poisson distribution model. The dispersions of the negative binomial model were estimated using common dispersions only in chapter 3 and both common and tagwise dispersions in chapter four. Using both common and tagwise dispersions ensured that genes with read counts which were more consistent between replicates were ranked to a higher extent than genes that were not. The data was normalised across library size between samples using the trimmed mean of M-values (TMM) normalization method (Robinson & Oshlack 2010). The Benjamini-Hochberg false

discovery rate (FDR) was used to achieve differential gene expression with an adjusted P -value of < 0.1 (Benjamini & Hochberg 1995).

A plot was constructed that displayed the relations between samples based on multidimensional scaling (MDS). In chapter three 6 samples, 3 animals paired at two time-points: 15 and 30 DPP, were analysed using the MDS plot. In chapter four firstly comparisons were made within groups, sub-clinically endometritic (SCE) and healthy cows over time 7DPP v 21DPP and genes were either significantly up at 7 DPP or up at 21 DPP. The second comparison was between groups (SCE v healthy) at 7 DPP and 21 DPP and genes were either significantly up in the SCE or up in healthy cows.

2.4.7 Representation of bovine genes in pathway analysis

Chapter 3 – Bovine Ensembl genes were converted to human Ensembl orthologs using the Biomart tool on the Ensembl website (www.ensembl.org/biomart/martview). Genes differentially expressed with an adjusted p -value < 0.1 and $P < 0.05$ were utilised in the downstream pathway analysis. The bioconductor R software (version 2.14.0) package GoSeq (version 1.6.0) (Young *et al.* 2010) pathway analysis tool was implemented to identify pathways enriched by significantly differentially expressed genes.

Chapter 4 - All bovine genes present in the Ensembl (version 70) annotation of the bovine genome were converted to human Ensembl orthologs using the BioMart application on the Ensembl website (<http://www.ensembl.org/biomart/martview/>). Genes differentially expressed with an adjusted p -value < 0.1 were utilised in the downstream pathway analysis. Bioconductor, an open source and open development software project, provide the R software (version 2.15.2) package GoSeq (version 1.10.0) for implementation in the analysis of enriched pathway analysis of data from this study.

In both chapters 3 and 4 to achieve significantly enriched KEGG pathways the gene length bias is first quantified by calculating the Probability Weighting Function (PWF) which determines the probability that a gene will be differentially expressed only based on its length. The P - values for over-represented (enriched) and under-represented pathways were achieved using the GoSeq default method: “Wallenius” by the Wallenius non-central hypergeometric distribution (Young *et al.* 2010).

The advanced KEGG Pathway mapping tool “Search&Colour Pathway”, in the KEGG Pathway Database, was used to produce graphical images depicting differentially expressed (DE) genes

within the enriched pathways. Significantly differentially expressed Ensembl gene IDs were converted to Entrez IDs using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.7) (Huang *et al.* 2008) Gene ID Conversion Tool. Within an excel file the significant DE genes with Entrez IDs were allocated colours depending on the comparison being made. The Entrez gene IDs with their corresponding colours were entered into the advanced KEGG Pathway mapping tool “Search&Colour Pathway” in the KEGG Pathway Database.

2.4.8 Gene filtering measures for candidate gene selection in chapter 4

Stringency measures:

There were three different levels of stringency, which differed from initial differential gene expression analysis, applied to determine the top most significantly expressed genes for selection of candidate biomarkers for diagnosis of subclinical endometritis. Differential gene expression analysis was repeated as explained previously. (1) The Benjamini-Hochberg false discovery rate (FDR) was used to achieve differential gene expression with an adjusted *P*-value of < 0.05 (Benjamini & Hochberg 1995). (2) Low expression tags were filtered using the Bioconductor package EdgeR (version 3.0.8) (Robinson *et al.* 2010) in R software (version 2.15.2) which involved the removal of significant genes with on average 10 read counts in all samples within the group which had the lowest sample number of the groups being compared. To achieve this all read counts within each sample library were summed (LRC) then divided by 1 million, resulting in counts per million (CPM) for that library, the average CPM across all libraries was calculated. To achieve at least 10 read counts in the specified number of samples (eg. 6), 10 was divided by the average CPM across all libraries and the resulting low expression tags filter (LETF) factor (eg. 150.5) was assigned to the command: >keep <- rowSums (cpm(D)>150.5) >=6 (Table 2.4-1).

Table 2.4-1: Method to filter significant (adjusted $p < 0.1$) genes where less than “n” number of samples had less than 10 read counts on average using EdgeR software. “n” was determined by the group which had the lowest sample number of the two groups being compared. Groups that were compared: subclinical endometritic (SCE) cows 7 days postpartum (DPP), SCE cows 21 DPP, healthy cows 7 DPP and healthy cows 21 DPP. The library read count (LRC) was converted to counts per million (CPM) for each sample for all significant genes, average read count (ARC) was the target and the low expression tags filter (LETf) factor was used in the command line to remove genes where less than “n” samples had less than 10 reads.

	7 DPP SCE v Healthy			21 DPP SCE v Healthy			SCE 7 v 21 DPP			Healthy 7 v 21 DPP		
	Total N = 15			Total N = 15			Total N = 12			Total N = 18		
	Lowest n = 6			Lowest n = 6			Lowest n = 6			Lowest n = 9		
	LRC	CPM	ARC	LRC	CPM	ARC	LRC	CPM	ARC	LRC	CPM	ARC
Average	66428	0.066	10	7E+05	0.742	10	1358	0.001	10	4E+06	4.324	10
LETf Factor	150.5			13.5			7364			2.3		

(3) Genes with a Log₂ fold change greater than +2 and less than -2 were kept and genes with Log₂ fold changes between and not including -2 and +2 were removed.

2.4.8.1 Heat maps with significantly differentially expressed genes, stringently filtered

Heat maps were constructed with stringently filtered genes from the previous section that were significantly differently expressed using read counts as dependant variables, genes identified the rows and cow IDs identified the columns. Four heat maps in total were created comparing gene expression (1) 7 DPP to 21 DPP in the SCE group (2) 7 DPP to 21 DPP in the healthy group (3) between SCE and healthy groups at 7 DPP and (4) between SCE and healthy groups at 21 DPP.

The gplots package (version 2.11.0) was used and employed in R software (version 2.15.2) using the heatmap.2 option to produce the heat maps. Initially both the rows and columns were mixed and then clustered based on the Euclidean distance and dendograms were inserted based on this hierarchical clustering.

The rows were then scaled based on Z-scores, which measured the relationship of read counts to the mean read count within a gene across all samples. A Z-score of 0 indicated that the read count was the same as the mean read count for that sample compared to all samples for the same gene. The Z-score was either positive or negative, indicating that it was either above or below the mean

and by how many standard deviations. In the graphs produced, red indicated a negative Z-score, black was nearer the mean and green was a positive Z-score.

2.4.8.2 Genes commonly and uniquely elevated within and between groups

Comparing within groups (7 v 21 DPP) genes that were significantly elevated at the same time point (eg. 7 DPP) in each group were categorised as either unique (to the SCE or healthy group) or common (to both the SCE and Healthy groups). Comparing between groups (SCE v healthy), significant genes elevated at each time point within the same group (eg. SCE) were categorised as either unique (at 7 DPP or 21 DPP) or common (to both 7 and 21 DPP). Genes uniquely elevated 7 DPP in the SCE cows over time (7 v 21 DPP) and genes commonly elevated in the SCE cows (SCE v healthy) at each time point which were not elevated in healthy cows were selected as candidate genes to identify unique differences in sub-clinical endometritic cows compared to healthy cows.

2.4.8.3 Identification of candidate genes with predicted secretory domains

Genes uniquely elevated gene expression 7 DPP in the SCE cows over time (7 v 21 DPP) and genes commonly up in the SCE cows compared to the healthy cows at each time point were submitted to the Ensembl (version 70) application BioMart (<http://www.ensembl.org/biomart/martview/>). Genes with signal peptide cleavage sites in their amino acid sequences and Gene Ontology information for these genes were provided in the output. Ensembl uses the SignalP server (Nielsen *et al.* 1997) to predict the presence and location of a signal peptide cleavage sites in the amino acid sequences and the Ensembl Ontology database is populated with the most up to date version available from the Gene Ontology database (<http://www.geneontology.org/>). The proteins with signalling peptides and potential to act as biomarkers were those that were secreted extracellularly and this information was attained from the Gene Ontology (GO) cellular component domain for each gene with a transcribed signalling peptide. If the GO cellular component domain for a particular gene was named “extracellular space”, which is defined as “That part of a multicellular organism outside the cells proper, usually taken to be outside the plasma membranes, and occupied by fluid”, it was selected as a potential biomarker for subclinical endometritis.

2.5 microRNA-Seq Library Preparation, Sequencing and Data Analysis

2.5.1 microRNA purification, quantity assessment

The sample size for microRNA-Seq library preparation was 20 samples from 10 animals 7 and 21 days postpartum. These samples and animals were also sequenced by mRNA-Seq and chosen for microRNA sequencing because the total RNA from their biopsies had the highest RIN values.

Purification: The retained flow through from the Qiagen RNeasy® Plus Mini kit containing RNA fragments less than 200 nucleotides was purified using the Qiagen RNeasy® MinElute® Cleanup Kit (Qigen Ltd). The 700ul flow-through from the RNeasy® Plus Mini kit procedure and 455µl (0.65 volumes) of 100% ethanol were added to a 2ml reaction tube and mix thoroughly by vortexing. 700µl of sample was added to an RNeasy MinElute Spin Column, centrifuged at 8,000 x g for 15sec and the flow-through was discarded. The remaining 455µl of sample was added to the same column and centrifugation was repeated. 500µl of Buffer RPE was added to the RNeasy MinElute spin column and centrifuge for 15 sec at 8000 x g, both the flow-through and collection tube were discarded. The RNeasy minElute spin column was placed in a new 2ml collection tube and lid was left open and centrifuge for 1min at 8000 x g. The RNeasy minElute spin column was placed in a

1.5 mL collection tube, 14µl of RNase-free water was added directly to the spin column membrane and centrifuge for 1min at 8000 x g to elute the RNA (small RNA containing miRNA).

Quantity assessment: The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the quantity of miRNA extracted and purified. Based on total RNA concentrations from the NanoDrop spectrophotometer reading, 1µl of miRNA was diluted 10 times in nuclease-free H₂O. Reagents were equilibrated to room temperature for 30min pre-use. 350µl RNase Zap and RNase-free water were used to clean the electrodes in the bioanalyser. The gel was prepared by the addition of 650µl Small RNA gel matrix into a spin column to filter and centrifuged for 15min at 10,000 x g. The Small RNA dye was vortexed for 10sec, spun down and 2µl of dye was added to a 0.5ml microcentrifuge tube. 40µl of the filtered gel was added to the dye, mixed by pipetting and centrifuged at 13,000 x g for 10min at room temperature. A Small RNA chip was placed in the chip priming station and 9µl of the gel-dye mixture was added into appropriate well. The attached syringe was adjusted to 1ml and the plunger was pressed down until held by a catch. After 60sec the catch was released and after 5sec the plunger was slowly released to the 1ml mark. The chip priming station was opened and 9µl of the gel mix into two appropriate wells on chip. 9µl of Small RNA conditioning solution was added to the appropriate well and 5µl of the Small RNA marker was added into the ladder well and each of 11 sample wells. 1µl of sample and Small RNA ladder were added to the appropriate wells. The chip was vortexed for 60sec in the vortex mixer (Agilent Technologies), after which the chip was run in the Bioanalyser.

2.5.2 microRNA library preparation and sequencing

Library preparation and sequencing was outsourced to GATC-biotech (European Genome and Diagnostics Centre, Jakob-Stadler-Platz 7, 78467 Konstanz, Germany). The Illumina® TruSeq™ Small RNA sample preparation kit was used to prepare 20 cDNA sequencing libraries according to the manufacturer's instructions. Samples were allocated indexed adapter sequences to enable multiplexing (Appendices: Table A-5). Samples were sequenced on an Illumina® HiSeq™ 2000 and produced up to 50 base pair (bp) single end reads.

2.5.3 microRNA-Seq data analysis

*The quality assessment and filtering of sequence files and summarizing of read counts per gene for miRNA-Seq data was completed by a collaborator Dr. David Lynn in Teagasc Grange.

*Quality assessment and filtering of sequence files:

Adapter sequences were trimmed using the Cutadapt software (version 1.1) (<http://www.cutadapt/>). Subsequently reads shorter than 18 nucleotides were discarded. The fastq quality filter (http://hannonlab.cshl.edu/fastx_toolkit/) (version 0.0.13) removed reads which had at least 50% of bases with a Phred score of 20. Additionally reads with bases which had a Phred score of less than 20 were trimmed. High quality reads were aligned to the bovine genome (UMD 3.1) with Novoalign (version 2.07.11) (<http://www.novocraft.com>) implementing the “-m” miRNA mode. Reads that aligned to more than one position in the genome were discarded.

*Summarizing read counts per gene:

HtSeq-count	version	0.5.3p9
-------------	---------	---------

(<http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html>) was used to summarised the number of aligned reads per exon (-t exon) using the union mode (-m union) and the Ensembl (version 66) annotation of the bovine genome and miRNA annotation (separately). Ensembl gene ID's (-i gene_id) were used in the output file to identify the number of read counts per gene. The data analysed was not strand specific therefore the option “-s” was selected as “no”.

Normalisation and differential gene expression:

Bioconductor package EdgeR (version 3.0.8) (Robinson *et al.* 2010) was employed in R software (version 2.15.2). To account for biological and technical variation data was modelled as a negative binomial distribution using a generalisation of the Poisson distribution model. The dispersions of the negative binomial model were estimated using common and tagwise dispersions ensuring that genes which more consistent between replicates were ranked to a higher extent than genes that were not. The data was normalised across library size between samples using the trimmed mean of M-values normalization method (Robinson & Oshlack 2010). The Benjamini-Hochberg false discovery rate (FDR) was used to achieve differential gene expression with an adjusted *P*-value of < 0.1 (Benjamini & Hochberg 1995). A plot was constructed that displayed the relations between samples based on multidimensional scaling (MDS). Firstly comparisons were made within groups, sub-clinically endometritic (SCE) and healthy cows over time 7DPP v 21DPP and

genes were either significantly up at 7 DPP or up at 21 DPP. The second comparison was between groups (SCE v healthy) at 7 DPP and 21 DPP and genes were either significantly up in the SCE or up in healthy cows.

2.5.4 Crossover between mRNA-Seq and microRNA-Seq expression

To assess the crossover of miRNA expression between the mRNA-Seq and microRNA-Seq data sets, significant genes with a false discovery rate (FDR) of < 0.1 were selected and their biotype attained using the BioMart application on the Ensembl website (<http://www.ensembl.org/biomart/martview/>). The microRNAs which were identified in the mRNA-Seq data set were cross referenced with the microRNA-Seq data set for each comparison, within groups SCE and Healthy comparing 7 to 21 DPP and between SCE and healthy at either 7 or 21 DPP.

2.6 Quantitative Real Time PCR (qRT-PCR)

2.6.1 Candidate Gene Selection and Primer Design

In chapter three there were 19 candidate genes selected from the results generated by mRNA Seq differential gene expression and pathway analysis which were subsequently assessed by real-time qRT-PCR. Genes were selected based on a combination of criteria including: an adjusted *P*-value <0.1, read counts, pathway analysis and fold change.

The Coding Sequence (CDS) downloaded from Ensembl for each gene and its transcripts were submitted to the UCSC BLAT genome aligner to allow visualisation of exonic sequence separated by intronic sequence (<http://genome.ucsc.edu/cgi-bin/hgBlat>). To ensure coverage across multiple gene transcripts, exons common to all transcripts of the gene were selected for primer design. To assess for genomic DNA contamination, primers were designed to be intron spanning. Primers for real-time qRT-PCR were designed using the Primer3 (version 0.4.0) software program (Rozen & Skaletsky 2000) to measure expression of the candidate and reference genes. The Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to ensure the specificity of the primers within the bovine genome (Appendices: Table A-1).

2.6.2 cDNA Synthesis

Initially total RNA samples were diluted to a concentration of 0.05 µg/µL in final volume of 10 µL to achieve a total RNA yield of 0.5 µg. The High-Capacity cDNA Reverse Transcription Kit was used as per manufactures instructions, (Applied Biosystems, Warrington, UK) to synthesise first strand complementary DNA (cDNA) with random hexamers by reverse transcribing 0.5 µg of RNA extracted. Initially a 2X reverse transcription master mix was produced: 2 µL 10X RT buffer, 0.8 µL 25X dNTP Mix (100 mM), 2 µL 10X RT Random Primers, 1 µL MultiScribe Reverse Transcriptase and 4.2 µL nuclease-free water. Into each nuclease-free 0.5 mL tube: 10 µL of the 2X reverse transcription master mix and 10 µL of each 0.5 µg RNA sample were added. The mixture was gently pipetted in and out 3 times and the tubes were sealed and briefly centrifuged. The tubes were placed in a thermal cycler and the following process was carried out: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C and hold at 4 °C. Samples were subsequently stored at -20 °C. This procedure was also carried out for 10 test samples and repeated with an additional 8 samples. The resulting cDNA from the additional 4 animals was pooled for use as an inter-plate calibrator and to achieve the efficiencies of the genes to be used downstream. The pooled samples consisted of cDNA from cows biopsied at 15DPP and 30DPP.

2.6.3 Real-time qRT-PCR Reaction

Initially cDNA working stocks were made by diluting total cDNA 1 in 5 with nuclease free H₂O. qRT-PCR reactions were carried out in 96 well plates with a final reaction volume of 20 µL to include: 10 µL SYBR FAST Green Mastermix, 1 µL cDNA, 1 µL of primer mix (forward and reverse primers with a concentration of 250nM each in the final reaction volume) and 8 µL nuclease free water. Real-time PCR measurements were performed in triplicate to calculate the gene efficiencies and in duplicate to measure the gene expression levels in each sample. The Applied Biosystems Fast 7500 version 2.0.1 instrument was used with the following cycling parameters: 95 °C for 20 sec; 40 cycles of 95 °C for 3 sec; 60 °C for 30 sec, followed by amplicon dissociation (95 °C for 15 sec; 60 °C for 60 sec; 95 °C for 15 sec and 60 °C for 15 sec). Disassociation curves were examined for the presence of a single PCR product for each gene amplified. A non-template control (NTC) was in each plate where water replaced cDNA and all other reagents were used.

To assess the effect of standardising the concentration of input cDNA on the results, all samples were first diluted 1 in 5 as above and their concentrations were achieved using the nanodrop with setting specified for DNA. The concentration range was between 329.8 and 549.5 ng/µL, taking this into consideration, 300 ng/µL working cDNA stocks were constructed for all samples. The real

time qRT-PCR reaction and plate was prepared and run as above for two genes (*GATA2* and *DEFB6*) with the standardised cDNA concentrations for all samples.

2.6.4 Quantitative Real Time PCR Reaction Efficiency and Inter-Plate Calibration

The reaction efficiencies for 19 primer sets using the pooled cDNA sample were calculated using a 5-fold dilution series to generate a standard curve. Triplicate samples for each cDNA dilution in the series were used and the average raw cycle threshold (Ct) value from the real-time qRT-PCR reaction was then calculated. The standard curve was constructed in excel and the efficiency of the reaction was calculated with the formula: $10^{(-1/m)-1}$ where *m* is the slope of the line. An acceptable efficiency (*E*) was between 0.9 and 1.1. The efficiency correction was then carried out on the average raw cycle threshold (Ct) values, from the 12 test samples (1 in 5 dilution of cDNA from each cow) in duplicate, using the software package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden). Variation in PCR efficiency was calculated by the GenEx software.

A multitude of plates were used and to account for inter-plate variation, pooled cDNA acted as an inter-plate calibrator (IPC) and the gene *ACTB* was amplified on each plate in triplicate. The software package GenEx 5.2.1.3 was used to normalise all samples for the inter-plate variation.

2.6.5 Relative Gene Expression Levels

Relative gene expression levels were determined with the use of highly stable reference genes. The stability of 5 different reference genes was examined across all samples using qRT-PCR. The reference genes included glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β -actin (*ACTB*), suppressor of zeste 12 homolog (*SUZ12*), zinc finger protein 131 (*ZNF131*) and ribosomal protein S9 (*RPS9*) (Walker *et al.* 2009; Coyne *et al.* 2011). Data was analyzed using geNorm (Vandesompele *et al.* 2002 accessible through MultiD Analyses AB, Gothenburg, Sweden) to measure the overall stability of the reference genes under examination. The software calculated and compared the M-value for all reference genes, eliminated the gene with the highest M-value and repeated the process to achieve the two most stable reference genes. Results indicated that *GAPDH* and *ACTB* were the most stable reference genes and had a stability value (M-value) of 0.6, and as a result were chosen as the reference genes (Figure 2.6-1).

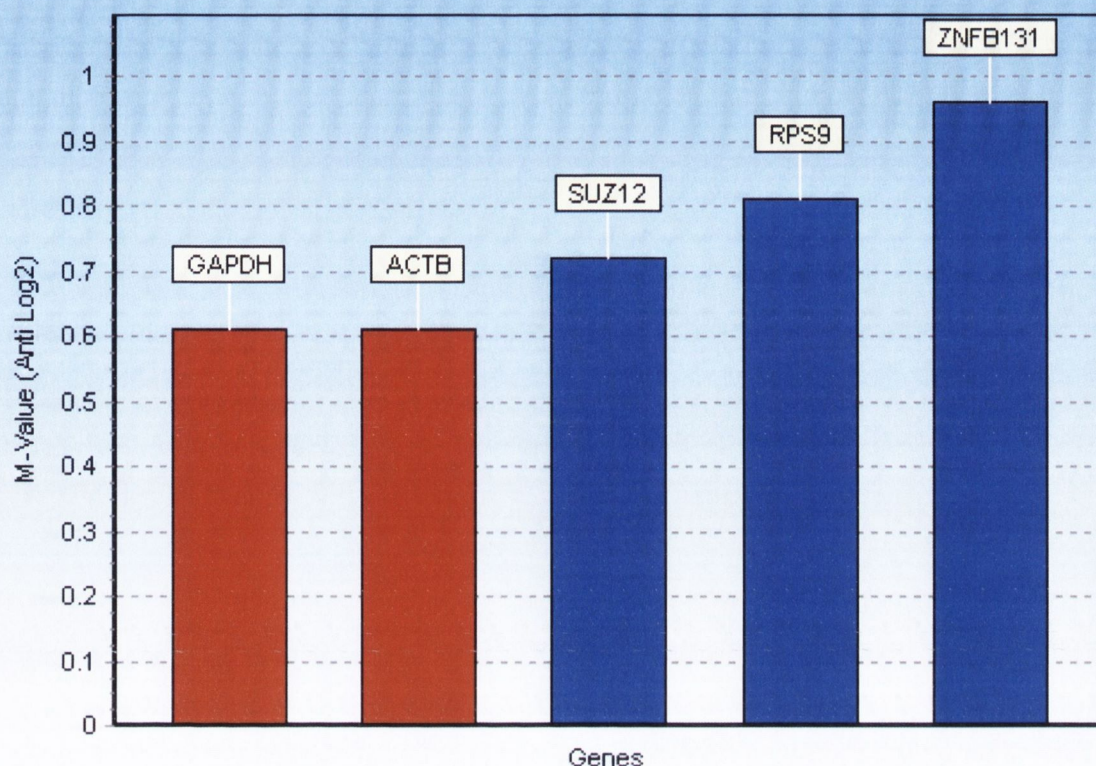


Figure 2.6-1: Graph produced by geNorm – the stability (M-value) of reference genes under examination for qRT-PCR. The stability of 5 different reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β -actin (ACTB), suppressor of zeste 12 homolog (SUZ12), zinc finger protein 131 (ZNF131) and ribosomal protein S9 (RPS9) was examined across all samples. GAPDH and ACTB (red) in were the most stable reference genes (M-value of 0.6).

The GenEx 5.2.1.3 software program was used to normalise test samples in the 15DPP group with reference samples in the 30DPP group pair-wise using the “Normalise to reference samples” application. Following this the expression values were converted to Log_2 scale. Significant differences between 15 DPP and 30 DPP sample groups were analysed using a Student’s *t*-test with the *GraphPad Prism version 3.0* software package on the *Ct* values achieved post normalising to reference samples. This data was log transformed to insure homogeneity of variance.

2.7 Histopathology

In chapter 4 formalin fixed uterine tissues from twenty two cows were paraffin-embedded, sectioned at 5-8µm thickness, mounted on a glass microscope slide and stained with haematoxylin and eosin (H&E). This processing carried out in collaboration with Professor Sean Callanan at the Pathobiology and Infectious Disease Unit in the School of Agriculture, Food Science & Veterinary Medicine, University College Dublin.

The degree of inflammation in endometrial biopsies was graded blind without knowledge of sample ID or background. Endometrial inflammation was classified based on previous criteria outlined by Chapwanya *et al.* 2010, which focused on intensity of inflammatory cell infiltrating the stroma, in combination with a recent study by Meira *et al.* 2012, which incorporated the numbers of polymorphonuclear neutrophils in the endometrial epithelium. Both the Chapwanya *et al.* 2010 and Meira *et al.* 2012 criteria focused on biopsies taken after 14 days and 21 days postpartum, in the present study biopsies were taken after 7 days postpartum. Therefore criteria for grading endometrial inflammation was defined which combined components of both Chapwanya *et al.* 2010 and Meira *et al.* 2012. Each sample was graded firstly on the intensity of epithelial polymorphonuclear (PMN) infiltrate and secondly on the intensity of stromal leukocyte cell infiltrate, the greater the number of PMN in the epithelium the higher the score which ranged between 0 and 3 (Table 2.7-1). Three samples were removed from further analysis as they could not be adequately graded resulting in a sample size of 17.

Table 2.7-1: Histopathology grading criteria adapted from Chapwanya *et al* 2009 and Meira *et al* 2012. The presence or absence of polymorphonuclear (PMN)/mononuclear cells was graded separately in the epithelium and stroma of endometrial biopsies 21 days postpartum on a scale from 0, with little or no inflammation, to 3, with severe inflammation. Histology sections were graded at x40 high power field (hpf) magnification.

Epithelium		
Score	Inflammatory cell type	Infiltrate intensity
3	PMN	Severe (>10 cells / hpf x40)
2	PMN	Moderate (≥6-10 cells / hpf x40)
1	PMN	Mild (≤5 cells / hpf x40)
0	Absent	0
Stroma		
Score	Inflammatory cell type	Infiltrate intensity
3	PMN and/or Mononuclear	Severe inflammation, high infiltration of polymorphonuclear cells.
2	PMN and/or Mononuclear	Moderate inflammation showing prominent leukocyte infiltration.
1	PMN and/or Mononuclear	Low grade inflammation, little infiltration of lymphocytes and plasma cells.
0	PMN and/or Mononuclear	No evidence of inflammation.

2.8 Microbiology

In chapter four the terminal-restriction fragment length polymorphism (T-RFLP) technique was used to differentiate microbial populations within the uterus between 28 samples (14 cows all of which were also used in downstream mRNA-Seq analysis with uterine biopsies) at 7 and 21 DPP. This technique involved the extraction of total DNA from uterine swabs, amplification of the 16S rRNA gene with fluorescently-labelled primers and digestion of the PCR product with restriction enzymes which generated fluorescently-labelled terminal restriction fragments. The output was a series of peaks (fragments) ranging in sizes and heights representative of the various microbial species present. Analysis of the data did not define specific bacterial communities yet highlighted how different the microbial communities within each sample were from other samples using a Principal Coordinates Ordination (PCO) plot and dendrogram based on the S17 Bray-Curtis similarity test. The data was also transformed with the square root and a permanova test was carried out to determine if there was a significant difference between the microbial populations in healthy compared to sub-clinically endometritic cows. The analysis of these samples and data was carried out in the School Of Biomolecular & Biomed Science, Conway Institute, University College Dublin, in collaboration with Professor Wim Meijer.

2.9 Whole blood haematology analysis

In chapter five blood was collected from the coccygeal vein into one sodium fluoride/Na₂ EDTA and one spray coated K₂EDTA Vacutainer®. The tubes were inverted repeatedly and transported to the lab within 3 hours of collection. 1 ml of blood from the 10 ml of blood in a spray-coated K₂EDTA Vacutainer® was removed and added to a 6 ml spray-coated K₂EDTA Vacutainer® for Haematology analysis. The haematology analysis was outsourced to the animal health laboratory in Teagasc Grange. Total leukocyte, neutrophil, lymphocyte, monocyte, eosinophil and basophil were determined from K₂EDTA anti-coagulated blood with an automated haematology analyser (ADVIA 2120, Bayer Healthcare, Siemens, UK).

Statistics: Initially a Kruskal-Wallis test, a non-parametric one way analysis of variance (ANOVA), was performed comparing pre-calving (n=8), sub-clinically endometritic cows (n=6) at 7 and 21 DPP and healthy cows (n=9) at 7 and 21 DPP. Subsequently, under the assumptions of non-normality, a Mann-Whitney test was used to determine significance between groups and Wilcoxon paired test was used to determine significance over time.

2.10 Blood plasma protein quantification

In chapter five the remaining 9 ml of blood in a spray-coated K₂EDTA Vacutainer® was centrifuged at 2000 x g for 15 min at 4°C and plasma were collected and placed in 1.5 µL tubes and stored at -80°C.

Plasma was analysed for the presence of acute phase proteins (APPs) serum amyloid A (SAA) and haptoglobin (HP). The analysis of these APPs was outsourced to the animal health laboratory in Teagasc Grange. SAA was measured using the PHASE™ Serum Amyloid A Assay (SAA) kit, a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA), following the manufacturer's protocol and was supplied by Tridelata Development Ltd, Maynooth, Co. Kildare, Ireland, cat. no. TP-802. HP was measured using the Tridelata PHASE™ Haptoglobin Assay kit, a colorimetric assay, following the manufacturer's protocol (supplied by Tridelata Development Ltd, Maynooth, Co. Kildare, Ireland, cat. no. TP-801). Sample absorbances were read using the GloMax®-Multi Detection System from Promega.

ELISAs were performed on blood plasma from 14 cows 7 and 21 days postpartum (DPP) and 8 of these cows pre-calving and these assays were done by lab technicians in Teagasc Grange. IL-17A (GenWay Biotech, Inc 6777 Nancy Ridge Drive, San Diego, CA 92121, USA) was predicted as a potential systemic biomarker for sub-clinical endometritis as a result of local transcriptomic

analysis which was secreted into the extracellular space. Other plasma proteins associated with inflammation were also assessed IL-6 and IL-1 (ThermoScientific®, 3747 N. Meridian Rd. Rockford, IL 61101, United States).

Statistics: Initially a Kruskal-Wallis test, a non-parametric one way analysis of variance (ANOVA), was performed comparing pre-calving (n=8), sub-clinically endometritic cows (n=5) 7 and 21 DPP and healthy (n=9) cows 7 and 21 DPP. Subsequently, under the assumptions of non-normality, a Mann-Whitney test was used to determine significance between groups and Wilcoxon paired test was used to determine significance over time.

2.11 Blood plasma metabolite quantification

4 ml of blood from a sodium fluoride/Na₂ EDTA Vacutainer® was centrifuged at 2000 x g for 15 min at 4°C and plasma were collected and placed in 1.5 µL tubes and stored at -80°C. Plasma was analysed for the blood metabolites, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose and urea. The analysis of these metabolites was outsourced to the animal health laboratory in Teagasc Grange. Glucose was measured using the hexokinase method, the kit supplied by Audit Diagnostics, Cork, Ireland Cat no. AAU706 and was performed on Beckman Coulter AU 400 clinical analyser using test protocol supplied by kit manufacturer. The kinetic method measured urea concentration; the kit was supplied by Audit Diagnostics Cat no. AAU812 and was performed on Beckman Coulter AU 400 clinical analyser using the protocol supplied by kit manufacturer. Kinetic method was used to measure β-hydroxybutyrate and the kit used was supplied by Randox Laboratories, Belfast, Northern Ireland cat no RB1007 and was performed on Beckman Coulter AU 400 clinical analyser using test protocol supplied by kit manufacturer. The colourimetric method measured non-esterified fatty acids; the kit used was supplied by Randox Laboratories cat no. FA115, and performed on Beckman Coulter AU 400 clinical analyser using test protocol supplied by kit manufacturer.

Statistics: Initially a Kruskal-Wallis test, a non-parametric one way analysis of variance (ANOVA), was performed comparing pre-calving (n=8), sub-clinically endometritic (n=5) cows at 7 and 21 DPP and healthy (n=9) cows at 7 and 21 DPP. Subsequently, under the assumptions of non-normality, a Mann-Whitney test was used to determine significance between groups and Wilcoxon paired test was used to determine significance over time.

Chapter 3

3 Global endometrial transcriptomic profiling in healthy postpartum beef cows

3.1 Introduction

Uterine involution is the time frame after parturition, within which the uterus returns to a physiological functioning state becoming receptive to and supportive of a new conceptus. Completion of involution encompasses a variety of processes on a physiological and histological level to involve uterine size reduction, contraction, caruncle shedding, necrosis, and rejuvenation of endometrial tissue (Gier & Marion 1968). In addition to the intrinsic physiological processes of involution, all bovine uteri undergo consistent bacterial contamination, clearance and recontamination within two weeks postpartum (Azawi 2008; Sheldon & Dobson 2004). The spontaneous clearance of bacteria, following a normal parturition, occurs within 10 – 15 days postpartum (Singh *et al.* 2008).

Uterine involution requires the involvement of inflammatory process and the immune response to deal with intrinsic uterine tissue damage and an extrinsic influx of bacteria into the uterus (Chapwanya *et al.* 2010; Gabler *et al.* 2010; Llewellyn *et al.* 2008; Salamonsen 2003). The usual inflammatory response results in protection of the host and a phase of resolution and repair, rather than in the establishment of chronic inflammation leading to loss of tissue (Medzhitov 2008; Serhan & Savill 2005; Serhan *et al.* 2007; Serhan *et al.* 2008). Acute inflammation which is not resolved in some cows may result in chronic inflammation which can lead to metritis or endometritis. Numerous recent studies have highlighted the role of the inflammatory response during uterine involution at a cellular level which is characterised by the presence of a neutrophil-rich inflammatory cell influx to the endometrium (Bondurant 1999; Chapwanya *et al.* 2012; Gabler *et al.* 2010) an essential component of acute wound healing (Hart 2002a; Hart 2002b).

A reduction in neutrophil numbers as involution progresses in healthy animals is the result of inflammatory resolution in the endometrium (Chapwanya *et al.* 2012; Gabler *et al.* 2010; Kasimanickam *et al.* 2005) mirrored by molecular changes consisting of extensive immune gene expression and regulation (Chapwanya *et al.* 2009; Davies *et al.* 2008; Herath *et al.* 2009; Martins *et al.* 2011). The postpartum endometrial inflammatory response and its allied immune gene activation is a transient feature of normal physiological attributes associated with uterine involution (Chapwanya *et al.* 2012).

The timeframe of uterine involution has been shown to be extended significantly from 40 to between 50 and 60 days postpartum due to damage of the endometrium, degree of infection and pathological uterine discharge (Hajurka *et al.* 2005). Previous studies have revealed a large proportion of cows fail to spontaneously clear bacterial infections (40%) within 7 days postpartum (Sheldon *et al.* 2006; Sheldon *et al.* 2009) and dysregulation of the immune response in these animals may lead to prolonged inflammation and development of disease (Herath, Lilly, Santos, *et al.* 2009; Herath, Lilly, Fischer, *et al.* 2009). Clinical endometritis occurs 4 - 6 weeks postpartum in 15 - 20% of cows and 30 - 35% of cows acquire subclinical endometritis between weeks 4 and 9 postpartum. During uterine infection, endometritis causes infertility in cows and even after successful clearance of endometritis subfertility is observed in these cows (LeBlanc *et al.* 2002; LeBlanc 2008; Sheldon *et al.* 2008; Sheldon *et al.* 2006)

Previously by histopathological assessments of uterine biopsies, we have described endometrial infiltration by leukocytes early during involution which was significantly reduced by 30 DPP. In parallel, we showed significant temporal reduction in toll-like receptor, leukocyte surface receptor, pro-inflammatory and antimicrobial gene transcription during later stages of involution (Chapwanya *et al.* 2012). In the current study we hypothesised that repeat uterine biopsies, at two postpartum time points in healthy cows undergoing involution, would reveal distinct temporal gene expression profiles identifying molecular changes associated with bacterial clearance and the resolution of inflammation.

Recent publications in this area have elucidated the mechanisms of bovine endometrial infection and immune responses in primary cells *in vitro* (Cronin *et al.* 2012; Herath, Lilly, Fischer, *et al.* 2009; Sheldon *et al.* 2010). However, the complexity of the uterine bacterial milieu and immune responses observed *in vivo* are influenced by a variety of animal factors, such as energy balance (Wathes *et al.* 2010) which cannot be accurately replicated *in vitro*. Therefore, postpartum changes over time, in endometrial biopsies *ex vivo*, are likely to reflect a more accurate profile of the immune response in healthy compared to diseased animals (Galvão *et al.* 2011). Interestingly, new approaches have used endometrial explants to characterise endometrial immune function *ex vivo* in response to specific bacterial stimuli (Borges *et al.* 2012). These studies focus on specific gene subsets whereas the current study takes a more comprehensive approach by analysing the entire transcriptome of endometrial biopsies providing a novel and important insight into the regulation of inflammation postpartum.

This is the first study to use next generation sequencing to assess bovine endometrial inflammation postpartum and the temporal resolution of this inflammation during involution in

healthy cows. The dataset achieved highlighted the value of large scale genomic approaches toward understanding the immune regulatory networks associated with physiological bacterial clearance and the resolution of inflammation in the postpartum cow that may form the basis for future diagnosis of delayed clearance and perturbed immune regulation in diseased animals.

3.2 Aim

The aim of this study was to characterise the temporal transcriptomic changes over two time points early postpartum in endometrial biopsies from healthy cows using mRNA-Seq during uterine involution.

3.3 Results

3.3.1 RNA quality & quantity and mRNA-SEQ cDNA libraries

The total RNA, extracted from 3 cows at 15 and 30 DPP for mRNA-Seq library preparation and from 4 additional cows for qRT-PCR (total N=5), had an RNA Integrity Number (RIN) which averaged 7.9 ranging between 6.5 – 9 and RNA yields on average of 11.5 µg ranging between 2 – 21 µg. The highest quality total RNA was used as input for the preparation of mRNA-Seq cDNA libraries.

3.3.2 Next Generation Sequencing

3.3.2.1 Reads Aligned to the Bovine Genome & Summary of Read Counts per Gene

Sequencing files that were returned from the sequencing facility were of high quality as they satisfied the criteria of the FastQC software which analysed sequence quality. The Sequencing fastQC files have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE40312. Across 6 sequenced libraries the number of reads input prior to processing with TopHat was 26,785,987 on average. The percentage of these reads that achieved “at least one reported alignment” was 71% (18,894,871 reads) on average which were subsequently used in downstream analysis. The percentage of reads that failed to align to the bovine genome was 26% (738,402 reads) on average and 2% of reads (507,093 reads) were suppressed due to the option “-m” (Table 3.3-1).

The sorted output file of aligned sequence reads from TopHat and a GFF file containing genomic features were used as input files for HTSeq-Count. A genomic feature represented the unified exons of a gene and the amount of reads that aligned to each feature was counted. On average there were 3,678,861 reads with “no feature” (reads not assigned to any feature and may have either the entire read or partial sections of their read within intronic regions). Reads that were assignable to more than one feature were “ambiguous”, on average 165,175 reads were ambiguous. The average number reads counted per library and used in downstream analysis was 7,214,431 (Table 3.3-2).

Table 3.3-1: Tophat and Bowtie software processed all reads outputted from next generation sequencing of 6 endometrial transcriptomes from 3 different cows 15 and 30 days postpartum (DPP). Reads were aligned to the bovine genome, keeping reads with at least one reported alignment and discarding reads with greater than one reported alignment (-m) and reads of poor quality. The percentage of reads that failed to align to the bovine genome was 26% (738,402 reads) on average and 2% of reads (507,093 reads) were suppressed due to the option “-m”.

	Cow	Reads processed	Reads with at least one reported alignment	%	Reads that failed to align	%	Reads with alignments suppressed due to -m	%
15 DPP	1	29861283	23651998	79	5671978	19	537307	2
	2	29263720	19269559	66	9522331	33	471830	2
	3	32562297	23241962	71	8716484	27	603851	2
30 DPP	1	5699743	4791981	84	753534	13	154228	3
	2	35625106	27448452	77	7414082	21	762572	2
	3	27703774	14965278	54	12225721	44	512775	2

Table 3.3-2: HtSeq-Count summarised read counts for each gene with the output from Tophat and Bowtie for 6 endometrial transcriptomes from 3 different cows 15 and 30 days postpartum (DPP). Reads that were discarded at this step in the data analysis pipeline included: (A) reads not assigned to any feature or with either the entire read or partial sections within intronic regions were defined as “no feature” and (B) reads that were assignable to more than one feature were “ambiguous”. Reads that were assignable to more than one feature were “ambiguous”, on average 165,175 reads were ambiguous. The average number reads counted per library and used in downstream analysis was 7,214,431.

	Cow	(A) Reads - no feature	(B) Reads - ambiguous	Reads Counted
15DPP	1	2404531	33073	4121695
	2	3199801	88287	5175957
	3	3017806	268572	6719833
30DPP	1	4739860	219035	10041873
	2	3790904	208421	8076896
	3	4920268	173665	9150337

3.3.2.2 mRNA-Seq Differential Gene Expression

After trimmed mean of M-values (TMM) normalization of library size between samples (Table 3.3-3), differential gene expression was achieved. TMM EdgeR assigned values of significance (*P* – adjusted value) and expression (Log₂ fold change) to each gene based on the read counts between groups (15 DPP and 30 DPP).

Table 3.3-3: Trimmed mean of M-values (TMM) normalization method in the software package EdgeR normalised library read count size between for 6 endometrial transcriptomes from 3 different cows 15 and 30 days postpartum (DPP).

	Cow	Initial Library Size (read counts)	Normalised Library Size (read counts)
15 DPP	1	4,198,311	3,131,962
	2	29,133,235	10,217,486
	3	14,992,194	5,466,911
30 DPP	1	22,815,931	9,597,417
	2	19,078,650	7,195,164
	3	21,966,787	8,806,913

Two output files were constructed from the EdgeR results, based on different levels of stringency. Using a P-value cut off of 0.05, 2,856 significantly differentially expressed genes were identified, of which 752 were elevated 15 DPP and 2,104 elevated 30 DPP. With an adjusted P-value of 0.1, the second output file contained 1,107 genes of which 73 were significantly increased 15 DPP and 1,034 significantly increased 30 DPP (Figure 3.3-1).

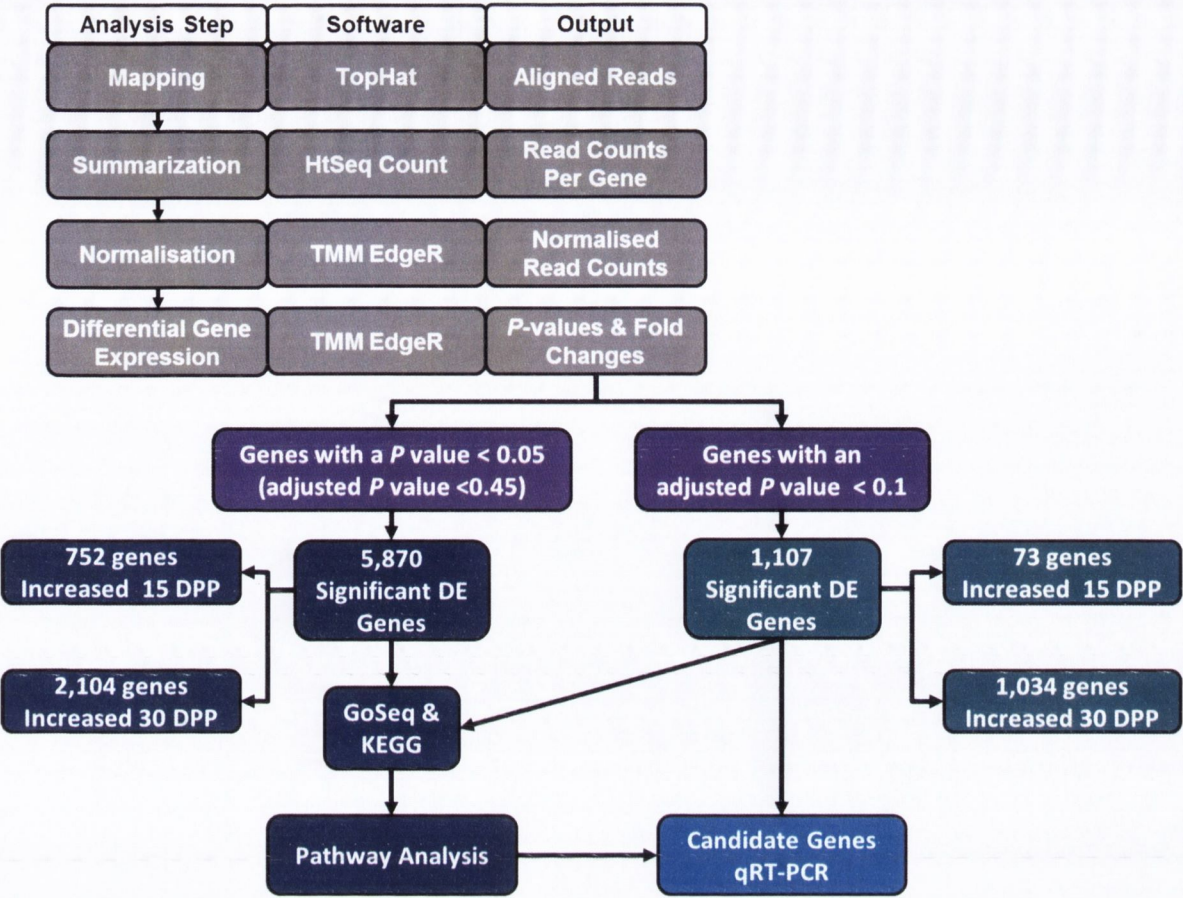


Figure 3.3-1: Flow chart from mRNA-Seq data analysis pipeline to differential gene expression results. The number of differentially expressed (DE) genes between the same healthy cows (n=3) 15 and 30 days postpartum (DPP) were under two different filtering criteria: more stringent filtering (adjusted *P* value <0.1) for candidate gene selection and more relaxed filtering (adjusted *P* value <0.45) for GoSeq and KEGG pathway analysis.

To assess the contribution of changes in endometrial cell subpopulations to the differential gene expression patterns detected between the two postpartum time points, the expression of cell surface markers associated with specific leukocyte cell subsets were analysed at a molecular level. The mRNA-Seq data set showed that six genes encoding leukocyte cell surface markers *CD11d* (also known as *ITGAD*), *CD18* (also known as *ITGB2*), *CD45* (also known as *PTPRC*), *CD48*, *CD53* and *CD62L* (also known as *SELL*) were significantly elevated at 15 DPP with a *P*-value of < 0.05 but were not significant using an adjusted *P*-value of < 0.1 (Table 3.3-4).

Table 3.3-4: Differentially elevated gene expression 15 days relative to 30 days postpartum (DPP) in the mRNA-Seq transcriptomic data set of endometrial biopsies for specific leukocyte markers. Significance was determined with a *P*-value < 0.05.

Gene Name	Cellular Expression	Bovine ID	<i>P</i> Value	Adjusted <i>P</i> Value
<i>CD11d</i>	Leukocytes	ENSBTAG000000019524	0.01	0.20
<i>CD18</i>	Leukocytes	ENSBTAG000000017060	0.01	0.15
<i>CD45</i>	All Hematopoietic Cells	ENSBTAG000000023144	0.01	0.19
<i>CD48</i>	Leukocytes	ENSBTAG000000011238	0.03	0.32
<i>CD53</i>	Leukocytes	ENSBTAG000000006466	0.03	0.32
<i>CD14</i>	Neutrophils	ENSBTAG000000015032	0.72	1.00
<i>CD16</i>	Neutrophils, NK cells, Macrophages	ENSBTAG000000002096	0.08	0.58

3.3.2.3 GoSeq KEGG pathway analysis

GoSeq is designed to account for gene length bias and was therefore selected to identify enriched pathways in the dataset. To account for the under representation of bovine genes in GoSeq KEGG pathway analysis, genes with a $P < 0.05$ were initially used. Additional analysis was also performed using the more stringent gene set (adjusted P of < 0.1) which reduces the risk of false positive genes in GoSeq KEGG pathway analysis.

Five pathways were enriched 15 DPP by genes with an adjusted P of < 0.1 , all of which are also present in the list of enriched pathways containing genes with a $P < 0.05$. Nineteen pathways were enriched 30 DPP containing genes with an adjusted $P < 0.1$ and eighteen of these pathways are also present in the list of enriched pathways containing genes with a $P < 0.05$.

The majority of the top enriched pathways (adjusted $P < 0.1$) 15 DPP from both gene datasets are immune associated such as T cell receptor signalling pathway, cytokine-cytokine receptor interaction (Figure 3.3-2), natural killer cell mediated cytotoxicity, PPAR signalling pathway, rheumatoid arthritis, graft-versus-host disease, allograft rejection and autoimmune thyroid disease (Table 3.3-5). A different functional profile is observed 30 DPP depicted by the top enriched pathways (adjusted $P < 0.1$) at this time (Table 3.3-6), which are indicative of proliferation and repair such as pathways in cancer, focal adhesion, wingless type (wnt) signalling pathway (Figure 3.3-3) and extracellular matrix (ECM)-receptor interaction (Figure 3.3-4).

Table 3.3-5: The most highly enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated pathways (adjusted $P < 0.1$) with significantly increased genes 15 days postpartum ($P < 0.05$; adjusted $P < 0.1$).

Enriched KEGG Pathways 15 DPP	Adjusted P -value	
	Over Represented	Under Represented
Input - Genes with a P -value < 0.05		
Primary immunodeficiency	3.26E-17	1.00E+00
T cell receptor signalling pathway	5.63E-14	1.00E+00
Natural killer cell mediated cytotoxicity	9.16E-14	1.00E+00
Hematopoietic cell lineage	1.90E-13	1.00E+00
Cytokine-cytokine receptor interaction	1.38E-11	1.00E+00
Input - Genes with an adjusted P -value < 0.1		
PPAR signalling pathway	6.19E-03	1.00E+00
Rheumatoid arthritis	7.63E-03	1.00E+00
Graft-versus-host disease	3.36E-02	9.99E-01
Allograft rejection	3.86E-02	9.99E-01
Autoimmune thyroid disease	4.19E-02	9.99E-01

Table 3.3-6: The most highly enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated pathways (adjusted P<0.1) with significantly increased genes 30 days postpartum (P<0.05; adjusted P<0.1).

Enriched KEGG Pathways 30 DPP	Adjusted <i>P</i> -value	
	Over Represented	Under Represented
Input - Genes with a <i>P</i>-value < 0.05		
Focal adhesion	4.80E-08	1.00E+00
Axon guidance	4.62E-06	1.00E+00
Hedgehog signalling pathway	4.75E-06	1.00E+00
ECM-receptor interaction	1.07E-05	1.00E+00
Basal cell carcinoma	1.83E-04	1.00E+00
Wnt signalling pathway	2.59E-04	1.00E+00
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	2.60E-04	1.00E+00
Melanogenesis	9.64E-04	1.00E+00
Tight junction	9.69E-04	1.00E+00
Cell adhesion molecules (CAMs)	1.35E-03	9.99E-01
Input - Genes with an adjusted <i>P</i>-value < 0.1		
Hedgehog signalling pathway	6.03E-08	1.00E+00
Focal adhesion	3.75E-07	1.00E+00
Basal cell carcinoma	1.20E-06	1.00E+00
ECM-receptor interaction	1.01E-05	1.00E+00
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	1.51E-05	1.00E+00
Melanogenesis	1.51E-05	1.00E+00
Wnt signalling pathway	4.60E-05	1.00E+00
Axon guidance	7.34E-05	1.00E+00
Pathways in cancer	8.55E-04	1.00E+00
Protein digestion and absorption	1.24E-03	1.00E+00

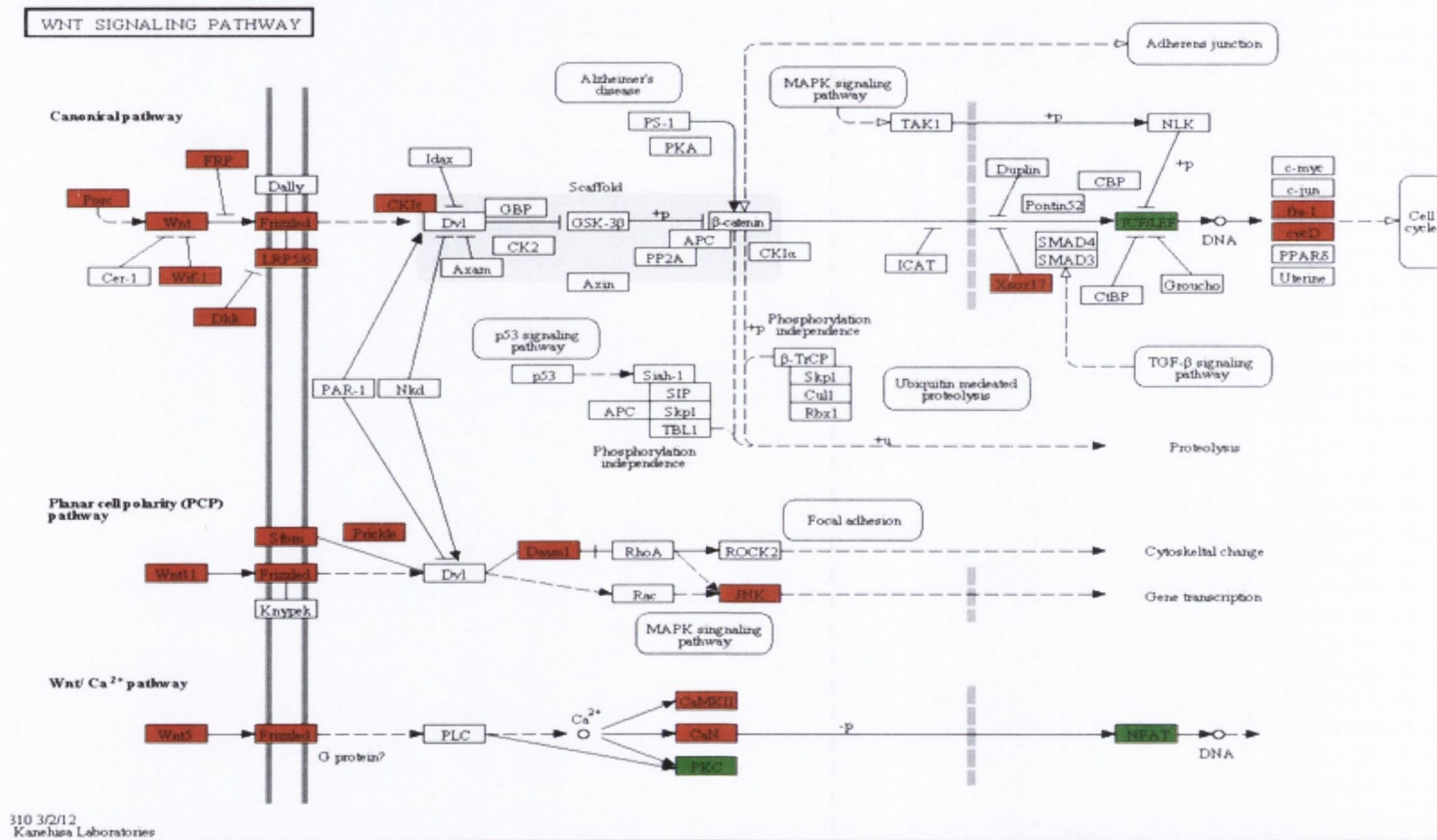
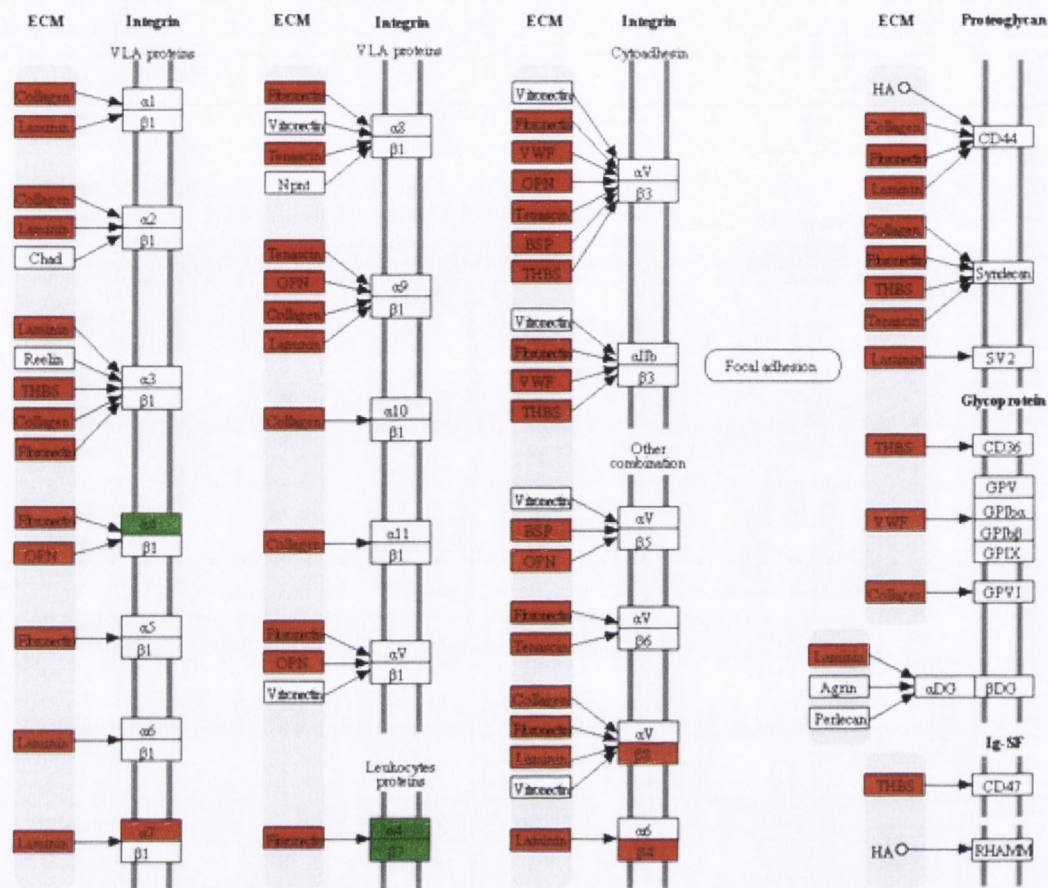


Figure 3.3-3: Differentially expressed genes within the Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Wnt Signalling pathway. Genes significantly elevated ($P < 0.05$) 15 DPP are in green and 30 DPP are in red.

ECM-RECEPTOR INTERACTION



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Figure 3.3-4: Differentially expressed genes within the Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated ECM (extra cellular matrix) receptor interaction pathway. Genes significantly elevated ($P < 0.05$) 15 DPP are in green and 30 DPP are in red.

3.3.3 Quantitative expression of candidate genes in additional animals

An additional panel of animals (5 cows) from the original sample set were used for qRT-PCR assessment of 19 candidate genes selected based on a variety of criteria (adjusted $P < 0.1$, read counts, pathway analysis and $\log_2$ fold change). Thirteen of these genes were annotated in the KEGG pathways: *IGF1* (fold change 7.7), *MGAT3* (fold change 7.4), *PDGFRA* (fold change 6.8), *RASGRP2* (fold change 4.7), *RDH10* (fold change 4.6), *WNT5A* (fold change 5.9), *SHC2*, *BMP6*, *CTF1*, *CLDN4* (fold change 8.9), *CDH5* (fold change 5.9), *TNFRSF13* (fold change 6.0) and *CD22* (fold change 4.9). The three candidate genes with no pathway annotation in the KEGG database were: *PLAC9* (fold change 6.4), *GATA2* (fold change 8.4) and *RARRES2* (fold change 6.6). Eleven of these candidate genes (adjusted $P < 0.1$) were represented in the enriched pathways ($P < 0.05$), with the exception of *MGAT3* and *RDH10*.

The qRT-PCR gene expression profile across this gene set corroborated the profile detected using mRNA-seq, with 4 of the 19 genes increased in expression 15 DPP and 15 genes increased in expression 30 DPP for both expression analysis platforms. However the magnitude of the $\log_2$ fold change was lower for qRT-PCR compared to mRNA-Seq for the majority of candidate genes which is probably due to the larger sample size used for qRT-PCR introducing greater biological variation (Figure 3.3-5).

Of the 19 candidate genes assessed by qRT-PCR, 5 were significantly increased 30 DPP: Serum Amyloid A - 2 (*SAA1/2*) [$P = 0.002$, $\log_2$ fold change 8.5], Insulin Growth Factor 1 (*IGF1*) [$P = 0.021$, $\log_2$ fold change 7.3], GATA binding protein 2 (*GATA2*) [$P = 0.001$, $\log_2$ fold change 6.4], serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 14 (*SERPINA14*) [$P = 0.031$, $\log_2$ fold change 5.4] and Src homology 2 domain containing transforming protein C2 (*SHC2*) [$P = 0.016$, $\log_2$ fold change 3.6] (Fig. 4). Other genes were also differentially expressed however these did not achieve statistical significance - *WNT5A* ($P = 0.053$), *PDGFRA* ($P = 0.077$) and *MGAT3* ($P = 0.057$).

Of note, six of the nineteen candidate genes selected from NGS data analysis have 0 reads aligned at one of the time points across all samples. Five genes, *SHC2*, *SERPINA14*, *BMP6*, *CTF1* and *SAA1/2*, have no aligned reads 15 DPP and have an average of 500, 216, 140, 136 and 80 reads respectively, aligned 30 DPP across all animals. One gene (*DEFB6*) has no reads aligned 30 DPP and an average of 28 reads aligned 15 DPP. It is not possible to calculate the fold change in these instances, therefore for illustrative purposes twice the highest $\log_2$ fold change was set as the fold change (Figure 3.3-5).

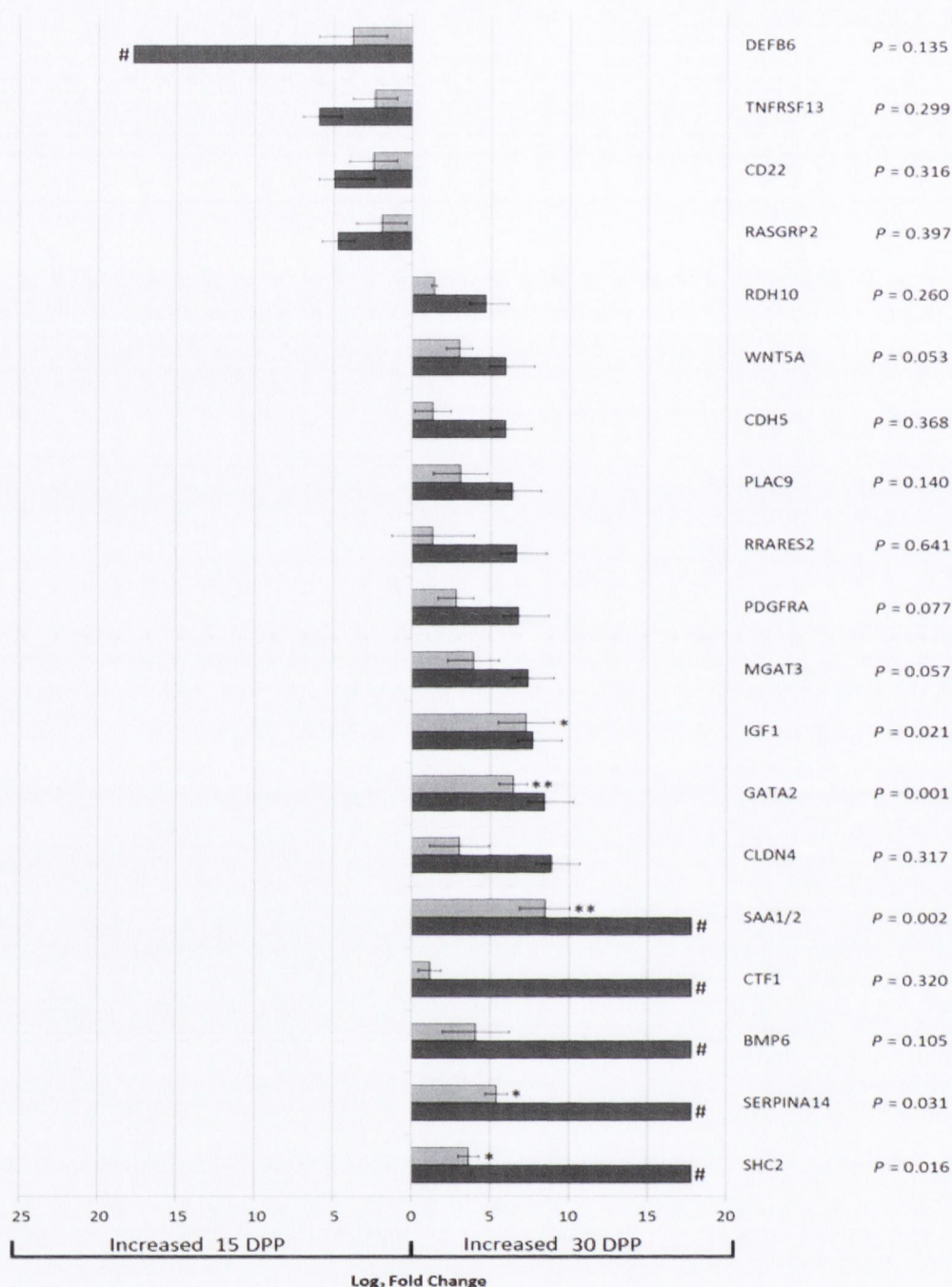


Figure 3.3-5: Log₂ fold changes from quantitative real-time PCR and mRNA-Seq for 19 differentially expressed genes in the endometrium of healthy cows between 15 and 30 days postpartum (DPP). Genes selected were significantly differentially expressed by mRNA-Seq (dark grey bars) (adjusted $P < 0.1$) and the P -values on the graph are those for qRT-PCR results (light grey bars) (* = $P < 0.05$, ** = $P < 0.005$). The error bars are representative of the standard error of the mean (SEM). Gene denoted by # have 0 reads at one time point across all samples and for illustrative purposes are represented on this graph as twice the size of the largest Log₂ fold change. Log₂ fold changes to the left of 0 are increased 15 DPP and Log₂ fold changes to the right of 0 are increased 30 DPP.

3.4 Discussion

Increasingly there is evidence at both a cellular and molecular level for the existence of an intimate role for the immune response during uterine involution. The neutrophilic infiltration of the endometrium is commonly associated with beneficial bacterial clearance, however in some situations there is evidence that their phagocytic activity may be hindered in animals susceptible to disease (Kim *et al.* 2005). During the early postpartum period recent literature has shown there is enrichment of genes within many immune pathways, Toll-like Receptors (TLRs) (Davies *et al.* 2008; Herath, Lilly, Santos, *et al.* 2009; Martins *et al.* 2011), inflammatory genes (Gabler *et al.* 2010), antimicrobial peptides and acute phase proteins (Chapwanya *et al.* 2009; Chapwanya *et al.* 2012). TLR4 is an example of a receptor, for bacterial lipopolysaccharide (LPS), shown to be temporally elevated in the postpartum uterus during involution (Chapwanya *et al.* 2012; Martins *et al.* 2011), between high and low fertility cows (Herath, Lilly, Fischer, *et al.* 2009) and in the epithelial and stromal cells of the endometrium in response to LPS *in vitro* (Davies *et al.* 2008).

However the extent to which inflammation is activated may become unregulated and can lead to disease (Maybin, Critchley, *et al.* 2011) therefore a balanced inflammatory immune response is key to sufficient bacterial clearance and the restoration of an endometrial environment capable of supporting a new pregnancy (Jabbour *et al.* 2009). Inflammatory resolution and the subcellular pathways involved in the restoration of uterine homeostasis have not been extensively explored in healthy cows. There is support for the hypothesis that dysregulation of the immune response (possibly as a consequence of inadequate bacterial clearance) is a contributory factor to disease development as significantly elevated expression of inflammatory genes has been previously documented in cows with endometritis at various time points postpartum (Galvão *et al.* 2011).

A previous study by our group determined the grade of endometrial inflammation based on the number of leukocytes present in each endometrial biopsy of the current study and the resulting candidate gene expression analysis showed significant temporal differences in this limited gene dataset (such as *IL16*, *TNF*, *IFNG*, *IL8*, *TAP*, *DEFB5*) between the two time points across involution (Chapwanya *et al.* 2012). Subsequent to this study it was hypothesised that global gene expression differences over time would reveal the pathways involved in the inflammatory immune response activation early postpartum, in the regulation of inflammation and the restoration of homeostasis in healthy cows. In the current study using the same animals, we use next generation sequencing to define the genome wide temporal changes in gene expression at two time points during involution in the postpartum cow.

3.4.1 Evidence at a molecular level of an elevated pro-inflammatory immune response 15 days postpartum (DPP) which undergoes a temporal change towards greater tissue proliferation and growth 30 DPP

In the current study changes in cellular subpopulations are likely to be reflected in the changes of the endometrial transcriptome. The resolution of inflammation detected in the healthy cows in this study will not be detected in animals that develop disease probably resulting from sustained cellular infiltration into uterine tissue associated with inflammation. Genes (*CD11d*, *CD18*, *CD45*, *CD48* and *CD53*) associated with specific leukocyte cell subsets were significantly elevated at 15 DPP with a *P*-value of < 0.05 however they did not have an adjusted *P*-value of < 0.1 and were not significant by this criteria.

Approximately only 27% of bovine genes are currently represented in the KEGG database, and therefore using a *P*-value cut-off of < 0.05 , 2,856 genes were found to be significantly differentially expressed between endometrial biopsies from the same animals across time points, 15 DPP and 30 DPP. Using increased stringency of an adjusted $P < 0.1$, over 1,100 genes were found to be significantly differentially expressed. In both sets of results, the immune system was predominantly represented by enriched pathways at 15 DPP. These pathways included T cell receptor signalling, cytokine-cytokine receptor interaction, natural killer cell mediated cytotoxicity, graft-versus-host disease and allograft rejection. This result concurs with related studies which have examined the expression of a limited number of candidate immune genes to show the activation of an inflammatory immune response in the postpartum endometrium (Chapwanya *et al.* 2009; Chapwanya *et al.* 2012; Gabler *et al.* 2010; Herath, Lilly, Santos, *et al.* 2009).

However, the majority of the differentially expressed genes detected in this study were significantly increased at the 30 DPP time point. The resolution of inflammation, as defined by histopathological assessment in these cows (Chapwanya *et al.* 2012), was supported by the significant decrease in expression of immune pathways as involution proceeds. At this time point (30 DPP) enriched pathways, such as focal adhesion, wnt signalling and ECM-receptor interaction, were associated with tissue regeneration and proliferative activity, reflecting repair processes. This complete temporal change in the endometrial transcriptomic profile from a pro-inflammatory immune response phenotype to a tissue regenerative profile shows rapid but transient immune induction in the uterus to clear bacteria and reduce associated inflammation within a 2 week period in healthy cows.

Endometrial receptivity has also been associated with an increase in extracellular matrix (ECM) remodelling pathways (Bauersachs *et al.* 2006; Klein *et al.* 2006), which is also one of the top enriched pathways 30 DPP in this study. “Pathways in cancer” is another of the top enriched pathways 30 DPP which highlights the proliferative capacity of the endometrium in the current study. Gene-expression patterns linked with the regeneration of damaged tissue closely resemble that of highly malignant tumours, as there is significant enrichment of genes involved in cell proliferation in both instances (Chang *et al.* 2004). In addition to cell proliferative processes, the endometrium is also undergoing regulation of cell growth and differentiation, and tissue homeostasis and repair 30 DPP, evident by the enrichment of the wnt signalling pathway (Zhao *et al.* 2009; Lin *et al.* 2010).

3.4.2 Candidate gene expression emphasises the molecular transition of the endometrium towards greater growth and proliferation in parallel with inflammatory resolution at a cellular level

Five candidate genes involved in this temporal transcriptomic change (*SAA1/2*, *IGF1*, *GATA2*, *SERPINA14* and *SHC2*), were found to be significantly differentially expressed 30 DPP using qRT-PCR. However the magnitude of the Log₂ fold change was lower for qRT-PCR compared to mRNA-Seq for the majority of candidate genes which is probably due to the larger sample size used for qRT-PCR introducing greater biological variation. This trend was also observed in another study that compared qRT-PCR and RNA-Seq data from an Illumina sequencer (Beane *et al.* 2011). The elevation of *SAA1/2* expression 30 DPP, suggests it has a role in postpartum inflammatory resolution in the endometrium of healthy cows. Serum amyloid A (SAA) is an acute phase protein (APP) produced and released by hepatocytes but it is also expressed in extrahepatic bovine tissues (Eckersall *et al.* 2006; Suffredini *et al.* 1999), constitutively in healthy endometria (Berg *et al.* 2011; Lecchi *et al.* 2012) and during inflammation (Chapwanya *et al.* 2009; Chapwanya *et al.* 2012). Studies have shown that SAA functions to preserve tissue maintenance and homeostasis (Urieli-shoval *et al.* 1998; Mitchell *et al.* 1991) and *SAA1/2* in particular has been shown in murine studies to be involved in the provision of immune homeostasis in mucosal tissue (Eckhardt *et al.* 2010). The elevated expression of *SAA1/2* 30 DPP may be indicative of the re-establishment of immune homeostasis later in involution as inflammation resolves.

Results also show the significant increased expression of *GATA2* as involution progresses in the postpartum uterus. Gene expression of interferon-tau (IFN- τ), a luteotrophic molecule, is regulated by the expression of *GATA2* in the bovine trophoblast (Bai *et al.* 2009), and is also

expressed in endometrial epithelial cells during the peri-attachment period of the conceptus in sheep (Bai *et al.* 2012). *GATA2* has also been recently shown to regulate the gene expression of endomucin, which is critical for cell growth, migration and angiogenesis, to ensure endothelial cell maintenance and physiological function (Kanki *et al.* 2011).

At systemic and local levels the insulin growth factor (IGF) system is implicated in endometrial repair and healing during involution (Llewellyn *et al.* 2008; Wathes *et al.* 2011). Decreased expression of *IGF1* has been observed in the previously gravid compared to the non-gravid uterine horn of the same cow 14 DPP (Llewellyn *et al.* 2008). In the present study we compared the progression of involution in the previously gravid uterine horn to a later stage of involution in the same horn and demonstrated that the expression of *IGF1* was elevated 30 DPP. In a murine study it has been shown that bioavailable IGF1 stimulates uterine growth therefore functioning to increase uterine size (Elis *et al.* 2011). A recent bovine study has suggested that an increase of IGF1 bioavailability has a negative effect on oocyte developmental competence (Velazquez *et al.* 2011). An increase in *IGF1* gene expression 30 DPP in the present study may infer proliferative effects on the uterus.

SHC2 gene expression was increased 30 DPP and is one of many genes enriching the focal adhesion pathway at this time, suggesting a role for *SHC2* in biological processes such as cell differentiation, motility, regulation of gene expression, cell proliferation, and cell survival in the endometrium later in involution. The Src family kinases are non-receptor tyrosine kinases involved in the mediation of intracellular signal transduction to initiate biological processes such as adhesion, migration, invasion, epithelial-to-mesenchymal transition, angiogenesis, apoptosis resistance and proliferation. Src members are activated by the binding of ligands to either their Src homology 2 (SH2) or 3 (SH3) domains (Johnson & Gallick 2007). In bovine endometrial epithelial cells it has been hypothesised that epidermal growth factor receptor may aid in the amplification of oxytocin signalling and activate c-Src resulting in the elevation of prostaglandin F2 α production, which is a luteolytic event (Krishnaswamy *et al.* 2010).

Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 14 (*SERPINA14*) belongs to the serpin superfamily of serine peptidase inhibitors (serpins) (Ing & Roberts 1989) and has also been previously called uterine milk protein (UTMP). Serine proteases are associated with immune functions involving inflammation, tissue remodelling, pathogen clearance and apoptosis, the over production of which causes pathologies in auto-immune diseases, tumour metastasis and allergies (Heutinck *et al.* 2010). Serpins limit the activity of serine proteases thereby regulating the severity of their immune functions (Heutinck *et al.* 2010). Gene expression of *UTMP*

has been observed predominantly in the bovine endometrium, ovary and caruncle tissues and the differential allelic expression of which has been associated with longevity in dairy cattle (Khatib *et al.* 2007). Expression of *SERPINA14* has been previously shown to be elevated by estrogens during estrus in cattle (Ulbrich *et al.* 2009; Bauersachs *et al.* 2005) and also during pregnancy (Klein *et al.* 2006). During pregnancy *UTMP* is thought to have a role in maternal immune modulation, by inhibiting NK-like activity and thus protecting the conceptus *in utero* (Tekin & Hansen 2002). Importantly, another study in sheep suggests that the expression of *UTMP* in the endometrium is a marker of differentiated and functional glandular epithelium (Gray *et al.* 2003). In the present study an increase of *SERPINA14* expression 30 DPP possibly indicates a greater degree of glandular epithelium repair within the endometrium at this time.

In conclusion this was the first study to assess global temporal transcriptional differences, using next generation sequencing, in the endometrium of healthy cows early postpartum. A genome-wide transcriptomic profile of endometrial biopsies 15 and 30 DPP was generated with mRNA-Seq and subsequently GoSeq KEGG pathway analysis identified enriched gene networks. This data, plus validation of specific gene expression patterns in additional postpartum animals provides evidence for a transcriptomic switch from a pro-inflammatory gene expression phenotype 15 DPP to a tissue regenerative and proliferative phenotype 30 DPP, despite the heterogeneity of cell type within the biopsy and bacterial species within the uterus. This transcriptomic switch from inflammation towards tissue proliferation may be a key attribute of a healthy endometrium early postpartum.

Chapter 4

4 Regulation of the local endometrial immune response in healthy and sub-clinically endometritic dairy cows early postpartum.

4.1 Introduction

An increase in the average herd size is predicted due to the abolition of Irish milk quotas in 2015. The pressure to achieve yearly spring compact calving will be amplified as farmers try to impregnate more cows within a larger herd during the recommended 12 week breeding season. Practical, cost-effective and rapid measures to detect health issues early in advance of the mating start date will be vital to improve farm sustainability, animal welfare and herd fertility. Additionally measures to identify and treat subclinical uterine disease will be a definite advantage considering that the rates of subclinical endometritis are approximately 30% of all cows (LeBlanc *et al.* 2002; LeBlanc 2008; Sheldon *et al.* 2006; Sheldon *et al.* 2008).

Previously we have highlighted the physiological role of inflammation early in involution which reduces over time in healthy cows, aiding in the elimination of ubiquitous bacterial contamination of the uterus (Chapwanya *et al.* 2012). However an elevated bacterial load in the uterus 7 and 21 DPP (Sheldon *et al.* 2002) and sustained endometrial inflammation around 21 DPP (Fischer *et al.* 2010) are a feature of endometritic infection. At parturition, contaminating bacteria can reside in the uterus without developing into an infection. When luteal progesterone down-regulates immune functions from approximately 12 to 21 days postpartum, bacteria proliferate which may result in uterine infection (Lewis 2003). Elevated bacterial growth scores in the uterus 7 and 21 days postpartum were associated with a reduced selection of first or second dominant follicles, resulting in slower growth of the first postpartum dominant follicle and delayed ovulation (Sheldon *et al.* 2002) and is indicative of subfertility (Opsomer *et al.* 2000). In endometritic cows neutrophil counts were >5% whereas in healthy cows these counts were <5% at 21 and 27 DPP (Fischer *et al.* 2010). In healthy cows, the number of endometrial neutrophils have been shown to decrease from around 21 DPP to 47 DPP (Fischer *et al.* 2010; Kasimanickam *et al.* 2005; Chapwanya *et al.* 2012). Indicating that at this time point postpartum there is a divergence occurring in the inflammatory profile of the endometrium with some cows resolving inflammation and others enduring sustained inflammation.

In the current study this divergence in the inflammatory profile of the endometrium was characterised at a cellular level using histology to grade the extent of inflammation 21 DPP and at a molecular level by implementing transcriptomics to unravel differences underpinning this

divergence from 7 to 21 DPP. The uterus is a complex organ, composed of multiple layers of specialised cell types. In order to capture the contribution from each of these layers, uterine biopsies are essential. Taken correctly and carefully, these valuable research tools do not have a detrimental impact on subsequent fertility (Chapwanya *et al.* 2010). Histology provides a visual appreciation for tissue integrity and the density of immune cell infiltrate in the epithelial and stromal layers of endometrial biopsies so as to facilitate the categorisation of cows as either healthy or at risk of sub-clinical endometritis 21 DPP. The combination of histology and transcriptomic assessment for each individual biopsy is invaluable for accurately evaluating the effect of time and the immune response in healthy compared to diseased animals on endometrial gene expression *ex vivo* (Galvão *et al.* 2011). The microbiomic profiling of the postpartum uterus in the current study is a complementary technique that may shed light on variations in bacterial populations over time or between healthy and sub-clinically endometritic cows.

Inflammatory responses that occur in the post-partum cow are critical for returning the uterus to a normal physiological state capable of supporting a subsequent pregnancy. Large numbers of genes have been shown to be differentially expressed in the post-partum uterus; and our work has shown a shift from the activation of immune pathways early post-partum to the upregulation of tissue repair and proliferation pathways in healthy cattle (Foley *et al.* 2012). Uterine inflammation is therefore part of the normal tissue remodelling process that occurs postpartum in healthy animals and may be driven by bacterial colonisation. Dysregulation of this immune response may contribute to sustained inflammation and development of chronic infection.

The integration of multiple layers including clinical, cellular, microbiological and molecular information, will define the mechanisms responsible for effective bacterial clearance from the postpartum uterus. In parallel, the signature of response that predicts failure to eliminate uterine bacteria and the point at which sub-clinically endometritic cows diverge from healthy cows will be characterised. The emergence of new reliable diagnostics will depend on a detailed understanding of the role the endometrial immune response plays in this process, and how dysregulation of the endometrial immune response contributes to the development of infection. A comprehensive picture of the role of local immunity, as well as interactions between the immune and other systems in the post-partum bovine uterus, will identify potential targets for early therapeutic intervention and improving downstream fertility.

4.2 Aim

The research aim of this experiment was to assess the local profile of the postpartum endometrium by investigating differential mRNA and microRNA expression in endometrial biopsies and characterise differences in the regulation of the host's immune response between sub-clinically endometritic and healthy cows 7 and 21 days postpartum.

4.3 Results

4.3.1 Clinical data and pregnancy diagnosis

Twenty two cows were sampled at 7 and 21 days postpartum (DPP) and on each occasion clinical information was recorded which included body condition score, temperature, vaginal mucosal character and smell. The definition of clinical endometritis is the presence of vaginal mucoid discharge from 21 DPP, in this study there were 6 out of 22 cows clinically diagnosed as endometritic.

The average rectal temperature across twenty two cows 7 DPP was 38.68°C (range 37.2°C – 39.7°C) and 21 DPP was 38.54°C (range 38.1°C – 39.1°C). There were two cows with a rectal temperature of $\geq 39.5^\circ\text{C}$ (39.7°C and 39.5°C) exclusively at 7 DPP. The systemic profile measurement of haptoglobin for cow 12 was below 1 g/l indicating no signs of systemic inflammation which occurs in 7.28% cows within 10 days after calving (Burfeind *et al.* 2012). The concentrations of haptoglobin in cow 16 was greater than 1 g/l indicating systemic inflammation which may be due to metritis as there was mucoid discharge from this cow at 7 DPP. All cows had rectal temperatures $\leq 39.5^\circ\text{C}$ at 21 DPP and consequently did not have a fever. The average body condition score (BCS) at 7 DPP was 2.5 (range 2.25 – 3) and at 21 DPP was 2.5 (range 2 – 3). After 60 days post artificial insemination (AI) pregnancy diagnosis was carried out for all cows, 13 were pregnant and 9 were not at the time of diagnosis (Table 4.3-1).

Two cows were removed from the study due to health complications, cow 3 had a severe womb infection and a bad heart receiving a selection of drugs for both problems and cow 19 had retained the placenta after birth and was treated with Solvasol for 5 days. Cows 10 and 15 received local administration of Synulox Lactating Cow tubes (Pfizer Animal Health, Ringaskiddy, County Cork, Ireland) to treat mastitis but these cows remained in the study as the withdrawal period of 7 days was surpassed when the biopsies were collected.

Table 4.3-1: Clinical results obtained from each cow (n=22) prior to sampling at 7 and 21 days postpartum (DPP). This included body condition score (BCS), rectal temperature (Temp), vaginal mucosal character and smell. Pregnancy Diagnosis (PD) was undertaken using ultrasound 60 days after artificial insemination (DAAI) and a cow was classified either pregnant (+) or not pregnant (-).

Cow ID	7 DPP				21 DPP				PD > 60 DAAI
	BCS	Temp	Mucosa Character	Mucosa Smell	BCS	Temp	Mucosa Character	Mucosa Smell	
1	2.75	38.80	Mucoid	Yes	2.50	38.30	Mucoid		-
2	2.25	38.10	Bloody	Yes	2.50	39.10	Mucoid	Yes	+
3	2.25	38.96	Mucoid & Bloody	Yes	2.00	38.51	Mucoid	Yes	-
4	2.50	38.30	Bloody		2.75	38.40			-
5	3.00	38.50	Bloody		2.75	38.40			+
6	2.25	38.80	Bloody		2.75	38.50			-
7	2.25	38.60	Bloody		2.25	38.30			+
8	2.50	37.65	Bloody		2.50	38.20			-
9	2.75	38.82	Mucoid & Bloody	Yes	2.75	38.10			-
10	2.50	38.40			3.00	38.40			+
11	2.50	38.10	Bloody		2.25	39.11			-
12	2.75	39.71	Bloody		2.50	38.40			+
13	2.50	37.20			2.50	38.70			+
14	2.50	39.06	Bloody		2.50	38.91			+
15	2.75	38.92	Mucoid & Bloody	Yes	2.50	38.60	Mucoid		+
16	2.25	39.50	Mucoid & Bloody	Yes	2.25	38.50	Mucoid & Blood		-
17	2.50	38.97			2.50	38.70			+
18	2.75	38.63	Bloody		2.75	38.67			+
19	2.50	38.70	Mucoid & Bloody	Yes	2.50	38.10	Mucoid		-
20	2.50	38.91	Bloody	Yes	2.25	38.70			+
21	2.75	38.88			2.50	38.39			+
22	2.50	39.43	Bloody		2.50	38.79	Bloody		+

4.3.2 Histopathology

The histological tissue sections were assessed using a conventional light microscope at an x 400 high power field (hpf) magnification allowing the visualisation of the endometrial epithelial and stromal layers at 7 and 21 DPP. The extent of inflammation in the endometrium was classified based on previous criteria outlined by Chapwanya *et al.* 2010, which focused on intensity of inflammatory cell infiltrating the stroma, in combination with a recent study by Meira *et al.* 2012, which incorporated the numbers of polymorphonuclear neutrophils in the endometrial epithelium (Table 4.3-2).

The majority of tissue sections at 7 DPP did not have an intact epithelial layer which did not make it possible to grade these sections appropriately. One cow (14) whose endometrial biopsy section did not have an intact epithelial layer 21 DPP however the stromal infiltrate was such that it was evident that the endometrium was not severely inflamed. Sub-clinical endometritis is diagnosed from 21 DPP and therefore these biopsy sections were graded and cows were grouped based the classification of inflammation at this time point. There were three biopsy sections (cows 20, 21 and 22) that either had little or no endometrial tissue present to score and were therefore removed from the study. Cows allocated a score of 4 or more were assigned to the sub-clinically endometritic group of which there were 6 and cows with an allocated score of 3 or less were assigned to the healthy group of which there were 9 (Table 4.3-3).

Table 4.3-2: Histology images of the bovine endometrium 7, 21 and 60 days postpartum at 20 x and 40 x high power field (hpf) magnification. (1) Reference of non-inflamed endometrial tissue ~ 60 days postpartum (DPP) (2) non-inflamed endometrial tissue 21 DPP and (3) severely inflamed endometrial tissue 21 DPP. Scoring criteria as outlined in Table 2.7-1.

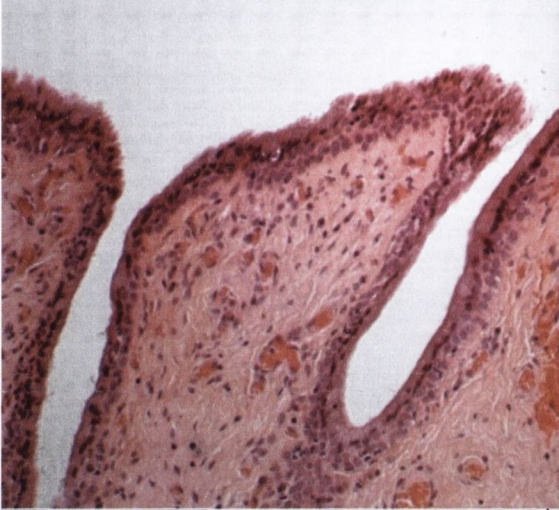
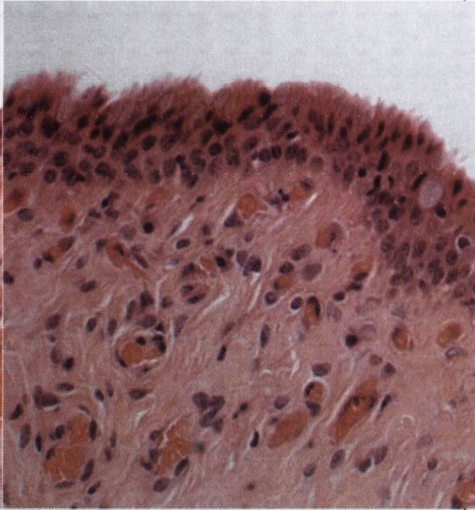
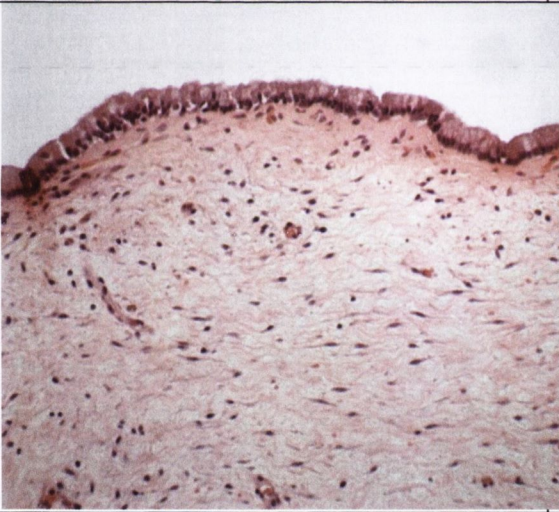
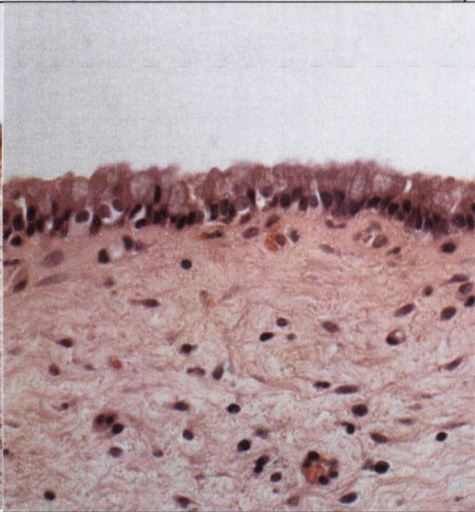
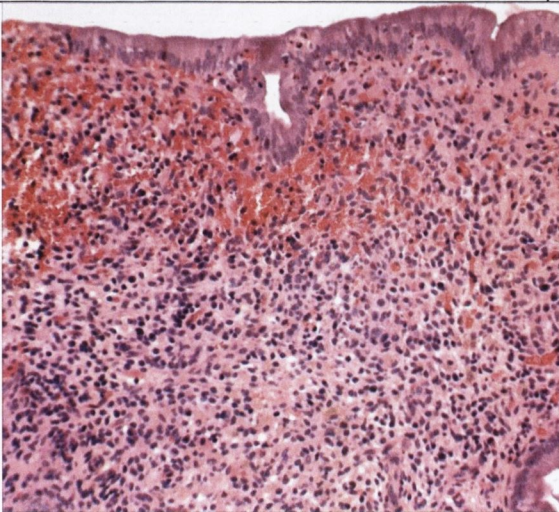
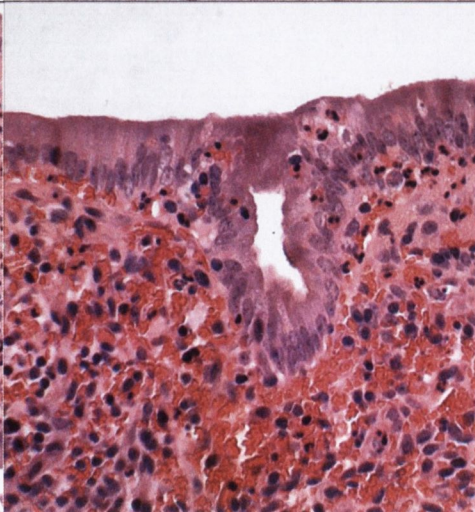
20 x	40 x	(Image), Cow ID, DPP, Score
		(1) example ~60 DPP 0
		(2) 4 21 DPP 0
		(3) 16 21 DPP 5

Table 4.3-3: Scores associated with the extent of inflammation 21 days postpartum in histology sections of endometrial biopsies based on the intensity of immune cell infiltration in the epithelium and stroma. Visual scoring criteria as demonstrated in Table 2.7-1. Total score ≥ 4 defined sub-clinically endometritic cows (n=6) and total score ≤ 3 defined healthy cows (n=9).

	Sample ID	Epithelium	Stroma	Score
Sub-Clinically Endometritic	1	3	3	6
	2	3	3	6
	8	3	3	6
	16	3	2	5
	5	3	2	5
	15	1	3	4
Healthy	10	1	2	3
	11	1	2	3
	13	1	2	3
	7	0	2	2
	9	0	2	2
	6	0	1	1
	12	0	1	1
	14	no epithelium	1	1
	4	0	0	0

4.3.3 Microbiology

The terminal-restriction fragment length polymorphism (T-RFLP) technique and Principal Coordinates Ordination (PCO) was used to identify differences in 16S rRNA profiles of microbial populations within the uterus between 28 samples (14 cows) 7 and 21 DPP. The output was a series of 16S rRNA peaks (fragments) ranging in sizes and heights representative of the various microbial species. Analysis of the data did not define specific bacterial communities yet highlighted how different the microbial communities within each sample were from other samples. A significant difference ($p = 0.04$) in the microbial communities present between healthy and sub-clinically endometritic cows was detected. A dendrogram (Figure 4.3-1) and Principal Coordinates PCO plot (Figure 4.3-2) were constructed to allow visualisation of the distribution of the variation within the data set. There is a slight clustering of sub-clinically endometritic (SCE) cows to the right of the dendrogram with the majority of healthy samples to the left. In the PCO plot 16.9% of the variation was accounted for on the x-axis and 8.4% on the y-axis, our data set highlighted a clustering of SCE samples to the left of the x-axis and a clustering of healthy samples to the right of the x-axis where more of the total variation is accounted for compared to the separation based on the y-axis.

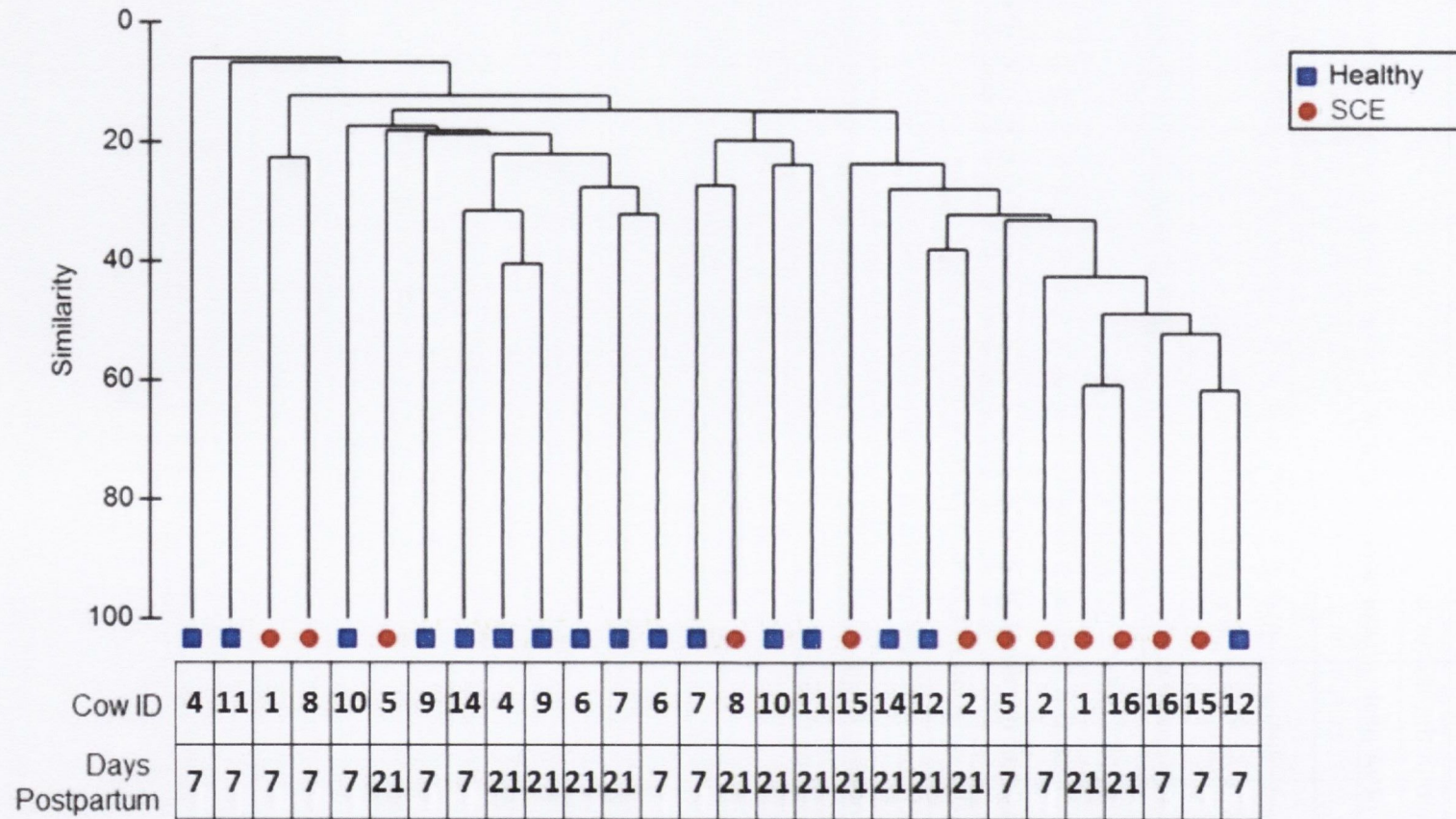


Figure 4.3-1: Dendrogram representing differences in microbial populations between uterine swab samples 7 and 21 days postpartum. Cows that were classified as healthy 21 days postpartum, subsequent to histopathology assessment, are square and blue and sub-clinically endometritic (SCE) are circular and red. Microbial communities were significantly different ($p = 0.04$) between healthy and SCE cows. (This graph was produced by a group led by Professor Wim Meijer in the School Of Biomolecular & Biomed Science, Conway Institute, University College Dublin, Ireland).

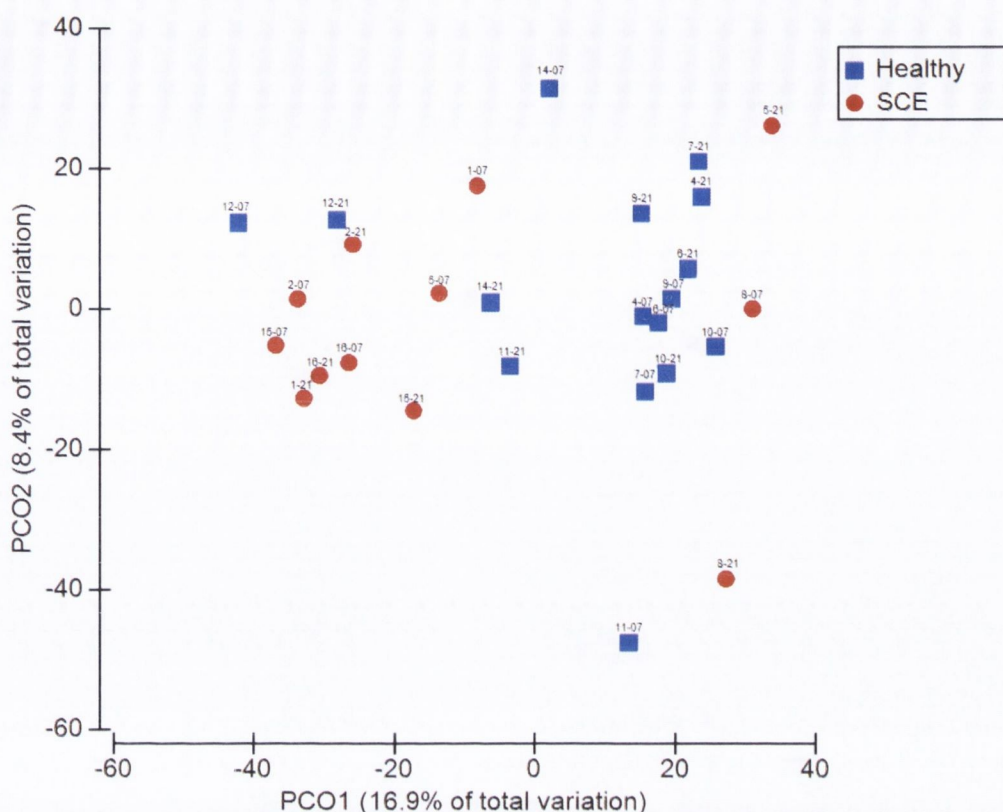


Figure 4.3-2: Principal coordinates ordination (PCO) plot representing differences in microbial populations between uterine swab samples 7 and 21 days postpartum. Cows that were classified as healthy 21 days postpartum, subsequent to histopathology assessment, are square and blue and sub-clinically endometritic (SCE) are circular and red. Microbial communities were significantly different ($p = 0.04$) between healthy and SCE cows. (This graph was produced by a group led by Professor Wim Meijer in the School Of Biomolecular & Biomed Science, Conway Institute, University College Dublin, Ireland).

4.3.4 RNA isolation

Optimisation of RNA isolation:

The isolation of RNA from endometrial biopsies was optimised using endometrial biopsies, from cows which were removed from the experiment due to poor biopsy histology quality, and also from liver samples. Initially method 2 was used to extract RNA from 4 endometrial biopsies with weights between 43 – 101 mg, however this method did not produce RNA of sufficient quality, RNA integrity number (RIN) less than 5, and quantity, between 603 and 843 ng and 675 ng on average, for downstream analysis.

Therefore to independently assess the quality and quantity of RNA isolated using method 1 and compare it to method 2, four technical replicates from an individual liver sample were used. RNA was isolated from two of the technical replicates with weights of 115 and 132 mg using method 2 and two other technical replicates with weights 96 and 95 mg using method 1. Both methods produced RNA of high quality, RIN 7.5 – 8.1, and quantity, 45369 – 68442 ng, and on average produced between 478.5 and 595.3 ng per mg of tissue used. These results suggested that the input weight of endometrial tissue may have been too low when RNA was initially isolated using method 2.

The input weight of endometrial tissue used was increased, 166 and 243 mg, by using the entire biopsy at once and method 1 was used to isolate the RNA. The isolated RNA was of high enough quality, RIN 6.1 – 8.1, and quantity, 24780 – 49002 ng, for use in downstream analysis. As a result of this optimisation the entire biopsy and method 1 were used to isolate RNA from test endometrial biopsies (Figure 4.3-4).

Table 4.3-4: The quantity and quality of RNA isolated from endometrial and liver tissue during the optimisation of the method required to isolate RNA. The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the quality of RNA extracted which calculated the RNA integrity number (RIN) as a measure of RNA quality. The NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc.) calculated RNA concentration based on absorbance readings. Each sample letter identifies a different cow. Methods 1 and 2 are the different RNA isolation methods under investigation.

Sample	Tissue	Method	Tissue weight	RNA concentration		RNA yield		RNA quality	RNA per mg of tissue	
			mg	ng/μL	Average	ng	Average	RIN	ng RNA per mg tissue	Average
A	Endometrium	2	101	21.3	22.5	639	675	< 5	6.3	11.4
B	Endometrium	2	43	20.1		603		< 5	14.0	
C	Endometrium	2	70	28.1		843		< 5	12.0	
D	Endometrium	2	46	20.5		615		< 5	13.4	
E	Liver	2	115	1768.9	1974.8	53067	59244	8.1	461.5	478.5
E	Liver	2	132	2180.7		65421		7.5	495.6	
E	Liver	1	96	2281.4	1896.9	68442	56906	7.9	712.9	595.3
E	Liver	1	95	1512.3		45369		8.1	477.6	
F	Endometrium	1	116	1633.4	1229.7	49002	36891	6.1	422.4	262.2
G	Endometrium	1	243	826.0		24780		8.1	102.0	

RNA quantity and quality assessment:

RNA was isolated from endometrial biopsies of 17 cows at both 7 and 21 DPP which accumulated to 34 test biopsies in total. However the quality and quantity of RNA isolated from two cows, 17 and 18, was too low at either or both times points for use in downstream analysis and these samples were removed from the study resulting in a sample size of 15. With the exclusion of these cows the isolated RNA from the remaining biopsies, weighing on average 199 mg and ranging between 61 – 438 mg, was of high quality, with an average RIN value of 8 and ranging between a RIN of 6 and 9.9, and sufficiently high quantity, with an average of 68173 ng and ranging between 23847 – 116589 ng, to be used in downstream analysis (Table 4.3-5).

Table 4.3-5: The quantity and quality of RNA isolated from test endometrial biopsies using the optimised method for RNA isolation. Each number in the cow ID column represents a specific cow which was sampled 7 and 21 days postpartum (DPP). The RNA integrity number (RIN) was produced with the Agilent 2100 Bioanalyser (Agilent Technologies) and NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc.) achieved the 260/230 ratio and RNA concentration. * These samples were not used in downstream analysis as either cow had a RIN value less than 6 at either one or both time points under investigation.

Cow ID	DPP	Tissue weight	RNA concentration	RNA yield	RNA per mg of tissue		RNA quality	
		mg	ng/µl	ng	ng/µl per mg tissue	ng per mg tissue	RIN	260/230
1	7	170	1158.0	34740.0	6.8	204.4	7.8	2.1
1	21	330	2119.6	63588.0	6.4	192.7	8.3	2.0
2	7	214	1481.6	44448.0	6.9	207.7	7.3	2.3
2	21	178	2533.5	76005.0	14.2	427.0	8.5	2.2
4	7	117	1074.1	32223.0	9.2	275.4	6.0	2.3
4	21	306	2971.2	89136.0	9.7	291.3	6.9	2.2
5	7	153	1542.4	46272.0	10.1	302.4	7.9	1.8
5	21	355	1750.3	52509.0	4.9	147.9	7.8	2.1
6	7	155	2413.1	72393.0	15.6	467.1	7.7	2.0
6	21	330	1980.2	59406.0	6.0	180.0	8.2	1.8
7	7	187	955.9	28677.0	5.1	153.4	7.0	2.3
7	21	284	3884.4	116532.0	13.7	410.3	7.0	1.9
8	7	438	3679.5	110385.0	8.4	252.0	6.6	2.1
8	21	61	1872.8	56184.0	30.7	921.0	7.8	2.1
9	7	147	2719.9	81597.0	18.5	555.1	7.1	2.1
9	21	167	3718.7	111561.0	22.3	668.0	7.8	2.0
10	7	245	2097.1	62913.0	8.6	256.8	6.2	2.3
10	21	322	3836.6	115098.0	11.9	357.4	6.2	2.0
11	7	123	1357.6	38691.6	11.0	314.6	6.5	2.2
11	21	203	2619.4	78582.0	12.9	387.1	8.1	1.8
12	7	123	2766.2	78836.7	22.5	640.9	8.8	2.2
12	21	77	3486.1	104583.0	45.3	1358.2	7.3	2.0
13	7	229	3886.3	116589.0	17.0	509.1	7.9	2.0
13	21	150	1969.1	59073.0	13.1	393.8	8.1	2.2
14	7	134	1817.5	54525.0	13.6	406.9	9.9	2.2
14	21	142	1240.1	37203.0	8.7	262.0	8.9	1.5
15	7	91	794.9	23847.0	8.7	262.1	7.1	1.2
15	21	228	2414.1	72423.0	10.6	317.6	7.5	1.7
16	7	157	1591.8	47754.0	10.1	304.2	8.6	2.2
16	21	142	2647.6	79428.0	18.6	559.4	7.9	2.1
17*	7	192	803.6	24108.0	4.2	125.6	4.9	1.5
17*	21	195	1318.0	39540.0	6.8	202.8	5.6	2.3
18*	7	356	0.8	24.0	0.0	0.1	0.0	0.0
18*	21	200	2412.0	72360.0	12.1	361.8	7.3	2.2

4.3.5 mRNA-Seq library preparation and cDNA quantity assessment

Prior to using test samples to construct cDNA libraries for mRNA-Seq the mRNA-Seq library preparation was carried out to discern the optimal input RNA concentration and the least number of PCR enrichment cycles that could be used to achieve an adequate quantity of cDNA.

Optimisation of cDNA library preparation for sequencing:

RNA previously isolated from liver was used to optimise the amount of input RNA for cDNA library preparation. From the same pool 2, 4 and 6 µg of RNA was used as input for the construction of 3 different libraries, using the protocol as per manufactures instruction, which produced cDNA libraries of concentration 34.9, 62.0 and 51.5 ng/µl, respectively . The concentration of each cDNA sample had to be ≥ 2.98 nM to ensure that it could be sequenced on the Illumina HiSeq 2000 sequencer. The estimated concentration (nM) for each sample was more than 100 times the required concentration for library preparation at 303.7, 307.7 and 311.6 nM respectively. Based on these results it was decided to progress with an input of 4 µg of RNA for cDNA library preparation so as to get the maximum concentration of cDNA while sparing RNA for potential repeats (Table 4.3-6).

Table 4.3-6: cDNA sequencing library concentrations achieved from the optimisation of the mRNA-Seq library preparations using varying RNA input concentrations isolated from liver tissue. The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the quality of input RNA which calculated the RNA integrity number (RIN) as a measure of RNA quality. The NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc.) calculated input RNA concentration based on absorbance readings. The quantity of cDNA in the final sequencing cDNA library was assessed using the Quant-iT™ dsDNA HS assay kit and the Qubit™ fluorometer.

Input RNA	Tissue	Liver					
	Sample	E					
	RIN	8.1					
	Concentration	2 µg		4 µg		6 µg	
Output cDNA		ng/µl	nM	ng/µl	nM	ng/µl	nM
		34.9	303.7	62.0	307.7	51.5	311.6

RNA previously isolated from two endometrial biopsies was used to discover how few enrichment cycles could be used to achieve a cDNA library concentration of ≥ 2.98 nM for sequencing so as to avoid introducing unnecessary PCR bias in downstream analysis. The input RNA concentration was 4 µg for all library preparations of which there were 8 in total, two samples and four different cycling treatments. The library preparation of sample F using 12 PCR cycles failed during the

library preparation, from the samples that were successful using 10 PCR enrichment cycles produced cDNA libraries with concentrations between 55.2 and 75.7 nM which was sufficient for downstream sequencing. Subsequent to these results the cDNA library construction for test samples incorporated a PCR enrichment step with 10 cycles (Table 4.3-7).

Table 4.3-7: cDNA sequencing library concentrations achieved from the optimisation of the mRNA-Seq library preparations with variations in the number of PCR enrichment cycles using RNA isolated from endometrial tissue. The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the quality of input RNA which calculated the RNA integrity number (RIN) as a measure of RNA quality. The NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc.) calculated input RNA concentration based on absorbance readings. The quantity of cDNA in the final sequencing cDNA library was assessed using the Quant-iT™ dsDNA HS assay kit and the Qubit™ fluorometer.

	Tissue	Endometrial			
	Sample	F		G	
Input RNA	RIN	6.1		8.1	
	Concentration	4 µg		4 µg	
Output cDNA		ng/µL	nM	ng/µL	nM
	10 Cycles	10.1	55.2	17.1	75.7
	12 Cycles	1.2	x	33.9	153.6
	14 Cycles	18.7	91.5	48.8	258.4
	15 Cycles	57.0	274.5	60.1	299.7

cDNA sequencing library quantity and purity assessment:

The input RNA concentration for all library preparations was 4 µg in a final volume of 50 µl and the first round of library preparations resulted in average cDNA concentrations of 17.57 ng/µl. However there were a number of samples with low cDNA concentrations and it was decided to repeat these libraries. By including these repeats in the place of their first round libraries the average concentration of cDNA increased to 20.41 ng/µl, with a range of between 9.6 and 31.4 ng/µl (Table 4.3-8).

Table 4.3-8: cDNA sequencing library concentrations achieved from mRNA-Seq library preparations using RNA isolated from test endometrial biopsies. Each number in the cow ID column represents a specific cow which was sampled 7 and 21 days postpartum (DPP). The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the quality of input RNA which calculated the RNA integrity number (RIN) as a measure of RNA quality. The NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc.) calculated input RNA concentration based on absorbance readings. The quantity of cDNA in the final sequencing cDNA library was assessed using the Quant-iT™ dsDNA HS assay kit and the Qubit™ fluorometer.

Cow ID	DPP	RNA Concentration		4 ug		Final Volume	cDNA Library (Qubit™) Concentration	
		ng/μl	ug/μl	RNA (μl)	H2O (μl)	μl	ng/μL	Repeats ng/μL
1	7	1158	1.2	3.5	46.5	50	6.5	27.6
1	21	2119.6	2.1	1.9	48.1	50	22.4	
2	7	1481.6	1.5	2.7	47.3	50	17.6	
2	21	2544.5	2.5	1.6	48.4	50	15.7	
4	7	1074.1	1.1	3.7	46.3	50	10.1	14.1
4	21	2971.2	3.0	1.3	48.7	50	13.2	
5	7	1542.4	1.5	2.6	47.4	50	23.2	
5	21	1750.3	1.8	2.3	47.7	50	24.2	
6	7	2413.1	2.4	1.7	48.3	50	9.4	18.9
6	21	1980.2	2.0	2.0	48.0	50	31.2	
7	7	955.9	1.0	4.2	45.8	50	17.4	
7	21	3884.4	3.9	1.0	49.0	50	19.8	
8	7	3679.5	3.7	1.1	48.9	50	19.7	
8	21	1872.8	1.9	2.1	47.9	50	17.8	
9	7	2719.9	2.7	1.5	48.5	50	4.5	21.8
9	21	3718.7	3.7	1.1	48.9	50	5.4	14.5
10	7	2097.1	2.1	1.9	48.1	50	18.7	21.0
10	21	3836.6	3.8	1.0	49.0	50	6.4	14.2
11	7	1357.6	1.4	2.9	47.1	50	17.1	
11	21	2619.4	2.6	1.5	48.5	50	13.1	
12	7	2766.2	2.8	1.4	48.6	50	8.3	22.4
12	21	3486.1	3.5	1.1	48.9	50	9.6	
13	7	3886.3	3.9	1.0	49.0	50	22.0	
13	21	1969.1	2.0	2.0	48.0	50	25.4	
14	7	1817.5	1.8	2.2	47.8	50	31.4	
14	21	1240.1	1.2	3.2	46.8	50	18.4	
15	7	794.9	0.8	5.0	45.0	50	18.2	
15	21	2414.1	2.4	1.7	48.3	50	28.4	
16	7	1591.8	1.6	2.5	47.5	50	27.8	
16	21	2647.6	2.6	1.5	48.5	50	24.2	

Sample pooling QC:

Once cDNA libraries were constructed 5 were pooled together in equimolar concentrations (10 nM each) to achieve a final pool concentration of 10 nM. Note that cow 3 is included in pool 2 and three samples were duplicated (cows 1 - 7DPP, 1 - 21DPP and 6 - 21DPP), in order to achieve a balance of 5 samples within all pools, however as addressed earlier cow 3 was not included in downstream analysis due to health complications. Once the pools were constructed their concentrations were measured (average 1.92 ng/μl and range 1.83 – 2.02 ng/μl) and molarity calculated (average 8.98 nM and range 8.25 – 9.58 nM).

These pools were then sent for sequencing and upon arrival at the sequencing facility the pooled concentrations were assessed. The results were similar to those that we achieved in house, averaging 2.07 ng/μl and ranging between 1.99 – 2.40 ng/μl. However the sequencing facility performed a separate protocol to assess the final molarity of cDNA within each pool which resulted in an average of 36.13 nM ranging between 34 and 38.5 nM. In addition to this we used the cDNA concentration and base pair sizes for each pool achieved by the sequencing facility to estimate the molarity of the pool using the method of calculation we used previously. Using this criterion the average molarity of the cDNA pools was 11.69 nM ranging between 9.27 and 12.84 nM which was closer to our own data (average 8.98 nM and range 8.25 – 9.58 nM) and it was decided to proceed with the sequencing (Table 4.3-9).

Table 4.3-9: The quantity of pooled cDNA libraries and the average cDNA fragment size within each pool as calculated in Teagasc Grange (In House QC) and by the sequencing facility (BGI QC). Each number in the cow ID column represents a specific cow which was sampled 7 and 21 days postpartum (DPP). Technical replicates were used across different pools – technical replicate pool (TRP). The quantity of cDNA in the final sequencing cDNA library was assessed using the Quant-iT™ dsDNA HS assay kit and the Qubit™ fluorometer. The Agilent 2100 Bioanalyser (Agilent Technologies) was used to assess the average base pair (bp) fragment size of the cDNA library.

Cow ID	DPP	Pool	In House QC				BGI QC				
			Qubit		Bioanalyzer	nM	Qubit		Bioanalyzer	In House	BGI
			ng/μL	μg	bp		ng/μl	μg	bp	nM	nM
1 (TRP1)	7	Pool 1	1.90	0.07	333	8.79	2.24	0.07	272	12.69	36.4
1 (TRP1)	21										
2	7										
6 (TRP1)	21										
2	21										
3	7	Pool 2	2.02	0.08	325	9.58	2.40	0.07	288	12.84	36.4
3	21										
4	7										
4	21										
1 (TRP2)	21										
5	7	Pool 3	1.88	0.07	351	8.25	2.10	0.06	271	11.94	35.6
5	21										
6	7										
1 (TRP3)	7										
6 (TRP3)	21										
7	7	Pool 4	1.93	0.08	321	9.26	1.99	0.06	270	11.36	34.0
7	21										
8	7										
8	21										
9	7										
9	21	Pool 5	1.89	0.07	320	9.10	2.12	0.06	268	12.19	38.5
10	7										
10	21										
11	7										
11	21										
12	7	Pool 6	2.00	0.08	324	9.51	2.04	0.06	272	11.56	34.7
12	21										
13	7										
13	21										
14	7										
14	21	Pool 7	1.83	0.07	336	8.39	1.63	0.05	271	9.27	37.3
15	7										
15	21										

Table 4.3-10: The number of reads sequenced at the sequencing facility for each cDNA sequencing library. Each number in the cow ID column represents a specific cow which was sampled 7 and 21 days postpartum (DPP). Technical replicates were used across different pools – technical replicate pool (TRP).

Cow ID	DPP	Total Reads Sequenced
1 (TRP1)	7	41,416,581
1 (TRP3)	21	41,324,681
1 (TRP1)	7	38,633,375
1 (TRP2)	21	41,271,330
2	7	40,078,567
2	21	32,849,221
4	7	40,764,252
4	21	34,628,629
5	7	33,315,144
5	21	37,908,053
6	7	42,835,916
6 (TRP1)	21	43,009,073
6 (TRP3)	7	39,636,649
7	21	28,951,871
7	7	41,728,621
8	21	38,292,356
8	7	32,512,905
9	21	43,631,452
9	7	38,704,938
10	21	42,268,870
10	7	32,760,099
11	21	43,306,451
11	7	36,638,312
12	21	47,845,466
12	7	40,037,502
13	21	39,239,991
13	7	37,654,676
14	21	32,500,641
14	7	39,634,033
15	21	38,951,770
15	7	33,398,231
16	21	43,693,587
16	7	37,834,060

4.3.6 mRNA-Seq data analysis

4.3.6.1 Sequence quality with FastQC

An initial examination of read quality was carried out using the FastQC software which flags aspects of the data that should be attended to in greater detail by presenting graphs and tables for eleven different quality control modules. A graph displayed the mean quality score at each base across all reads, a score above 20 and ideally above 28 at each base in the read was considered to be of high quality (Figure 4.3-3). The average quality score for all bases in each read of each library in our data set was above 28 which indicated that our data was of high quality and there was no need to remove bases at either end of the read.

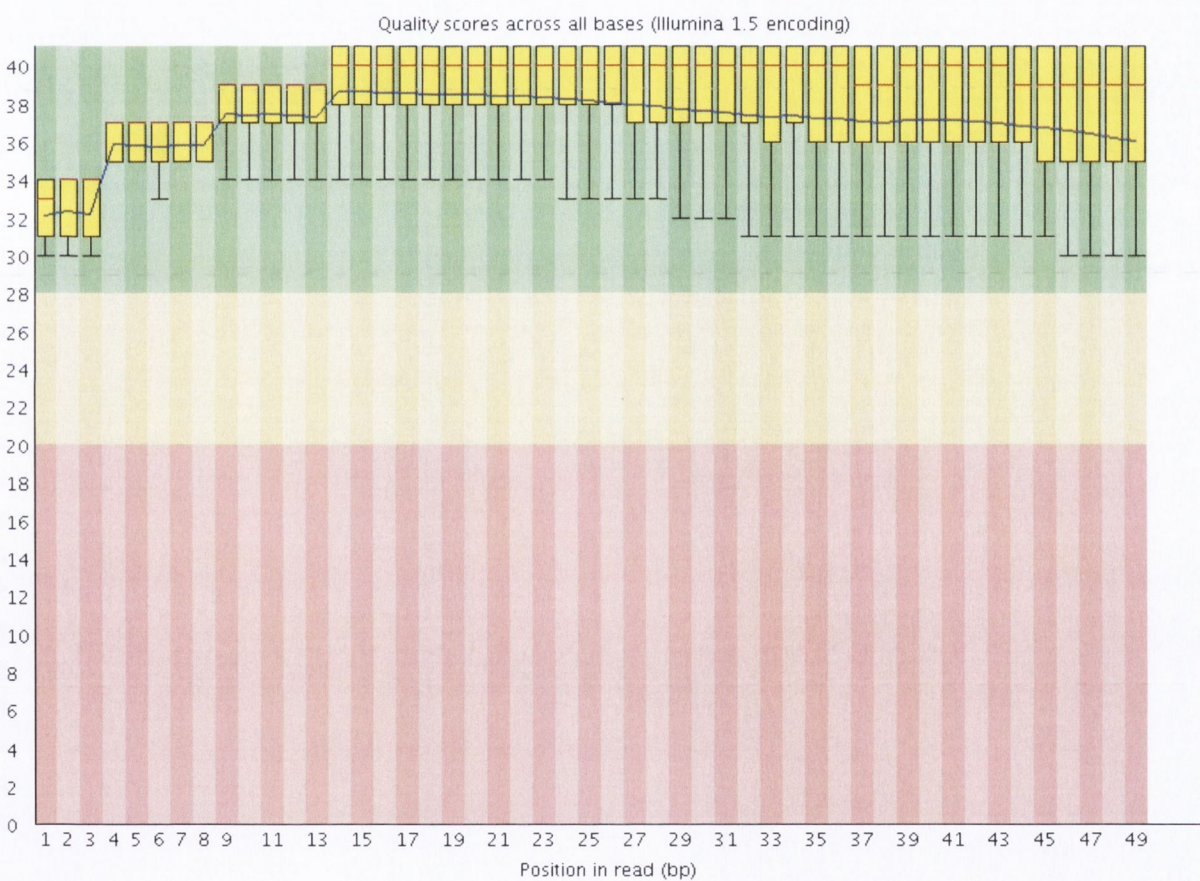


Figure 4.3-3: The average quality scores across all bases for all reads within a sample produced by the software FastQC. The x-axis is each position in the read and the y-axis is the quality score. Scores above 28 in the green segment of the graph are of exceptionally good quality.

4.3.6.2 Estimating Insert Sizes

Prior to aligning sequenced reads to the genome the mean insert size between paired reads and its standard deviation was calculated for each library. These values could then be used as part of the command required for aligning reads within a library to the genome. The average insert size was 191 base pairs (bp) and ranged between 163 to 221 bp (Table 4.3-11).

Table 4.3-11: The estimated mean insert size and standard deviation between reads in a pair for paired end mRNA-Seq data. The mean insert distance and standard deviation was measured in base pairs (bp) using Bowtie2 and Picard-tools software. Each number in the cow ID column represents a specific cow which was sampled 7 and 21 days postpartum (DPP). Technical replicates were used across different pools – technical replicate pool (TRP).

Cow ID	DPP	Mean Insert Size (bp)	Standard Deviation (bp)
1 (TRP1)	7	197	92
1 (TRP3)	7	197	92
1 (TRP1)	21	192	87
1 (TRP2)	21	192	88
2	7	197	84
2	21	195	88
4	7	184	82
4	21	183	73
5	7	185	79
5	21	189	81
6	7	194	93
6 (TRP1)	21	203	98
6 (TRP3)	21	203	98
7	7	197	92
7	21	193	88
8	7	171	65
8	21	200	92
9	7	174	67
9	21	188	83
10	7	192	86
10	21	186	80
11	7	181	78
11	21	165	60
12	7	163	59
12	21	185	82
13	7	188	81
13	21	185	83
14	7	208	104
14	21	202	100
15	7	209	101
15	21	201	102
16	7	221	124
16	21	198	92

4.3.6.3 Mapping reads to the bovine genome

The software program TopHat was used in combination with Bowtie2 to map reads to the bovine genome. The data analysed was paired end and therefore two input files were simultaneously aligned with a specified insert size and standard deviation between paired reads. The input and aligned number of reads for both sequence files attributed to a single library/sample was reported in the TopHat “log” folder. The average input read count per count per sequence file was 38,668,748 and the average number of reads that aligned per sequence file was 33,502,198, therefore 86.6% of reads sequenced aligned to the bovine genome on average. There was one sample (cow 7 sampled at 7 DPP) with a sequenced read count of 28,906,144 on average which was below the ENCODE recommendations, however the number of reads that aligned to the genome for this sample was 25,432,718 which subsequently reached the target specified in the ENCODE guidelines for mRNA-SEQ.

A separate tool named FlagStat was used to estimate the percentage of reads that properly paired with each other taking into consideration the mean insert size specified for each library. The results of this assessment revealed that 81% of reads were properly paired on average across all libraries which equates to 27,038,304 reads per library on average (Table 4.3-12).

4.3.6.4 Summarizing read counts per gene

Once aligned to the bovine genome the location of each read was specified however it was unknown if these reads were aligned to exonic or intronic positions in the genome. To capture this information a software program named HtSeq-Count was used to determine the number of reads that aligned to predicted exonic regions of the bovine genome. If the reads did not align to these regions they were discarded and counted as having “no feature”. If reads aligned to segments of overlapping genes for example these were also discarded and counted as “ambiguous”. The average number of reads that aligned to exonic features was 17,771,547 this was 65.7% of properly paired reads (Table 4.3-12).

Table 4.3-12: Summary of all reads input prior and post aligning to the bovine genome (Bowtie2 and Tophat), the number of reads properly paired (FlagStat) and found within annotated and predicted genes (HtSeq-Count). Each number in the cow ID column represents a specific cow which was sampled 7 and 21 days postpartum (DPP). Technical replicates were used across different pools – technical replicate pool (TRP).

Cow ID	DPP	Bowtie2 & TopHat									FlagStat - Properly Paired			HtSeq-Count		
		Left Kept Reads			Right Kept Reads			Total			% Properly Paired	Total Reads Properly Paired	Paired Reads Properly Paired	No Feature	Ambiguous	Total Read Count Per Library
		Input Reads	Aligned Reads	% Aligned	Input Reads	Aligned Reads	% Aligned	Input Reads	Aligned Reads	% Aligned						
1 (TRP1)	7	41415715	36353617	87.78%	41299002	35906352	86.94%	82714717	72259969	87.36%	80.61%	58248761	29124381	7524242	167400	19636310
1 (TRP3)	21	41323832	36272682	87.78%	41200735	35802939	86.90%	82524567	72075621	87.34%	80.64%	58121781	29060890	7504614	164500	19593620
1 (TRP1)	7	38620029	33136488	85.80%	38513852	32684064	84.86%	77133881	65820552	85.33%	79.85%	52557711	26278855	6576675	152518	17760978
1 (TRP2)	21	41255699	35350715	85.69%	41187842	34938822	84.83%	82443541	70289537	85.26%	79.99%	56224601	28112300	6958409	162017	18949244
2	7	40077197	36617206	91.37%	39964265	36112965	90.36%	80041462	72730171	90.87%	70.88%	51551145	25775573	8779917	134023	13254197
2	21	32848012	27547389	83.86%	32755407	27208945	83.07%	65603419	54756334	83.47%	77.39%	42375927	21187963	5101232	128028	12943889
4	7	40760341	35203124	86.37%	40692118	34842469	85.62%	81452459	70045593	86.00%	81.07%	56785962	28392981	7243988	165251	17759370
4	21	34623875	30780378	88.90%	34566986	30438252	88.06%	69190861	61218630	88.48%	82.49%	50499248	25249624	7377723	133614	15295508
5	7	33313974	28860732	86.63%	33215618	28458144	85.68%	66529592	57318876	86.16%	80.90%	46370971	23185485	6032928	126109	16308197
5	21	37907268	33179073	87.53%	37795831	32741161	86.63%	75703099	65920234	87.08%	78.98%	52063801	26031900	7739215	133444	16144098
6	7	42835387	37300460	87.08%	42710237	36825058	86.22%	85545624	74125518	86.65%	81.17%	60167683	30083841	7424668	154567	21007509
6 (TRP1)	21	43008508	37543804	87.29%	42888124	37086891	86.47%	85896632	74630695	86.88%	80.12%	59794113	29897056	8228017	154833	19613495
6 (TRP2)	7	39636127	34595267	87.28%	39518824	34157260	86.43%	79154951	68752527	86.86%	80.05%	55036398	27518199	7608440	145614	18242472
7	21	28947441	25615017	88.49%	28864847	25250420	87.48%	57812288	50865437	87.98%	79.88%	40631311	20315656	5837087	126075	13590152
7	7	41723086	36486548	87.45%	41602537	35939898	86.39%	83325623	72426446	86.92%	81.18%	58795789	29397894	8402051	146126	19629558
8	21	38286593	32235397	84.20%	38175652	31790547	83.27%	76462245	64025944	83.74%	80.68%	51656132	25828066	7228737	153303	15859061
8	7	32508483	27467768	84.49%	32415052	27074163	83.52%	64923535	54541931	84.01%	78.07%	42580886	21290443	5047483	130701	13173426

9	21	43625333	38027958	87.17%	43498608	37499255	86.21%	87123941	75527213	86.69%	81.94%	61886998	30943499	8949913	137417	20675596
9	7	38697935	34065426	88.03%	38675288	33794624	87.38%	77373223	67860050	87.70%	82.85%	56222051	28111026	8312089	140044	18908094
10	21	42262794	36999423	87.55%	42237086	36702873	86.90%	84499880	73702296	87.22%	81.66%	60185295	30092647	7779796	150015	20244296
10	7	32755449	29059843	88.72%	32735216	28818145	88.03%	65490665	57877988	88.38%	80.81%	46771202	23385601	6217345	123964	14048221
11	21	43300665	37949504	87.64%	43274797	37627293	86.95%	86575462	75576797	87.30%	83.01%	62736299	31368150	8457161	142634	20880647
11	7	36633008	32002065	87.36%	36610484	31747022	86.72%	73243492	63749087	87.04%	82.46%	52567497	26283749	6856753	113443	15501275
12	21	47840674	41034488	85.77%	47827225	40636529	84.97%	95667899	81671017	85.37%	81.52%	66578213	33289107	8192370	166489	24016183
12	7	39235971	34102543	86.92%	39225265	33780408	86.12%	78461236	67882951	86.52%	82.99%	56336061	28168031	7292959	135511	17074780
13	21	40033731	35042054	87.53%	40022566	34722693	86.76%	80056297	69764747	87.14%	82.66%	57667540	28833770	7388298	146826	19431253
13	7	32497471	28597489	88.00%	32488370	28340175	87.23%	64985841	56937664	87.62%	82.04%	46711660	23355830	7353339	123065	15491634
14	21	37651195	32603010	86.59%	37640566	32289423	85.78%	75291761	64892433	86.19%	81.70%	53017118	26508559	7434913	128262	19895424
14	7	39630847	34817587	87.85%	39612566	34502052	87.10%	79243413	69319639	87.48%	81.66%	56606417	28303209	8372327	164331	19113563
15	21	38949495	33143709	85.09%	38931705	32846575	84.37%	77881200	65990284	84.73%	78.30%	51670392	25835196	6338070	158987	16211297
15	7	33396317	29197125	87.43%	33380964	28925190	86.65%	66777281	58122315	87.04%	81.53%	47387123	23693562	5844596	182503	15312929
16	21	43690759	37501069	85.83%	43670788	37165472	85.10%	87361547	74666541	85.47%	82.00%	61226564	30613282	7614432	186547	22823584
16	7	37831435	33041431	87.34%	37814319	32758610	86.63%	75645754	65800041	86.98%	81.30%	53495433	26747717	7247484	151554	18071194

4.3.6.5 Differential gene expression between technical replicates

Differential gene expression was assessed between six technical replicates from three different samples to examine if there were effects associated with comparing samples in different pools. The three samples randomly selected to act as technical replicates were: cow 1 at 7 DPP in pools 3 and 1, cow 1 at 21 DPP in pools 1 and 2 and cow 6 at 21 DPP in pools 1 and 3. Even though the replicates were sequenced in different pools they clustered together on a multi-dimensional scaling (MDS) plot. The differential gene expression of two groups (1): cow 1 at 7 DPP in pool 1, cow 1 at 21 DPP in pool 1, cow 6 at 21 DPP in pool 1 and (2): cow 1 at 7 DPP in pool 3, cow 1 at 21 DPP in pool 2 and cow 6 in pool 3, was compared and there were no significantly differentially expressed genes. This indicated that there was no pool effect on the differential gene expression observed for test samples (Figure 4.3-4).

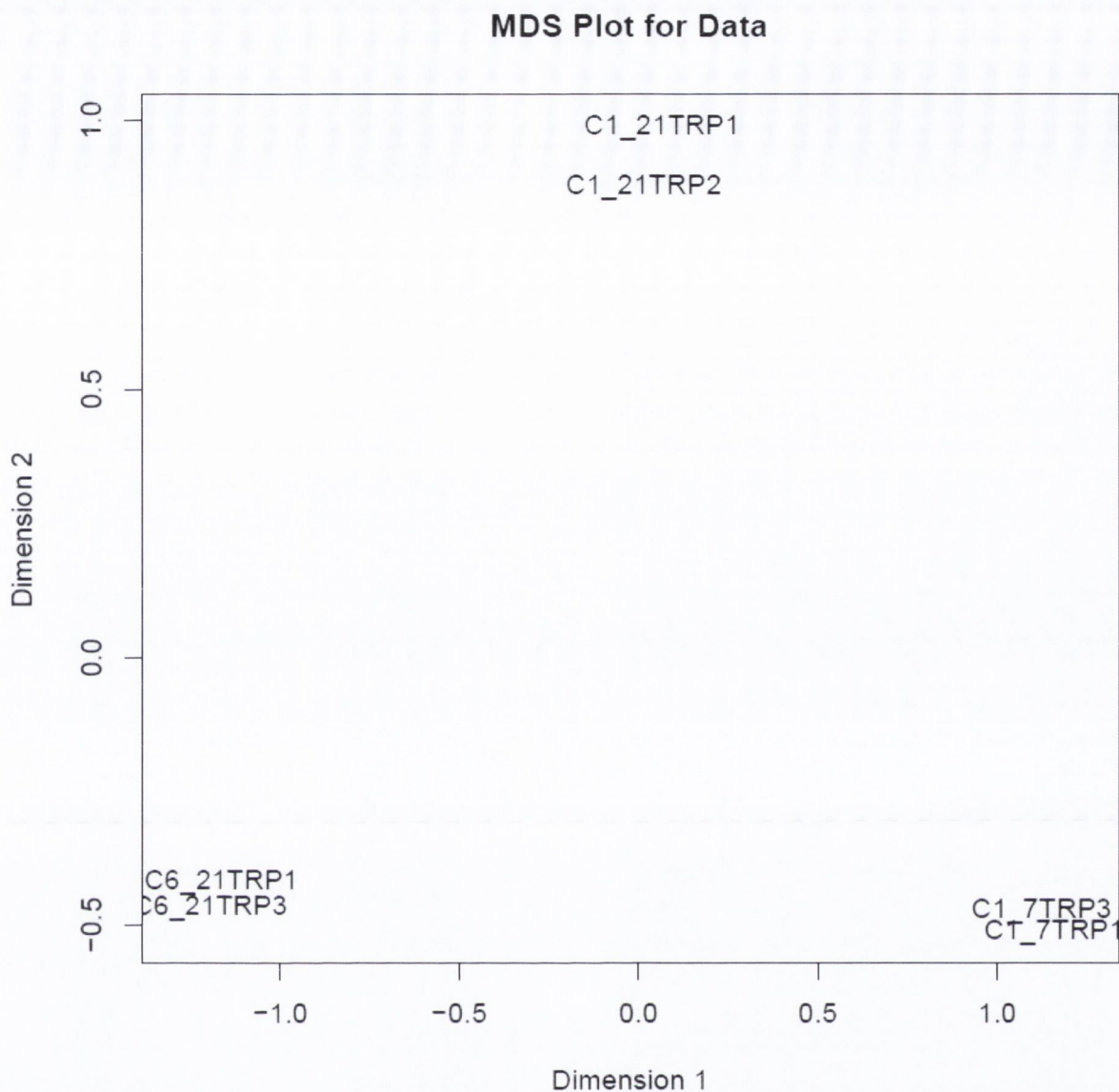


Figure 4.3-4: A multi-dimensional scaling (MDS) plot displaying clustering of technical replicates for mRNA-Seq data. Cow 1 was assessed 7 days postpartum (DPP) (C1_7) in pools 3 and 1 (TRP1 and 3) and 21 DPP (C1_21) in pools 3 and 2 (TRP1 and 2) and cow 6 was assessed 21 DPP (C6_21) in pools 1 and 3 (TRP1 and 3).

4.3.7 (A) mRNA-Seq expression within groups, between 7 and 21 days postpartum

Differences between the transcriptional profiles of sub-clinically endometritic (n=6) and healthy (n=9) cows were analysed within each group and compared over time between 7 and 21 days postpartum (DPP). The software program EdgeR was used to uncover the most highly differently expressed genes over time. A multi-dimensional scaling (MDS) plot was created using the software program EdgeR which displayed how different samples were from each other (Figure 4.3-5).

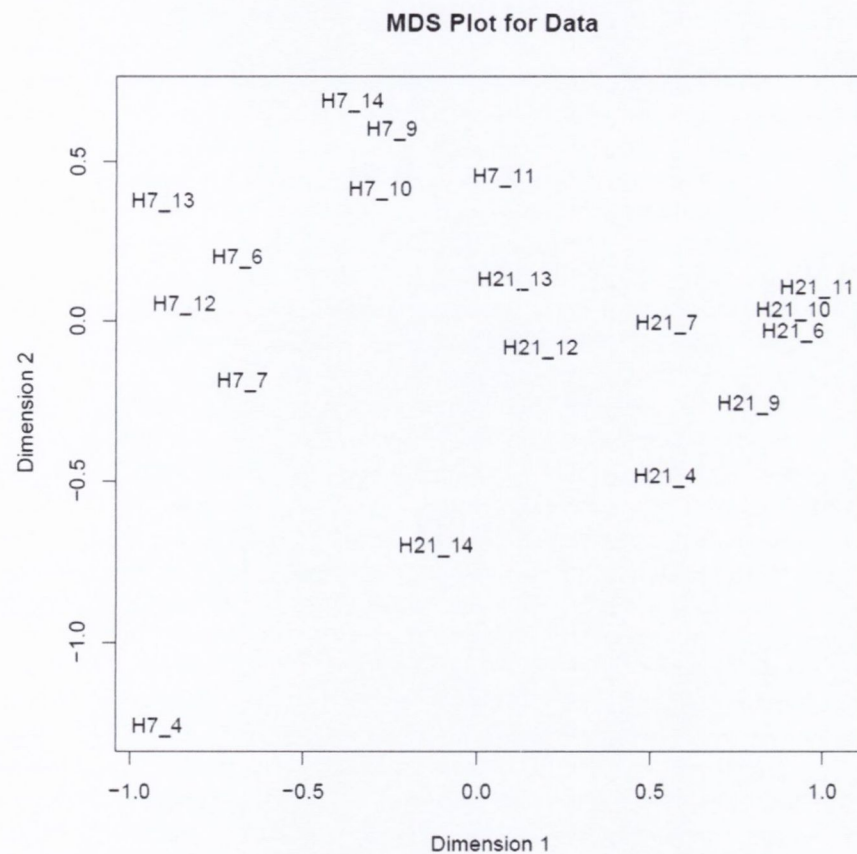
On close inspection of the MDS plot for healthy cows there was a slight clustering of 21 DPP samples towards the bottom right of the plot and 7 DPP samples towards the top left of the graph. This general trend was observed except for cow 4 at 7 DPP which appeared to be an outlier from visual inspection of the MDS plot. However this sample was not removed from downstream analysis as there was no auxiliary data to indicate that this sample had cause to be removed. In the MDS plot for sub-clinically endometritic cows there was no obvious clustering observed when 7 and 21 DPP samples were compared.

Genes were significantly differentially expressed with a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1. The number of genes significantly differentially expressed between 7 and 21 DPP in sub-clinically endometritic cows was 95, of these 66 were elevated 7 DPP and 29 were elevated 21 DPP, and in healthy cows was 4068, of these 2495 were elevated 7 DPP and 1364 were elevated 21 DPP (Table 4.3-13).

Table 4.3-13: The number of genes significantly differentially expressed in the bovine endometrium between 7 and 21 days postpartum (DPP) in the sub-clinically endometritic (SCE) and healthy cowswith a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1. Genes were elevated (↑) 7 and 21 DPP within the SCE and healthy group of cows.

			Gene Numbers	
			Total	Elevated at each time point
SCE	7 v 21 DPP	↑ 7 DPP	95	66
		↑ 21 DPP		29
Healthy		↑ 7 DPP	4068	2495
		↑ 21 DPP		1573

Healthy 7 v 21DPP



SCE 7 v 21DPP

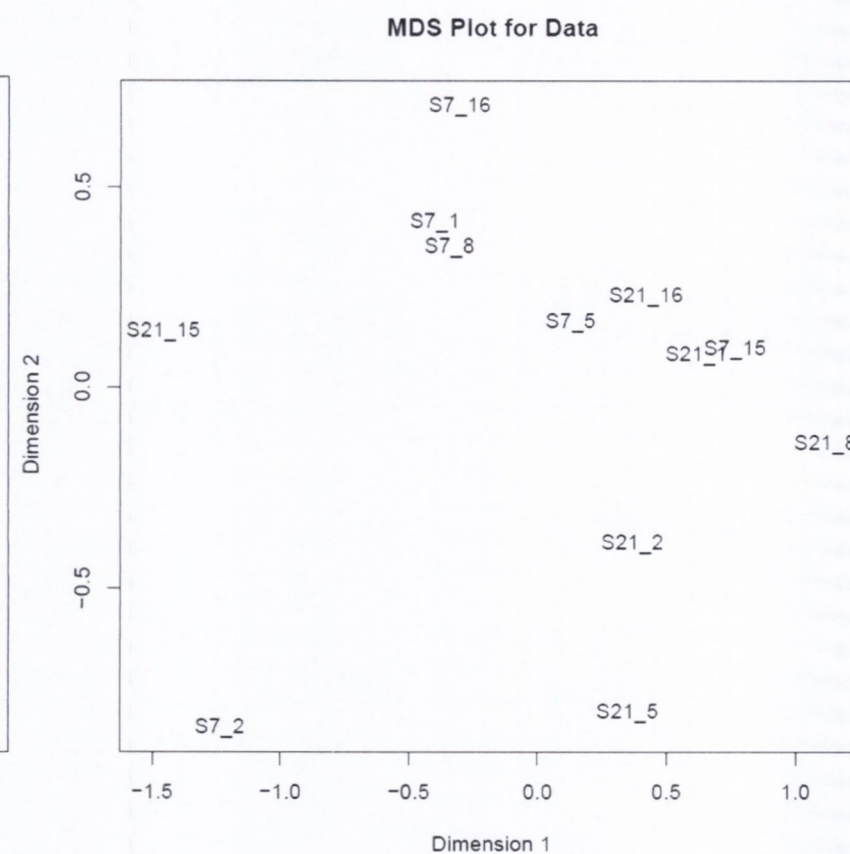


Figure 4.3-5: Multi-dimensional scaling (MDS) plots produced with EdgeR comparing samples 7 and 21 days postpartum (DPP) in the healthy (H) and sub-clinically endometritic (S) groups.

Pathway analysis

All genes significantly differentially expressed between 7 and 21 DPP were either elevated 7 DPP or 21 DPP in both sub-clinically endometritic and healthy cows with an FDR < 0.1. These genes were converted to human one to one orthologs which reduced the number of genes in both the SCE and healthy group by 14%. These human Ensembl gene IDs were converted to KEGG IDs by the GoSeq software program, which predicted over represented KEGG pathways, in some instances there were multiple KEGG IDs for an individual human gene ID which are represented as extra KEGG IDs in the table below. The conversion of human to KEGG IDs did not increase the number of genes in the SCE group and 24 extra genes were gained in the healthy group (Table 4.3-14).

Table 4.3-14: The number of significantly differentially expressed genes (FDR < 0.1) in the bovine endometrium between 7 and 21 days postpartum (DPP) that were used in downstream Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis. Genes were elevated (↑) 7 days and 21 DPP in both sub-clinically endometritic (SCE) and healthy groups.

			Bovine Genes		Human Genes		Human ID to KEGG IDs		KEGG IDs		
			Total		Total		Total		Total		Extra
SCE	7 v 21 DPP	↑ at 7 DPP	95	66	82	56	82	56	82	56	0
		↑ at 21 DPP		29		26		26		26	0
Healthy		↑ at 7 DPP	4068	2495	3502	2138	3482	2133	3530	2157	24
		↑ at 21 DPP		1573		1364		1349		1373	24

The most highly enriched pathways were selected from each analysis and within the SCE and healthy groups there were no common pathways elevated 7 DPP and elevated 21 DPP (Table 4.3-15). When comparing the most highly enriched pathways elevated 7 DPP in the SCE and Healthy groups there were four pathways in common, Rheumatoid arthritis, Toll-like receptor signalling (Figure 4.3-6 and Figure 4.3-7), Phagosome (Figure 4.3-8 and Figure 4.3-9) and Leishmaniasis. There were no enriched pathways elevated 21 DPP in common between the SCE and Healthy groups. Examples of pathways enriched 21 DPP relative to 7 DPP in healthy cows are Calcium Signalling (Figure 4.3-10) and Focal Adhesion (Figure 4.3-11).

Table 4.3-15: The most highly enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways with genes significantly expressed between 7 and 21 days postpartum (DPP) in the sub-clinically endometritic (SCE) and healthy cows. * These pathways are enriched 7 days postpartum in both the sub-clinically endometritic (SCE) and healthy groups.

7 DPP v 21 DPP			
Elevated 7 DPP in SCE		Elevated 21 DPP in SCE	
KEGG Pathway Name	Over represented <i>p</i> value	KEGG Pathway Name	Over represented <i>p</i> value
1 Rheumatoid arthritis*	0.002	1 Neuroactive ligand-receptor interaction	0.006
2 Toll-like receptor signalling pathway*	0.003		
3 Phagosome*	0.007		
4 Arachidonic acid metabolism	0.008		
5 Malaria	0.008		
6 Leishmaniasis*	0.017		
7 Epithelial cell signalling in Helicobacter pylori infection	0.020		
8 Small cell lung cancer	0.034		
9 ECM-receptor interaction	0.036		
10 Chagas disease (American trypanosomiasis)	0.044		
Elevated 7 DPP in Healthy		Elevated 21 DPP in Healthy	
KEGG Pathway Name	Over represented <i>p</i> value	KEGG Pathway Name	Over represented <i>p</i> value
1 Lysosome	7.30E-30	1 Calcium signalling pathway	5.02E-07
2 Rheumatoid arthritis*	2.79E-20	2 Salivary secretion	1.09E-06
3 Phagosome*	1.43E-19	3 Vascular smooth muscle contraction	1.80E-06
4 Osteoclast differentiation	1.37E-17	4 Dilated cardiomyopathy	4.45E-06
5 Cytokine-cytokine receptor interaction	1.24E-13	5 Hypertrophic cardiomyopathy (HCM)	1.38E-05
6 Chagas disease (American trypanosomiasis)	1.90E-13	6 Pancreatic secretion	2.53E-05
7 Leishmaniasis*	2.04E-13	7 Cardiac muscle contraction	6.25E-05
8 Toll-like receptor signalling pathway*	2.62E-13	8 Focal adhesion	1.48E-04
9 Amoebiasis	2.15E-11	9 Glycine, serine and threonine metabolism	1.58E-04
10 Complement and coagulation cascades	3.48E-11	10 Arrhythmogenic right ventricular cardiomyopathy (ARVC)	2.97E-04

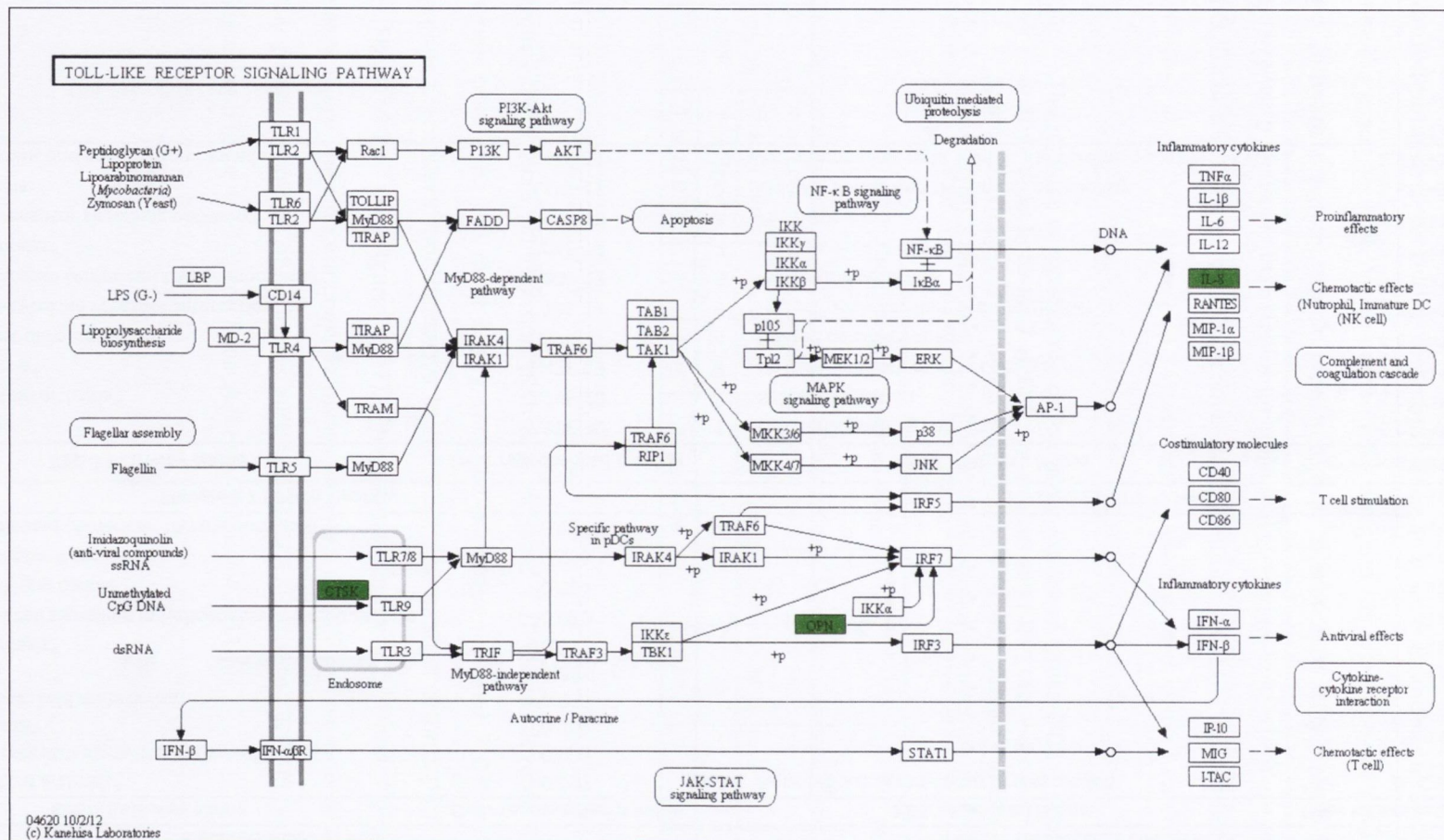


Figure 4.3-6: The Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Toll-Like Receptor Signalling Pathway enriched 7 days postpartum (DPP) in sub-clinically endometritic cows (SCE). Green boxes highlight the genes within the pathway that are elevated 7 DPP in the SCE group.

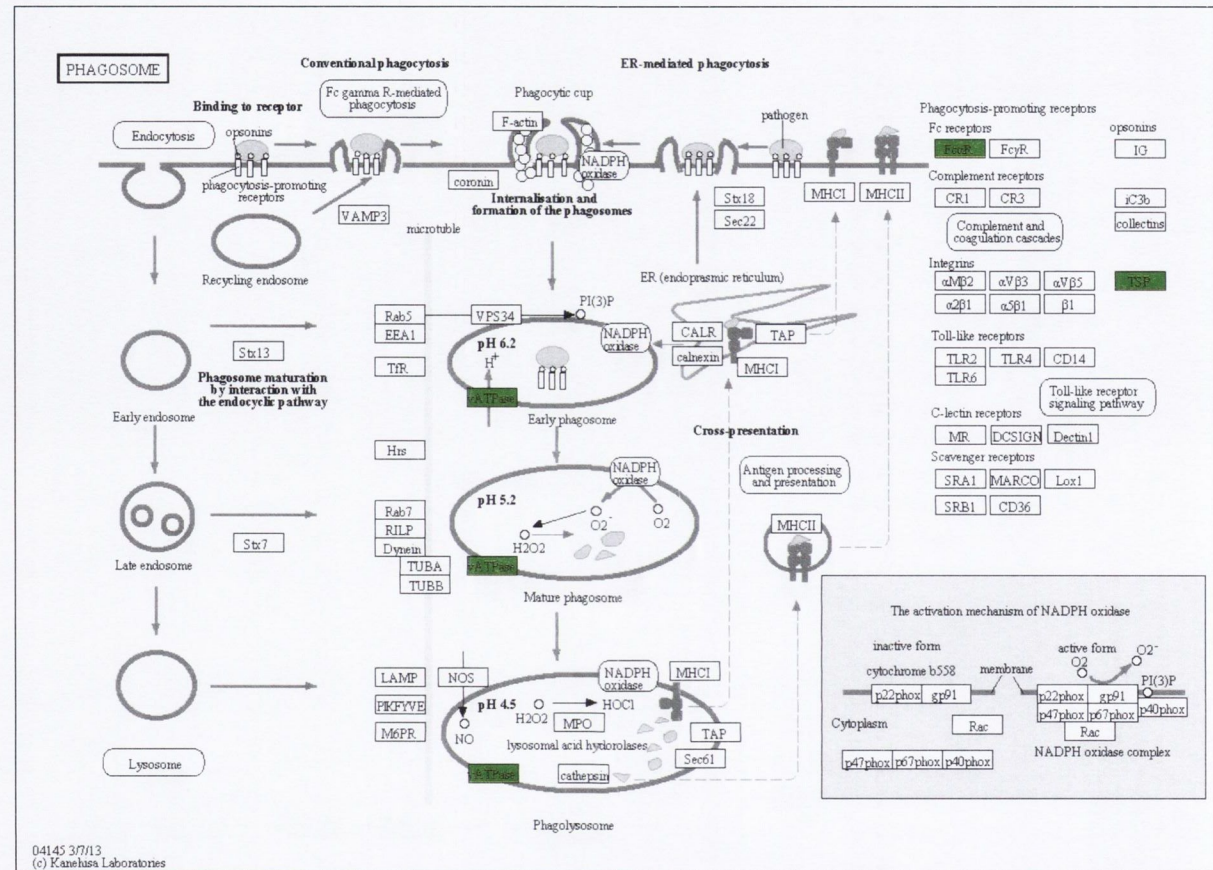


Figure 4.3-8: The Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Phagosome pathway enriched 7 days postpartum (DPP) in sub-clinically endometritic cows (SCE). Green boxes highlight the genes within the pathway that are elevated 7 DPP in the SCE group.

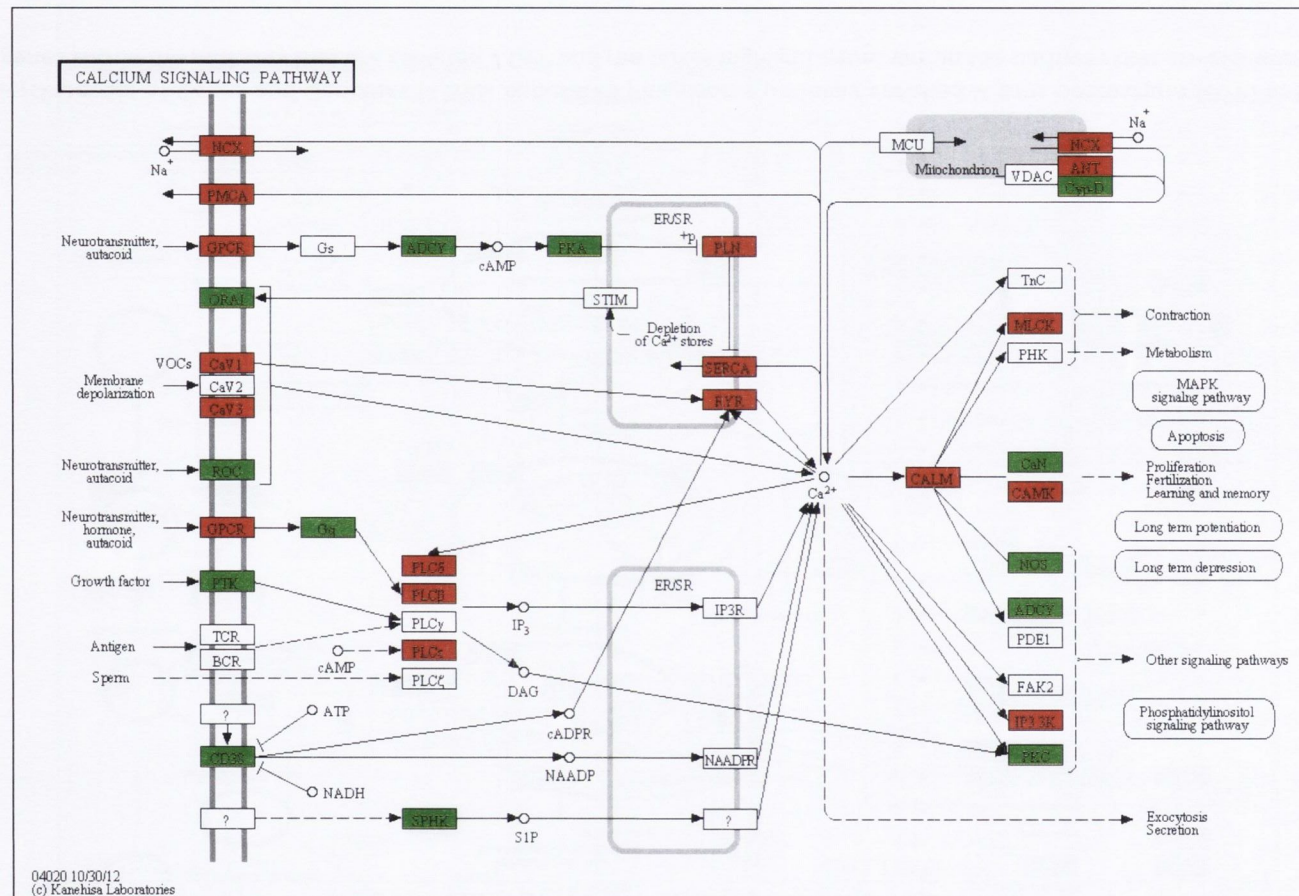


Figure 4.3-10: The Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Calcium Signalling pathway enriched 21 days postpartum (DPP) in healthy cows. Green boxes highlight the genes within the pathway that are elevated 7 DPP and red boxes highlight genes within the pathway that are elevated 21 DPP in healthy cows.

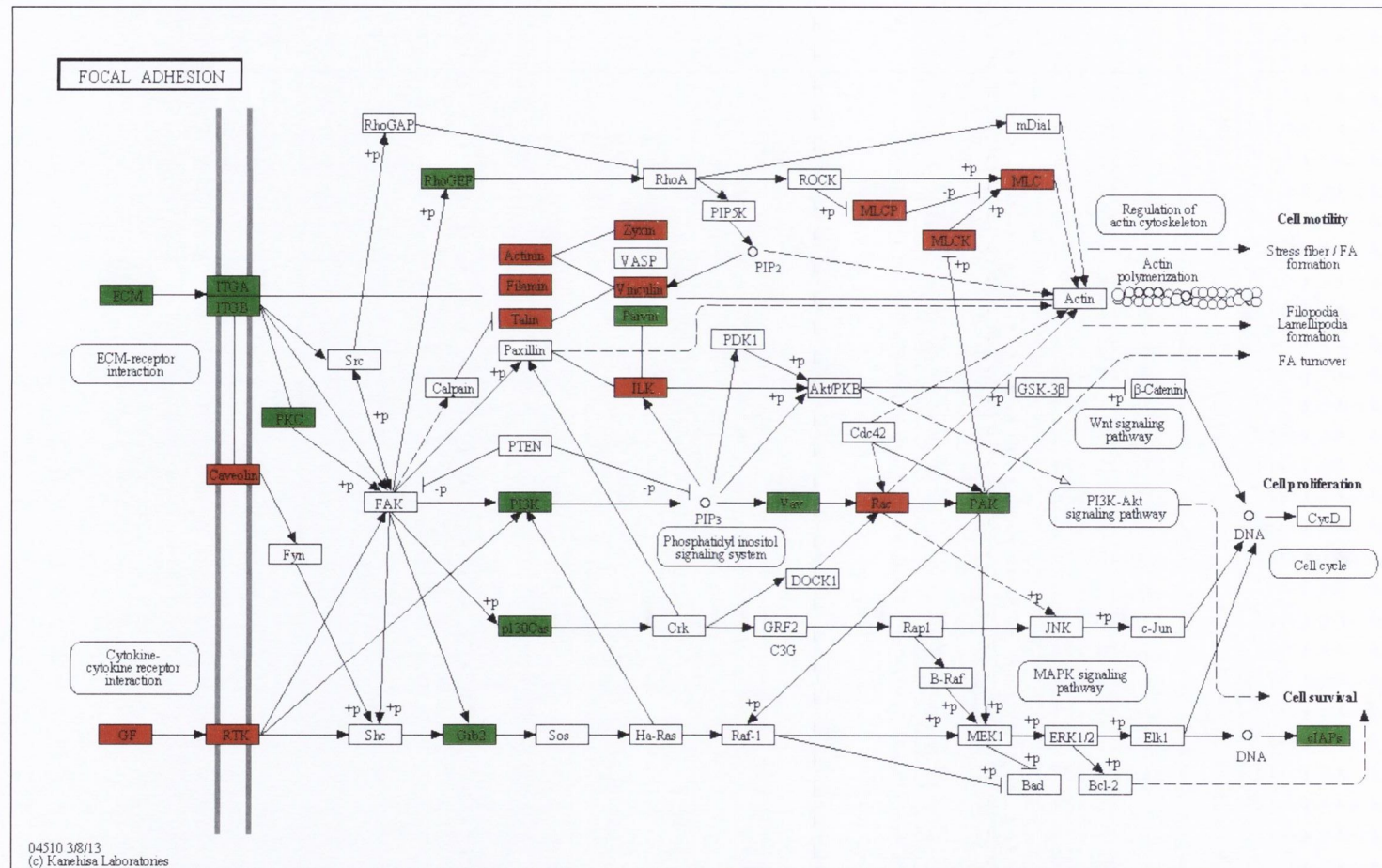


Figure 4.3-11: The Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Focal Adhesion pathway enriched 21 days postpartum (DPP) in healthy cows. Green boxes highlight the genes within the pathway that are elevated 7 DPP and red boxes highlight genes within the pathway that are elevated 21 DPP in healthy cows.

Table 4.3-16: Differentially elevated genes 7 and 21 days postpartum (DPP) in healthy cows within each enriched pathway.

7 DPP v 21 DPP			
Elevated 7 DPP in Healthy		Elevated 21 DPP in Healthy	
Ensembl Gene ID	Gene Name	Ensembl Gene ID	Gene Name
Lysosome		Salivary secretion	
ENSBTAG00000018207	<i>M6PR</i>	ENSBTAG00000010551	<i>ATP1A2</i>
ENSBTAG00000007399	<i>BT.104573</i>	ENSBTAG00000025652	<i>BEST2</i>
ENSBTAG00000011056	<i>IDS</i>	ENSBTAG00000014059	<i>ATP2B4</i>
ENSBTAG00000015520	<i>SLC11A1</i>	ENSBTAG00000000520	<i>ATP2B3</i>
ENSBTAG00000019218	<i>BT.68276</i>	ENSBTAG00000000952	<i>ATP2B1</i>
ENSBTAG00000000831	<i>CTNS</i>	ENSBTAG00000013680	<i>ATP1B2</i>
ENSBTAG00000003450	<i>BT.61946</i>	ENSBTAG00000038523	<i>BT.106410</i>
ENSBTAG00000001124	<i>GALC</i>	ENSBTAG00000008059	<i>CHRM3</i>
ENSBTAG00000027337	<i>CTSA</i>	ENSBTAG00000014600	<i>BT.24461</i>
ENSBTAG00000012117	<i>ATP6AP1</i>	ENSBTAG00000013300	<i>KCNMA1</i>
ENSBTAG00000005592	<i>BT.26509</i>	ENSBTAG00000001305	<i>ATP2B2</i>
ENSBTAG00000001189	<i>BT.35586</i>	ENSBTAG00000014576	<i>GUCY1A1</i>
ENSBTAG00000016053	<i>ARSA</i>	ENSBTAG00000020921	<i>PRKCB</i>
ENSBTAG00000046979	<i>LGMN</i>	ENSBTAG00000024508	<i>CALML6</i>
ENSBTAG00000018784	<i>CTSZ</i>	ENSBTAG00000040253	<i>BT.27996</i>
ENSBTAG00000019256	<i>GLA</i>	ENSBTAG00000018777	<i>ADCY5</i>
ENSBTAG00000004826	<i>ACPS</i>	ENSBTAG00000008338	<i>PLCB1</i>
ENSBTAG00000018846	<i>CLN5</i>	ENSBTAG00000017981	<i>ADRB3</i>
ENSBTAG00000007512	<i>PLA2G15</i>	ENSBTAG00000014583	<i>CALM</i>
ENSBTAG00000007896	<i>NAGPA</i>	ENSBTAG00000014583	<i>CALM</i>
ENSBTAG00000002352	<i>GGA2</i>	ENSBTAG00000014583	<i>CALM</i>
ENSBTAG00000010992	<i>CTSH</i>	ENSBTAG00000025642	<i>RYR3</i>
ENSBTAG00000011257	<i>ASAH1</i>	Vascular smooth muscle contraction	
ENSBTAG00000006241	<i>MANB</i>	ENSBTAG00000014609	<i>BT.41853</i>
ENSBTAG00000006082	<i>DNASE2</i>	ENSBTAG00000014567	<i>BT.105316</i>
ENSBTAG00000004263	<i>ATP6V0A4</i>	ENSBTAG00000007129	<i>MRVI1</i>
ENSBTAG00000003636	<i>BT.65903</i>	ENSBTAG00000011772	<i>PPP1R12B</i>
ENSBTAG00000047956	<i>NAGLU</i>	ENSBTAG00000047491	<i>CACNA1S</i>
ENSBTAG00000019761	<i>MANBA</i>	ENSBTAG00000011962	<i>PLA2G3</i>
ENSBTAG00000011100	<i>BT.49573</i>	ENSBTAG00000011473	<i>MYL9</i>
ENSBTAG00000000292	<i>TCIRG1</i>	ENSBTAG00000017847	<i>PPP1R12C</i>
ENSBTAG00000019409	<i>GNPTAB</i>	ENSBTAG00000015988	<i>BT.100599</i>
ENSBTAG00000018889	<i>ATP6V0B</i>	ENSBTAG00000002985	<i>KCNMB1</i>
ENSBTAG00000007622	<i>CATD</i>	ENSBTAG00000014600	<i>BT.24461</i>
ENSBTAG00000021955	<i>NPC2</i>	ENSBTAG00000013300	<i>KCNMA1</i>
ENSBTAG00000044053	<i>SLC17A5</i>	ENSBTAG00000010026	<i>BT.31482</i>
ENSBTAG00000007002	<i>IDUA</i>	ENSBTAG00000012217	<i>PLA2G2F</i>
ENSBTAG00000000133	<i>CD68</i>	ENSBTAG00000005268	<i>PLA2G4D</i>

ENSBTAG00000013367	<i>BT.101768</i>	ENSBTAG00000014614	<i>ACTA2</i>
ENSBTAG00000008849	<i>SORT1</i>	ENSBTAG00000014576	<i>GUCY1A1</i>
ENSBTAG00000000720	<i>CTSL1</i>	ENSBTAG00000020921	<i>PRKCB</i>
ENSBTAG00000017992	<i>CD164</i>	ENSBTAG00000006234	<i>BT.76843</i>
ENSBTAG00000017465	<i>BT.20235</i>	ENSBTAG00000024508	<i>CALML6</i>
ENSBTAG00000010956	<i>SCARB2</i>	ENSBTAG00000040253	<i>BT.27996</i>
ENSBTAG00000004568	<i>GALNS</i>	ENSBTAG00000018777	<i>ADCY5</i>
ENSBTAG00000015195	<i>NPC1</i>	ENSBTAG00000008338	<i>PLCB1</i>
ENSBTAG00000021035	<i>CTSK</i>	ENSBTAG00000011198	<i>PPP1CC</i>
ENSBTAG00000021092	<i>ATP6V0D2</i>	ENSBTAG00000021924	<i>PLA2G4E</i>
ENSBTAG00000014553	<i>ATP6V0D1</i>	ENSBTAG00000031217	<i>MYL6B</i>
ENSBTAG00000005477	<i>LAPTM5</i>	ENSBTAG00000014583	<i>CALM</i>
ENSBTAG00000017135	<i>CTSS</i>	ENSBTAG00000014583	<i>CALM</i>
ENSBTAG00000012442	<i>CATB</i>	ENSBTAG00000014583	<i>CALM</i>
ENSBTAG00000015628	<i>SMPD1</i>	Dilated cardiomyopathy	
ENSBTAG00000015403	<i>BT.35140</i>	ENSBTAG00000002036	<i>BT.37145</i>
ENSBTAG00000000704	<i>GUSB</i>	ENSBTAG00000012311	<i>CACNG5</i>
ENSBTAG00000015254	<i>GLB1</i>	ENSBTAG00000000589	<i>CACNG4</i>
ENSBTAG00000016021	<i>GAA</i>	ENSBTAG00000047491	<i>CACNA1S</i>
ENSBTAG00000014841	<i>GBA</i>	ENSBTAG00000022314	<i>EMD</i>
ENSBTAG00000015267	<i>SGSH</i>	ENSBTAG00000001865	<i>SGCG</i>
ENSBTAG00000026428	<i>ATP6V0C</i>	ENSBTAG00000006424	<i>TNNI3</i>
ENSBTAG00000010242	<i>LAMP1</i>	ENSBTAG00000012897	<i>ITGA7</i>
ENSBTAG00000002402	<i>BT.4977</i>	ENSBTAG00000005373	<i>TPM1</i>
ENSBTAG00000006768	<i>BT.29990</i>	ENSBTAG00000026986	<i>TTN</i>
ENSBTAG00000021499	<i>BT.42764</i>	ENSBTAG00000014600	<i>BT.24461</i>
ENSBTAG00000009506	<i>NAGA</i>	ENSBTAG00000010026	<i>BT.31482</i>
ENSBTAG00000005674	<i>NEU1</i>	ENSBTAG00000005714	<i>ACTC1</i>
ENSBTAG00000012981	<i>HEXA</i>	ENSBTAG00000008394	<i>MYL3</i>
Rheumatoid arthritis		ENSBTAG00000022000	<i>CACNB2</i>
ENSBTAG00000019218	<i>BT.68276</i>	ENSBTAG00000018777	<i>ADCY5</i>
ENSBTAG00000003450	<i>BT.61946</i>	ENSBTAG00000005353	<i>DES</i>
ENSBTAG00000012117	<i>ATP6AP1</i>	ENSBTAG00000013861	<i>SLC8A1</i>
ENSBTAG00000010303	<i>ICAM1</i>	ENSBTAG00000011424	<i>TPM2</i>
ENSBTAG00000005359	<i>TGFB2</i>	ENSBTAG00000012931	<i>BT.43673</i>
ENSBTAG00000047400	<i>IL11</i>	ENSBTAG00000022886	<i>RYR2</i>
ENSBTAG00000016309	<i>ATP6V1D</i>	ENSBTAG00000008248	<i>DMD</i>
ENSBTAG00000004826	<i>ACP5</i>	Hypertrophic cardiomyopathy (HCM)	
ENSBTAG00000020457	<i>TGFB1</i>	ENSBTAG00000002036	<i>BT.37145</i>
ENSBTAG00000004263	<i>ATP6V0A4</i>	ENSBTAG00000012311	<i>CACNG5</i>
ENSBTAG00000037811	<i>CCL2</i>	ENSBTAG00000000589	<i>CACNG4</i>
ENSBTAG00000000292	<i>TCIRG1</i>	ENSBTAG00000047491	<i>CACNA1S</i>
ENSBTAG00000015100	<i>ATP6V0E1</i>	ENSBTAG00000022314	<i>EMD</i>
ENSBTAG00000013118	<i>CD86</i>	ENSBTAG00000001865	<i>SGCG</i>

ENSBTAG00000002703	<i>ATP6V1A</i>	ENSBTAG00000002917	<i>PRKAG2</i>
ENSBTAG00000010349	<i>IL1A</i>	ENSBTAG00000006424	<i>TNNI3</i>
ENSBTAG00000021326	<i>CCL20</i>	ENSBTAG00000012897	<i>ITGA7</i>
ENSBTAG00000010620	<i>ATP6V1B1</i>	ENSBTAG00000005373	<i>TPM1</i>
ENSBTAG00000018889	<i>ATP6V0B</i>	ENSBTAG00000026986	<i>TTN</i>
ENSBTAG00000012004	<i>TGFB3</i>	ENSBTAG00000010026	<i>BT.31482</i>
ENSBTAG00000008924	<i>TNFSF11</i>	ENSBTAG00000005714	<i>ACTC1</i>
ENSBTAG00000018059	<i>BT.27986</i>	ENSBTAG00000008394	<i>MYL3</i>
ENSBTAG00000001321	<i>IL1B</i>	ENSBTAG00000022000	<i>CACNB2</i>
ENSBTAG00000014238	<i>ATP6V1E1</i>	ENSBTAG00000005353	<i>DES</i>
ENSBTAG00000000720	<i>CTSL1</i>	ENSBTAG00000013861	<i>SLC8A1</i>
ENSBTAG00000014921	<i>IL6</i>	ENSBTAG00000011424	<i>TPM2</i>
ENSBTAG00000006240	<i>TLR4</i>	ENSBTAG00000022886	<i>RYR2</i>
ENSBTAG00000008008	<i>TLR2</i>	ENSBTAG00000008248	<i>DMD</i>
ENSBTAG00000021035	<i>CTSK</i>	Pancreatic secretion	
ENSBTAG00000001927	<i>ATP6V1C2</i>	ENSBTAG00000010551	<i>ATP1A2</i>
ENSBTAG00000018646	<i>ATP6V1B2</i>	ENSBTAG00000014059	<i>ATP2B4</i>
ENSBTAG00000021092	<i>ATP6V0D2</i>	ENSBTAG00000000520	<i>ATP2B3</i>
ENSBTAG00000014051	<i>ANGPT1</i>	ENSBTAG00000009552	<i>ATP2B1</i>
ENSBTAG00000013513	<i>ATP6V1C1</i>	ENSBTAG00000014806	<i>BT.62377</i>
ENSBTAG00000014553	<i>ATP6V0D1</i>	ENSBTAG00000046169	<i>CPA1</i>
ENSBTAG00000017060	<i>ITGB2</i>	ENSBTAG00000011962	<i>PLA2G3</i>
ENSBTAG00000000130	<i>TNFSF13</i>	ENSBTAG00000013680	<i>ATP1B2</i>
ENSBTAG00000001570	<i>CSF2</i>	ENSBTAG00000038523	<i>BT.106410</i>
ENSBTAG00000004322	<i>FOS</i>	ENSBTAG00000008059	<i>CHRM3</i>
ENSBTAG00000000283	<i>CSF1</i>	ENSBTAG00000014600	<i>BT.24461</i>
ENSBTAG00000026428	<i>ATP6V0C</i>	ENSBTAG00000013300	<i>KCNMA1</i>
ENSBTAG00000025471	<i>TNF</i>	ENSBTAG00000001305	<i>ATP2B2</i>
Phagosome		ENSBTAG00000012217	<i>PLA2G2F</i>
ENSBTAG00000018207	<i>M6PR</i>	ENSBTAG00000020921	<i>PRKCB</i>
ENSBTAG00000007399	<i>BT.104573</i>	ENSBTAG00000018777	<i>ADCY5</i>
ENSBTAG00000015739	<i>MRC2</i>	ENSBTAG00000008338	<i>PLCB1</i>
ENSBTAG00000019218	<i>BT.68276</i>	ENSBTAG00000006541	<i>BT.62768</i>
ENSBTAG00000002885	<i>MSR1</i>	ENSBTAG00000022886	<i>RYR2</i>
ENSBTAG00000003450	<i>BT.61946</i>	Cardiac muscle contraction	
ENSBTAG00000003895	<i>CYBA</i>	ENSBTAG00000010551	<i>ATP1A2</i>
ENSBTAG00000004937	<i>SEC61A1</i>	ENSBTAG00000002036	<i>BT.37145</i>
ENSBTAG00000012117	<i>ATP6AP1</i>	ENSBTAG00000012311	<i>CACNG5</i>
ENSBTAG00000032719	<i>TFRC</i>	ENSBTAG00000000589	<i>CACNG4</i>
ENSBTAG00000014269	<i>SCARB1</i>	ENSBTAG00000047491	<i>CACNA1S</i>
ENSBTAG00000010193	<i>BT.41314</i>	ENSBTAG00000013680	<i>ATP1B2</i>
ENSBTAG00000017139	<i>STX7</i>	ENSBTAG00000006424	<i>TNNI3</i>
ENSBTAG00000013755	<i>ITGB5</i>	ENSBTAG00000038523	<i>BT.106410</i>
ENSBTAG00000007531	<i>NCF4</i>	ENSBTAG00000005373	<i>TPM1</i>

ENSBTAG00000016309	<i>ATP6V1D</i>	ENSBTAG00000010026	<i>BT.31482</i>
ENSBTAG00000004263	<i>ATP6V0A4</i>	ENSBTAG00000005714	<i>ACTC1</i>
ENSBTAG00000000292	<i>TCIRG1</i>	ENSBTAG00000008394	<i>MYL3</i>
ENSBTAG00000015100	<i>ATP6V0E1</i>	ENSBTAG00000022000	<i>CACNB2</i>
ENSBTAG00000002703	<i>ATP6V1A</i>	ENSBTAG00000013861	<i>SLC8A1</i>
ENSBTAG00000010620	<i>ATP6V1B1</i>	ENSBTAG00000021916	<i>BT.22263</i>
ENSBTAG00000008004	<i>NCF2</i>	ENSBTAG00000011424	<i>TPM2</i>
ENSBTAG00000018889	<i>ATP6V0B</i>	ENSBTAG00000022886	<i>RYR2</i>
ENSBTAG00000001192	<i>STX12</i>	Focal adhesion	
ENSBTAG00000017280	<i>BT.19562</i>	ENSBTAG00000021879	<i>BT.102875</i>
ENSBTAG00000014238	<i>ATP6V1E1</i>	ENSBTAG00000001462	<i>FLT4</i>
ENSBTAG00000000720	<i>CTSL1</i>	ENSBTAG00000014609	<i>BT.41853</i>
ENSBTAG00000006240	<i>TLR4</i>	ENSBTAG00000014567	<i>BT.105316</i>
ENSBTAG00000008008	<i>TLR2</i>	ENSBTAG00000018255	<i>ACTN1</i>
ENSBTAG00000002006	<i>THBS1</i>	ENSBTAG00000011772	<i>PPP1R12B</i>
ENSBTAG00000019929	<i>ITGAV</i>	ENSBTAG00000009696	<i>BT.49327</i>
ENSBTAG00000001927	<i>ATP6V1C2</i>	ENSBTAG00000011473	<i>MYL9</i>
ENSBTAG00000018646	<i>ATP6V1B2</i>	ENSBTAG00000045584	<i>CAV2</i>
ENSBTAG00000021092	<i>ATP6V0D2</i>	ENSBTAG00000017869	<i>CAV1</i>
ENSBTAG00000008592	<i>BT.54393</i>	ENSBTAG00000026273	<i>MYL10</i>
ENSBTAG00000032515	<i>PLA2R1</i>	ENSBTAG00000011706	<i>TNR</i>
ENSBTAG00000013513	<i>ATP6V1C1</i>	ENSBTAG00000002979	<i>PIK3R3</i>
ENSBTAG00000003305	<i>NCF1</i>	ENSBTAG00000013688	<i>PGF</i>
ENSBTAG00000014553	<i>ATP6V0D1</i>	ENSBTAG00000015650	<i>TNN</i>
ENSBTAG00000017060	<i>ITGB2</i>	ENSBTAG00000017847	<i>PPP1R12C</i>
ENSBTAG00000017135	<i>CTSS</i>	ENSBTAG00000006253	<i>FLNC</i>
ENSBTAG00000019289	<i>ITGA2</i>	ENSBTAG00000012897	<i>ITGA7</i>
ENSBTAG00000019953	<i>CYBB</i>	ENSBTAG00000022004	<i>FLNB</i>
ENSBTAG00000047238	<i>BT.35830</i>	ENSBTAG00000013155	<i>COL2A1</i>
ENSBTAG00000015032	<i>CD14</i>	ENSBTAG00000017970	<i>ZYX</i>
ENSBTAG00000014546	<i>BT.49447</i>	ENSBTAG00000009476	<i>FIGF</i>
ENSBTAG00000004547	<i>OLR1</i>	ENSBTAG00000000696	<i>ILK</i>
ENSBTAG00000014031	<i>BT.45021</i>	ENSBTAG00000020921	<i>PRKCB</i>
ENSBTAG00000017740	<i>BT.54364</i>	ENSBTAG00000000057	<i>THBS3</i>
ENSBTAG00000026428	<i>ATP6V0C</i>	ENSBTAG00000022927	<i>RAC3</i>
ENSBTAG00000010242	<i>LAMP1</i>	ENSBTAG00000003667	<i>TLN2</i>
ENSBTAG00000018810	<i>THBS2</i>	ENSBTAG00000022635	<i>BT.106077</i>
ENSBTAG00000021647	<i>FCAR</i>	ENSBTAG00000022699	<i>CAV3</i>
ENSBTAG00000023730	<i>TUBB3</i>	ENSBTAG00000011198	<i>PPP1CC</i>
Osteoclast differentiation		ENSBTAG00000014575	<i>COL4A5</i>
ENSBTAG00000019250	<i>BTK</i>	ENSBTAG00000003658	<i>BT.82217</i>
ENSBTAG00000007338	<i>TYROBP</i>	ENSBTAG00000011190	<i>FLNA</i>
ENSBTAG00000012544	<i>BT.49564</i>	ENSBTAG00000013760	<i>COL4A6</i>
ENSBTAG00000009381	<i>BT.74007</i>	ENSBTAG00000000601	<i>COL11A2</i>

ENSBTAG00000006909	<i>PIK3CB</i>	Glycine, serine and threonine metabolism	
ENSBTAG00000003895	<i>CYBA</i>	ENSBTAG00000001288	<i>MAOB</i>
ENSBTAG000000021709	<i>BT.58934</i>	ENSBTAG000000039719	<i>PHGDH</i>
ENSBTAG000000023929	<i>FOSL2</i>	ENSBTAG000000008434	<i>GCAT</i>
ENSBTAG00000006017	<i>BT.20288</i>	ENSBTAG000000004112	<i>GAMT</i>
ENSBTAG000000005359	<i>TGFB2</i>	ENSBTAG000000002110	<i>DMGDH</i>
ENSBTAG000000021358	<i>BT.24520</i>	ENSBTAG000000013960	<i>PSAT1</i>
ENSBTAG000000007275	<i>TREM2</i>	ENSBTAG000000002321	<i>AMT</i>
ENSBTAG000000007531	<i>NCF4</i>	ENSBTAG000000002255	<i>BHMT</i>
ENSBTAG000000016683	<i>BIKBA</i>	ENSBTAG000000000160	<i>CBS</i>
ENSBTAG000000004826	<i>ACP5</i>	ENSBTAG000000007946	<i>PIPOX</i>
ENSBTAG000000007599	<i>IKBKB</i>	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	
ENSBTAG000000038428	<i>RELB</i>	ENSBTAG000000002036	<i>BT.37145</i>
ENSBTAG000000020457	<i>TGFB1</i>	ENSBTAG000000018255	<i>ACTN1</i>
ENSBTAG000000020270	<i>BT.60919</i>	ENSBTAG000000012311	<i>CACNG5</i>
ENSBTAG000000010349	<i>IL1A</i>	ENSBTAG000000000589	<i>CACNG4</i>
ENSBTAG000000005273	<i>IL1R1</i>	ENSBTAG000000009696	<i>BT.49327</i>
ENSBTAG000000001086	<i>FHL2</i>	ENSBTAG000000047491	<i>CACNA1S</i>
ENSBTAG000000008004	<i>NCF2</i>	ENSBTAG000000022314	<i>EMD</i>
ENSBTAG000000008924	<i>TNFSF11</i>	ENSBTAG000000001865	<i>SGCG</i>
ENSBTAG000000009232	<i>PIK3CA</i>	ENSBTAG000000012897	<i>ITGA7</i>
ENSBTAG000000001321	<i>IL1B</i>	ENSBTAG000000001835	<i>GJA1</i>
ENSBTAG000000005978	<i>PIK3R5</i>	ENSBTAG000000010026	<i>BT.31482</i>
ENSBTAG000000021819	<i>IFNAR1</i>	ENSBTAG000000022000	<i>CACNB2</i>
ENSBTAG000000021035	<i>CTSK</i>	ENSBTAG000000005353	<i>DES</i>
ENSBTAG000000008592	<i>BT.54393</i>	ENSBTAG000000013861	<i>SLC8A1</i>
ENSBTAG000000010007	<i>MAPK13</i>	ENSBTAG000000022886	<i>RYR2</i>
ENSBTAG000000003305	<i>NCF1</i>	ENSBTAG000000008248	<i>DMD</i>
ENSBTAG000000015212	<i>IFNAR2</i>		
ENSBTAG000000012899	<i>IFNGR2</i>		
ENSBTAG000000015591	<i>BT.105440</i>		
ENSBTAG000000004767	<i>SYK</i>		
ENSBTAG000000019953	<i>CYBB</i>		
ENSBTAG000000004322	<i>FOS</i>		
ENSBTAG000000004380	<i>STAT2</i>		
ENSBTAG000000001051	<i>OSCAR</i>		
ENSBTAG000000012046	<i>JUNB</i>		
ENSBTAG000000018984	<i>PIK3CD</i>		
ENSBTAG000000006194	<i>BT.17885</i>		
ENSBTAG000000004736	<i>GRB2</i>		
ENSBTAG000000012771	<i>CSF1R</i>		
ENSBTAG000000000283	<i>CSF1</i>		
ENSBTAG000000008441	<i>SOCS3</i>		

ENSBTAG00000004386	<i>SOC51</i>
ENSBTAG00000005816	<i>BT.92462</i>
ENSBTAG000000025471	<i>TNF</i>
Cytokine-cytokine receptor interaction	
ENSBTAG000000012082	<i>BT.20111</i>
ENSBTAG000000010785	<i>FAS</i>
ENSBTAG000000012544	<i>BT.49564</i>
ENSBTAG000000024928	<i>TNFRSF1B</i>
ENSBTAG000000001602	<i>IL4R</i>
ENSBTAG000000005359	<i>TGFB2</i>
ENSBTAG000000047400	<i>IL11</i>
ENSBTAG000000016163	<i>BT.45038</i>
ENSBTAG000000021697	<i>PDGFB</i>
ENSBTAG000000009064	<i>CSF2RB</i>
ENSBTAG000000020736	<i>BT.42498</i>
ENSBTAG000000015362	<i>BMP7</i>
ENSBTAG000000001191	<i>CCL17</i>
ENSBTAG000000016284	<i>IL7</i>
ENSBTAG000000020457	<i>TGFB1</i>
ENSBTAG000000025782	<i>BT.44261</i>
ENSBTAG000000021462	<i>CSF3</i>
ENSBTAG0000000037811	<i>CCL2</i>
ENSBTAG000000014113	<i>CCL8</i>
ENSBTAG000000005215	<i>IL10RA</i>
ENSBTAG000000002804	<i>BT.74208</i>
ENSBTAG000000018105	<i>ACVR2B</i>
ENSBTAG000000010349	<i>IL1A</i>
ENSBTAG000000021326	<i>CCL20</i>
ENSBTAG000000006343	<i>IL1R2</i>
ENSBTAG000000005273	<i>IL1R1</i>
ENSBTAG000000001034	<i>IL18R1</i>
ENSBTAG0000000033748	<i>IL18RAP</i>
ENSBTAG000000013334	<i>CSF3R</i>
ENSBTAG000000012004	<i>TGFB3</i>
ENSBTAG000000047412	<i>TNFSF18</i>
ENSBTAG000000008924	<i>TNFSF11</i>
ENSBTAG000000001060	<i>CXCR4</i>
ENSBTAG000000015361	<i>CNTFR</i>
ENSBTAG000000001321	<i>IL1B</i>
ENSBTAG000000005111	<i>BMP2</i>
ENSBTAG000000007424	<i>LIF</i>
ENSBTAG000000006078	<i>IL15RA</i>
ENSBTAG000000019628	<i>EDAR</i>
ENSBTAG000000014921	<i>IL6</i>

ENSBTAG00000006685	<i>IL10</i>
ENSBTAG00000002081	<i>BMPRI1B</i>
ENSBTAG00000038639	<i>CXCL9</i>
ENSBTAG00000021819	<i>IFNAR1</i>
ENSBTAG00000001100	<i>BT.49121</i>
ENSBTAG00000033107	<i>BT.103407</i>
ENSBTAG00000006694	<i>CXCL14</i>
ENSBTAG00000020054	<i>TNFRSF21</i>
ENSBTAG00000014737	<i>VEGFC</i>
ENSBTAG00000015212	<i>IFNAR2</i>
ENSBTAG00000012899	<i>IFNGR2</i>
ENSBTAG00000007193	<i>CCL16</i>
ENSBTAG00000031998	<i>CXCL16</i>
ENSBTAG00000000130	<i>TNFSF13</i>
ENSBTAG00000032017	<i>BT.45028</i>
ENSBTAG00000001570	<i>CSF2</i>
ENSBTAG00000002923	<i>CX3CR1</i>
ENSBTAG00000015150	<i>IL12A</i>
ENSBTAG00000011067	<i>IL17RA</i>
ENSBTAG00000018069	<i>TNFSF15</i>
ENSBTAG00000012771	<i>CSF1R</i>
ENSBTAG00000000283	<i>CSF1</i>
ENSBTAG00000008197	<i>EPOR</i>
ENSBTAG00000020242	<i>CRLF2</i>
ENSBTAG00000025471	<i>TNF</i>
ENSBTAG00000008411	<i>PLEKHO2</i>
ENSBTAG00000027407	<i>TNFRSF6B</i>
Chagas disease (American trypanosomiasis)	
ENSBTAG00000010998	<i>FLIP</i>
ENSBTAG00000006894	<i>BT.23126</i>
ENSBTAG00000010785	<i>FAS</i>
ENSBTAG00000012544	<i>BT.49564</i>
ENSBTAG00000006909	<i>PIK3CB</i>
ENSBTAG00000019988	<i>BT.27662</i>
ENSBTAG00000015718	<i>CASP8</i>
ENSBTAG00000005359	<i>TGFB2</i>
ENSBTAG00000016683	<i>BIKBA</i>
ENSBTAG00000007599	<i>IKBKB</i>
ENSBTAG00000020457	<i>TGFB1</i>
ENSBTAG00000014465	<i>SERPINE1</i>
ENSBTAG00000037811	<i>CCL2</i>
ENSBTAG00000020270	<i>BT.60919</i>
ENSBTAG00000012004	<i>TGFB3</i>

ENSBTAG00000009232	<i>PIK3CA</i>
ENSBTAG00000001321	<i>IL1B</i>
ENSBTAG000000017280	<i>BT.19562</i>
ENSBTAG00000002714	<i>GNAI1</i>
ENSBTAG000000014921	<i>IL6</i>
ENSBTAG00000006685	<i>IL10</i>
ENSBTAG00000006240	<i>TLR4</i>
ENSBTAG00000008008	<i>TLR2</i>
ENSBTAG000000019079	<i>PLCB2</i>
ENSBTAG00000005978	<i>PIK3R5</i>
ENSBTAG000000010007	<i>MAPK13</i>
ENSBTAG000000012899	<i>IFNGR2</i>
ENSBTAG000000011193	<i>C1QC</i>
ENSBTAG000000009520	<i>ADCY1</i>
ENSBTAG000000012599	<i>SMAD3</i>
ENSBTAG000000018274	<i>FADD</i>
ENSBTAG000000021717	<i>BT.72991</i>
ENSBTAG000000015150	<i>IL12A</i>
ENSBTAG000000004322	<i>FOS</i>
ENSBTAG000000018984	<i>PIK3CD</i>
ENSBTAG00000000563	<i>BT.48995</i>
ENSBTAG000000011196	<i>C1QB</i>
ENSBTAG000000007153	<i>C1QA</i>
ENSBTAG000000014031	<i>BT.45021</i>
ENSBTAG000000039916	<i>SMAD2</i>
ENSBTAG000000016085	<i>IRAK1</i>
ENSBTAG000000025471	<i>TNF</i>
Leishmaniasis	
ENSBTAG000000006894	<i>BT.23126</i>
ENSBTAG000000012544	<i>BT.49564</i>
ENSBTAG000000003895	<i>CYBA</i>
ENSBTAG000000014127	<i>BT.104982</i>
ENSBTAG000000005359	<i>TGFB2</i>
ENSBTAG000000007531	<i>NCF4</i>
ENSBTAG000000016683	<i>BIKBA</i>
ENSBTAG000000001778	<i>BT.20178</i>
ENSBTAG000000020457	<i>TGFB1</i>
ENSBTAG000000020270	<i>BT.60919</i>
ENSBTAG000000020294	<i>PTPN6</i>
ENSBTAG000000010349	<i>IL1A</i>
ENSBTAG000000008004	<i>NCF2</i>
ENSBTAG000000012004	<i>TGFB3</i>
ENSBTAG000000001321	<i>IL1B</i>
ENSBTAG000000017280	<i>BT.19562</i>

ENSBTAG00000006685	<i>IL10</i>
ENSBTAG00000006240	<i>TLR4</i>
ENSBTAG00000008008	<i>TLR2</i>
ENSBTAG00000008592	<i>BT.54393</i>
ENSBTAG00000010007	<i>MAPK13</i>
ENSBTAG00000003305	<i>NCF1</i>
ENSBTAG00000012899	<i>IFNGR2</i>
ENSBTAG00000017060	<i>ITGB2</i>
ENSBTAG00000015150	<i>IL12A</i>
ENSBTAG00000047238	<i>BT.35830</i>
ENSBTAG00000004322	<i>FOS</i>
ENSBTAG00000000563	<i>BT.48995</i>
ENSBTAG00000046862	<i>MARCKSL1</i>
ENSBTAG00000016085	<i>IRAK1</i>
ENSBTAG00000025471	<i>TNF</i>
Toll-like receptor signalling pathway	
ENSBTAG00000010576	<i>MAP2K3</i>
ENSBTAG00000006909	<i>PIK3CB</i>
ENSBTAG00000015718	<i>CASP8</i>
ENSBTAG00000016683	<i>BIKBA</i>
ENSBTAG00000020736	<i>BT.42498</i>
ENSBTAG00000007599	<i>IKBKB</i>
ENSBTAG00000011600	<i>MAP3K8</i>
ENSBTAG00000020270	<i>BT.60919</i>
ENSBTAG00000013118	<i>CD86</i>
ENSBTAG00000005260	<i>SPP1</i>
ENSBTAG00000018059	<i>BT.27986</i>
ENSBTAG00000009232	<i>PIK3CA</i>
ENSBTAG00000001321	<i>IL1B</i>
ENSBTAG00000004989	<i>IRF5</i>
ENSBTAG00000016864	<i>BT.24181</i>
ENSBTAG00000013475	<i>TRAF3</i>
ENSBTAG00000014921	<i>IL6</i>
ENSBTAG00000006240	<i>TLR4</i>
ENSBTAG00000006378	<i>RIPK1</i>
ENSBTAG00000008008	<i>TLR2</i>
ENSBTAG00000038639	<i>CXCL9</i>
ENSBTAG00000005978	<i>PIK3R5</i>
ENSBTAG00000021819	<i>IFNAR1</i>
ENSBTAG00000021035	<i>CTSK</i>
ENSBTAG00000008864	<i>MD2</i>
ENSBTAG00000010007	<i>MAPK13</i>
ENSBTAG00000015212	<i>IFNAR2</i>
ENSBTAG00000018274	<i>FADD</i>

ENSBTAG00000015150	<i>IL12A</i>
ENSBTAG00000004322	<i>FOS</i>
ENSBTAG00000015032	<i>CD14</i>
ENSBTAG00000018984	<i>PIK3CD</i>
ENSBTAG00000000563	<i>BT.48995</i>
ENSBTAG00000014031	<i>BT.45021</i>
ENSBTAG00000016085	<i>IRAK1</i>
ENSBTAG00000000477	<i>TLR5</i>
ENSBTAG00000022161	<i>TLR7</i>
ENSBTAG00000025471	<i>TNF</i>
Amoebiasis	
ENSBTAG00000006894	<i>BT.23126</i>
ENSBTAG00000011975	<i>SERPINB1</i>
ENSBTAG00000017473	<i>LAMC3</i>
ENSBTAG00000006909	<i>PIK3CB</i>
ENSBTAG00000027181	<i>LAMA3</i>
ENSBTAG00000000793	<i>LAMC2</i>
ENSBTAG00000019988	<i>BT.27662</i>
ENSBTAG00000010193	<i>BT.41314</i>
ENSBTAG00000001945	<i>BT.22399</i>
ENSBTAG00000005359	<i>TGFB2</i>
ENSBTAG00000020457	<i>TGFB1</i>
ENSBTAG00000020270	<i>BT.60919</i>
ENSBTAG00000016149	<i>C9</i>
ENSBTAG00000008300	<i>BT.23418</i>
ENSBTAG00000006343	<i>IL1R2</i>
ENSBTAG00000005273	<i>IL1R1</i>
ENSBTAG00000012004	<i>TGFB3</i>
ENSBTAG00000009232	<i>PIK3CA</i>
ENSBTAG00000001321	<i>IL1B</i>
ENSBTAG00000025210	<i>COL4A2</i>
ENSBTAG00000014921	<i>IL6</i>
ENSBTAG00000006685	<i>IL10</i>
ENSBTAG00000006240	<i>TLR4</i>
ENSBTAG00000008008	<i>TLR2</i>
ENSBTAG00000019079	<i>PLCB2</i>
ENSBTAG00000005978	<i>PIK3R5</i>
ENSBTAG00000001061	<i>BT.62458</i>
ENSBTAG00000017060	<i>ITGB2</i>
ENSBTAG00000015874	<i>CASP3</i>
ENSBTAG00000001570	<i>CSF2</i>
ENSBTAG00000009520	<i>ADCY1</i>
ENSBTAG00000015150	<i>IL12A</i>
ENSBTAG00000047238	<i>BT.35830</i>

ENSBTAG00000015032	<i>CD14</i>
ENSBTAG00000018984	<i>PIK3CD</i>
ENSBTAG00000014367	<i>PRKX</i>
ENSBTAG00000012849	<i>BT.32410</i>
ENSBTAG00000016542	<i>LAMB3</i>
ENSBTAG00000025471	<i>TNF</i>
Complement and coagulation cascades	
ENSBTAG00000013125	<i>PLAUR</i>
ENSBTAG00000003572	<i>F11</i>
ENSBTAG00000007052	<i>BT.62541</i>
ENSBTAG00000001244	<i>PLAT</i>
ENSBTAG00000014465	<i>SERPINE1</i>
ENSBTAG00000016149	<i>C9</i>
ENSBTAG00000005122	<i>KNG1</i>
ENSBTAG00000038496	<i>CR2</i>
ENSBTAG00000007101	<i>F3</i>
ENSBTAG00000001271	<i>PLG</i>
ENSBTAG00000005947	<i>PLAU</i>
ENSBTAG00000009876	<i>C4BPA</i>
ENSBTAG00000007268	<i>BT.19195</i>
ENSBTAG00000017280	<i>BT.19562</i>
ENSBTAG00000011193	<i>C1QC</i>
ENSBTAG00000007450	<i>C2</i>
ENSBTAG00000020859	<i>SERPINF2</i>
ENSBTAG00000021717	<i>BT.72991</i>
ENSBTAG00000022120	<i>FGF</i>
ENSBTAG00000019741	<i>C3AR1</i>
ENSBTAG00000011196	<i>C1QB</i>
ENSBTAG00000007153	<i>C1QA</i>
ENSBTAG00000002758	<i>THBD</i>
ENSBTAG00000020199	<i>BT.69975</i>
ENSBTAG00000004840	<i>C1S</i>
ENSBTAG00000023652	<i>PROS1</i>
ENSBTAG00000006984	<i>BT.91089</i>
ENSBTAG00000020872	<i>BT.34208</i>
ENSBTAG00000034501	<i>BT.64790</i>

Table 4.3-17: Differntially elevated genes 7 and 21 days postpartum (DPP) in sub-clinically endometritic (SCE) cows within each enriched pathway.

7 DPP v 21 DPP			
Elevated 7 DPP in SCE		Elevated 21 DPP in SCE	
Ensembl Gene ID	Gene Name	Ensembl Gene ID	Gene Name
Rheumatoid arthritis		Neuroactive ligand-receptor interaction	
ENSBTAG000000021035	CTSK	ENSBTAG000000016645	GABRA4
ENSBTAG000000021092	ATP6V0D2	ENSBTAG000000047586	NPY1R
ENSBTAG000000019716	IL-8	ENSBTAG000000010306	RXFP1
Toll-like receptor signalling pathway			
ENSBTAG000000005260	SPP1		
ENSBTAG000000021035	CTSK		
ENSBTAG000000019716	IL-8		
Phagosome			
ENSBTAG000000021092	ATP6V0D2		
ENSBTAG000000018810	THBS2		
ENSBTAG000000021647	FCAR		
Arachidonic acid metabolism			
ENSBTAG000000014127	BT.104982		
ENSBTAG000000043553	GPX3		
Malaria			
ENSBTAG000000019716	IL-8		
ENSBTAG000000018810	THBS2		
Leishmaniasis			
ENSBTAG000000006894	BT.23126		
ENSBTAG000000014127	BT.104982		
Epithelial cell signalling in Helicobacter pylori infection			
ENSBTAG000000021092	ATP6V0D2		
ENSBTAG000000019716	IL-8		
Small cell lung cancer			
ENSBTAG000000006894	BT.23126		
ENSBTAG000000014127	BT.104982		
ECM-receptor interaction			
ENSBTAG000000005260	SPP1		
ENSBTAG000000018810	THBS2		
Chagas disease (American trypanosomiasis)			
ENSBTAG000000006894	BT.23126		
ENSBTAG000000019716	IL-8		

Gene filtering within groups

The number of genes was filtered by increasing the stringency which defined whether a gene was significantly differentially expressed or not. The first level of filtering kept genes with a false discovery rate (FDR) of less than 0.1 and these genes were used in pathway analysis as above. The second level of stringency retained genes with an FDR < 0.05, 46 genes remained in the SCE group and 3271 genes in the healthy group. These genes were then passed through the third filter which involved removing genes which had less than 10 read counts on average in 6 samples for the SCE group and 9 samples for the healthy group. The number of genes kept after the third filter was 19 in the SCE group and 2732 in the healthy group. The fourth and final filtering criteria retained genes with a Log₂ fold change of greater than 2 and less than -2, resulting in 8 genes in the SCE group and 502 in the healthy group.

Table 4.3-18: The number of genes present after each stage of filtering that were significantly differentially expressed between 7 and 21 days postpartum (DPP) in sub-clinically endometritic (SCE) and healthy cows.

			1		2		3		4	
SCE	7 v 21 DPP	↑ 7DPP	95	66	46	32	19	5	8	5
		↑ 21DPP		29		14		3		3
Healthy		↑ 7DPP	4068	2495	3271	2048	2732	321	502	321
		↑ 21DPP		1573		1223		181		181

- 1 – genes with a false discovery rate (FDR) of less than 0.1.
- 2 – genes with a false discovery rate (FDR) of less than 0.05.
- 3 – genes with more than 10 read counts on average in 6 samples for the SCE group and 9 samples for the healthy group.
- 4 – genes with a Log₂ fold change of greater than 2 and less than -2.

The most highly differentially expressed genes which remained after the fourth filtering step were examined using heat maps and hierarchal clustering to determine if the differences between samples were similar to those observed previously using multi-dimensional scaling plots prior to gene filtering (Figure 4.3-12). The positions of genes and samples were reordered so that these independent variables were placed nearer to similar and further from different genes and samples. Dendograms illustrated how close or distant the relationship was between individual genes and samples. The rows were scaled based on z-scores, where red indicated lower number of read counts than the mean and green indicated a higher number of reads than the mean across all samples for each gene.

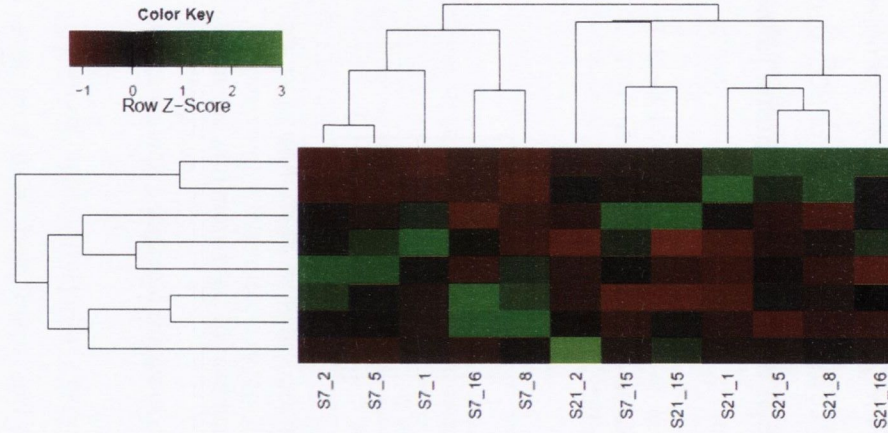
Within the SCE group over time there appears to be a clustering of samples by time point with the majority of samples at 7DPP to the left of the graph and at 21 DPP to the right of the graph.

However there is one sample that does not conform to this clustering, cow 15 at 7 DPP is clustering with the 21 DPP samples.

Within the Healthy group over time the samples cluster together based on time point postpartum with the majority of samples at 7 DPP clustering to the right and 21 DPP clustering to the left of the graph. There is an outlier, cow 4 at 7DPP, which appears to be different from both the 7 and 21 DPP clustering's and it is interesting that this same animal is also an outlier for the same comparison using multi-dimensional scaling prior to gene filtering.

An additional observation was the sides of the graphs to which either 7 or 21 DPP samples clustered when comparing the heat maps for SCE and healthy cows. In the heat map for SCE cows the 7 DPP samples cluster to the left whereas in the healthy cows the 7 DPP samples cluster to the right and the same is true for the 21 DPP samples.

SCE 7 v 21 DPP



Healthy 7 v 21 DPP

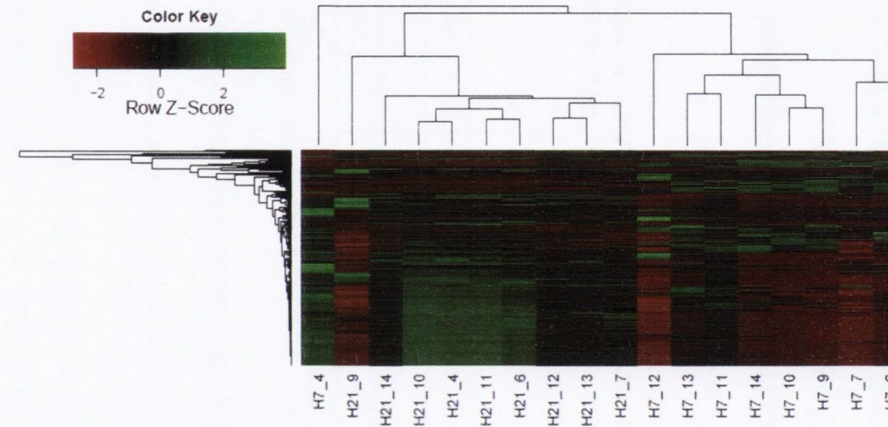


Figure 4.3-12: Heatmaps and hierarchical clustering of significant differentially expressed genes, comparing read counts 7 and 21 days postpartum (DPP) in sub-clinically endometritic (SCE and S) and healthy (H) cows. Genes were significantly differentially expressed with a FDR <0.05 and Log₂ fold change of greater than 2 and less than -2. The Z-score was used to visualise read counts lower or higher than the mean across all samples for a gene as red and green, respectively.

The number of genes significantly differentially expressed was greater in the healthy group compared to the SCE group at each level of stringency imposed. There were a greater number of genes significantly differentially elevated 7 DPP than 21 DPP and this trend was observed in both groups and at each stage of filtering.

A further assessment was carried out to determine what genes were commonly and uniquely significantly differentially expressed between 7 and 21 DPP in the SCE and healthy groups. In total there were 3 genes common to both the SCE and healthy groups, 5 unique to the SCE group and 499 unique to the healthy group. Genes that were significant differentially elevated at 7 DPP in both SCE and healthy groups were compared, 2 genes were common to both groups, 3 were unique to SCE cows and 319 were unique to healthy cows. Significant differentially elevated genes at 21 DPP in both the SCE and healthy group were also compared, 1 was common to both groups, 2 were unique to the SCE group and 180 were unique to the healthy group. Genes that were uniquely expressed in the SCE group, of which there were 5, were used in the selection of potential biomarkers for subclinical endometritis (Figure 4.3-13).

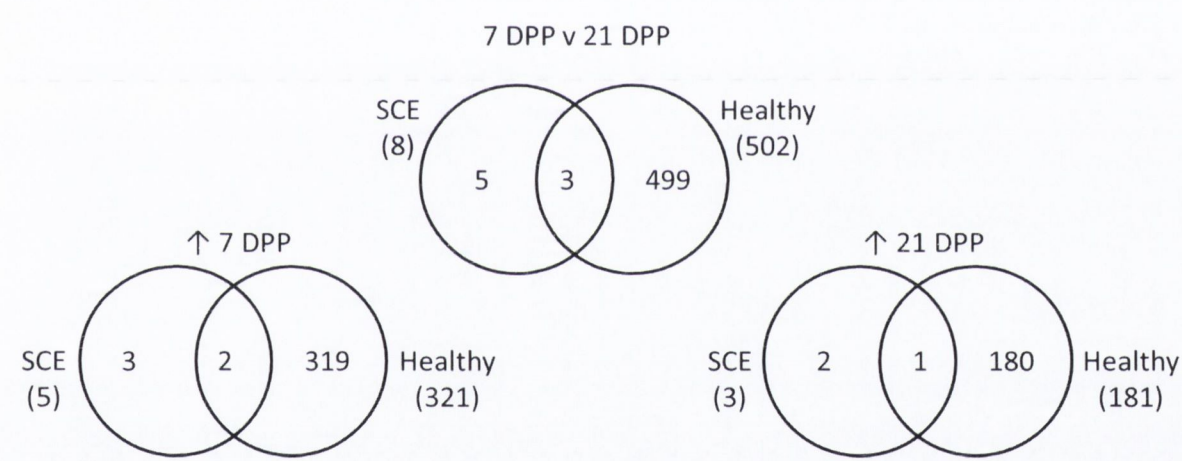


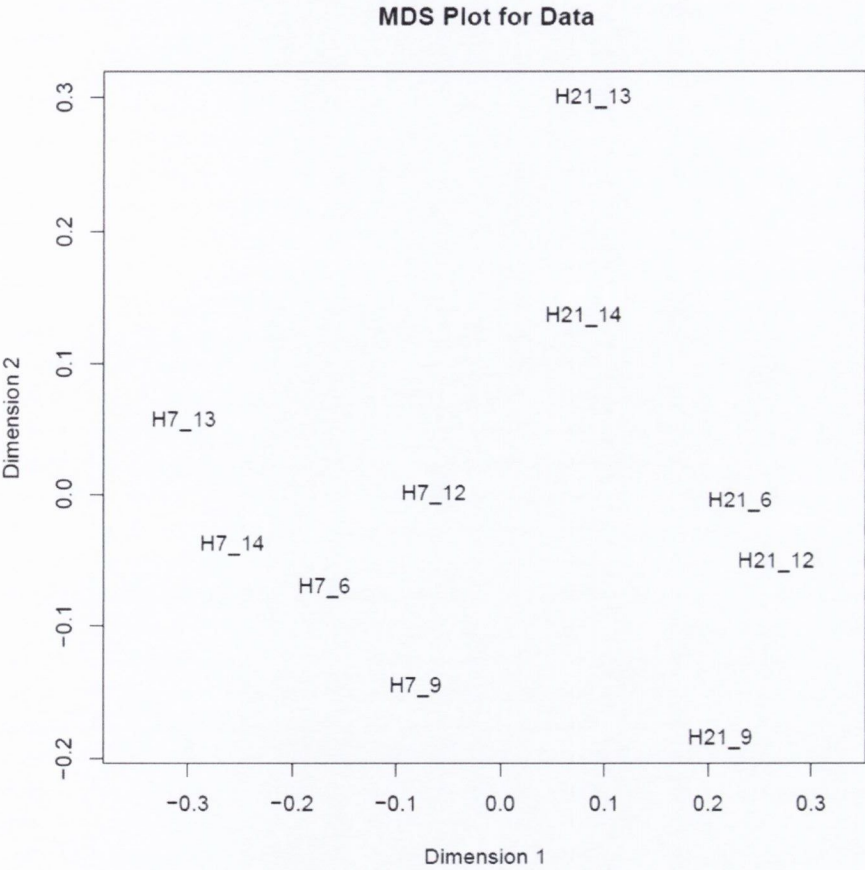
Figure 4.3-13: Venn diagrams representing the number of significantly differentially expressed genes that are common and unique to sub-clinically endometritic (SCE) and healthy groups 7 and 21 days postpartum (DPP). Genes were significantly differentially expressed with a FDR <0.05 and Log₂ fold change of greater than 2 and less than -2.

4.3.8 (A) microRNA-Seq expression within groups, between 7 and 21 days postpartum

Differences in MicroRNA transcriptional profiles between sub-clinically endometritic (n=5) and healthy cows (n=5) were analysed within each group and compared over time between 7 and 21 days postpartum (DPP). The software program EdgeR was used to uncover the most highly differentially expressed genes over time. A multi-dimensional scaling (MDS) plot was created using the software program EdgeR which displayed how different samples were from each other.

On close inspection of the MDS plot for healthy cows there was clustering of 21 DPP samples towards right side of the plot and 7 DPP samples towards the left side of the graph in the first dimension (Figure 4.3-14). Clustering of the healthy 7 DPP samples was tighter towards the bottom of the graph, in the second dimension, than that observed 21 DPP. In the SCE group there was separation in the first dimension with the 7 DPP samples clustering to the left side of the MDS plot and 21 DPP samples clustering to the right side of the graph. In the SCE groups 21 DPP samples clustered tighter in the second dimension than the 7 DPP samples did.

Healthy 7 v 21DPP



SCE 7 v 21DPP

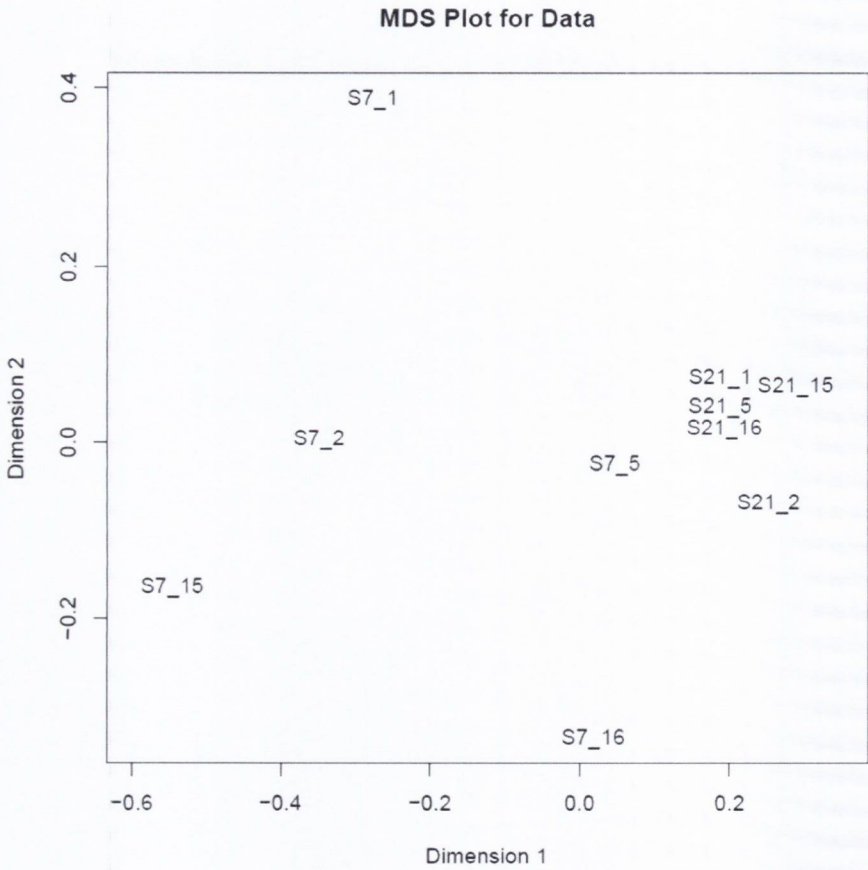


Figure 4.3-14: Multi-dimensional scaling (MDS) plots produced with EdgeR comparing samples 7 and 21 days postpartum (DPP) in the healthy (H) and sub-clinically endometritic (S) groups.

Genes were significantly differentially expressed with a false discovery rate (FDR) (or adjusted p -value) of less than 0.1. The number of genes significantly differentially expressed between 7 and 21 DPP in sub-clinically endometritic cows was 37, of these 30 were elevated 7 DPP and 7 were elevated 21 DPP, and 16 in healthy cows, of these 6 were elevated 7 DPP and 10 were elevated 21 DPP.

A further assessment was carried out to determine what genes were commonly and uniquely significantly differentially expressed between 7 and 21 DPP in the SCE and healthy groups. In total there were 4 genes common to both the SCE and healthy groups, 33 unique to the SCE group and 12 unique to the healthy group. Genes that were significant differentially elevated at 7 DPP in both SCE and healthy groups were compared, 2 genes were common to both groups, 28 were unique to SCE cows and 4 were unique to healthy cows. Significant differentially elevated genes at 21 DPP in both the SCE and healthy group were also compared, 2 were common to both groups, 5 were unique to the SCE group and 8 were unique to the healthy group (Figure 4.3-15,

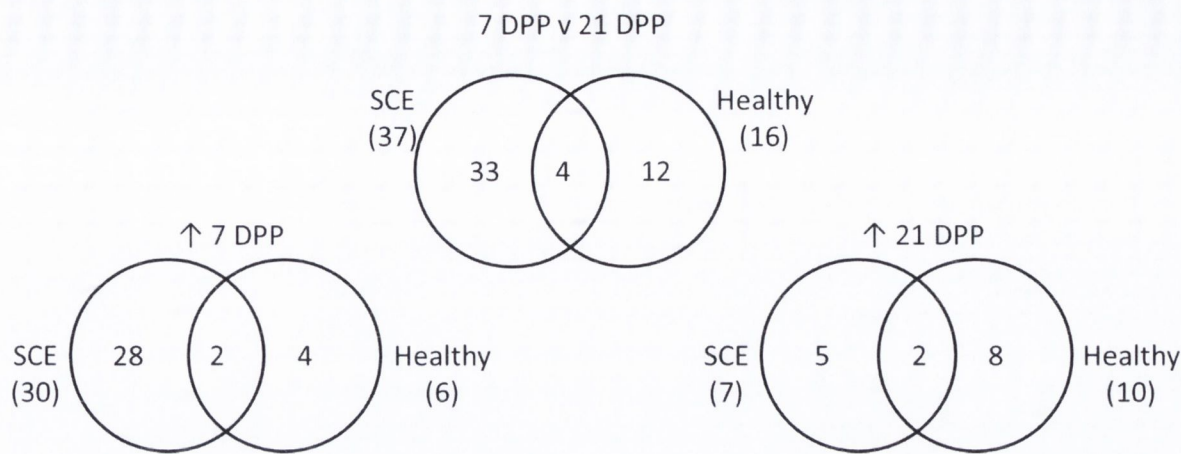


Figure 4.3-15: Venn diagrams representing the number of significantly differentially expressed genes that are common and unique to sub-clinically endometritic (SCE) and healthy groups at 7 and 21 days postpartum (DPP). Significantly differentially expressed genes had a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1

Table 4.3-19: MicroRNA genes significantly elevated 7 days postpartum (DPP) in both sub-clinically endometritic (SCE) and healthy cows, uniquely in the sub-clinically endometritic (SCE) and healthy cows. Significantly differentially elevated (↑) genes had a false discovery rate (FDR) of less than 0.1).

↑ 7 DPP					
Unique to SCE		Common to both SCE and healthy		Unique to healthy	
Ensembl ID	microRNA Name	Ensembl ID	microRNA Name	Ensembl ID	microRNA Name
ENSBTAG00000029854	<i>bta-mir-205</i>	ENSBTAG00000036407	<i>bta-mir-211</i>	ENSBTAG00000044456	<i>bta-mir-2307</i>
ENSBTAG00000029763	<i>bta-mir-375</i>	ENSBTAG00000036380	<i>bta-mir-184</i>	ENSBTAG00000029910	<i>bta-mir-20b</i>
ENSBTAG00000029959	<i>bta-mir-200c</i>			ENSBTAG00000030111	<i>bta-mir-763</i>
ENSBTAG00000036414	<i>bta-mir-451</i>			ENSBTAG00000036418	<i>bta-mir-223</i>
ENSBTAG00000047544	<i>bta-mir-31</i>				
ENSBTAG00000044589	<i>bta-mir-2313</i>				
ENSBTAG00000038882	<i>bta-mir-1249</i>				
ENSBTAG00000036422	<i>bta-mir-95</i>				
ENSBTAG00000045183					
ENSBTAG00000029977	<i>bta-mir-96</i>				
ENSBTAG00000029998	<i>bta-mir-200a</i>				
ENSBTAG00000030062	<i>bta-mir-200b</i>				
ENSBTAG00000029950	<i>bta-mir-505</i>				
ENSBTAG00000037264	<i>bta-mir-486</i>				
ENSBTAG00000029953	<i>bta-mir-144</i>				
ENSBTAG00000030118	<i>bta-mir-182</i>				
ENSBTAG00000029762	<i>bta-mir-183</i>				
ENSBTAG00000036395	<i>bta-mir-429</i>				
ENSBTAG00000036410	<i>bta-mir-141</i>				
ENSBTAG00000036401	<i>bta-mir-147</i>				
ENSBTAG00000046985	<i>bta-mir-652</i>				
ENSBTAG00000046437	<i>bta-mir-210</i>				
ENSBTAG00000029811	<i>bta-mir-378-1</i>				
ENSBTAG00000029862	<i>bta-mir-204</i>				
ENSBTAG00000029833	<i>bta-mir-326</i>				
ENSBTAG00000029803	<i>bta-mir-221</i>				
ENSBTAG00000030089	<i>bta-mir-149</i>				
ENSBTAG00000048241	<i>bta-mir-3431</i>				

Table 4.3-20: MicroRNA genes significantly elevated 21 DPP relative to 7 DPP in both sub-clinically endometritic (SCE) and healthy cows, uniquely in the SCE and healthy cows. Significantly differentially elevated (↑) genes had a false discovery rate (FDR) of less than 0.1

↑ 21 DPP					
Unique to SCE		Common to both SCE and healthy		Unique to healthy	
Ensembl ID	microRNA Name	Ensembl ID	microRNA Name	Ensembl ID	microRNA Name
ENSBTAG00000036379	<i>bta-mir-379</i>	ENSBTAG00000030051	<i>bta-mir-449c</i>	ENSBTAG00000030010	<i>bta-mir-92b</i>
ENSBTAG00000029829	<i>bta-mir-136</i>	ENSBTAG00000030083	<i>bta-mir-670</i>	ENSBTAG00000029827	<i>bta-mir-125b-2</i>
ENSBTAG00000036419	<i>bta-mir-424</i>			ENSBTAG00000029892	<i>bta-mir-10a</i>
ENSBTAG00000029800	<i>bta-mir-335</i>			ENSBTAG00000029912	<i>bta-let-7c</i>
ENSBTAG00000030024	<i>bta-mir-542</i>			ENSBTAG00000030027	<i>bta-mir-449b</i>
				ENSBTAG00000037292	<i>bta-mir-873</i>
				ENSBTAG00000030121	<i>bta-mir-449a</i>
				ENSBTAG00000029926	<i>bta-mir-1-2</i>

4.3.8.1 Cross over between mRNA-Seq and microRNA-Seq gene expression data

In mRNA-Seq gene expression analysis it is common to find a variety of gene biotypes that significantly differentially expressed in addition to protein coding genes such as processed pseudogenes, pseudogenes, rRNAs, snoRNAs, snRNAs and microRNAs within the data set. This was the case with the present data set and of particular interest to this study were the microRNAs that were expressed in the mRNA-Seq data set and also microRNAs that were common to both mRNA-Seq and microRNA-Seq data sets.

The biotypes for all genes significantly expressed with a false discovery rate of < 0.1 were attained for the mRNA-Seq data. Genes differentially expressed between 7 and 21 DPP in the SCE group did not have any microRNA biotypes. In the healthy group there were 12 differentially elevated genes 7 DPP and 5 differentially elevated genes 21 DPP that had a microRNA biotype (Table 4.3-21). These genes were then compared to the microRNA-Seq data set and two microRNAs were found to be common in the healthy group and differentially elevated 7 DPP, bta-mir-184 and bta-mir-223 (Table 4.3-22).

Table 4.3-21: microRNAs significantly differentially elevated in the mRNA-Seq data set in sub-clinically endometritic (SCE) and healthy cows 7 and 21 days postpartum (DPP). Significantly differentially elevated (↑) genes had an FDR or adjusted *p*-value of less than 0.1.

7 DPP v 21 DPP							
SCE				Healthy			
↑ 7 DPP		↑ 21 DPP		↑ 7 DPP		↑ 21 DPP	
Ensembl Gene ID	microRNA Name	Ensembl Gene ID	microRNA Name	Ensembl Gene ID	microRNA Name	Ensembl Gene ID	microRNA Name
				ENSBTAG00000029857	<i>bta-mir-145</i>	ENSBTAG00000029857	<i>bta-mir-145</i>
				ENSBTAG00000029897	<i>bta-mir-21</i>	ENSBTAG00000029906	<i>bta-mir-133a-2</i>
				ENSBTAG00000029906	<i>bta-mir-133a-2</i>	ENSBTAG00000030114	<i>bta-mir-143</i>
				ENSBTAG00000029930	<i>bta-mir-22</i>	ENSBTAG00000036361	<i>bta-mir-152</i>
				ENSBTAG00000029938	<i>bta-mir-138-2</i>	ENSBTAG00000043695	<i>bta-mir-450b</i>
				ENSBTAG00000030114	<i>bta-mir-143</i>		
				ENSBTAG00000036361	<i>bta-mir-152</i>		
				ENSBTAG00000036380	<i>bta-mir-184</i>		
				ENSBTAG00000036418	<i>bta-mir-223</i>		
				ENSBTAG00000043695	<i>bta-mir-450b</i>		
				ENSBTAG00000044284	<i>bta-mir-2357</i>		
				ENSBTAG00000046795			

Table 4.3-22: microRNAs significantly differentially elevated in both the mRNA-Seq and microRNA data sets in sub-clinically endometritic (SCE) and healthy cows 7 and 21 days postpartum (DPP). Significantly differentially elevated (↑) genes had a FDR or adjusted *p*-value of less than 0.1)

7 DPP v 21 DPP	
Healthy	
↑ 7 DPP	
microRNA	
ENSBTAG00000036380	<i>bta-mir-184</i>
ENSBTAG00000036418	<i>bta-mir-223</i>

4.3.9 (B) mRNA-Seq expression between groups, 7 and 21 days postpartum

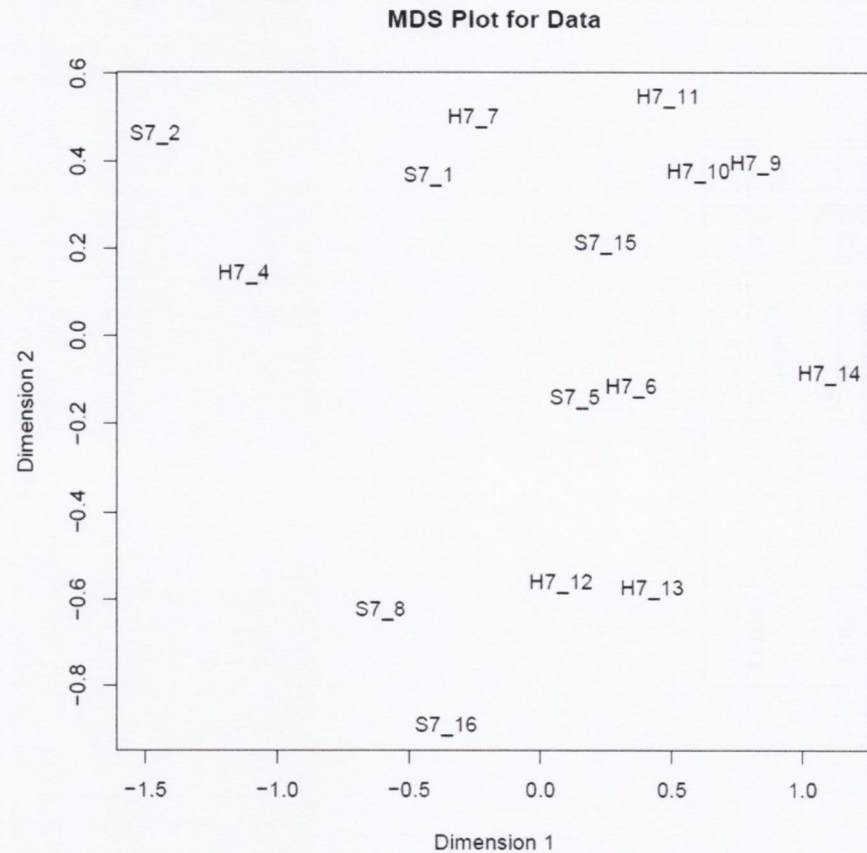
Differences between the transcriptional profiles of sub-clinically endometritic (n=6) and healthy (n=9) cows were analysed 7 days and 21 days postpartum (DPP). The software program EdgeR was used to uncover the most highly differently expressed genes between groups. A multi-dimensional scaling (MDS) plot was created using the software program EdgeR which displayed how different samples were from each other. In the MDS plot comparing sub-clinically endometritic and healthy cows 7 and 21 DPP there was no obvious clustering observed (Figure 4.3-16).

Genes were significantly differentially expressed with a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1. The number of genes significantly differentially expressed between SCE and healthy cows 7 DPP was 182, of these 147 were elevated in healthy cows and 35 were elevated in SCE cows, and 21 DPP was 1219, of these 991 were elevated in healthy cows and 228 were elevated in SCE (Table 4.3-23).

Table 4.3-23: The number of genes significantly differentially elevated genes in sub-clinically endometritic (SCE) or healthy cows at both 7 and 21 days postpartum (DPP) Significantly differentially elevated (↑) genes had a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1.

			Total	
7 DPP	SCE v Healthy	↑ Healthy	182	35
		↑ SCE		147
21 DPP		↑ Healthy	1219	228
		↑ SCE		991

7DPP SCE v Healthy



21DPP SCE v Healthy

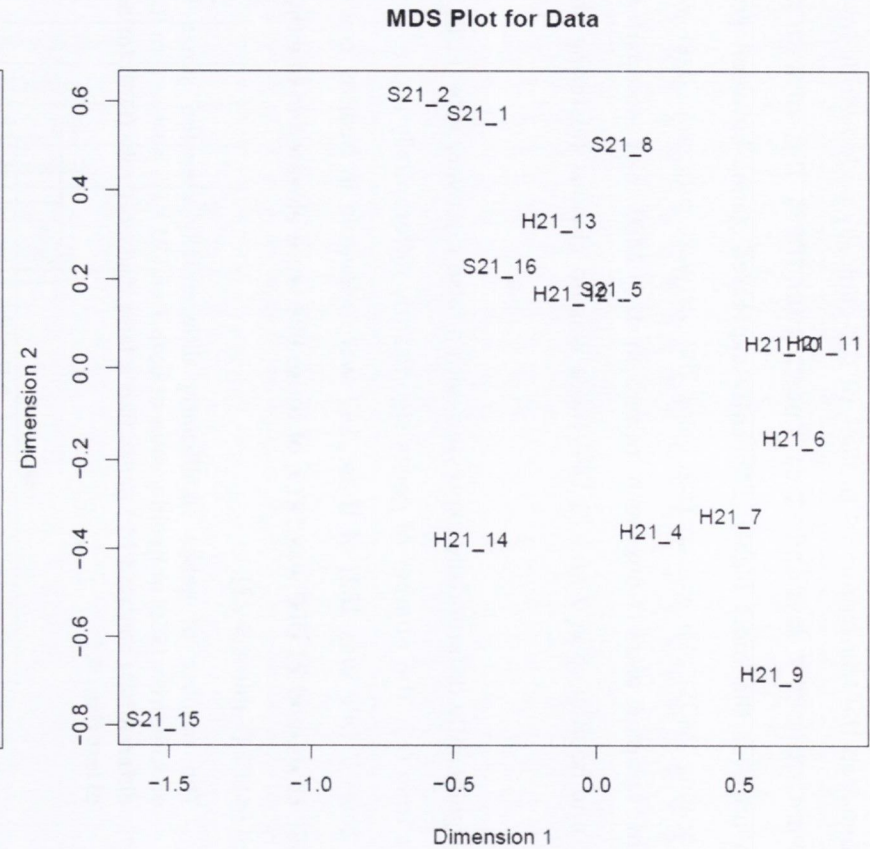


Figure 4.3-16: Multi-dimensional scaling (MDS) plots produced with EdgeR comparing healthy (H) and sub-clinically endometritic (S) samples 7 and 21 days postpartum (DPP).

Pathway analysis

All genes significantly differentially expressed between SCE and healthy cows were either elevated in SCE cows or healthy cows both 7 and 21 DPP with an FDR < 0.1. These genes were converted to human one to one orthologs which reduced the number of genes both 7 and 21 DPP by 27% and 14%, respectively. These human Ensembl gene IDs were converted to KEGG IDs by the GoSeq software program, which predicted over represented KEGG pathways, in some instances there were multiple KEGG IDs for an individual human gene ID which are represented as extra KEGG IDs in the table below. The conversion of human to KEGG IDs resulted in the gaining 2 extra genes 7 DPP and an extra 8 genes at 21 DPP (Table 4.3-24).

Table 4.3-24: The number of significantly differentially elevated genes in sub-clinically endometritic (SCE) and healthy cows 7 and 21 days postpartum (DPP) used in downstream Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis. Genes were significantly elevated (↑) with a false discovery rate of <0.1. ^aBovine ensembl gene ids were converted to human othologs, ^bhuman Ensembl gene IDs were converted to KEGG IDs and ^dthere were multiple KEGG ids for some human ids and these are denoted as "Extra".

			^a Bovine Genes		^b Human Genes		^c KEGG to Human IDs		^d KEGG IDs		
			Total		Total		Total		Total		Extra
7 DPP	SCE v Healthy	↑ in Healthy	182	147	130	99	123	92	125	93	1
		↑ in SCE		35		31		31		32	1
21 DPP		↑ in Healthy	1219	991	1041	810	1009	807	1017	815	8
		↑ in SCE		228		204		202		202	0

The most highly enriched pathways were selected from each analysis and elevated 7 and 21 DPP there were no common pathways up in both the SCE and healthy groups (Table 4.3-25). When comparing the most highly enriched pathways up in the SCE group 7 and 21 DPP there were two pathways in common, the Cytokine-cytokine receptor interaction (Figure 4.3-17 and Figure 4.3-18) and Jak-STAT signalling pathways. There were no commonly enriched pathways in the healthy group which were elevated at both 7 and 21 DPP.

Table 4.3-25: The most highly enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways with genes significantly expressed between sub-clinically endometritic (SCE) and healthy cows 7 and 21 days postpartum (DPP).

SCE v Healthy			
Elevated in SCE 7 DPP		Elevated in Healthy 7 DPP	
KEGG Pathway Name	Over represented <i>p</i> value	KEGG Pathway Name	Over represented <i>p</i> value
1 Cytokine-cytokine receptor interaction*	0.0002	1 Retinol metabolism	0.0014
2 Primary immunodeficiency	0.0011	2 Pathways in cancer	0.0243
3 Intestinal immune network for IgA production	0.0013		
4 alpha-Linolenic acid metabolism	0.0039		
5 Linoleic acid metabolism	0.0041		
6 Ether lipid metabolism	0.0156		
7 Fat digestion and absorption	0.0209		
8 Vascular smooth muscle contraction	0.0214		
9 Arachidonic acid metabolism	0.0260		
10 Jak-STAT signalling pathway*	0.0386		
Elevated in SCE 21 DPP		Elevated in Healthy 21 DPP	
KEGG Pathway Name	Over represented <i>p</i> value	KEGG Pathway Name	Over represented <i>p</i> value
1 Cytokine-cytokine receptor interaction*	7.98E-20	1 Dilated cardiomyopathy	0.002
2 Malaria	3.45E-08	2 Hypertrophic cardiomyopathy (HCM)	0.006
3 Rheumatoid arthritis	2.44E-07	3 ECM-receptor interaction	0.011
4 Osteoclast differentiation	5.34E-07	4 Renin-angiotensin system	0.013
5 Amoebiasis	6.19E-07	5 Calcium signalling pathway	0.015
6 Hematopoietic cell lineage	2.43E-06	6 Vascular smooth muscle contraction	0.015
7 Jak-STAT signalling pathway*	2.49E-06	7 Protein digestion and absorption	0.016
8 Toll-like receptor signalling pathway	7.58E-06	8 Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.019
9 Pathways in cancer	1.25E-05	9 Salivary secretion	0.022
10 Staphylococcus aureus infection	1.32E-05	10 Focal adhesion	0.024

* These pathways are enriched in the sub-clinically endometritic (SCE) groups both 7 days and 21 days postpartum (DPP).

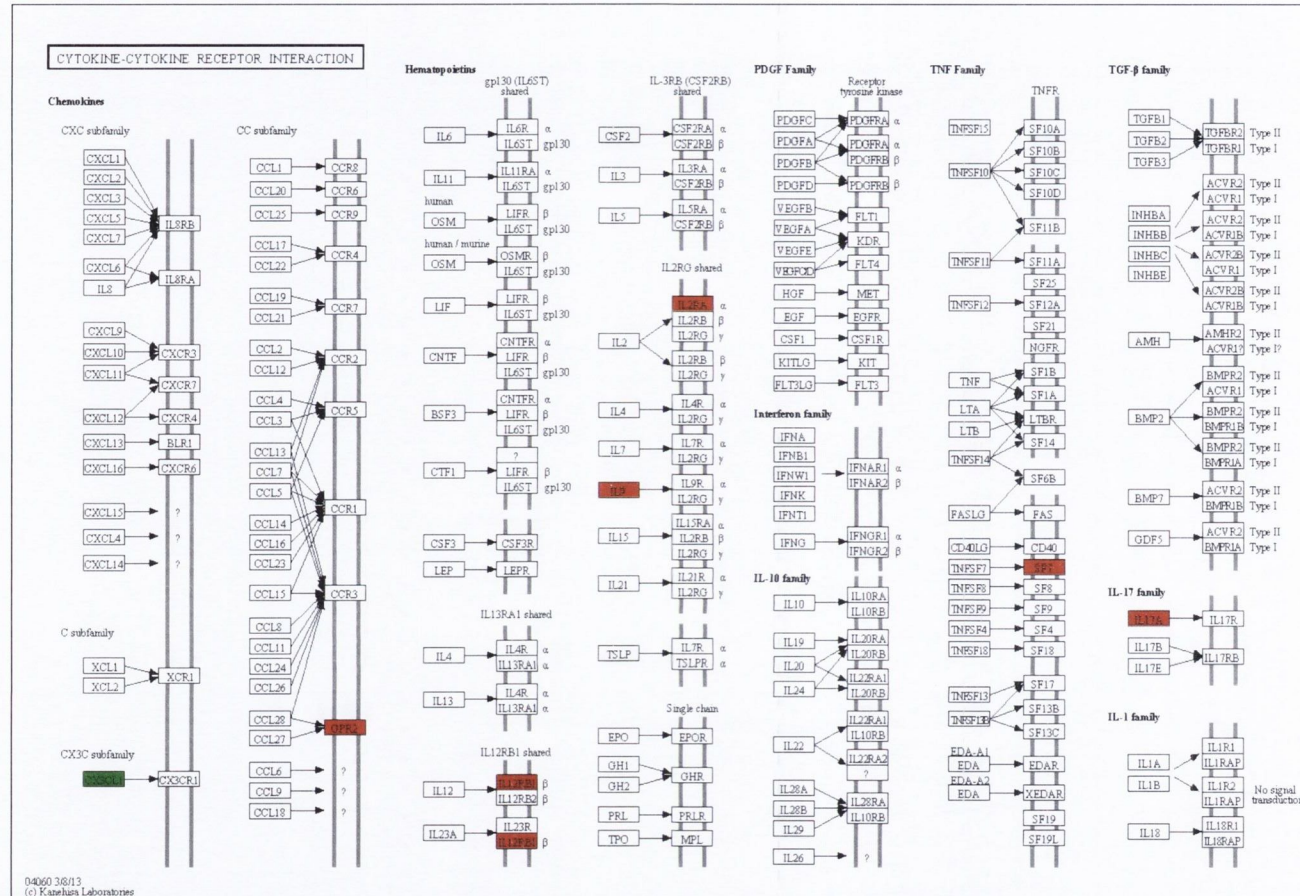


Figure 4.3-17: The Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Cytokine-Cytokine Receptor Interaction pathway enriched in the sub-clinically endometritic cows (SCE) 7 days postpartum (DPP). Genes within the pathway that are significantly elevated 7 DPP in the SCE group are red and in the healthy group are green.

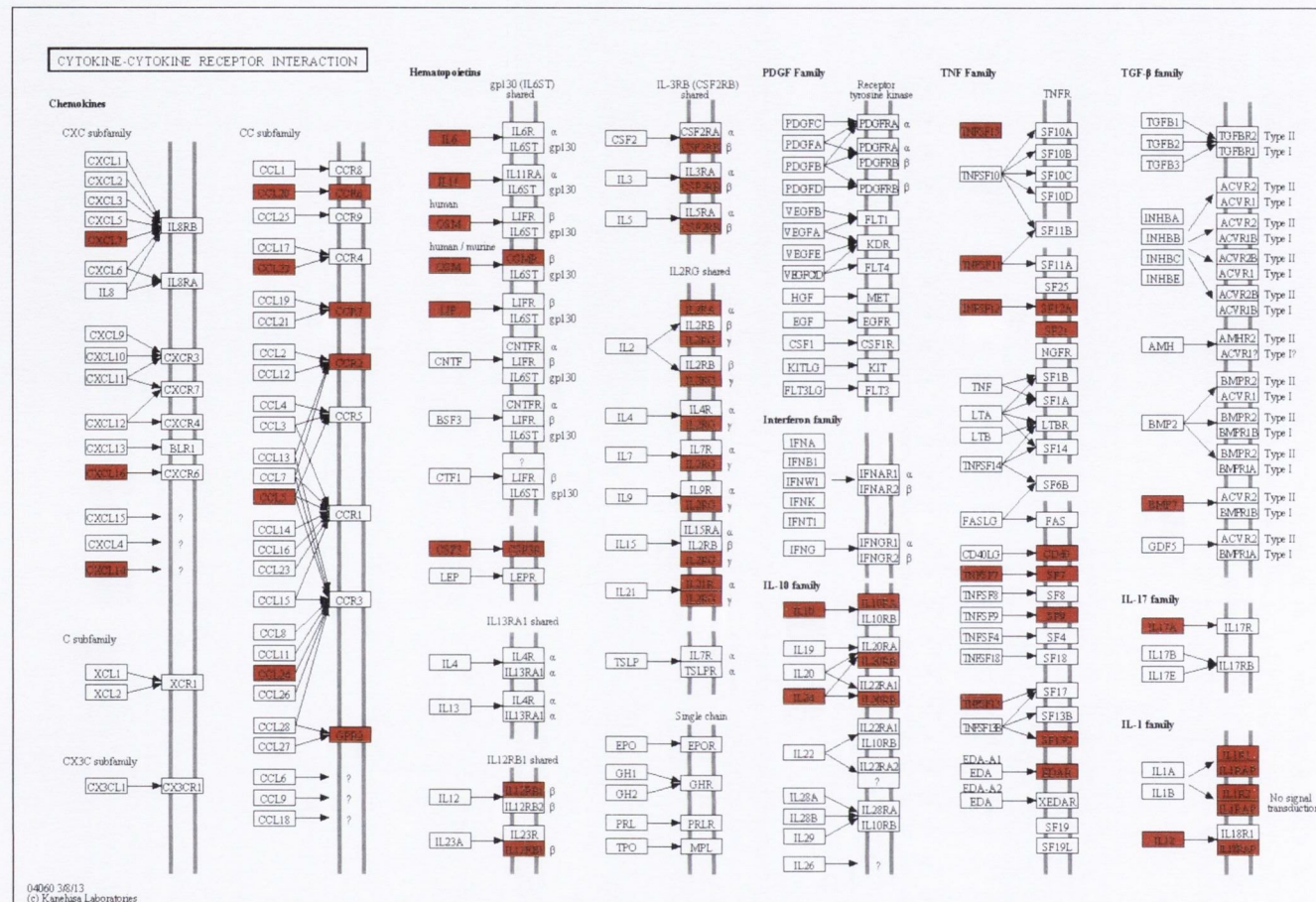


Figure 4.3-18: The Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotated Cytokine-Cytokine Receptor Interaction pathway enriched in the sub-clinically endometritic cows (SCE) 21 days postpartum (DPP). Genes within the pathway that are significantly elevated 21 DPP in the SCE group are red.

Table 4.3-26: Differentially elevated genes in sub-clinically endometritic (SCE) and healthy cows 7 days postpartum (DPP) within each enriched pathway.

SCE v Healthy			
Elevated in SCE 7 DPP		Elevated in Healthy 7 DPP	
Ensembl Gene ID	Gene Name	Ensembl Gene ID	Gene Name
Cytokine-cytokine receptor interaction		Retinol metabolism	
ENSBTAG000000011481	IL12RB1	ENSBTAG000000021118	CYP26A1
ENSBTAG000000002150	IL17	ENSBTAG000000010119	ALDH1A2
ENSBTAG000000020892	IL2RA	Pathways in cancer	
ENSBTAG000000014725	CD27	ENSBTAG000000021310	COL4A4
ENSBTAG000000018290	IL9	ENSBTAG000000005198	FGF1
ENSBTAG000000019095	CCR10	ENSBTAG000000000570	RET
ENSBTAG000000015298	TNFRSF13B	ENSBTAG000000021316	COL4A3
Primary immunodeficiency			
ENSBTAG000000001882	CD79A		
ENSBTAG000000021596	ICOS		
ENSBTAG000000015298	TNFRSF13B		
Intestinal immune network for IgA production			
ENSBTAG000000021596	ICOS		
ENSBTAG000000019095	CCR10		
ENSBTAG000000015298	TNFRSF13B		
alpha-Linolenic acid metabolism			
ENSBTAG000000039122	BT.84824		
ENSBTAG000000021924	PLA2G4E		
Linoleic acid metabolism			
ENSBTAG000000039122	BT.84824		
ENSBTAG000000021924	PLA2G4E		
Ether lipid metabolism			
ENSBTAG000000039122	BT.84824		
ENSBTAG000000021924	PLA2G4E		
Fat digestion and absorption			
ENSBTAG000000039122	BT.84824		
Vascular smooth muscle contraction			
ENSBTAG000000039122	BT.84824		
ENSBTAG000000002985	KCNMB1		
ENSBTAG000000021924	PLA2G4E		
Arachidonic acid metabolism			
ENSBTAG000000039122	BT.84824		
ENSBTAG000000021924	PLA2G4E		
Jak-STAT signalling pathway			
ENSBTAG000000011481	IL12RB1		
ENSBTAG000000020892	IL2RA		
ENSBTAG000000018290	IL9		

Table 4.3-27: Differentially elevated genes in sub-clinically endometritic (SCE) and healthy cows 21 days postpartum (DPP) within each enriched pathway.

SCE v Healthy			
Elevated in SCE 21 DPP		Elevated in Healthy 21 DPP	
Ensembl Gene ID	Gene Name	Ensembl Gene ID	Gene Name
Malaria		Dilated cardiomyopathy	
ENSBTAG00000007307	SELE	ENSBTAG00000001865	SGCG
ENSBTAG000000020736	BT.42498	ENSBTAG000000022000	CACNB2
ENSBTAG000000004630	BT.3034	ENSBTAG000000018777	ADCY5
ENSBTAG000000021462	CSF3	ENSBTAG000000011424	TPM2
ENSBTAG000000011154	SDC1	ENSBTAG000000022886	RYR2
ENSBTAG000000014921	IL6	ENSBTAG000000008248	DMD
ENSBTAG000000006685	IL10	Hypertrophic cardiomyopathy (HCM)	
ENSBTAG000000008008	TLR2	ENSBTAG00000001865	SGCG
ENSBTAG000000000277	IL18	ENSBTAG000000022000	CACNB2
ENSBTAG000000020755	SELP	ENSBTAG000000011424	TPM2
ENSBTAG000000003220	BT.27077	ENSBTAG000000022886	RYR2
ENSBTAG000000025471	TNF	ENSBTAG000000008248	DMD
Rheumatoid arthritis		ECM-receptor interaction	
ENSBTAG000000012082	BT.20111	ENSBTAG000000011706	TNR
ENSBTAG000000003313	BT.49346	ENSBTAG000000027074	SV2B
ENSBTAG0000000047400	IL11	ENSBTAG000000014575	COL4A5
ENSBTAG000000011481	IL12RB1	ENSBTAG000000013760	COL4A6
ENSBTAG000000016163	BT.45038	ENSBTAG000000012837	COL6A6
ENSBTAG000000009064	CSF2RB	Renin-angiotensin system	
ENSBTAG000000020736	BT.42498	ENSBTAG000000012393	AGT
ENSBTAG000000015362	BMP7	ENSBTAG000000016881	BT.27994
ENSBTAG000000017718	CCL22	Calcium signalling pathway	
ENSBTAG000000019567	IL21R	ENSBTAG000000012653	CAMK2B
ENSBTAG000000026275	BT.32408	ENSBTAG000000014059	ATP2B4
ENSBTAG000000021462	CSF3	ENSBTAG000000000520	ATP2B3
ENSBTAG000000005215	IL10RA	ENSBTAG000000007169	P2RX1
ENSBTAG000000002150	IL17	ENSBTAG000000026461	CACNA1H
ENSBTAG000000014496	BT.106551	ENSBTAG000000022886	RYR2
ENSBTAG000000021326	CCL20	ENSBTAG000000025642	RYR3
ENSBTAG000000006343	IL1R2	Vascular smooth muscle contraction	
ENSBTAG000000005273	IL1R1	ENSBTAG000000014609	BT.41853
ENSBTAG000000033748	IL18RAP	ENSBTAG000000007129	MRVI1
ENSBTAG000000013334	CSF3R	ENSBTAG000000002985	KCNMB1
ENSBTAG000000008924	TNFSF11	ENSBTAG000000018777	ADCY5
ENSBTAG000000013586	BT.63814	ENSBTAG000000011377	KCNMB3
ENSBTAG000000009752	CD70	Protein digestion and absorption	
ENSBTAG000000015133	CCR7	ENSBTAG000000020391	BT.29198
ENSBTAG000000007424	LIF	ENSBTAG000000014575	COL4A5

ENSBTAG00000020892	<i>IL2RA</i>	ENSBTAG00000011741	<i>COL13A1</i>
ENSBTAG00000019628	<i>EDAR</i>	ENSBTAG00000013760	<i>COL4A6</i>
ENSBTAG00000014921	<i>IL6</i>	ENSBTAG00000012837	<i>COL6A6</i>
ENSBTAG00000006685	<i>IL10</i>	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	
ENSBTAG00000014725	<i>CD27</i>	ENSBTAG00000001865	<i>SGCG</i>
ENSBTAG00000033107	<i>BT.103407</i>	ENSBTAG00000022000	<i>CACNB2</i>
ENSBTAG00000006694	<i>CXCL14</i>	ENSBTAG00000022886	<i>RYR2</i>
ENSBTAG00000020054	<i>TNFRSF21</i>	ENSBTAG00000008248	<i>DMD</i>
ENSBTAG00000007626	<i>IL2RG</i>	Salivary secretion	
ENSBTAG00000000277	<i>IL18</i>	ENSBTAG00000014059	<i>ATP2B4</i>
ENSBTAG00000026489	<i>TNFRSF13C</i>	ENSBTAG00000000520	<i>ATP2B3</i>
ENSBTAG00000007191	<i>CCL5</i>	ENSBTAG00000018777	<i>ADCY5</i>
ENSBTAG00000031998	<i>CXCL16</i>	ENSBTAG00000025642	<i>RYR3</i>
ENSBTAG00000000130	<i>TNFSF13</i>	Focal adhesion	
ENSBTAG00000007851	<i>IL24</i>	ENSBTAG00000014609	<i>BT.41853</i>
ENSBTAG00000032425	<i>PPBP</i>	ENSBTAG00000011706	<i>TNR</i>
ENSBTAG00000008299	<i>IL20RB</i>	ENSBTAG00000006253	<i>FLNC</i>
ENSBTAG00000018069	<i>TNFSF15</i>	ENSBTAG00000003667	<i>TLN2</i>
ENSBTAG00000019095	<i>CCR10</i>	ENSBTAG00000014575	<i>COL4A5</i>
ENSBTAG00000013205	<i>BT.28708</i>	ENSBTAG00000013760	<i>COL4A6</i>
ENSBTAG00000025471	<i>TNF</i>	ENSBTAG00000012837	<i>COL6A6</i>
ENSBTAG00000015298	<i>TNFRSF13B</i>		
ENSBTAG00000027407	<i>TNFRSF6B</i>		
Osteoclast differentiation			
ENSBTAG00000019250	<i>BTk</i>		
ENSBTAG00000007338	<i>TYROBP</i>		
ENSBTAG00000021709	<i>BT.58934</i>		
ENSBTAG00000021358	<i>BT.24520</i>		
ENSBTAG00000004826	<i>ACP5</i>		
ENSBTAG00000020848	<i>PIK3CG</i>		
ENSBTAG00000005273	<i>IL1R1</i>		
ENSBTAG00000008924	<i>TNFSF11</i>		
ENSBTAG00000008182	<i>FOSB</i>		
ENSBTAG00000005978	<i>PIK3R5</i>		
ENSBTAG00000010007	<i>MAPK13</i>		
ENSBTAG00000003305	<i>NCF1</i>		
ENSBTAG00000004767	<i>SYK</i>		
ENSBTAG00000004322	<i>FOS</i>		
ENSBTAG00000012046	<i>JUNB</i>		
ENSBTAG00000006194	<i>BT.17885</i>		
ENSBTAG00000008441	<i>SOC3</i>		
ENSBTAG00000002103	<i>PLCG2</i>		
ENSBTAG00000025471	<i>TNF</i>		

Amoebiasis			
ENSBTAG00000011975	<i>SERPINB1</i>		
ENSBTAG00000017473	<i>LAMC3</i>		
ENSBTAG00000027181	<i>LAMA3</i>		
ENSBTAG00000000793	<i>LAMC2</i>		
ENSBTAG00000001945	<i>BT.22399</i>		
ENSBTAG00000020848	<i>PIK3CG</i>		
ENSBTAG00000016149	<i>C9</i>		
ENSBTAG00000006343	<i>IL1R2</i>		
ENSBTAG00000005273	<i>IL1R1</i>		
ENSBTAG00000014921	<i>IL6</i>		
ENSBTAG00000006685	<i>IL10</i>		
ENSBTAG00000008008	<i>TLR2</i>		
ENSBTAG00000005978	<i>PIK3R5</i>		
ENSBTAG00000015874	<i>CASP3</i>		
ENSBTAG00000015032	<i>CD14</i>		
ENSBTAG00000016542	<i>LAMB3</i>		
ENSBTAG00000025471	<i>TNF</i>		
Hematopoietic cell lineage			
ENSBTAG00000013569	<i>CD38</i>		
ENSBTAG00000014764	<i>CD9</i>		
ENSBTAG00000011578	<i>CD44</i>		
ENSBTAG00000047400	<i>IL11</i>		
ENSBTAG00000004270	<i>FCER2</i>		
ENSBTAG00000020427	<i>DNTT</i>		
ENSBTAG00000021462	<i>CSF3</i>		
ENSBTAG00000006343	<i>IL1R2</i>		
ENSBTAG00000005273	<i>IL1R1</i>		
ENSBTAG00000013334	<i>CSF3R</i>		
ENSBTAG00000020892	<i>IL2RA</i>		
ENSBTAG00000014921	<i>IL6</i>		
ENSBTAG00000006452	<i>CD3D</i>		
ENSBTAG00000015032	<i>CD14</i>		
ENSBTAG00000025471	<i>TNF</i>		
Jak-STAT signalling pathway			
ENSBTAG00000047400	<i>IL11</i>		
ENSBTAG00000011481	<i>IL12RB1</i>		
ENSBTAG00000016163	<i>BT.45038</i>		
ENSBTAG00000009064	<i>CSF2RB</i>		
ENSBTAG00000019567	<i>IL21R</i>		
ENSBTAG00000020848	<i>PIK3CG</i>		
ENSBTAG00000021462	<i>CSF3</i>		
ENSBTAG00000005215	<i>IL10RA</i>		
ENSBTAG00000013334	<i>CSF3R</i>		

ENSBTAG00000007424	LIF		
ENSBTAG00000020892	IL2RA		
ENSBTAG00000014921	IL6		
ENSBTAG00000006685	IL10		
ENSBTAG00000000396	PIM1		
ENSBTAG00000005978	PIK3R5		
ENSBTAG00000033107	BT.103407		
ENSBTAG00000007626	IL2RG		
ENSBTAG00000007851	IL24		
ENSBTAG00000008299	IL20RB		
ENSBTAG00000008441	SOC3		
ENSBTAG00000017678	SPRED3		
Toll-like receptor signalling pathway			
ENSBTAG00000015718	CASP8		
ENSBTAG00000020736	BT.42498		
ENSBTAG00000020848	PIK3CG		
ENSBTAG00000013118	CD86		
ENSBTAG00000018059	BT.27986		
ENSBTAG00000016864	BT.24181		
ENSBTAG00000014921	IL6		
ENSBTAG00000008008	TLR2		
ENSBTAG00000005978	PIK3R5		
ENSBTAG00000008864	MD2		
ENSBTAG00000010007	MAPK13		
ENSBTAG00000007191	CCL5		
ENSBTAG00000004322	FOS		
ENSBTAG00000015032	CD14		
ENSBTAG00000025471	TNF		
Pathways in cancer			
ENSBTAG00000014400	E2F2		
ENSBTAG00000017473	LAMC3		
ENSBTAG00000027181	LAMA3		
ENSBTAG00000003012	TRAF1		
ENSBTAG00000000793	LAMC2		
ENSBTAG00000015718	CASP8		
ENSBTAG00000021709	BT.58934		
ENSBTAG00000007164	FGFR3		
ENSBTAG00000020935	HIF1A		
ENSBTAG00000020676	MMP9		
ENSBTAG00000020848	PIK3CG		
ENSBTAG00000003711	EPAS1		
ENSBTAG00000013334	CSF3R		
ENSBTAG00000008353	CDKN1A		
ENSBTAG00000011043	RAC2		

ENSBTAG00000008172	<i>EGLN3</i>		
ENSBTAG00000002341	<i>ETS1</i>		
ENSBTAG00000014921	<i>IL6</i>		
ENSBTAG00000010427	<i>BT.22323</i>		
ENSBTAG00000004013	<i>FGF7</i>		
ENSBTAG00000005978	<i>PIK3R5</i>		
ENSBTAG00000016412	<i>BT.45725</i>		
ENSBTAG00000039397	<i>BT.71501</i>		
ENSBTAG00000004742	<i>RUNX1</i>		
ENSBTAG00000022671	<i>WNT4</i>		
ENSBTAG00000000783	<i>TGFA</i>		
ENSBTAG00000015874	<i>CASP3</i>		
ENSBTAG00000003362	<i>HSP90B1</i>		
ENSBTAG00000004322	<i>FOS</i>		
ENSBTAG00000009532	<i>WNT7B</i>		
ENSBTAG00000016542	<i>LAMB3</i>		
ENSBTAG00000002103	<i>PLCG2</i>		
Staphylococcus aureus infection			
ENSBTAG00000031851	<i>SELPLG</i>		
ENSBTAG00000017280	<i>BT.19562</i>		
ENSBTAG00000006685	<i>IL10</i>		
ENSBTAG00000007450	<i>C2</i>		
ENSBTAG00000027051	<i>PTAFR</i>		
ENSBTAG00000020755	<i>SELP</i>		
ENSBTAG00000004840	<i>C15</i>		
ENSBTAG00000020872	<i>BT.34208</i>		
ENSBTAG00000034501	<i>BT.64790</i>		

Gene filtering between groups

The number of genes was filtered by increasing the stringency which defined whether a gene was significantly differentially expressed or not. The first level of filtering kept genes with a false discovery rate (FDR) of less than 0.1 and these genes were used in pathway analysis as above. The second level of stringency retained genes with an FDR < 0.05, 129 genes remained 7 DPP and 874 genes remained 21 DPP. These genes were then passed through the third filter which involved removing genes which had less than 10 read counts on average in 6 samples. The number of genes kept after the third filter was 98 at 7 DPP and 736 at 21 DPP. The fourth and final filtering criteria retained genes with a Log₂ fold change of greater than 2 and less than -2, resulting in 86 genes 7 DPP and 236 genes 21 DPP (Table 4.3-28).

Table 4.3-28: The number of significantly differentially expressed genes between sub-clinically endometritic (SCE) and healthy cows, 7 and 21 days postpartum (DPP).present after each stage of filtering

			1		2		3		4	
7DPP	SCE v Healthy	↑ SCE	182	147	129	107	98	77	86	72
		↑ Healthy		35		22		21		14
21DPP		↑ SCE	1219	991	874	751	736	631	236	205
		↑ Healthy		228		123		105		31

- 1 – genes with a false discovery rate (FDR) of less than 0.1.
2 – genes with a false discovery rate (FDR) of less than 0.05.
3 – genes with more than 10 read counts on average in 6 samples for the SCE group and 9 samples for the healthy group.
4 – genes with a Log₂ fold change of greater than 2 and less than -2.

The most highly differentially expressed genes which remained after the fourth filtering step were examined using heat maps and hierarchal clustering to determine if the differences between samples were similar to those observed previously using multi-dimensional scaling plots prior to gene filtering. The positions of genes and samples were reordered so that these independent variables were placed nearer to similar and further from different genes and samples. Dendograms illustrated how close or distant the relationship was between individual genes and samples. The rows were scaled based on z-scores, where red indicated lower number of read counts and green indicated a higher number of reads across all samples for each gene.

At both 7 and 21 DPP there was no distinct separation of SCE from healthy cows based on the clustering presented by either dendogram. 7 DPP there was a clustering of five healthy cows (cows 6, 9, 11, 13 and 14) to the left of the graph, however the remaining four healthy cows (4, 7, 10 and 12) also clustered with the SCE cows. It was more difficult to discern any patterns of

clustering 21 DPP however it was clear that cow 15 had a very different gene expression profile compared to the other cows 21 DPP (Figure 4.3-19).

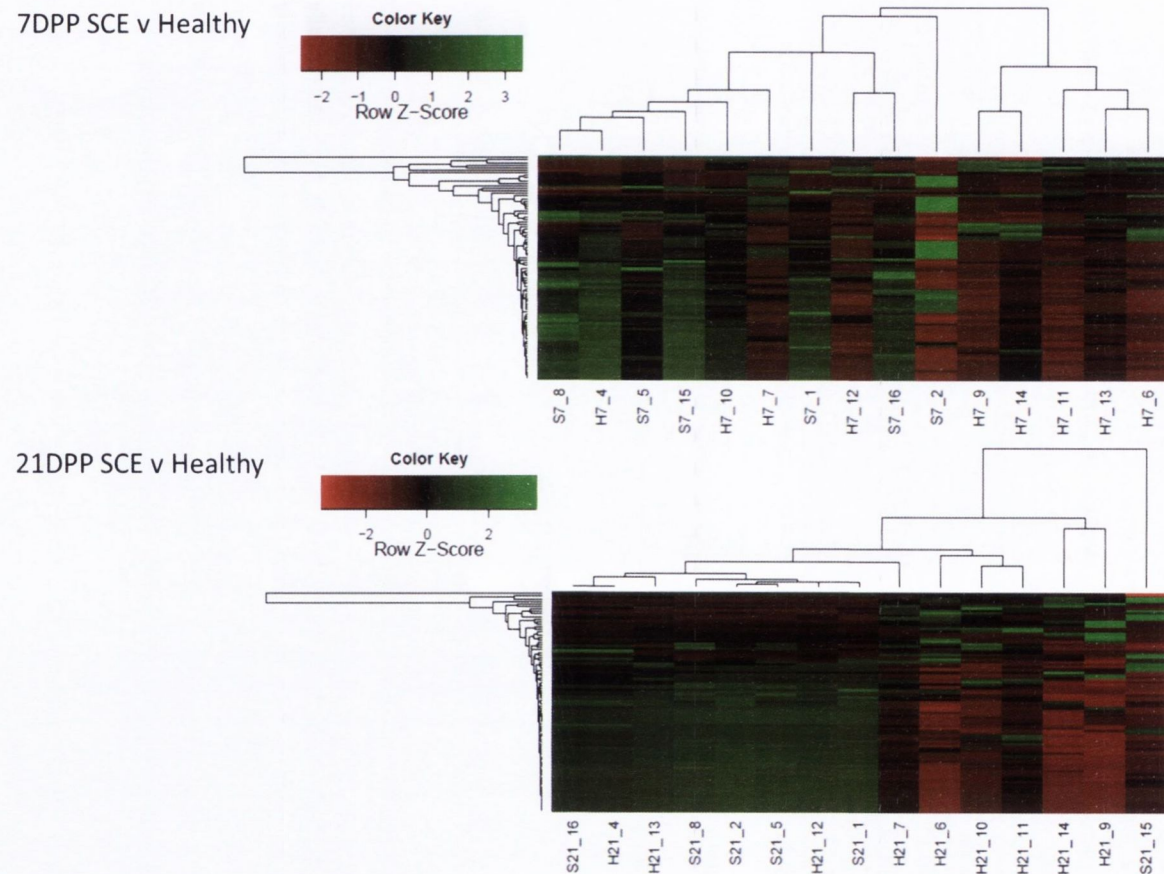


Figure 4.3-19: Heatmaps and hierarchical clustering of significant differentially expressed genes, comparing read counts in sub-clinically endometritic (SCE and S) and healthy (H) cows 7 and 21 days postpartum (DPP). The Z-score was used to visualise read counts lower or higher than the mean across all samples for a gene as red and green, respectively. Genes were significantly differentially expressed with a FDR <0.05 and Log₂ fold change of greater than 2 and less than -2.

The number of genes significantly differentially expressed between groups was greater 21 DPP compared to 7 DPP at each level of stringency imposed. There were a greater number of genes significantly differentially elevated in the SCE group compared to the healthy group and this trend was observed at both time points and at each stage of filtering.

A further assessment was carried out to determine what genes were commonly and uniquely significantly differentially expressed in the SCE and healthy groups 7 and 21 DPP. In total there were 27 genes common both 7 and 21 DPP, 59 uniquely differentially expressed 7 DPP and 209 uniquely differentially expressed 21 DPP. Genes that were significantly differentially elevated in the SCE group both 7 and 21 DPP were compared, 26 genes were common both 7 and 21 DPP, 46 were uniquely elevated 7 DPP and 179 genes were uniquely elevated 21 DPP. Significant differentially elevated genes in the healthy group both 7 and 21 DPP were also compared, 1 gene was common to both groups, 13 genes were uniquely elevated 7 DPP and 30 genes were uniquely elevated 21 DPP (Figure 4.3-20).

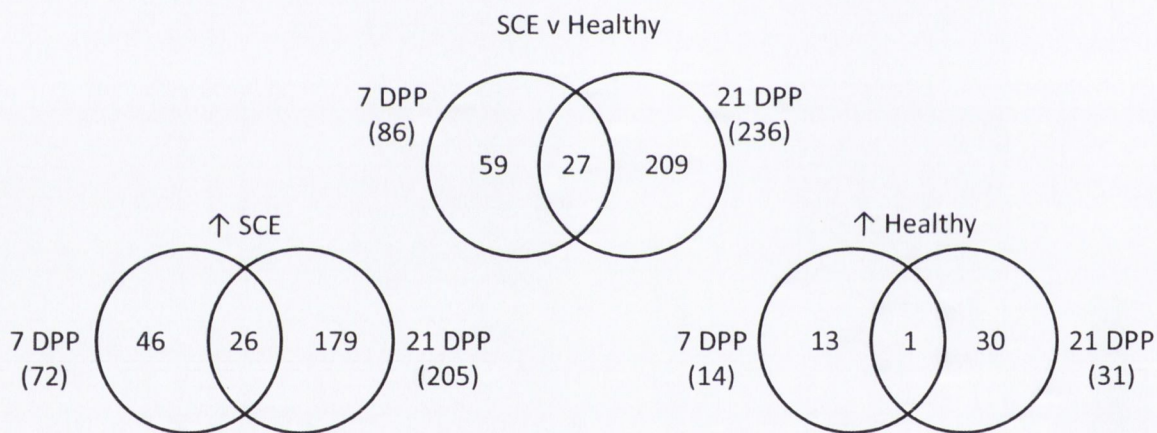


Figure 4.3-20: Venn diagrams representing the number of significantly differentially expressed genes that are common and unique 7 and 21 days postpartum (DPP) in sub-clinically endometritic (SCE) and healthy groups. Genes were significantly differentially expressed with a FDR <0.05 and Log₂ fold change of greater than 2 and less than -2.

Genes that were significantly differentially elevated both 7 and 21 DPP in the SCE group, of which there were 26, were used in the selection of potential biomarkers for subclinical endometritis.

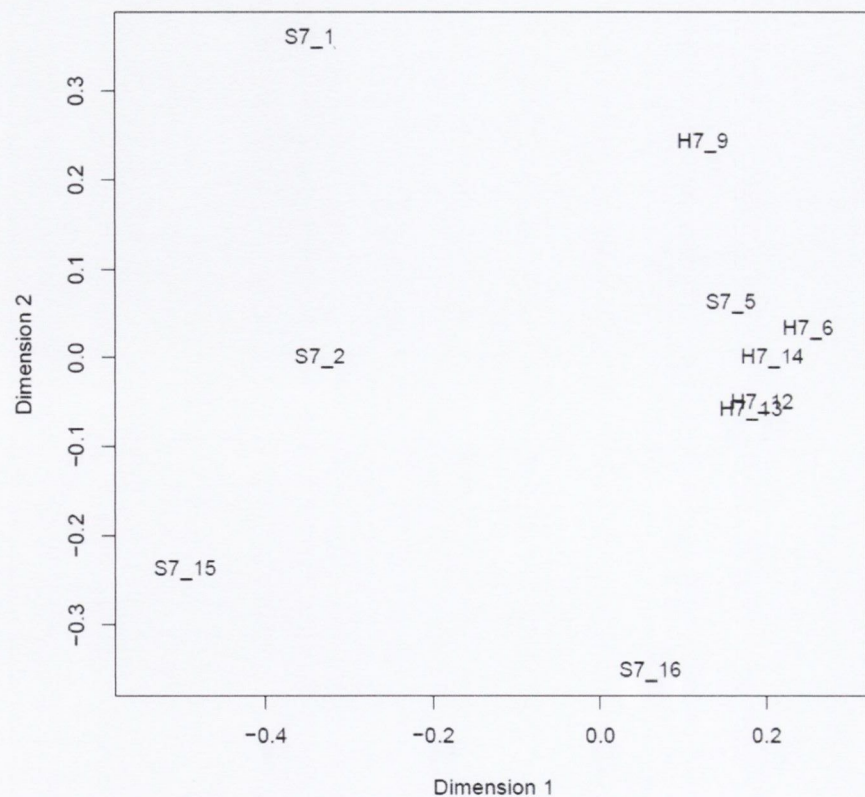
4.3.10 (B) MicroRNA-Seq expression between groups, 7 and 21 days postpartum

Differences in microRNA transcriptional profiles between sub-clinically endometritic (n=5) and healthy cows (n=5) were analysed 7 and 21 DPP and compared between SCE and healthy groups. The software program EdgeR was used to uncover the most highly differently expressed genes between groups. A multi-dimensional scaling (MDS) plot was created using the software program EdgeR which displayed how different samples were from each other.

On close inspection of the MDS plots 7 DPP and 21 DPP there was not a clear separation of samples from each other. 7 DPP the healthy cows appear to cluster more tightly together than the SCE cows did, however within this cluster there cow 5 from the SCE groups is closer to the healthy cows than cow 9 from the healthy group. 21 DPP no apparent clustering occurs (Figure 4.3-21).

7DPP SCE v Healthy

MDS Plot for Data



21DPP SCE v Healthy

MDS Plot for Data

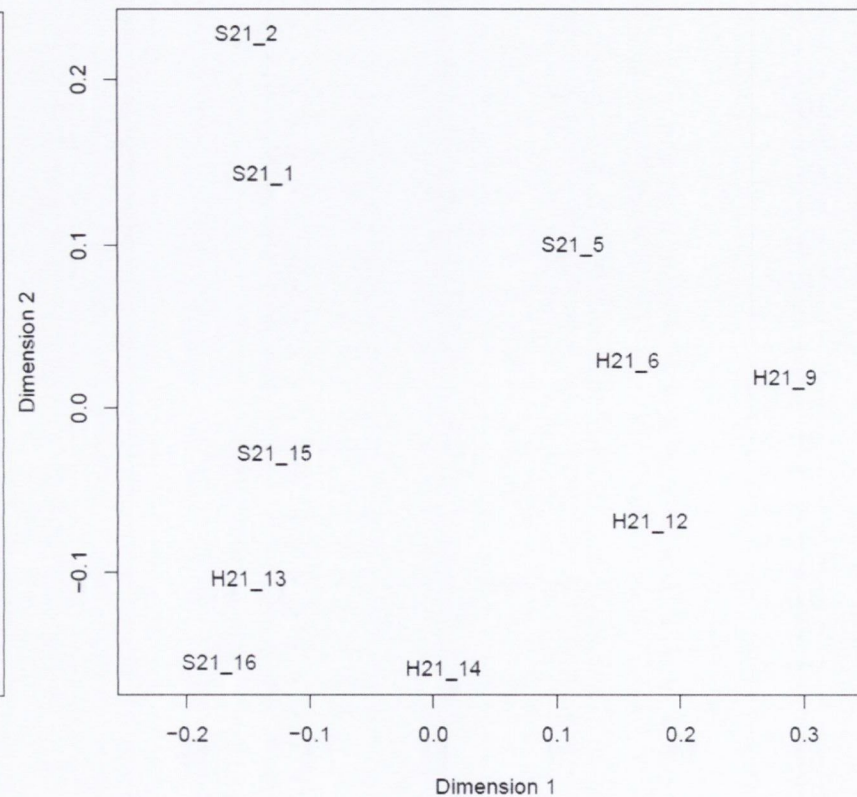


Figure 4.3-21: Multi-dimensional scaling (MDS) plots produced with EdgeR comparing healthy (H) and sub-clinically endometritic (S) samples 7 and 21 days postpartum (DPP).

Genes were significantly differentially expressed with a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1. The number of genes significantly differentially expressed between the SCE and healthy groups 7 DPP was 3, of these 3 were elevated in the SCE group and none were up in the healthy group, and no microRNA were differentially expressed 21 DPP between groups (Table 4.3-29 and Table 4.3-30).

Table 4.3-29: The number of genes significantly differentially elevated in sub-clinically endometritic (SCE) and healthy cows 7 and 21 days postpartum (DPP). Genes were significantly differentially elevated (↑) with a false discovery rate (FDR) (or adjusted *p*-value) of less than 0.1.

			Gene Numbers	
7DPP	SCE v Healthy	↑ SCE	3	3
		↑ Healthy		0
21DPP		↑ SCE	0	0
		↑ Healthy		0

Table 4.3-30: microRNA genes uniquely elevated (↑) in the sub-clinically endometritic (SCE) relative to healthy cows 7 DPP. False discovery rate (FDR) (or adjusted *p*-value) of less than 0.1.

↑ SCE	
7 DPP	
Ensembl ID	microRNA Name
ENSBTAG000000029854	<i>bta-mir-205</i>
ENSBTAG000000029763	<i>bta-mir-375</i>
ENSBTAG000000029921	<i>bta-mir-197</i>

4.3.10.1 Cross over between mRNA-Seq and microRNA-Seq gene expression data

In mRNA-Seq gene expression analysis it is common to find a variety of gene biotypes that significantly differentially expressed in addition to protein coding genes such as processed pseudogenes, pseudogenes, rRNAs, snoRNAs, snRNAs and microRNAs within the data set. This was the case with the present data set and of particular interest to this study were the microRNAs that were expressed in the mRNA-Seq data set and also microRNAs that were common to both mRNA-Seq and microRNA-Seq data sets.

The biotypes for all genes significantly expressed with a false discovery rate of < 0.1 were attained for the mRNA-Seq data. Genes differentially elevated in the healthy group both 7 and 21 DPP did not have any microRNA biotypes. 7 DPP there were 18 differentially elevated genes in the SCE groups and 4 differentially elevated genes 21 DPP in the SCE group which had a microRNA biotype. These genes were then compared to the microRNA-Seq data set and no microRNAs found in common (Table 4.3-31).

Table 4.3-31: microRNAs significantly differentially elevated in the mRNA-Seq data set in sub-clinically endometritic (SCE) and healthy cows 7 and 21 days postpartum (DPP). Genes were significantly elevated (↑) with a False discovery rate <0.1.

SCE v Healthy							
7 DPP				21 DPP			
↑ Healthy		↑ SCE		↑ Healthy		↑ SCE	
Ensembl Gene ID	microRNA Name	Ensembl Gene ID	microRNA Name	Ensembl Gene ID	microRNA Name	Ensembl Gene ID	microRNA Name
		ENSBTAG000000045494	<i>bta-mir-2904-3</i>			ENSBTAG000000029897	<i>bta-mir-21</i>
		ENSBTAG000000045632	<i>bta-mir-2904-3</i>			ENSBTAG000000036418	<i>bta-mir-223</i>
		ENSBTAG000000045657	<i>bta-mir-2904-2</i>			ENSBTAG000000045836	
		ENSBTAG000000045710	<i>RN28S1</i>			ENSBTAG000000045926	
		ENSBTAG000000045836					
		ENSBTAG000000045965	<i>bta-mir-2904-3</i>				
		ENSBTAG000000046351	<i>RN28S1</i>				
		ENSBTAG000000046537	<i>bta-mir-2904-3</i>				
		ENSBTAG000000046695					
		ENSBTAG000000047042	<i>bta-mir-2904-3</i>				
		ENSBTAG000000047046	<i>RN28S1</i>				
		ENSBTAG000000047154	<i>RN28S1</i>				
		ENSBTAG000000047381	<i>bta-mir-2904-3</i>				
		ENSBTAG000000047501	<i>bta-mir-2887-2</i>				
		ENSBTAG000000047663	<i>bta-mir-2887-2</i>				
		ENSBTAG000000047852	<i>bta-mir-2904-3</i>				
		ENSBTAG000000048000	<i>bta-mir-2904-3</i>				
		ENSBTAG000000048191	<i>bta-mir-2887-1</i>				

4.3.11 Candidate genes selected as potential biomarkers

Candidate genes expressed locally in the endometrium that differentiated sub-clinical endometritic from healthy cows and the protein products of which could potentially be detected systemically in blood plasma, were selected. The candidate genes that differentiated SCE and healthy cows were (a) 5 uniquely expressed in the SCE group between 7 and 21 DPP, and (b) 26 elevated in the SCE group compared to the healthy group both 7 and 21 DPP (Figure 4.3-22).

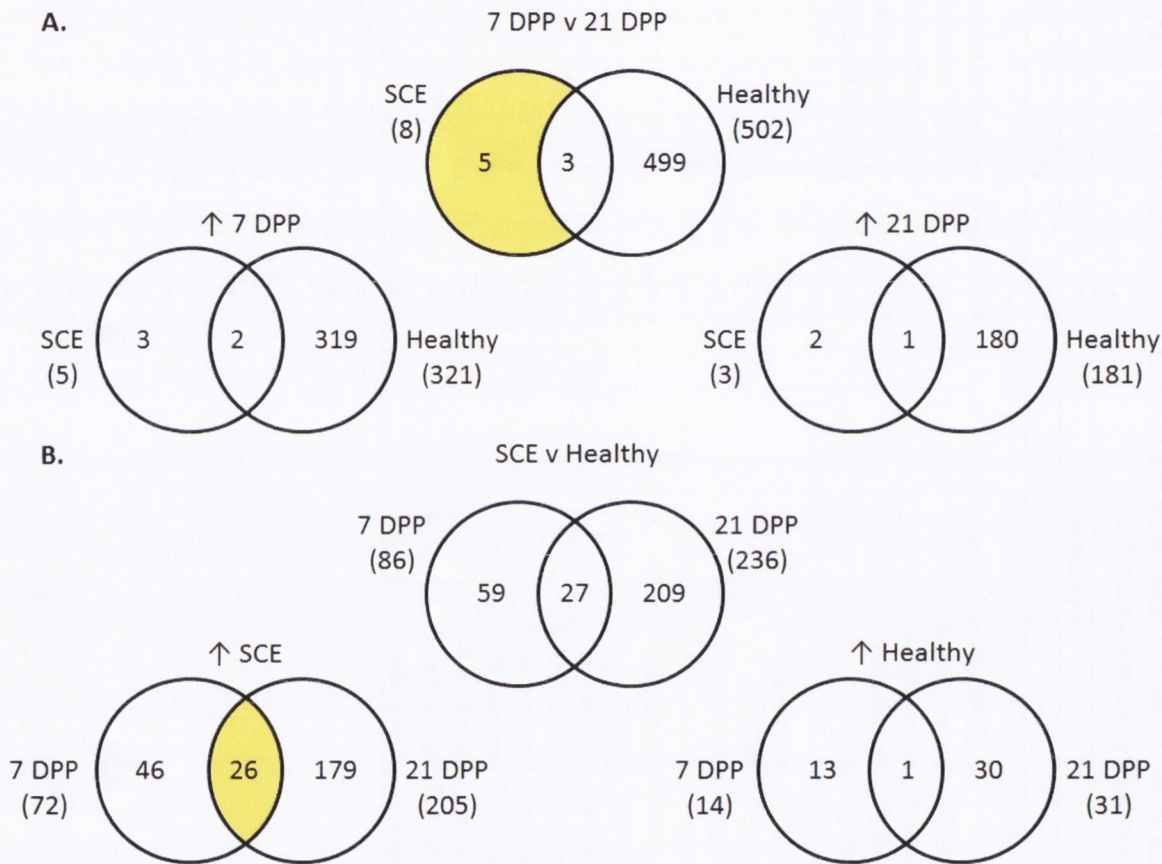


Figure 4.3-22: Venn diagrams displaying the number of candidate genes significantly differentiating sub-clinically endometritic (SCE) from healthy cows (A) uniquely expressed in the SCE group between 7 and 21 days postpartum (DPP) and (B) uniquely elevated in the SCE group compared to the healthy group both 7 and 21 DPP. Genes were significantly differentially expressed with a FDR <0.05 and Log₂ fold change of greater than 2 and less than -2. Number of candidate genes are highlighted in yellow.

These genes were then assessed to uncover which had a signalling peptide and if they did which of these peptides were excreted to the extracellular space and potentially into the circulatory system. Of the 31 candidate genes 17 had signalling peptides, two of these were secreted to the extracellular space and therefore IL17 and IL17F were selected as potential biomarkers for subclinical endometritis.

Table 4.3-32: Candidate genes that significantly differentiated sub-clinically endometritic (SCE) from healthy cows either elevated both 7 and 21 days postpartum (DPP) or uniquely in SCE cows and not in healthy cows when comparing 7 and 21 DPP in either group. This table is sorted based on whether the protein products of these genes contained signalling peptides and were potentially secreted to the extracellular space. Genes were significantly differentially expressed with a false discovery rate (FDR) <0.05 and Log₂ fold change of greater than 2 and less than -2. The log₂ fold change (FC), *p* value and FDR for genes with elevated expression (↑) SCE cows 7 and 21 DPP.

SCE v Healthy				↑ in SCE cows at 7 DPP			↑ in SCE cows at 21 DPP		
Up at both 7 and 21 DPP	Associated Gene Name	Signal Peptide	GO Term Name	log ₂ FC	<i>P</i> Value	FDR	log ₂ FC	<i>P</i> Value	FDR
ENSBTAG00000016835	<i>IL17F</i>	Signal peptide	extracellular space	2.8	2.6E-04	5.0E-02	3.2	2.5E-08	1.0E-05
ENSBTAG00000002150	<i>IL17</i>	Signal peptide	extracellular space	3.4	1.8E-04	4.0E-02	3.1	1.7E-06	2.6E-04
ENSBTAG00000046076		Signal peptide		2.2	6.4E-06	4.3E-03	2.1	2.7E-10	2.5E-07
ENSBTAG00000047529	<i>VL1X</i>	Signal peptide		2.3	6.1E-06	4.2E-03	2.0	5.1E-08	1.7E-05
ENSBTAG00000000720	<i>CTSL1</i>	Signal peptide		2.3	2.3E-04	4.6E-02	2.6	1.4E-08	7.1E-06
ENSBTAG00000014466	<i>VTCN1</i>	Signal peptide		2.3	1.4E-04	3.4E-02	2.8	4.9E-05	3.6E-03
ENSBTAG00000014725	<i>CD27</i>	Signal peptide		2.7	4.9E-06	4.0E-03	2.4	2.4E-08	1.0E-05
ENSBTAG00000047986		Signal peptide		2.7	4.3E-06	3.7E-03	2.9	4.3E-13	1.8E-09
ENSBTAG00000048030	<i>BT.90712</i>	Signal peptide		2.7	2.3E-05	1.1E-02	3.5	1.1E-14	8.9E-11
ENSBTAG00000021057	<i>TMEM190</i>	Signal peptide		2.8	5.4E-06	4.2E-03	2.1	4.4E-06	5.7E-04
ENSBTAG00000047121		Signal peptide		2.9	7.7E-06	4.8E-03	2.7	3.6E-11	5.6E-08
ENSBTAG00000038337	<i>PACAP</i>	Signal peptide		3.0	5.8E-06	4.2E-03	3.1	5.0E-11	6.9E-08
ENSBTAG00000001882	<i>CD79A</i>	Signal peptide		3.1	4.6E-07	6.0E-04	2.4	4.3E-07	9.1E-05
ENSBTAG00000044204	<i>CD79B</i>	Signal peptide		3.3	6.1E-06	4.2E-03	2.7	1.5E-06	2.5E-04
ENSBTAG00000040394	<i>PRSS27</i>	Signal peptide		4.5	1.8E-08	1.2E-04	3.4	6.7E-06	7.9E-04
ENSBTAG00000002240	<i>GPR18</i>			2.3	4.0E-05	1.5E-02	2.5	6.3E-08	2.0E-05
ENSBTAG00000048135	<i>BT.97040</i>			2.4	3.5E-05	1.4E-02	2.2	3.0E-06	4.2E-04
ENSBTAG00000046509	<i>FAM46C</i>			2.4	1.3E-06	1.3E-03	2.1	2.2E-07	5.5E-05
ENSBTAG00000018224	<i>GLP1R</i>			2.6	3.9E-05	1.5E-02	2.9	3.5E-06	4.6E-04

ENSBTAG00000046010	<i>BHLHA15</i>			2.6	1.5E-05	8.3E-03	2.6	1.1E-06	1.9E-04
ENSBTAG00000019390	<i>CA12</i>			2.7	1.6E-05	8.8E-03	2.9	4.2E-07	9.1E-05
ENSBTAG00000002929	<i>IRF4</i>			2.9	5.6E-06	4.2E-03	2.3	8.7E-05	5.5E-03
ENSBTAG00000005128	<i>VPREB3</i>			3.3	1.1E-04	2.9E-02	3.4	4.8E-10	4.1E-07
ENSBTAG00000046544	<i>BT.97125</i>			3.5	2.3E-07	4.1E-04	2.1	6.0E-06	7.4E-04
ENSBTAG00000006282	<i>POU2AF1</i>			3.7	1.0E-07	2.3E-04	2.9	9.1E-08	2.7E-05
ENSBTAG00000039967	<i>KRT78</i>			4.1	2.2E-06	2.1E-03	4.4	4.3E-07	9.1E-05
7 DPP v 21 DPP				↑ 7 DPP in SCE cows			↑ 21 DPP in SCE cows		
Uniquely up in SCE	Associated Gene Name	Signal Peptide	GO Term Name	logFC	PValue	FDR	logFC	PValue	FDR
ENSBTAG00000033248	<i>CDH3</i>	Signal peptide		2.6	3.7E-05	3.4E-02			
ENSBTAG00000039046	<i>MGC127538</i>	Signal peptide		2.0	4.1E-05	3.4E-02			
ENSBTAG00000021685	<i>EEF1A2</i>			3.6	2.3E-05	3.2E-02			
ENSBTAG00000046322							2.4	1.8E-05	3.1E-02
ENSBTAG00000047586	<i>NPY1R</i>						2.5	1.3E-05	2.6E-02

4.4 Discussion

4.4.1 A high relative density of leukocyte cells in endometrial histology sections identifies cows with sub-clinical endometritis.

Inflammation is a physiological event in the postpartum endometrium of all cows due to an influx of contaminating bacteria and intrinsic tissue damage as a result of parturition. The majority of cows resolve this inflammation transiting towards endometrial repair, restoring functioning capacity of the uterus to be receptive to and supportive of a new conceptus.

Bovine endometritis is defined as endometrial inflammation from 21 days postpartum (DPP) and is manifested either both clinically, with mucoid vaginal discharge, and sub-clinically or exclusively at a sub-clinical level. Both of these conditions have been linked with an increased susceptibility of sub-fertility in postpartum cows. Sub-clinical endometritis is detected using either endometrial cytology smears or histology sections based on the number of leukocyte cells present in particular polymorphonuclear (PMN) cells such as neutrophils. There are numerous examples in the literature defining different criteria specific for the diagnosis of sub-clinical endometritis (Barlund *et al.* 2008; Gabler *et al.* 2010; Gilbert *et al.* 2005; Kasimanickam *et al.* 2005; Santos *et al.* 2009; Sheldon & Dobson 2004). Without a clear standardised method to definitively diagnose sub-clinical endometritis histology was used to examine the degree of relative endometrial inflammation in this study. On the basis of recent publications equal weight was attributed to the density of leukocytes in the stroma and neutrophils in the epithelium of endometrial sections (Chapwanya *et al.* 2010; Meira *et al.* 2012).

Initially endometrial biopsies both 7 days and 21 DPP were assessed however substantial epithelial loss was observed in the majority of sections 7 DPP creating difficulties grading inflammation. Six cows out of fifteen had the highest density of leukocyte cells, within the epithelium and stroma of endometrial histology sections 21 DPP. Four of these six cows also displayed symptoms of clinical endometritis therefore cows with a high relative density of leukocyte cells within the epithelium and stroma, of endometrial biopsies, have a high risk of developing sub-clinical endometritis. Although some animals had both sub-clinical and clinical endometritis, both conditions involve inflammation of the endometrium and only differ in relation to clinical appearance (Fischer *et al.* 2010).

4.4.2 Microbial populations significantly differentiate sub-clinically endometritic and healthy cows

Following an uncomplicated normal parturition consistent bacterial clearance and recontamination within the first two weeks postpartum in the absence of clinical signs of puerperal metritis occurs and spontaneously clears 10 to 15 days postpartum (Azawi 2008; Sheldon & Dobson 2004; Singh *et al.* 2008). A previous study by our group confirmed a bacterial presence in all animals sampled 15 DPP which reduced by half 30 DPP (Chapwanya *et al.* 2012). An elevated bacterial load 7 days and 21 DPP resulted in slower growth of the first postpartum dominant follicle and delayed ovulation which was suggested to be associated with sub-fertility (Sheldon *et al.* 2002). Bacterial species have been isolated by culture dependant methods from the uterus of clinically endometritic cows which included: *Arcanobacterium pyogenes* (*A. pyogenes*), *Escherichia coli* (*E. coli*) and different gram negative (GN) obligate anaerobic bacteria: *Fusobacterium necrophrum* (*F. necrophrum*), *Prevotella* spp. and *Bacteroides* spp. (Földi *et al.* 2006; Huszeniczal *et al.* 1999; Zerbe *et al.* 2001). *A. pyogenes* contributes to clinical and reproductive consequences of uterine disease either alone or in combination with other bacteria such as *E. coli* and GN obligate anaerobes. The enhancement of uterine disease severity is achieved by the synergistic action of *A. pyogenes* and; *F. necrophrum*, *Bacteroides* spp. and *Prevotella* spp. (Földi *et al.* 2006; Sheldon & Dobson 2004; Williams *et al.* 2005). Cultured aerobic and anaerobic bacteria known to be associated with pathogenicity in the postpartum uterus have been categorised into three different categories “(1) bacteria that frequently cause endometritis, (2) bacteria that were an infrequent cause of endometritis, and (3) bacteria not recognised as uterine pathogens” (Sheldon *et al.* 2004).

Although numerous studies have identified specific bacterial species associated with endometritic uterine environments, the majority used culture dependent techniques. By using culture-independent techniques to characterise bacterial diversity of the uterus, may provide additional information that has not yet been uncovered due to the limitation of growing specific bacteria with culture dependent techniques. Santos *et al.* (2011) performed a culture-independent evaluation of the uterine microbial populations, achieving a metagenomic profile of bacteria in the uteri of both healthy and metritic postpartum cows. Five major phylum of bacteria were defined: Gammaproteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, and Tenericutes. In the metritic cows Fusobacteria, Bacteroidetes, and Proteobacteria (particularly Gammaproteobacteria) were classified. Interestingly, using culture-independent methods

sequences for *A. pyogenes* and *E. coli* were not observed in either healthy or metritic cows (Santos *et al.* 2011).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a culture independent technique which assesses the DNA sequence encoding the 16S rRNA gene to differentiate populations of bacteria in a uterine swab for example. In the current study uterine swabs taken at 7 and 21 DPP were analysed with T-RFLP in both sub-clinical endometritic and healthy cows to determine relative changes in these populations. It is evident based on the results of this study that there are differential microbial populations within the SCE uterus that are either present or absent in the healthy uterus early postpartum. The extent of sub-clinical inflammation graded in this study may be due to the presence of specific microbial populations causing such inflammation or the lack of other microbial populations preventing such inflammation. However it is also clear that not all sub-clinical inflammation in this study can be attributed directly with the presence or absence of specific microbial populations as some SCE samples cluster closely with healthy samples which may indicate that the host response may in fact be causing and driving sustained endometrial inflammation early postpartum in some cows.

4.4.3 Temporal transcriptomic changes in the endometrium of healthy cows contrasts with sub-clinically endometritic cows

RNA-Seq analysis of the bovine endometrial transcriptome was used to understand the molecular mechanisms and signalling pathways controlling the regulation of early postpartum bacterial clearance and local inflammation. The temporal endometrial transcriptomic profile was defined by differential gene expression between 7 days and 21 days postpartum (DPP) within either the healthy or sub-clinically endometritic (SCE) group. The magnitude of the global transcriptomic change over time in either group of cows was captured by the numbers of differentially expressed genes and whether these genes were elevated 7 days or 21 DPP. In healthy cows a larger number of genes were differentially expressed (4068) compared to SCE cows (95). Due to the low number of genes differentially expressed in SCE cows, distinct biological and functional differences, highlighted with pathway analysis, may not be clearly discernible. Therefore assessing differentially expressed genes that distinguish SCE from healthy cows at each time point would be more informative, which will be addressed in later sections of this discussion.

In healthy cows there were vast differences in biological and functional activities in the endometrium 7 DPP compared to 21 DPP. Inflammation was enriched 7 days and by 21 DPP a distinct transition towards greater regulation of calcium signalling was evident which is essential

in eliciting and regulating a number of cellular functions including, cell proliferation and differentiation, axonal guidance and cell migration, cell death, gene expression and many more.

4.4.3.1 A transition towards regulation of calcium signalling from inflammation in the healthy postpartum endometrium

Calcium signalling pathways are one of the predominant pathways activated 21 DPP relative to 7 DPP in healthy cows postpartum. The genes significantly differentially expressed in this pathway included: *ATP2A1*, *ATP2A3*, *PLN*, *SLC8A1*, *SLC8A2*, *CHRM3*, *PLCB1*, *RYR2*, *RYR3*, *CALM*, *CALM6*, *ATP2B1*, *ATP2B2*, *ATP2B3*, *ATP2B4*, *PRKCB*, *CACNA1S*, *CACNA1D*, *CACNB1*, *CNB2*, *ADCY5*, *ADCY8*, *PLA2G3*, *PLA2G2F*, *PLA2G4E* and *PLA2G4D*. Calcium signalling involves regulating cytosolic concentrations of calcium in various cellular locations which is critical to many cellular processes and the efficiency of a cell's response to stimuli. When cytosolic calcium increases above basal levels and reaches an optimum concentration for a particular cellular response a signalling cascade ensues, resulting in the activation of transcription factors initiating gene expression for example. However prior to possessing the ability to increase calcium concentration and subsequently respond to this calcium signal the appropriate infrastructure must be in place to maintain basal levels of cytosolic calcium by methods such as compartmentalisation or expulsion of calcium from the cell. The plasma membrane and either the endoplasmic or sarcoplasmic reticulum act as barriers to allow compartmentalisation of calcium and different channels such as voltage-gated Ca^{2+} and store/receptor-operated Ca^{2+} channels, allow either an influx or efflux of calcium (Karlstad *et al.* 2012).

Store-operated Ca^{2+} entry (SOCE) is the main pathway of Ca^{2+} entry in electrically non-excitabile cells whereby intracellular Ca^{2+} store depletion activates Ca^{2+} influx and is essential in regulating cell exocytosis, enzyme control, gene regulation, proliferation and cell growth, and central to eukaryotic cell physiology. SOCE relies on multiple channels and proteins within the cell to relay signals from the endoplasmic reticulum (ER) to Ca^{2+} channels on the cell membrane to allow extracellular Ca^{2+} entry to the cytoplasm in order to refill low ER Ca^{2+} stores. Additionally SOCE may also elicit a Ca^{2+} response through a G protein-coupled receptor (GPCR) on the plasma membrane by agonist binding, inducing a signal transduction cascade which results in the efflux of Ca^{2+} from the ER, thus elevating cytosolic Ca^{2+} concentration (Karlstad *et al.* 2012; Parekh & Putney 2005).

In this study the differential expression of genes associated with the infrastructure required for SOCE are elevated 21 days compared to 7 DPP in healthy cows. Sarcoendoplasmic reticular Ca^{2+}

ATPase (SERCA pumps) are located on the endoplasmic reticulum and pump Ca^{2+} into the endoplasmic reticulum from the cytosol expending ATP^2 in the process. In addition to SERCA pumps the ER membrane also contains an ion exchanger known as $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) which allows the exchange of one Ca^{2+} ion into the ER at the same time as three Na^+ ions leave the ER. NCX function compliments that of a SERCA pump as both channels can direct Ca^{2+} into the ER from the cytoplasm (Fameli *et al.* 2007; Wang *et al.* 2002). The elevation of the gene encoding SERCA pumps *ATP2A1* and *ATP2A3* and NCXs *SLC8A1* and *SLC8A2* in healthy cows 21 DPP suggests that cell homeostasis has returned to a greater extent as cells possess an enhanced ability to compartmentalise Ca^{2+} in the ER prior to or after an influx of calcium to the cytosol. In its unphosphorylated state the phospholamban (PLN) protein inhibits SERCA function however once phosphorylated its inhibitory actions are removed (Gorski *et al.* 2013). This protein is encoded by the gene *PLN* and although its subsequent actions post-translation are beyond the scope of this study its elevated gene expression suggests there is potentially greater regulation of SOCE 21 days compared to 7 DPP in healthy cows which is not observed in SCE cows.

21 DPP healthy endometrial tissue may have the infrastructure in place to facilitate an elevation in cytosolic concentrations of calcium by releasing it from the ER in response to an extracellular stimulus, as genes involved in this process (*CHRM3* and *PLCB1* (phospholipase C beta 1)) are elevated 21 days relative to 7 DPP. ER stores of calcium can also be released due to signal transduction initiated by an agonist binding to a GPCR on the plasma membrane which hydrolyses receptor subunits thus activating phospholipase C (PLC) to cleave phosphatidylinositol 4, 5 bisphosphate (PIP2) into diacylglycerol (DAG) and 1, 4, 5 inositol triphosphate (IP_3). ER receptors known as 1, 4, 5 inositol triphosphate receptors (IP_3Rs) change their conformation once IP_3 binds which allows the release of Ca^{2+} into the cytosol (Karlstad *et al.* 2012). The *CHRM3* gene encodes the protein better known as the muscarinic acetylcholine receptor M3 which is a muscarinic acetylcholine receptor. It has been shown that activation of the muscarinic acetylcholine receptor M3 induces the release of Ca^{2+} from IP_3 -sensitive Ca^{2+} stores increasing intracellular Ca^{2+} concentrations (Fohrman *et al.* 1993; Wojcik 2011). Another receptor on the ER, that involves release of Ca^{2+} into the cytosol, is the ryanodine receptor (RYP). There are a number of molecules that regulate RYPs either directly or indirectly, such as the $\text{CaV}1.1/1.2$ channels, protein kinase A (PKA), FK506 binding proteins (FKBP12 and 12.6), calmodulin (CaM) and Ca_2p /calmodulin-dependent protein kinase II (CaMKII). In particular calmodulin when bound to calcium will inhibit RYPs releasing Ca^{2+} from the ER and when unbound calmodulin is a partial agonist of RYP allowing Ca^{2+} release from the ER (Lanner *et al.* 2010). The results of our study indicate elevated expression of genes encoding RYP (*RYP2* and *RYP3*), $\text{CaV}1.1$ (*CACNA1S*) and calmodulin (*CALM* and *CALM6*)

proteins in healthy cows 21 days compared to 7 DPP highlighting an increased ability to regulate calcium release from the ER.

Regulating physiological concentrations of cytosolic calcium maintains cell homeostasis which involves the extrusion of Ca^{2+} from the cytosol when levels rise above basal levels. A number of genes involved in the maintenance of cell homeostasis by the extrusion of Ca^{2+} from the cytosol are elevated 21 days compared to 7 DPP in healthy cows. The plasma membrane has multiple channels that allow calcium entry and two systems for extrusion of Ca^{2+} which are the $\text{Na}^+ / \text{Ca}^{2+}$ exchanger (NCX) and the plasma membrane Ca^{2+} -ATPase (PMCA). As mentioned earlier the NCX can also allow Ca^{2+} entry however this occurs when the cytosolic or SR/ER concentrations of Na^+ rise from basal levels. The PMCA pump has poor affinity for calcium when cytosolic concentrations are at basal levels. Mechanisms that directly increase Ca^{2+} affinity and activate the PMCA pump include calmodulin binding and protein kinase C (PKC) phosphorylation of the carboxy-terminal tail. However depending on the isoform of PMCA present PKC may also inhibit or have no effect (Brini & Carafoli 2011; Di Leva *et al.* 2008). The elevated expression of genes encoding PMCAs (*ATP2B1*, *ATP2B2*, *ATP2B3* and *ATP2B4*), calmodulin (*CALM* and *CALM6*) and PKC (*PRKCB*) was evident 21 days compared to 7 DPP in healthy cows.

Calcium influx through the plasma membrane is also permitted by voltage-gated/operated/dependent calcium channels which are complexes containing several subunits (α_1 , $\alpha_2\delta$, β_{1-4} and γ), the subunit α_1 is divided into subfamilies such as the Ca_v1 family which are channels that conduct L-type Ca^{2+} currents. The β subunit is tightly bound to the α_1 subunit in the cytosol and regulates the modulation of Ca_v1 channels by G proteins and protein kinases. Once depolarisation of the plasma membrane occurs, voltage-gated Ca^{2+} channels permit the influx of extracellular Ca^{2+} to the cytosol (Buraei & Yang 2010; Fodor *et al.* 2013). The genes encoding proteins for the Ca_v1 family α_1 subunits, 1.1 and 1.3 (*CACNA1S* and *CACNA1D*), and β subunits, 1 and 2 (*CACNB1* and *CACNB2*), are elevated in healthy cows 21 days compared to 7 DPP. This once again highlights the capacity of the healthy endometrium by 21 DPP to facilitate calcium influx and the potential to increase cytosolic concentrations of calcium.

The concentration of cytosolic calcium is determined by a balance between reactions that allow entry into the cytosol, through the plasma membrane or from the ER/SR, and reactions that remove calcium from the cytosol to the extracellular space or ER/SR. The elevation of cytosolic calcium results in a proportion of calcium binding to effectors responsible for the stimulation of multiple Ca^{2+} dependant processes which may take microseconds, in the case of synaptic junction exocytosis, or minutes to hours to induce events such as cell proliferation or gene transcription.

“Ca²⁺ induced Ca²⁺ signalling remodelling” is an interesting concept whereby calcium itself activates the transcription of genes involved in calcium signalling. Transcription factors can be indirectly activated by an increase of cytosolic calcium concentrations to initiate gene transcription (Berridge *et al.* 2003). Calcium activated adenylyl cyclases (AC) produce 3', 5'-adenosine monophosphate (cAMP) which activates the transcription factor cAMP response element-binding protein (CREB) through a signalling cascade promoting gene transcription. In our data set the gene encoding the enzyme adenylyl cyclase 8 (AC8), *ADCY8* was elevated 21 days compared to 7 DPP in healthy cows. The activation of AC8 requires the binding of Ca²⁺ bound to calmodulin to produce cAMP. However the activity of another adenylyl cyclase AC5 is inhibited by calcium yet is activated by protein kinase C (PKC). This mechanism of action could be interpreted as indirect calcium activation of AC5 as PKC itself is activated by Ca²⁺ and DAG (Halls & Cooper 2011; Lipp & Reither 2011). In healthy cows 21 DPP the expression of genes encoding the proteins AC5 (*ADCY5*) and PKC (*PRKCB*) are elevated compared to 7 DPP.

It is evident from the examples discussed above that in cows with a healthy postpartum endometrium an enhanced potential to regulate cytosolic calcium concentration was observed 21 days compared to 7 DPP. This transient change in the endometrial gene expression signature is not independently replicated at the same postpartum time points in SCE cows emphasising the necessity to regulate cytosolic calcium concentrations through calcium signalling pathways in the absence of inflammation.

4.4.3.2 Elevated calcium signalling may increase the potential of healthy endometria to produce luteolytic prostaglandin F_{2α} (PGF) 21 DPP

The uterus is the predominant producer of prostaglandin F_{2α} (PGF) which permits the regression of the corpus luteum (luteolysis) in the absence of an embryo at the end of the estrus cycle thus allowing a subsequent estrus cycle to progress. PGF production and secretion from the endometrium is elevated in response to oxytocin receptor activation which elicits a calcium signalling cascade, elevating cytosolic calcium concentrations (Asselin *et al.* 1996; Burns *et al.* 1998). Important components that link this change in calcium concentration and PGF synthesis, Phospholipase A2 (PLA₂) and prostaglandin F_{2α} synthase (PGFS) enzymes (Leslie 2004; Madore *et al.* 2003) are both elevated in healthy cows 21 days compared to 7 DPP in this study.

The liberation of arachidonic acid (AA) from cellular membranes is a key step in the synthesis of all prostaglandins and is orchestrated by PLA₂. The elevation of cytosolic Ca²⁺ concentration influences the intracellular location of PLA₂ as it contains an N-terminal C2 domain which binds

Ca²⁺ promoting its translocation to endoplasmic reticulum, golgi or nuclear membrane. Under high cytosolic concentrations of Ca²⁺, PLA₂ docks in the membrane and actively releases AA by the interaction of its catalytic domain and a phospholipid substrate in the membrane (Leslie 2004). Previous studies in bovine endometrial cells have highlighted the role of various calcium signalling molecules and elevated Ca²⁺ cytosolic concentrations in the synthesis of PGF. There have been numerous calcium signalling molecules proposed as mediators in the synthesis of PGF which include PLC, PKC, PIP₂ DAG and IP₃ (Burns *et al.* 1998; Krishnaswamy *et al.* 2010).

In the current study calcium signalling genes encoding PLA₂ (*PLA2G3*, *PLA2G2F*, *PLA2G4E* and *PLA2G4D*), PLC (*PLCB1*), PKC (*PRKCB*) and the gene encoding the prostaglandin F_{2α} synthase (PGFS) enzyme *FAM213B* were elevated 21 days compared to 7 DPP in healthy cows. The facilitation of calcium signalling and potential to produce PGF by healthy cows was not observed in SCE cows when comparing the same postpartum time points. As PGF is a vital component of the estrus cycle, a greater capacity to resolve endometrial inflammation by 21 DPP may enhance a cow's ability to elevate the production and secretion of PGF earlier, permitting normal cyclic progression compared to a cow experiencing sustained endometrial inflammation 21 DPP.

4.4.3.3 Elevated gene expression of Toll-like receptors and related downstream signalling molecules in the endometrium of healthy cows early postpartum enhances recognition and response to pathogens

Toll-like receptors (TLRs) are fundamental to the early innate immune response of the bovine endometrium as they detect pathogen associated molecular patterns (PAMPs) on bacteria and/or viruses, (Fu *et al.* 2013). In the current study elevated gene expression of *TLR2*, *TLR4*, *TLR5*, *TLR6* and *TLR7* was observed 7 days compared to 21 DPP in healthy cows. Four of these TLRs (2, 4, 5 and 6) are activated by different bacteria ligands and are present at the cell surface on the cell membrane, TLR4 recognises lipopolysaccharide (LPS) generally in gram-negative bacteria, TLR2 and 6 are stimulated by lipoteichoic acid in gram-positive bacteria and TLR5 is activated by flagellin present in some bacteria. However TLR7 is located on endosomal membranes and recognises single stranded viral RNA (Akira & Takeda 2004; Fu *et al.* 2013).

Upon recognition of a PAMP the TLR transmits a signal from the cellular membrane through a series of signalling molecules in the cytosol to the nucleus and promotes the transcription of various inflammatory effectors that directly and/or indirectly counter the bacterial challenge (Akira & Takeda 2004; Fu *et al.* 2013). The majority of TLRs enhance their ligand-recognition repertoire by dimerising with other TLRs such as TLR2/6 dimers which are required to respond to

peptidoglycan. Recognition of LPS involves an initial attachment of LPS-binding protein (LBP) which facilitates the binding of LPS with CD14 and once formed LBP dissociates. Subsequently TLR4 must interact with an accessory protein known as MD2 (or LY96) before it can form a heterodimer with the LPS-CD14 complex and transfer the signal of recognition from the extracellular to intracellular space (Werling *et al.* 2009). Our results indicate that endometria of healthy cows are capable of recognising the bacterial PAMP LPS to a greater extent at 7 days relative to 21 DPP as genes encoding proteins essential to this process, *LBP*, *MD2*, *CD14* and *TLR4*, are differentially elevated.

Adapter proteins assist in transmitting TLR signals from the membrane to the cytosol and specific selection of adapter proteins results in differential downstream signalling (O'Neill & Bowie 2007). The TLRs differentially expressed in the current study, TLR 2, 4, 5, 6 and 7, once stimulated recruit and transmit their signal to the adapter protein known as myeloid differentiation antigen 88 (MyD88) through the MyD88-dependent pathway (Akira & Takeda 2004; Fu *et al.* 2013). The initial recruitment of TLR adapter proteins provides a focus point from which a signal is transmitted to downstream signalling components such as IL-1 receptor associated kinases (IRAKs), TAK1-binding proteins (TABs), TGF- β activated kinase (TAK1) and TNF receptor-associated factor 6 (TRAF6). Subsequent to TLR stimulation, the activation of the transcription factor NF- κ B is facilitated by IRAKs, a family of serine/threonine kinases of which there are four IRAK1, IRAK2, IRAK4, and IRAK-M (Kawai & Akira 2006). IRAK1 is generally recruited by MyD88 and IRAK4 then activates it by phosphorylation (Li *et al.* 2010). This interaction is facilitated to a greater extent 7 days compared to 21 DPP in the endometrium of healthy cows as the expression of genes *IRAK1* and *MYD88* are elevated in the current study. The downstream effects of this interaction involving TRAF6 recruitment, stimulation of the I κ B kinase (IKK) complex and inhibition of the degradation of I κ B (κ B), permits NF- κ B release into the nucleus. NF- κ B induces the expression of a variety of cytokine genes and is therefore a key regulator of inflammatory immune responses, in addition to this NF- κ B can also enhance the expression of genes associated with cell proliferation or survival (Li *et al.* 2010). Therefore the potential to recognise a PAMP, transmit this recognition from the cellular membrane to the nucleus instigating the transcription of genes involved in inflammation and cell proliferation is greater in 7 days compared to 21 in healthy cows and not evident in SCE cows comparing the same postpartum time points.

In addition to facilitating TLR signalling healthy cows are also regulating TLR signalling more 7 days compared to 21 DPP as the gene expression of numerous phosphoinositide 3-kinases (PI3Ks), *PIK3CA*, *PIK3CB* and *PIK3CD*, is elevated. PI3K activation is dependent upon TLR and/or MyD88

stimulation and acts as a negative feedback regulator of TLR signalling as its activation has been shown to selectively suppress pro-inflammatory responses and enhance anti-inflammatory responses. Molecules of the PI3K-Akt pathway are components of TLR signalling and key regulators of cell proliferation and survival (Hazeki *et al.* 2007; Li *et al.* 2010). Although the innate immune response in the early postpartum cow has been characterised as a pro-inflammatory environment (Chapwanya *et al.* 2009; Davies *et al.* 2008; Herath, Lilly, Santos, *et al.* 2009; Martins *et al.* 2011) the results of the current study suggest that there is also an element of regulation which may allow expansion of immune cells required to fight infection yet simultaneously integrating pro-inflammatory responses with endometrial tissue repair. As the expression of PI3K genes is not enhanced in SCE cows 7 days relative to 21 DPP the physiological negative feedback regulating TLR signalling may not be occurring, thus leading to the sustained inflammation identified histologically as greater immune cell infiltrate in the endometrium of SCE cows.

It is evident from the results discussed that healthy cows have a greater potential to recognise and respond to an invading bacterial challenge 7 DPP which is regulated and then reduced by 21 DPP. However this physiological capability appears to either be absent or sustained when comparing gene expression at both time points in SCE cows 7 and 21 DPP.

4.4.3.4 Cytokines differentially elevated early postpartum in the endometrium of healthy cows activate and regulate the innate immune response

Pro-inflammatory cytokines that will be discussed in this section were differentially elevated 7 days compared to 21 DPP in healthy cows suggesting that a pro-inflammatory immune response early postpartum is significantly resolved by 21 DPP. In the bovine endometrium the production of cytokines such as TNF and IL1B and chemokine IL8 is triggered in response to TLR4 activation (Singh *et al.* 2008; Cronin *et al.* 2012). Tumour necrosis factor α (TNF) is a key mediator of the acute inflammatory response to gram-negative bacteria and the prominent source of TNF production is from mononuclear phagocytes, it is also produced from other cells to a lesser extent. TNF functions to recruit neutrophils and monocytes to the site of infection by increasing the expression of endothelial adhesion molecules, stimulating cytokine secretion from endothelial cells and chemokine secretion from macrophages and activating neutrophils and macrophages to initiate their microbicidal functions (Abbas *et al.* 2007; Sedgwick *et al.* 2000).

In a mixed population of bovine endometrial cells stimulated with the bacterial PAMP – LPS, an initial rise of *TNF* gene expression was probably responsible for inducing the subsequent rise in *IL1B* (Swangchan-Uthai *et al.* 2012). The expression of *TNF* and *IL1B* are also expressed

immediately after LPS stimulation in bovine endometrial epithelial cells (Chapwanya *et al.* 2013). In addition to TNF production and secretion by immune cells, TNF is also produced and secreted by epithelial, glandular epithelial and endothelial cells of the endometrium throughout the estrous cycle to regulate uterine function (Okuda *et al.* 2010). In the current study increased gene expression of both *TNF* and *IL1B* contribute to the overall inflammatory profile of the endometrium 7 days relative to 21 DPP in healthy cows.

TNF exerts its effects through its receptors TNFR1 and TNFR2. The cellular context and microenvironment regulate TNFR1 activation which can induce proliferative processes, necroptosis or apoptosis. TNFR1 is activated by both soluble TNF (sTNF) and membrane bound TNF (mTNF) whereas TNFR2 is only activated by mTNF. TNFR2 has been implicated in the inflammatory immune response, T cell survival, angiogenesis, cell migration and proliferation and it is considered a biomarker for some tumours and various inflammatory diseases. Although TNFR2 does not contain the death domain that can facilitate cell death as TNFR1 does, it can under specific circumstances cause cell death (Cabal-Hierro & Lazo 2012; Wang *et al.* 1998). In the current study TNFR2 gene expression (*TNFRSF1B*) and the expression of signalling molecules involved in this signalling pathway *TRAF1* and *TRAF3* were elevated 7 days relative to 21 DPP in healthy cows which may suggest that endometrial cells are potentially avoiding apoptosis in order to perform more inflammatory or proliferative functions early postpartum in response to TNF.

IL-1 is a mediator of the host's inflammatory immune response to infections and is produced primarily by mononuclear phagocytes, it also produced by macrophages, epithelial cells and endothelial cells. There are two forms of IL-1 namely IL-1 α , which is active in either a caspase-1 cleaved or non-cleaved form, and IL-1 β which is active in only its cleaved form. Low concentrations of IL-1 mediate local inflammation by stimulating increased expression of surface molecules associated with leukocyte adhesion. High concentrations of IL-1 influence the systemic inflammatory response to induce fever, APP synthesis in the liver, stimulation of IL-6 and the production of neutrophil and platelet in the bone marrow (Abbas *et al.* 2007).

During parturition levels of IL-1 in serum are elevated enhancing vasodilation which allows increased leukocyte migration to the endometrial tissue. Plasma calcium concentration is increased by IL-1 which stimulates contractions in the myometrium thus removing debris from the uterus. IL-1 stimulates synthesis of prostaglandin which improves contraction and evacuation of the uterus (Abbas *et al.* 2007; Kim *et al.* 2005). *IL1B* gene expression in healthy cows decreased over time postpartum, this change over time was not observed in endometritic cows (Galvão *et al.* 2011). Consistent with these results observed by Galvão *et al.*, in the current study both *IL1A* and

IL1B are differentially elevated 7 days compared to 21 DPP in healthy cows and this change over time was not observed in SCE cows.

IL-1 α and IL-1 β bind to IL-1 receptor type I (IL-1R1) which has a toll/interleukin-1 receptor (TIR) domain, which is also present in TLRs, that facilitates an intracellular signalling cascade resulting in the activation of NF- κ B once IL-1 α and IL-1 β are bound. IL-1 receptor II (IL-1R2) also binds IL-1 α and IL-1 β however it lacks the TIR domain and does not transmit an intracellular signal acting as a decoy and therefore buffering the effect of IL-1 α and IL-1 β (Contassot *et al.* 2012). In addition to the elevated expression of IL1A and IL1B, IL1R1 and IL1R2 were also differentially increased 7 days compared to 21 DPP in healthy cows. The ability to respond to IL-1 α and IL-1 β and simultaneously buffer the effects of these molecules was enhanced in healthy cows 7 DPP suggesting a degree of innate immune regulation at this time may be beneficial to allow greater resolution of inflammation by 21 DPP.

4.4.3.5 A bacterial presence and elevation of IL-17C may regulate the expression of the S100 family proteins early postpartum in the endometrium of healthy cows

In the current study of the bovine endometrium early postpartum the genes encoding the S100 family of proteins S100A8, S100A9 and S100A12 are elevated 7 days relative to 21 DPP in healthy cows. As these S100 family members have antimicrobial ability, healthy cows appear to require their expression early postpartum which is then reduced later postpartum.

A subset of T helper cells namely T_H17 cells, produce cytokines such as IL17-A, IL17-F and IL-22 which enhance the innate immune defences of leucocytes, epithelial cells, vascular endothelial cells, keratinocytes and fibroblasts in response to extracellular bacterial infection (Kolls *et al.* 2008; Kolls & Khader 2010). In addition to IL-17A and IL-17F, the IL-17 family of cytokines also include IL-17B, IL-17C, IL-17D and IL-17E (IL-25) which stimulates heterodimeric complexes of IL-17 receptors, IL-17RA to IL-17RE to elicit the biological effects of IL-17 cytokines (Iwakura *et al.* 2011).

A bacterial presence with agonists for TLR2 (lipopeptides) and TLR5 (flagellin) and the cytokines TNF and IL-1 β elevate IL-17C production and gene expression in epithelial cells. IL-17C stimulates the IL-17RA and IL-17RE receptors on epithelial cells to elevate the expression of genes associated with innate immunity such as cytokines, chemokines and antimicrobial peptides (AMPs) (Ramirez *et al.* 2011). IL-17C elevated the expression of S100A7A, S100A8 and S100A9 which are AMPs and part of the S100 family proteins involved in cellular processes such as cell growth and

differentiation, calcium-dependent cell signalling, cell cycle regulation and antimicrobial defence (Abtin *et al* 2010, Ramirez *et al* 2011).

Of the S100 family proteins microbicidal activity has been functionally characterised for S100A7c (psoriasin), a heterodimeric complex of S100A8 (calgranulin A) and S100A9 (calgranulin B) known as the calprotectin complex, S100A12 (calgranulin C), and S100A15 (Abtin *et al.* 2010). The gene expression of S100A8 and S100A9 is elevated in the presence of flagellin and dependant on TLR5 signalling (Abtin *et al.* 2010), in parallel with this IL-17C is upregulated by TLR5 recognition of flagellin and IL-17C which itself elevates the gene expression of S100A8 and S100A9 in human epithelial cells (Ramirez-Carrozzi *et al.* 2011). In bovine endometrial epithelial and stromal cells, gene expression of S100A8, S100A9 and S100A12 was elevated in response to LPS (Swangchan-Uthai *et al.* 2012).

The mechanism that results in the elevation of these genes cannot be precisely identified at present, yet due to the elevation of TLR5, IL-17C and IL-17RA, 7 days relative to 21 DPP, it could be speculated that the presence of bacterial flagellin or LPS could be stimulating the production of S100A8, S100A9 and S100A12. Other members of the S100 family of proteins are also elevated 7 days relative to 21 DPP in healthy cows, which include S100A2, S100A3, S100A5, S100A13 and S100A14 which may also be involved in regulating key cellular processes early postpartum.

4.4.3.6 Potential for prostaglandin E₂ production in the pro-inflammatory endometrial environment of healthy cows early postpartum

In addition to the physiological regulation of prostaglandin production in the bovine endometrium there is also regulation under pathological conditions (Godkin *et al.* 2008; MacKintosh *et al.* 2013; Tithof *et al.* 2007). It has been documented that the presence of the bacterial pathogen associated molecular pattern (PAMP), lipopolysaccharide (LPS), there is a switch from PGF production to prostaglandin E₂ (PGE) production (Herath, Lilly, Fischer, *et al.* 2009). PGE is a luteotrophic molecule which is associated with prolonged luteal phases contributing to sub-fertility (Opsomer *et al.* 2000; Williams *et al.* 2007). Although a recent study did show an increase in endometrial production of PGE and PGF in response to LPS treatment, the ratio of PGE to PGF remained the same (Swangchan-Uthai *et al.* 2012) which conflicts to a degree with the study mentioned previously (Herath, Lilly, Fischer, *et al.* 2009) yet jointly both studies highlight bacterial regulation of PGF and PGE production in the bovine endometrium. In the current study the gene encoding the enzyme prostaglandin E synthase, responsible for the final step in PGE production, *PTGES* is elevated 7 DPP in healthy cows relative to 21 DPP. This is consistent with the hypothesis

that an elevated bacterial load or specific population 7 days relative to 21 DPP may result in greater production of PGE. Once the inflammatory immune response has reduced and or removed this bacterial load or specific species the synthesis of PGF increases allowing regression the corpus luteum (CL) progressing towards the next estrus cycle.

In addition to its immune-regulatory role, TNF also is implicated in the regulation of PGF and PGE production in the bovine endometrium (Miyamoto *et al.* 2000; Murakami *et al.* 2001) and has the ability to both shorten and prolong the bovine estrus cycle through its regulation of PGF and PGE (Skarzynski *et al.* 2003; Skarzynski *et al.* 2000). At low concentrations in the uterus TNF has the ability to stimulate PGF production associated with luteolysis and at high concentrations TNF stimulates endometrial production of PGE which is lyteotrophic (Skarzynski *et al.* 2003). Seven days postpartum the gene expression of *TNF*, *PTGS2 (COX2)*, *PGES1*, *PTGER2* is elevated in the endometrium of healthy cows with respect to the level of expression 21 DPP of these genes. Therefore the potential to facilitate PGE synthesis in response to TNF is greater 7 days relative to 21 DPP in healthy cows which not observed in SCE cows over time.

An elevated IL-1 cytokine-cytokine receptor presence in the bovine endometrium early postpartum may also enhance the facilitation of PG production. In particular IL1A was shown to induce PGE production by elevating the gene expression of *PTGS2 (COX2)* and *PGES1* and their proteins in bovine endometrial stromal cells (Tanikawa *et al.* 2008) and IL-1A is a more potent inducer than IL-1B of PGE and PGF in bovine endometrial epithelial and stromal cells (Tanikawa *et al.* 2009). PGE acts through the prostaglandin receptors 2 and 4 (*PTGER2* and *PTGER4*), which are expressed on bovine endometrial cells and are usually associated with the down regulation of inflammation. This provides a feedback loop that may be important in the regulation of the immune response to *E. coli* in the bovine uterus (Herath *et al.* 2009, Sheldon *et al.* 2009, Sheldon *et al.* 2009). In the current study *IL1A*, *PTGS2 (COX2)*, *PGES1*, *PTGER2* and *PTGER4* are all elevated 7 days compared to 21 DPP indicates greater potential regulation of inflammation early postpartum in healthy cows that is not evident in SCE cows.

4.4.3.7 Low numbers of differentially expressed genes between 7 and 21 days postpartum in sub-clinically endometritic cows

The most striking revelation of this study was the extent of the difference in temporal transcriptional activity between days 7 and 21 postpartum in healthy cows compared to SCE cows. There were almost 43 times more genes differentially expressed between 7 and 21 DPP in healthy cows amounting to 4068 relative to only 95 differentially expressed genes in SCE cows.

This indicated that the endometrial transcriptome in cows that resolve inflammation by 21 DPP vastly changes from 7 to 21 DPP whereas in SCE cows it remains in either a sustained state of transcriptional activity where gene expression remains elevated at both time points or there is no transcriptional activity at either time point. This dilemma can be addressed by comparing the gene expression profiles of SCE and healthy cows at both 7 and 21 DPP. If the number of differentially expressed genes between SCE and healthy cows at either 7 or 21 DPP is greater than that observed in SCE cows between 7 and 21 DPP, it would indicate that in fact there is sustained temporal transcriptional activity in SCE cows.

4.4.4 The transcriptomic profile of sub-clinically endometritic cows may show a sustained inflammatory profile

The low numbers of genes differentially expressed between 7 and 21 DPP shows that the resolution of inflammation detected in healthy cows is absent from the SCE cow profiles. Previously the idea that transcription in SCE cows is sustained over time was highlighted as a possible explanation as to why there were low numbers of genes differentially expressed between 7 days and 21 DPP. The number of genes differentially elevated in SCE relative to healthy cows was greater both 7 days (147) and 21 DPP (991) and these gene numbers were also greater than the numbers differentially expressed between 7 days and 21 DPP (95) in SCE cows. This confirms that temporal gene expression was in fact sustained in SCE cows between 7 days and 21 DPP. It is evident from pathway analysis that the immune response is what functionally differentiates SCE from healthy cows at each postpartum time point. By evaluating the transcriptomic profile of the immune response at the level of individual gene expression an interesting trend has emerged.

The same genes that are differentially elevated 7 days relative to 21 DPP in healthy cows are also elevated in SCE cows relative to healthy cows 21 DPP. To help with this explanation take for example healthy cows 21 DPP as the control group and when comparing healthy cows 7 DPP and SCE cows 21 DPP to this control group the same genes are elevated in healthy cows 7 DPP and SCE cows 21 DPP relative to the control group. Therefore it is reasonable to suggest that the immune profile of SCE cows 21 DPP is similar to the immune profile of SCE and healthy cows 7 DPP. Following on from this a hypothesis from this current study that could inform future studies is that the immune response of SCE cows is delayed behind that of healthy cows.

4.4.4.1 Increased expression of genes encoding cell marker expression supports leukocyte recruitment 7 days postpartum (DPP) in healthy cows and 21 DPP in SCE cows exceeds that of healthy cows 21 DPP

Through histology analysis of the endometrial stromal and epithelial layers 21 DPP, SCE cows were identified as those with greater relative leukocyte infiltrate. This was reaffirmed at the molecular level with transcriptional analysis as cellular markers for a variety of leukocyte cells were differentially elevated in SCE relative to healthy cows 21 DPP. These differentially expressed cell markers identified leukocytes, *CD44* and *CD53*, neutrophils, *CD14*, activated T, B cells and macrophages, *CD23*, *CD25* and *CD80*, B cells, monocytes, macrophages, dendritic cells, and regulatory T cells, *CD25*, *CD40*, *CD80*, *CD86*. With the exception of *CD23* and *CD25*, all remaining cell markers were also elevated 7 days relative to 21 DPP in healthy cows. The elevated

transcription of cell markers listed above suggests an elevated presence of leukocytes 21 DPP in SCE cows. An elevated presence of leukocytes infers a requirement to either deal with a persistent bacterial contamination or tissue damage in SCE cows relative to healthy cows.

4.4.4.2 Elevated expression of genes involved in PGE₂ production in SCE relative to healthy cows 21 DPP may infer a sustained bacterial presence associated with endometritis and subfertility

As previously discussed prostaglandin E₂ (PGE) is a luteotrophic molecule which is associated with prolonged luteal phases contributing to sub-fertility in postpartum cows (Opsomer *et al.* 2000; Williams *et al.* 2007). There is well documented evidence that there is bacterial regulation of PGE production in the bovine endometrium early postpartum (Swangchan-Uthai *et al.* 2012; Herath, Lilly, Fischer, *et al.* 2009). The results of this study have shown that gene expression, of molecules required in the synthesis of PGE are differentially elevated in SCE cows relative to healthy 21 DPP.

PGE acts through the prostaglandin receptors 2 and 4 (*PTGER2* and *PTGER4*), which are expressed on bovine endometrial cells and are usually associated with the down regulation of inflammation. This provides a feedback loop that may be important in the regulation of the immune response to *E. coli* in the bovine uterus (Herath *et al.* 2009, Sheldon *et al.* 2009, Sheldon *et al.* 2009). In the current study *PTGER4* is elevated in SCE cows relative to healthy cows 21 DPP which indicates greater potential regulation of inflammation in healthy cows that is not evident in SCE cows 21 DPP.

4.4.4.3 A potential gram-positive bacterial presence and inability to regulate TLR signalling differentiates healthy and SCE cows 21 DPP

The elevated gene expression of the pattern recognition receptor (PRR) *TLR2* and its accessory recognition molecules, *LBP*, *MD2*, *CD14* and *TLR2*, may indicate a greater requirement to recognise gram-positive bacteria in SCE relative to healthy cows 21 DPP and also 7 days relative to 21 in healthy cows. Simultaneously at 7 days relative to 21 DPP in healthy cows there is an elevation of genes encoding phosphoinositide 3-kinases (PI3Ks), *PIK3CA*, *PIK3CB* and *PIK3CD*, which are involved in the negatively regulating TLR signalling which may act to protect the host from over activation (Hazeki *et al.* 2007; Li *et al.* 2010). Although genes, involved in recognition of PAMPs on gram-positive bacteria, are up regulated in SCE relative to healthy cows 21 DPP, PI3K genes are not differentially expressed. Therefore SCE cows may recognise PAMPs in gram-positive

bacteria however the safety mechanism of negative regulation of TLR signalling is not occurring which may result in over stimulation and an excessive inflammatory response.

4.4.4.4 Persistent bacteria contamination and IL-17C production may regulate the expression of the S100 family proteins differentiating healthy from SCE cows 21 DPP

A bacterial presence with agonists for TLR2 (lipopeptides) and the cytokines TNF and IL-1 β elevate IL-17C production and gene expression in epithelial cells. Subsequently IL-17C elevates the expression of S100A7A, S100A8 and S100A9 which are AMPs and part of the S100 family proteins involved in cellular processes such as cell growth and differentiation, calcium-dependent cell signalling, cell cycle regulation and antimicrobial defence (Abtin *et al.* 2010; Ramirez-Carrozzi *et al.* 2011). In the current study TLR2, TNF, IL-1 β , IL-17C, S100A8 and S100A9 are differentially elevated 7 days relative to 21 DPP in healthy cows, as previously discussed. However these genes are also elevated in SCE cows relative to healthy cows 21 DPP.

An elevated and sustained concentration of TNF α postpartum is associated with the presence of a uterine bacterial infection such as endometritis (Kim *et al.* 2005). Endometrial gene expression of TNF decreased one week postpartum (Galvão *et al.* 2011), was elevated between 10 and 45 DPP (Gabler *et al.* 2010) and also between 21 and 27 DPP (Fischer *et al.* 2010) in inflamed uteri or endometritic compared to control healthy cows. IL1B gene expression in healthy cows decreased over time postpartum, this change over time was not observed in endometritic cows (Galvão *et al.* 2011). IL1B gene expression in the endometrium was decreased one week postpartum, elevated 5 to 7 weeks postpartum (Galvão *et al.* 2011), 21 to 27 DPP (Fischer *et al.* 2010) and 10 to 54 DPP (Gabler *et al.* 2010) in inflamed uteri or endometritic cows compared to healthy cows.

The current study revealed the elevated expression of numerous S100A genes 7 days relative to 21 DPP in healthy cows and also in SCE cows relative to healthy cows 21 DPP, namely S100A2, S100A3, S100A5, S100A8, S100A9, S100A12 and S100A14. Future work with these genes and their proteins could reveal interesting insights into their functionality within an inflamed environment, in the host's immune response to gram-positive bacteria and as biomarkers for sub-clinical endometritis.

4.4.4.5 Genes that locally differentiate cows at risk of sub-clinical endometritis from healthy cows in the early postpartum endometrium

Genes expressed locally in the endometrium, secreted to the extracellular space and which differentiated healthy from sub-clinically endometritic cows, were selected as potential systemic

markers for cows at risk of developing sub-clinical endometritis. Although the products of the selected genes were potentially secreted by other tissues during the postpartum period, the local endometrial expression and secretion would amplify their effects by increasing circulating concentrations. Subsequently these elevated circulating concentrations may differentiate cows at risk of developing sub-clinically endometritis. There were 31 candidate genes which uniquely differentiated SCE cows from healthy cows, 17 of these contained signalling peptides however only two of these could be secreted to the extracellular space and these were IL17A and IL17F. As a result these genes were selected as potential systemic biomarkers for subclinical endometritis and will be discussed further in chapter 5.

4.4.5 MicroRNA expression in the postpartum endometrium reveals greater temporal change within groups than between SCE and healthy cows

MicroRNAs have been implicated in both physically normal and pathological events associated with the human endometrium (Pan & Chegini 2008). Interestingly the lack of the enzyme DICER1, required for miRNA biogenesis, results in female infertility in mice which highlights an essential role for miRNAs and female fertility (Pastorelli *et al.* 2009). Although a variety of groups have suggested a role for miRNAs in the regulation of inflammation in the human endometrium, biological significance of miRNA function during inflammation has not yet been established (Chegini 2010). In the bovine endometrium the differential expression of microRNAs has been attributed to the inflammatory state of sub-clinical endometritis. Using gene networks, canonical pathways and biological function analysis a previous study has demonstrated that genes regulated by differentially expressed microRNAs during sub-clinical endometritis are associated with signalling pathways that mediate inflammatory responses, cell movement, cellular proliferation, apoptosis and the cell cycle. In other words sub-clinical endometritis enhances the expression of microRNAs that reduce the translation of genes associated with biological functions such as cellular growth and proliferation, cell cycle, gene expression, cellular movement and cellular development. This highlights the potential regulatory role miRNAs in the bovine endometrium during the development and progression of postpartum sub-clinical endometritis (Hailemariam *et al.* 2013).

Recent human studies in the endometrium have revealed that miRNA expression regulates cellular proliferation, migration and/or invasion and the miR-200 family members assist in these processes (Nothnick 2012; Panda *et al.* 2012). Interestingly in the current study all five members of the mir-200 family, *mir-200a*, *mir-200b*, *mir-200c*, *mir-141* and *mir-429*, are significantly

elevated 7 days relative to 21 DPP in SCE cows which was not replicated in healthy cows. This would suggest a potential to negatively regulate cellular proliferation at a microRNA level in the endometrium (Panda *et al.* 2012) of sub-clinically endometritic cows which is not present in healthy cows 7 DPP.

In the current study miRNA profiling also found significant differences between groups 7 DPP as 3 miRNAs, *bta-mir-205*, *bta-mir-375* and *bta-mir-197*, were differentially elevated in SCE cows relative to healthy cows. Further studies need to be carried out to define the role these molecules play in the regulation of transcription within the bovine uterus.

Chapter 5

5 Characterisation of the systemic haematological, metabolite and immune profile in healthy and sub-clinically endometritic dairy cows early postpartum; towards the identification of early prognostic biomarkers

5.1 Introduction

Results from chapter 4 have demonstrated that at a transcriptomic level, clear differences are detectable between healthy and SCE cows at both 7 and 21 DPP. Detecting these changes at a systemic level would be a major step toward the development of cost-effective and easy-to-use diagnostic tests for uterine disease. However the extent to which local inflammation and infection will be reflected systemically remains unclear. Understanding the relationship between local and systemic changes in the early postpartum period is a prerequisite to advocating reliable prognostic or diagnostic biomarkers for uterine disease in dairy cows.

The National Cancer Institute (NCI) defines a biomarker as: “A biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition.” (<http://www.cancer.gov/dictionary?cdrid=45618>). In addition to this definition, biomarkers are molecules that can be quantified and, from a clinical perspective, preferably obtained through non-invasive means when possible. The areas to which biomarkers can be applied include: diagnostics (the presence of a disease) (Ziogas & Roukos 2011), prognostics (monitoring the response to therapeutics) (Pettersson *et al.* 2011), stage of disease progression (Oh *et al.* 2008) and clinical (Voelkerding *et al.* 2010). Biological molecules that can be quantified for application as biomarkers include: specific cells, proteins such as enzymes and antibodies, hormones, genes, transcripts, DNA, RNA and many more. There is no doubt that biomarkers have the potential to improve patient care and reduce medical costs. However in recent times, validation of fewer than 100 biomarkers for routine clinical practise out of more than 150,000 claimed biomarkers have been documented in the literature (Poste 2011).

An optimistic light in biomarker discovery and clinical application was shed by van 't Veer *et al.* 2002 when they constructed a 70-gene tumour expression profile which was proposed as a powerful prognostic biomarker of disease outcome in young breast cancer patients. Subsequently the same research group determined that the gene expression signature best predicted the development of future metastases during the first five years after treatment (van de Vijver *et al.* 2002). Glas *et al.* (2006) demonstrated that a small custom-made microarray, based on the

previously mentioned gene expression signature, was a robust and reproducible clinical tool to predict the outcome of disease in patients with breast cancer. In a separate study concerned with stage II and stage III colorectal cancer, a gene expression signature produced with microarray technology was shown to be associated with the risk of relapse. The validation of this prognostic classifier was carried out and it was promoted as a robust standardised assay for routine clinical practice (Salazar *et al.* 2011). Microarray technology has also been used to diagnose the origin of cancer of unknown primary with the use of gene expression profiling to determine a robust clinical classifier (Tothill *et al.* 2005).

The discovery of a local or systemic biomarker for postpartum subclinical endometritis and a convenient on-farm method of detection, would allow provision of early medical and therapeutic intervention, avoiding the economic deficits and infertility associated with the disease. This would improve a farmer's ability to achieve a synchronised annual calving interval so that all cows within the herd become pregnant within a 12 week breeding season, thus improving fertility and reducing costs associated with infertility related culling.

The systemic profile of the postpartum cow is characterised by the cellular and molecular components of peripheral blood which can reflect what is happening locally but is dependent on the stage of local inflammation/disease. The systemic immune profile in particular can be defined by assessing the numbers of circulating leukocytes and plasma proteins such as acute phase proteins (APPs), cytokines and chemokines. The physiological status of the postpartum cow has been associated with alterations in the systemic immune profile in the immediate days and weeks following parturition. The local tissue damage and bacterial contamination in the uterus present postpartum involves physiological inflammation (Chapwanya *et al.* 2012) which is the instigator of events leading to the systemic acute phase response (APR) (Ceciliani *et al.* 2012) and fluctuations in the numbers of peripheral circulating leukocyte cells (Kim *et al.* 2005; Zerbe *et al.* 2000). The role and function of the neutrophil has been investigated in the postpartum cow (Hoeben *et al.* 2000; Rinaldi *et al.* 2008; Revelo & Waldron 2010; Hammon *et al.* 2006), however other cells types have not been as extensively assessed.

Acute phase proteins are well recognised sentinels of tissue damage and inflammation. They have been widely used in veterinary medicine across a range of species (Petersen *et al.* 2004). Parturition is associated with an elevation of acute phase protein (APP) concentrations and as involution progresses it decreases. However APP concentrations are sustained in the blood of cattle with uterine bacterial contamination comprising *A. pyogenes* and *E.coli* in particular (Sheldon *et al.* 2001) and the acute phase response also precedes the clinical appearance of

uterine disease early postpartum (Huzzey *et al.* 2009). It has also been demonstrated that the acute phase proteins serum amyloid A (SAA) and haptoglobin (HP) were more sensitive at distinguishing between acute and chronic inflammation than neutrophil and white cell blood cell counts (Horadagoda *et al.* 1999).

Serum amyloid A (SAA) and haptoglobin (HP) are produced and released by hepatocytes into peripheral blood circulation in response to stimulation by the proinflammatory cytokines, IL-1, TNF and IL-6. Although the liver is the predominant source of SAA, it is also produced in extrahepatic tissues, for example in the mammary gland during mastitis (Eckersall *et al.* 2006, Suffredini *et al.* 1999). In previous studies by our group we have shown that the endometrial gene expression of *SAA*, *HP*, *IL-1*, *TNF* and *IL-6* is elevated 15 days relative to 30 days postpartum and this is (Chapwanya *et al.* 2012; Foley *et al.* 2012). It is the intent of the current study to quantify the protein concentrations of these molecules in the peripheral blood circulation to potentially differentiate sub-clinically endometritic cows from healthy cows early postpartum. As a result of transcriptomic analysis of the endometrium in chapter 4, IL-17 was identified as a potential biomarker that could discriminate sub-clinically endometritic (SCE) cows from healthy cows both 7 and 21 DPP. The current study will also assess if this elevated expression of IL-17 locally in the endometrium could be detected systemically in the same cows and also discriminate SCE cows from healthy cows.

The assessment of the metabolite profile was performed for two reasons – the first is that negative energy balance (NEB) is often offered as a predisposing factor for postpartum disease; and secondly because there is an interesting link in the use of metabolites as biomarkers for disease (Monteiro *et al.* 2013). As negative energy balance (NEB) is associated with the impairment of the immune system and decreased fertility it was also assessed in the cows within this study. Cows experience NEB in early lactation when the high energy demand of lactation is not supplied through nutrition. Excessive stores of fat and increased blood non esterified fatty acid (NEFA) concentrations are associated with many pro-inflammatory diseases in periparturient dairy cows such as mastitis and uterine disease (Sordillo *et al.* 2009). Because no individual system acts in isolation; the integration of metabolic, endocrine and immune data will be vital to defining pathological and healthy signatures of postpartum bovine immune responses.

5.2 Aim

The research aim of this experiment was to investigate if cows that became sub-clinically endometritic 21 DPP could be differentiated from healthy cows at 7 days postpartum (DPP) prior

to diagnosis of sub-clinical endometritis 21 DPP by characterising the systemic haematological, metabolite and immune profile of all cows at both time points.

5.3 Results

5.3.1 Whole blood cell counts

Whole blood cell counts were analysed in pre-calving cows ($n=8$), sub-clinically endometritic cows ($n=6$) 7 and 21 days postpartum (DPP) and healthy cows ($n=9$) 7 and 21 DPP. A one way ANOVA test highlighted differences between the means of five different groups. There were significant differences in the percentage neutrophils ($p = 0.0038$), eosinophils ($p = 0.0046$) and lymphocytes ($p = 0.0374$) and also in the absolute neutrophil ($p = 0.0261$) and eosinophil ($p = 0.0004$) cell counts. Subsequently the groups were compared to each other in a variety of different combinations and significant differences are represented in Table (Appendices). Although percentages of neutrophils, eosinophils and lymphocytes were significant in a number of comparisons the extent of these differences cannot be appreciated unless all cell types are assessed together, therefore absolute cell counts may be more informative as they can be quantified independent of other cells.

The absolute neutrophil cells counts 7 DPP were significantly lower in SCE cows and significantly higher in healthy cows compared to pre-calving cows. This difference between groups 7 DPP was supported as absolute neutrophil cell counts were significantly lower 7 DPP in the SCE cows compared to healthy cows. There was a significant increase in absolute neutrophil cell counts from pre-calving to 21 DPP in healthy cows which was not observed in the SCE cows. There was also no significant difference between SCE and healthy cows 21 DPP (Figure 5.3-1).

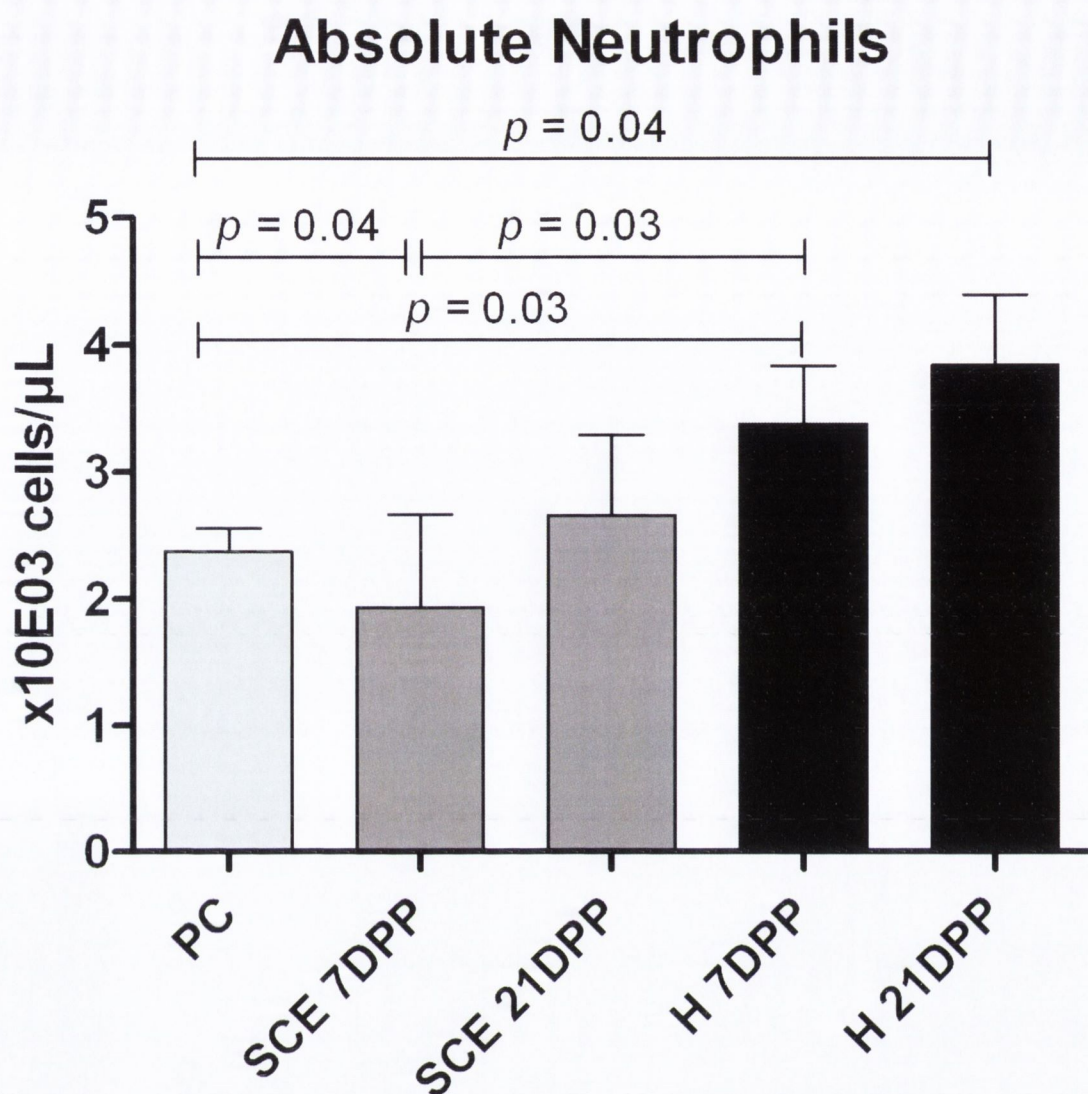


Figure 5.3-1: Absolute neutrophil cell counts in pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, H 7 DPP and H 21 DPP) .

In both healthy and sub-clinically endometritic cows peripheral eosinophil counts were significantly decreased 7 DPP compared to pre-calving and subsequently recovered to pre-calving levels by 21 DPP. There was also a significant increase in eosinophil numbers 21 DPP compared to 7 DPP in both sub-clinically endometritic and healthy cows. The only comparison that differentiated groups from one another was between groups at 7 DPP where there was significantly elevated eosinophil cell counts in the healthy group compared to the sub-clinically endometritic cows (Figure 5.3-2).

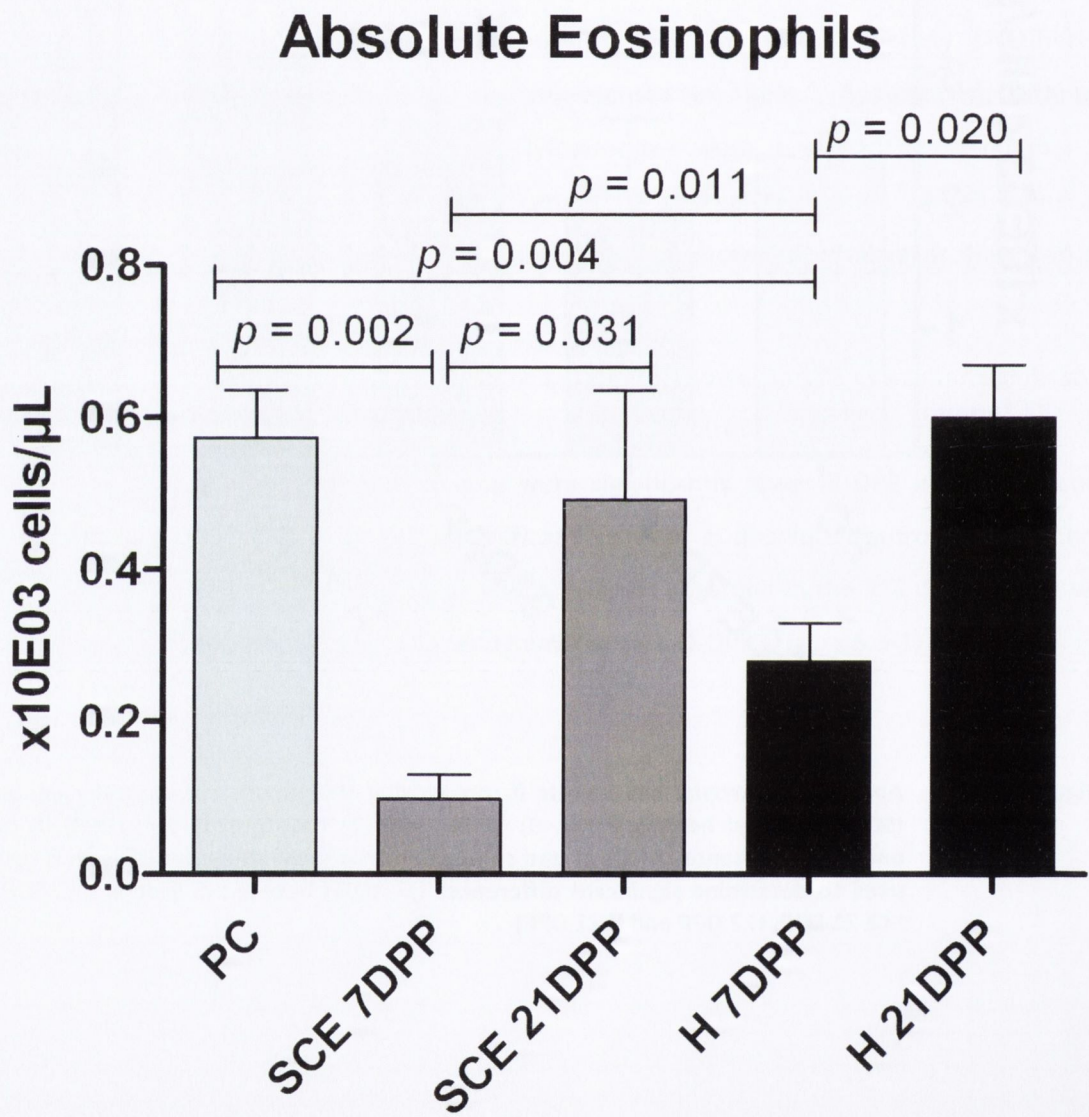


Figure 5.3-2: Absolute eosinophil cell counts in pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

5.3.2 Acute phase proteins serum amyloid A and haptoglobin

Serum amyloid A (SAA) and haptoglobin (HP) concentrations were analysed in the plasma of pre-calving cows (n=8), sub-clinically endometritic cows (n=5) 7 and 21 days postpartum (DPP) and healthy cows (n=9) 7 and 21 DPP. A one way ANOVA test of significance highlighted differences in the means between the five different groups for HP ($p = 0.004$) and SAA ($p = 0.037$). Subsequently the groups were compared to each other in a variety of different combinations and significant differences are represented in TabA-7 (Appendices).

Compared to pre-calving values both SCE and healthy cows had increased concentrations of SAA and HP 7 and 21 DPP. However this increase was only significantly different between pre-calving and 7 DPP in the SCE cows for both SAA ($p = 0.002$) and HP ($p = 0.007$) concentrations and between pre-calving and 7 DPP in the healthy cows for HP only ($p = 0.03$). Concentrations were significantly higher in SCE cows compared to healthy cows for both SAA ($p = 0.029$) and HP ($p = 0.019$) 7 DPP (Figure 5.3-3 and Figure 5.3-4).

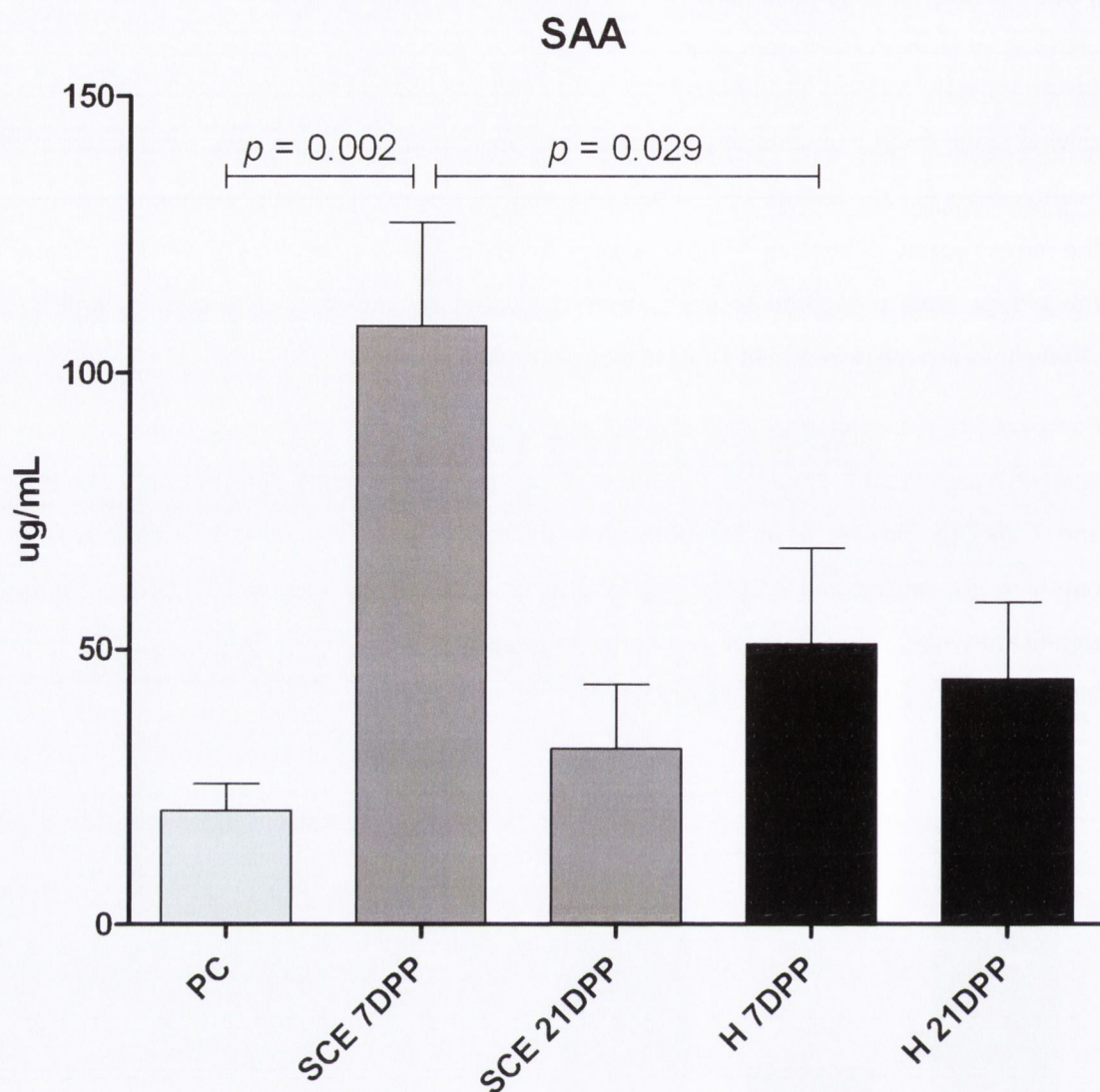


Figure 5.3-3: Serum amyloid A (SAA) concentrations in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=5) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, H 7 DPP and H 21 DPP).

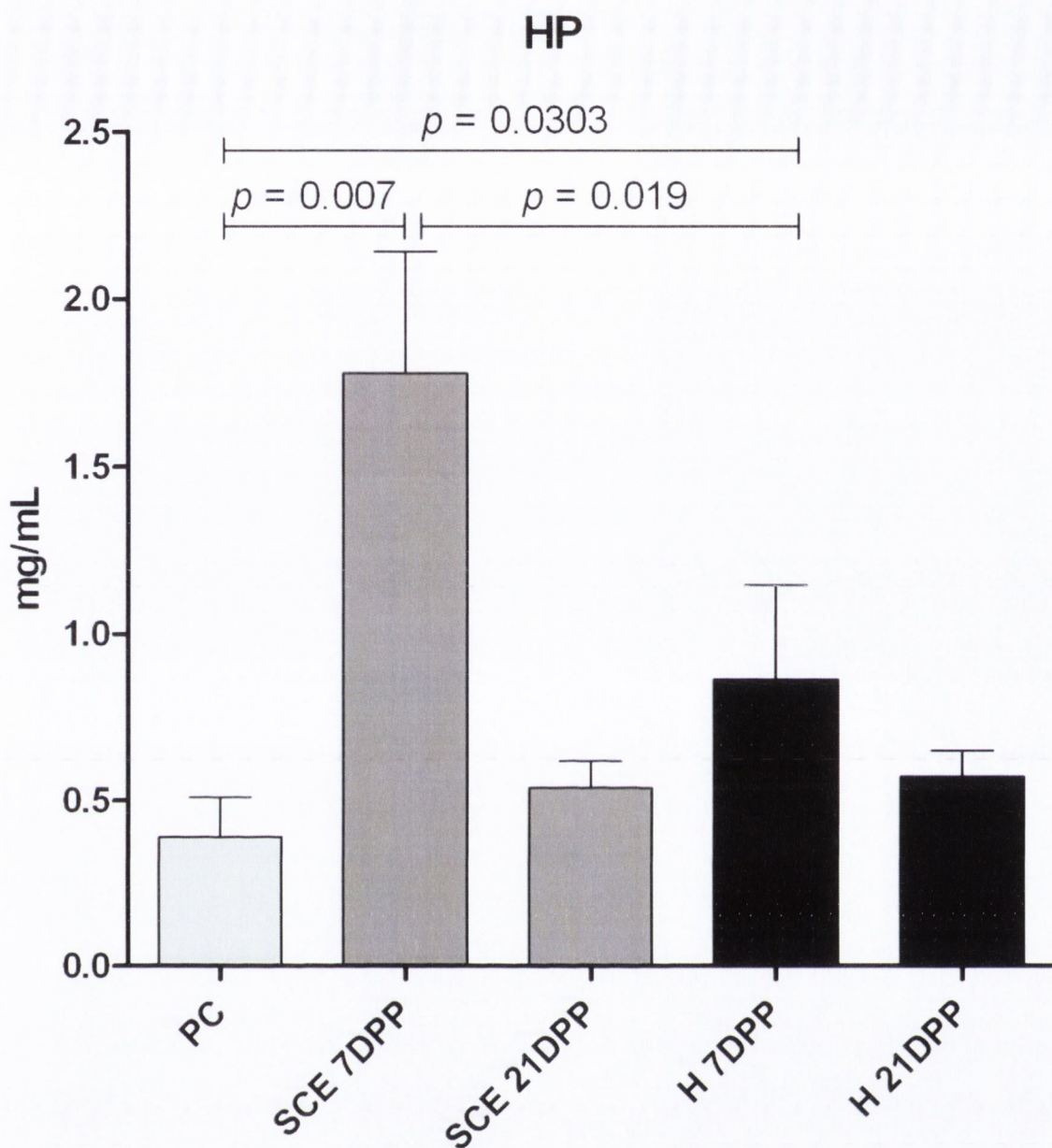


Figure 5.3-4: Haptoglobin (HP) concentrations in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=5) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, H 7 DPP and H 21 DPP).

5.3.3 Blood plasma protein ELISAs and potential biomarker assessment

Interleukin-1 (IL1) and Interleukin-6 (IL6)

A variety of cytokines can be detected in the plasma of peripheral blood and elevated levels are associated with an underlying inflammatory condition in the host. In particular IL-1 and IL-6 when elevated in the peripheral circulation can contribute to elevating the acute phase response. IL-1 and IL-6 protein concentrations were analysed in the plasma of pre-calving cows (n=8), sub-clinically endometritic cows (n=5) and healthy cows (n=9) 7 and 21 DPP. There was no expression of IL-1 present in plasma of cows assessed in this experiment. Initially a one way ANOVA test of significance highlighted differences in the means between the five different groups for IL-6 ($p = 0.0049$). The groups were then compared to each other in a variety of different combinations and significant differences are represented in Table (Appendices). There was a significant decrease in IL-6 concentrations in SCE cows 21 DPP compared to pre-calving levels (Figure 5.3-5).

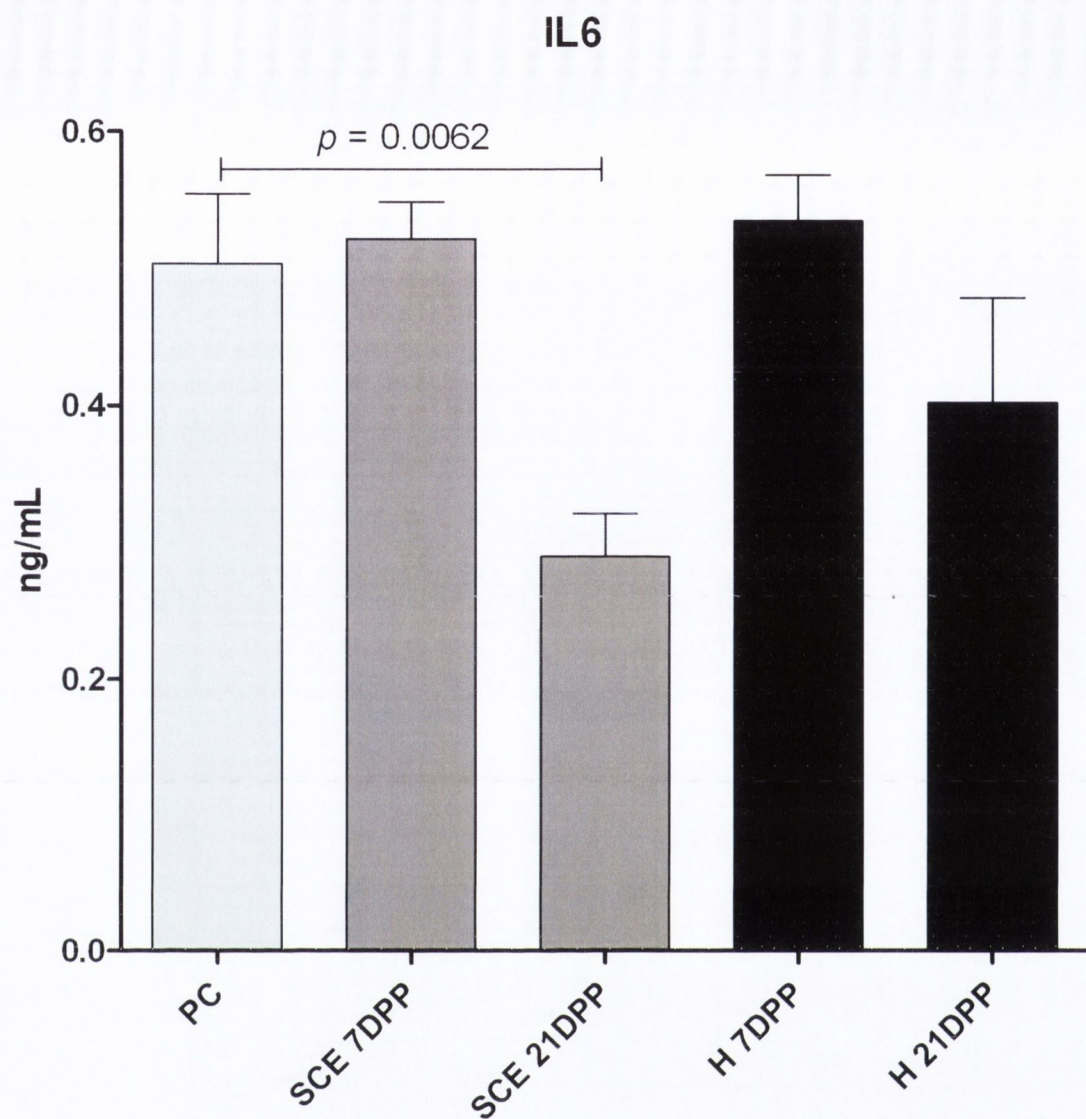


Figure 5.3-5: Interleukin-6 (IL-6) concentrations in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=5) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

Interleukin-17A (IL-17A)

Interleukin-17A (IL-17A) and interleukin-17F (IL-17F) were found to be highly significantly differentially expressed in the endometrium of sub-clinically endometritic cows 7 and 21 days postpartum and not in healthy cows. The protein products of IL-17A and IL-17F were predicted to be secreted into the extracellular space and therefore potentially detected systemically in blood plasma. However following the quantification of IL-17A in plasma concentrations were below detectable levels for the majority of cows, those cows with measureable concentrations are presented in Table 5.3-1.

Table 5.3-1: IL-17A concentrations in pre-calving (PC) cows (n=8) and postpartum sub-clinically endometritic (SCE) (n=5) and healthy (n=9) cows 7 and 21 days postpartum. A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

Cow ID	Time	Group	ng/ml
4	PC	Healthy	0.55
10	PC	Healthy	9.25
2	7 DPP	SCE	0.40
10	7 DPP	Healthy	3.10
12	7 DPP	Healthy	0.57
14	7 DPP	Healthy	0.62
10	21 DPP	Healthy	1.85

5.3.4 Blood metabolites

Blood metabolites, beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFAs), glucose and Urea, were analysed in pre-calving cows (n=8), sub-clinically endometritic cows (n=6) 7 and 21 days postpartum (DPP) and healthy cows (n=9) 7 and 21 DPP. A one way ANOVA test highlighted differences in the means between the five different groups and there were significant differences in BHB ($p = 0.0003$), NEFAs ($p = <0.0001$) and urea ($p = 0.0036$). There were no significant differences in the concentration of glucose between the groups. Subsequently the groups were compared to each other in a variety of different combinations and significant differences are represented in Table (Appendices).

The concentrations of BHB, NEFA and urea were significantly different between pre-calving and 7 DPP and pre-calving and 21 DPP in both the SCE and healthy cows. There were no significant differences between groups at either 7 or 21 DPP and also no significant differences were observed between time points in either group. Therefore energy balance was not different between sub-clinically endometritic and healthy cows (Figure 5.3-6, Figure 5.3-7 and Figure 5.3-8).

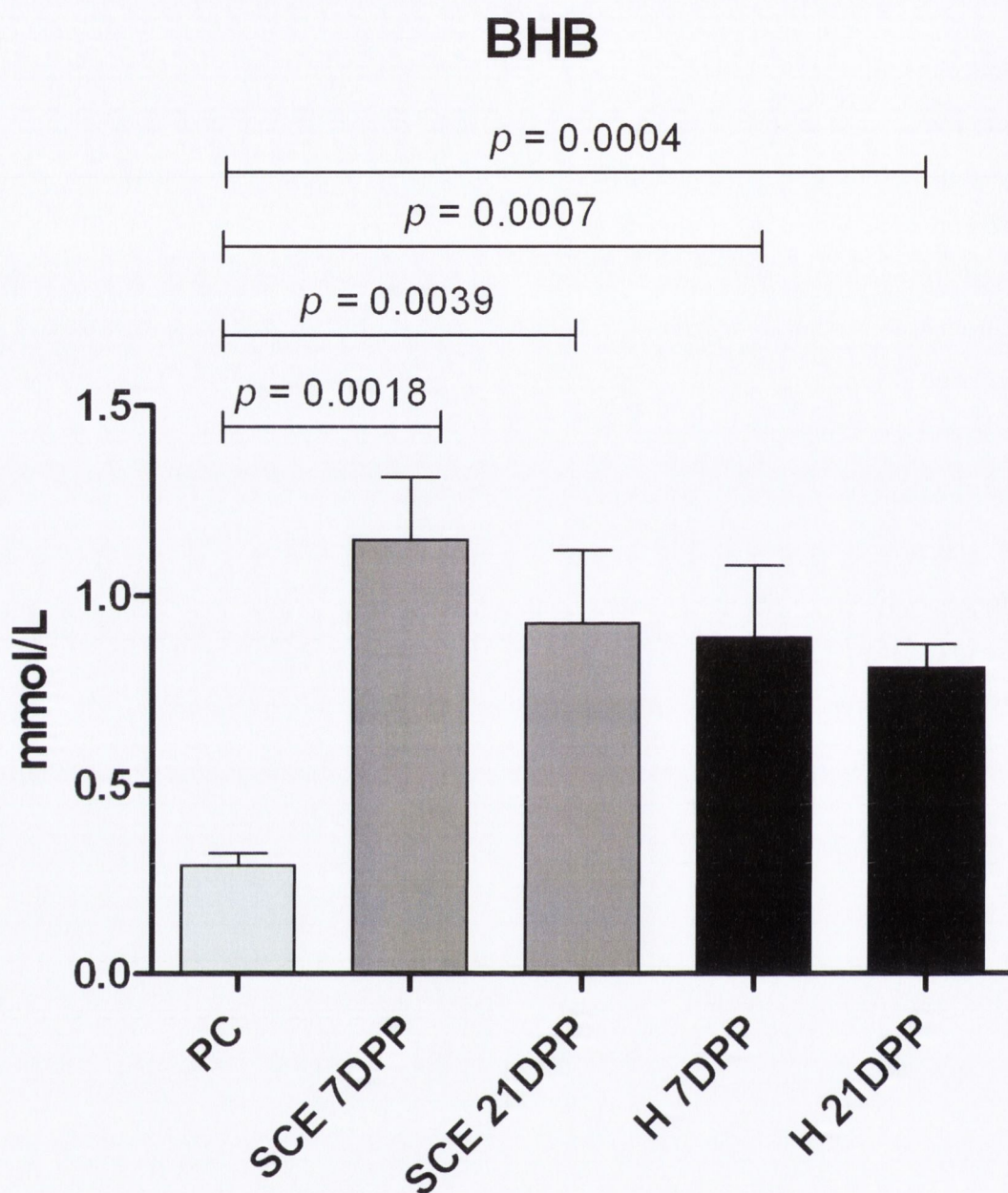


Figure 5.3-6: Beta-hydroxybutyrate (BHB) concentrations in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

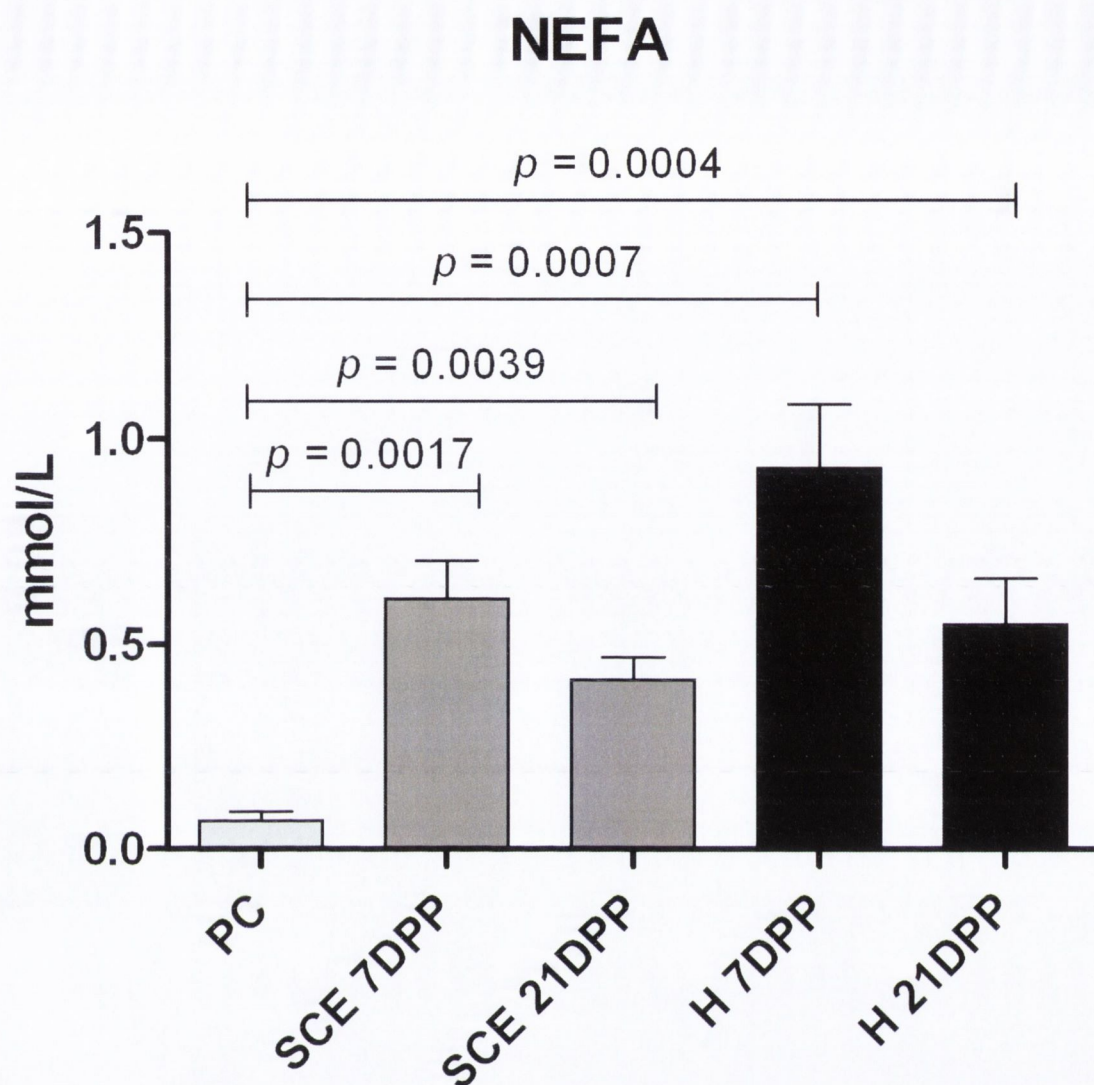


Figure 5.3-7: Non-esterified fatty acid (NEFA) concentrations in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

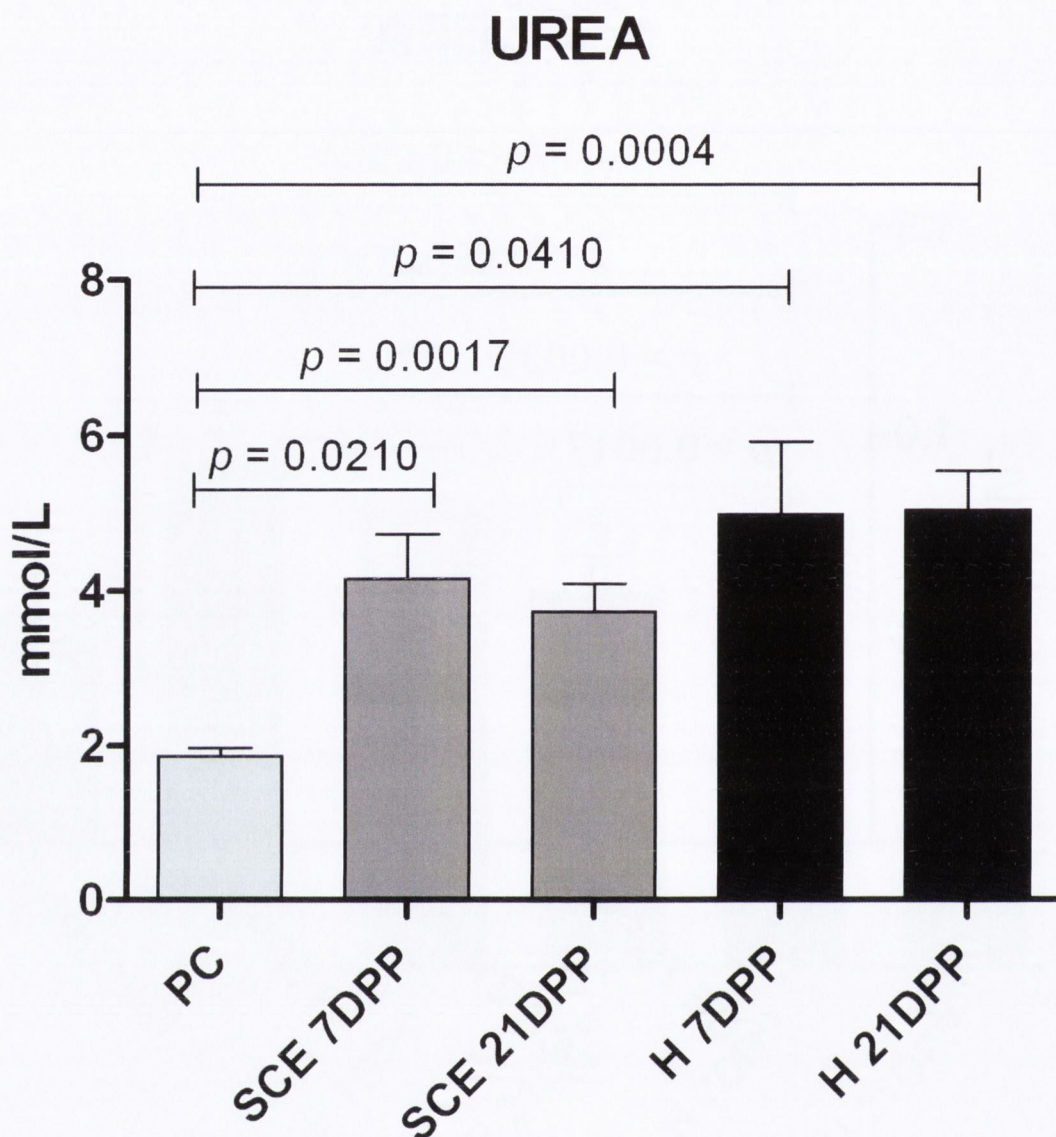


Figure 5.3-8: Urea concentrations in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (H) (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

5.4 Discussion

The diverse milieu in the postpartum uterus constitutes components of the host, pathogen and farm environment and the interactions of which determine whether a physiological immune response is resolved or sustained during the progression of involution. Local inflammation manifests itself systemically which is initiated by the secretion of proinflammatory cytokines, such as IL-1, TNF and IL-6 in the endometrium (Chapwanya *et al.* 2012), that travel via the peripheral blood system to the liver stimulating the production of acute phase proteins such as Serum amyloid A (SAA) and haptoglobin (HP) (Ceciliani *et al.* 2012). Therefore the local endometrial immune response has a significant influence on the systemic immune profile during involution in all postpartum cows. However variation in either the hosts immune response or bacterial load and population in the postpartum endometrium results in excessive or sustained local inflammation which if manifested systemically would allow systemic differentiation between healthy and sub-clinically endometritic cows. Previous studies have shown promising work in relation to systemic differentiation between healthy and sub-clinically endometritic cows as acute phase protein concentrations were shown to be elevated in the blood of cattle with uterine bacterial contamination comprising *A. pyogenes* and *E.coli* in particular (Sheldon *et al.* 2001) and the acute phase response also preceded the clinical appearance of uterine disease early postpartum (Huzzey *et al.* 2009).

Negative energy balance (NEB) is experienced by cows early in lactation and due to its impairment of the immune system and negative impact on fertility (Sordillo *et al.* 2009) it was also assessed in cows within this study. Excessive stores of fat and increased blood non esterified fatty acid (NEFA) concentrations are associated with many pro-inflammatory diseases in periparturient dairy cows such as mastitis and uterine disease (Sordillo *et al.* 2009). During severe negative energy balance it has been observed that the systemic concentrations of NEFAs and β -hydroxybutyrate (BHB) are elevated indicating lipid mobilization and fatty acid oxidation (Wathes *et al.* 2007) and circulating levels of glucose decrease (Wathes *et al.* 2009). In the current study healthy and SCE cows had peripheral concentrations of NEFAs and BHB below and glucose above those observed in severe negative energy balance (Wathes *et al.* 2009) indicating both groups of cows were not experiencing severe negative energy balance. In addition to this there were no significant differences in any of the blood metabolites analysed within and between groups 7 and 21 DPP. Therefore any effect of severe NEB on the systemic or local immune profiles can be ruled out for the current study.

Systemic detection of these biomarkers (e.g., in serum) would lead the way towards the development of diagnostics for uterine disease in cows. Identifying infection earlier prevents the reliance on the development of clinical symptoms for diagnosis of disease. Because no individual system acts in isolation; the integration of metabolic, endocrine and immune data will be vital to defining pathological and healthy signatures. The ultimate goal of our research is to develop biomarkers of early detection of uterine disease that may specifically identify sub-clinical endometritis by characterising and differentiating local endometrial inflammation, as revealed in chapter 4 that potentially manifests itself systemically.

5.4.1 A reduction in absolute neutrophil and eosinophil cell numbers in the periphery of sub-clinically endometritic cows relative to healthy cows 7 days postpartum prior to diagnosis of sub-clinical endometritis.

Decreased polymorphonuclear function has been associated with uterine health disorders (Hammon *et al.* 2006). Neutrophils account for the vast majority (~70%) of circulating leukocytes and are the primary cells which respond which respond and migrate to the site of infection in order to control bacteria but also to activate an immune response locally (Bondurant 1999). We have shown significant differences in neutrophil levels between healthy cows and those with sustained endometrial inflammation 21 DPP. It is thought that as more neutrophils migrate to the uterus, to fight infection, diseased animals become progressively neutropenic. Eosinophils are another population of innate immune leukocytes, normally residing at very low levels in the peripheral blood. However they play an important role in bridging innate and adaptive immunity as well as in the orchestration of tissue remodelling (Shamri *et al.* 2011).

Neutrophil chemotaxis from the bone marrow and systemic circulation to peripheral organs such as the uterus and the mammary gland is an important requirement in the postpartum cow to eliminate bacteria from these local environments following parturition. Fluctuations in peripheral numbers of neutrophils postpartum is a physiological event whereby a decrease has been observed in healthy cows within one week postpartum and subsequently increasing three weeks postpartum (Singh *et al.* 2008; Zerbe *et al.* 2000; Kim *et al.* 2005). In addition to eliminating bacterial contamination neutrophils may also have an additional physiological role in endometrial repair as demonstrated in a mouse model whereby neutrophil depleted mice displayed a significant delay in endometrial repair compared with controls (Kaitu'u-Lino *et al.* 2007). The decrease in peripheral blood leukocyte numbers in the first week postpartum is also observed in cows which developed postpartum endometritis (Kim *et al.* 2005) however what differentiates

healthy cows from those with intrauterine infection is an elevated bacterial load which has been correlated with the number of neutrophils migrated to the uterus (Zerbe *et al.* 2002). Eosinophils, like neutrophils, are granulocytic immune cells however are involved primarily in response to invading parasites, including helminths, and contribute to pathological processes in allergic diseases. They release inflammatory mediators such as lytic enzymes, superoxides and kallikreins enhancing endothelial permeability allowing the entry of circulating IgG into the uterine lumen. An abundance of eosinophils have been noted in the endometrium of heifers (Bondurant 1999) and in the corpus luteum post ovulation in cows (Reibiger & Spanel-Borowski 2000).

We have demonstrated locally that cows with an elevated density of endometrial neutrophils 21 DPP have an elevated inflammatory immune transcriptomic profile relative to healthy cows at both 7 and 21 DPP. An interesting finding of the current study was the significantly lower numbers of absolute neutrophils and eosinophils circulating in the periphery of sub-clinically endometritic (SCE) cows relative to healthy cows 7 DPP, which would suggest that there was greater recruitment of neutrophils and eosinophils from the periphery in SCE than healthy cows at this time. Subsequently by 21 DPP the numbers of neutrophils in the periphery of SCE cows does increase however not beyond the absolute number of neutrophils in healthy cows even at 7 DPP. In addition to this SCE cows have still not significantly increased or recovered their neutrophil reserves circulating in the periphery relative to pre-calving levels whereas even by 7 DPP in healthy cows there is a significant increase in circulating neutrophils compared to pre-calving cows. However in the eosinophil population absolute numbers have recovered in both SCE and healthy cows by 21 DPP. Taken together these observations suggest that there may still be a greater requirement in SCE cows even by 21 DPP to recruit neutrophils but not eosinophils from the periphery perhaps due to chemotactic signals from sustained inflammation in the uterus. Yet both neutrophil and eosinophil absolute cell numbers are significantly lower in SCE cows relative to healthy cows 7 DPP which may be an early warning sign for the potential development of sub-clinical endometritis 21 DPP.

5.4.2 Interleukin-17: potentially a local rather than systemic biomarker for sub-clinical endometritis

In chapter 4 cytokines interleukin (IL)-17A and IL-17F were the most differentially expressed genes in the postpartum endometrium to locally differentiate between healthy and sub-clinically endometritic cows both 7 and 21 DPP and whose protein products had the potential to be secreted into the extracellular environment. There are six members of the IL-17 cytokine family

which include IL-17A to IL-17F (Moseley *et al.* 2003). Subsequent to the discovery of the first IL-17 family member, IL-17A which was cloned from CD4⁺ memory cells (Rouvier *et al.* 1993), it was revealed that IL-17 producing cells were distinct from Th1 cells with potentially different functions in host immunity (Infante-Duarte *et al.* 2000) opposing the ideals of the Th1/Th2 paradigm (Kolls & Khader 2010; Mensikova *et al.* 2013). It is now established that a major subset of cells known as Th17 cells and also $\gamma\delta$ T-cells are the primary producers of IL-17A. Other cells that have the capacity to produce IL-17A include CD8⁺ T-cells, NK-cells and NKT-cells (Daughenbaugh *et al.* 2011; Dubin & Kolls 2008; Roark *et al.* 2008; Stepanova *et al.* 2012). IL-17A has been previously characterised in a variety of species other than human and mouse including cows (Mensikova *et al.* 2013; Riollet *et al.* 2006). The effects of IL-17 are initiated once recognised by its receptor IL-17RA which binds to at least three members of the IL-17 family including IL-17A and IL-17F (Gaffen 2009) and is expressed in almost all tissues (Moseley *et al.* 2003). Activation of the IL-17 receptor by its ligands stimulates somatic cells to produce chemokines such as IL-8, CXCL1, CXCL5, CXCL6 and MCP-1, antimicrobial peptides, G-CSF, IL-6, prostaglandin E2, ICAM-1 and matrix metalloproteinase, accumulatively resulting in an increased pro-inflammatory response during infections and autoimmune disease (Mensikova *et al.* 2013).

Interesting studies using knockout (KO) mice have shown that the lack of the IL-17RA can lead to the spontaneous development of *S. aureus* infection (Schwarzenberger & Kolls 2002) and increased susceptibility to pulmonary infection caused by Gram negative bacteria *K. pneumoniae* (Aujla *et al.* 2008) possibly caused by the reduction of neutrophil chemotaxis due to less chemokine production and reduced granulopoiesis due to less G-CSF production (Ye *et al.* 2001). Although the degree of IL-17A and IL-17F production maybe increased, unless the expression of IL-17RA on local tissue is adequate enough to recognise this increase, elevated IL-17 expression locally during an infection may not be as effective. This is evident in IL-17RA KO mice which were more susceptible to extracellular gram negative bacterial than were IL-17A KO mice (Aujla *et al.* 2008). It is interesting in our study that healthy cows have greater *IL-17RA* gene expression 7 days relative to 21 DPP whereas this is not the case in SCE cows comparing 7 and 21 DPP. This may suggest that healthy cows are potentially less susceptible to infection 7 days relative to 21 DPP whereas SCE cows are equally as susceptible at both 7 and 21 DPP. Yet this cannot be definitively confirmed by this study as *IL-17RA* gene expression was not significantly different between healthy and SCE cows at either postpartum time point and will require further study.

A role for IL-17 has been revealed during bovine mammary gland inflammation, particularly in epithelial cells which form the initial physical barrier against bacterial invasion, either alone or in

combination with TNF- α it elevated the expression of *IL-8*, *IL-6*, *Gro- α* , *CCL20*, *iNOS*, *TAP* and *SAA* (Riollet *et al.* 2006; Bougarn *et al.* 2011). During bovine mammary gland *S. aureus* mastitis, gene expression of *IL-17A* was found to be elevated in milk somatic cells (Tao & Mallard 2007) and different mammary gland tissues including alveolar, gland cistern, ductal and teat canal sections (Whelehan *et al.* 2011). Although it has a role to play locally IL-17 doesn't appear to influence the systemic response during mastitis as it was not found to be significantly different in the serum of dairy goats with mastitis (Jing *et al.* 2012) and either was its gene expression in systemic mononuclear cells differentially expressed in mastitic cows (Whelehan *et al.* 2011). The current study found that differences in endometrial gene expression of *IL-17A* and *IL-17F* differentiate SCE from healthy cows locally however IL-17 protein concentrations in blood serum could not differentiate SCE from healthy cows systemically. These results are similar to a recent study in mice whereby intrauterine infections with Group A *Streptococcus* (GAS) resulted in elevated IL-17A protein expression in the uterus however systemic levels of IL-17A in serum were not significantly different (Mason *et al.* 2013). Therefore the results of both the current study and work done by Manson *et al.* 2013 would suggest that IL-17A and IL17-F are potential local rather than systemic biomarkers of sub-clinical endometritis in the postpartum endometrium.

5.4.3 Elevated expression of systemic SAA and HP 7 DPP in cows that were subsequently diagnosed as sub-clinically endometritic 21 DPP

The potential of SAA, as an early prognostic and diagnostic for endometritis, was assessed measuring its concentration in healthy and infected cows postpartum. Within the first week postpartum, increased SAA concentrations were observed in the clinically healthy cows, after which concentrations decreased to baseline values. An elevated serum SAA concentration in cows with uterine infection, persisted for 2 months postpartum and then returned to baseline values (Chan *et al.* 2010). An association has been established between a poor prognosis of reproductive performance as a result of the uterine infection metritis and increased HP concentration in postpartum cows (Chan *et al.* 2004, Huzzey *et al.* 2009). A decrease in HP serum concentration in cows that had toxic puerperal metritis was associated with antimicrobial treatment (Smith *et al.* 1998).

In the current study cows that developed endometritis 21 DPP had significantly higher levels of SAA and HP 7 DPP compared to cows that resolved endometrial inflammation 21 DPP. Although systemic levels of SAA and HP were elevated in SCE relative to healthy cows 7 DPP, local expression of SAA and HP did not reflect this trend. In fact endometrial gene expression of *SAA1*,

SAA3, *MSAA3* and *HP* was significantly elevated in healthy cows 7 days relative to 21 DPP and this trend was not observed in SCE cows. Locally there was a significant elevation in *SAA1* gene expression between SCE and healthy cows 21 DPP however this was not reflected at a systemic level. The primary source of systemic serum amyloid A (SAA) and haptoglobin (HP) production is by hepatocytes in response to stimulation by the proinflammatory cytokines, IL-1, TNF and IL-6 released from sites of local inflammation which travel to the liver via the peripheral blood circulation (Eckersall *et al.* 2006). The current study was in agreement with previous studies by our group which demonstrated that local endometrial gene expression of *SAA*, *HP*, *IL-1*, *TNF* and *IL-6* were elevated earlier relative to later postpartum in healthy cows (Chapwanya *et al.* 2012; Foley *et al.* 2012). As the current study also examined the endometrial response in cows that developed sub-clinical endometritis, it has revealed the elevated gene expression of *SAA1*, *TNF* and *IL-6* in SCE cows relative to healthy cows 21 DPP. However at a systemic level there was no significant difference in SAA or IL-6 protein concentrations between groups 21 DPP. Therefore the local differential expression of acute phase proteins in the postpartum endometrium was not reflected at a systemic level which may indicate a more substantial role for these molecules locally rather than systemically in response to sub-clinical endometritis 21 DPP. However systemically it appears that an elevated acute phase response early postpartum may predispose cows to sub-clinical endometritis later postpartum.

Chapter 6

General Discussion

6 General Discussion

The impact of uterine disease and disrupted fertility to the European dairy industry has been estimated to cost €1.4 billion per annum (Sheldon *et al.* 2009). Understanding the role of the immune response in driving bacterial clearance during involution, but also in restoring homeostasis, is likely to lead to new methods for early diagnosis and treatment of uterine disease and improving fertility. However, uterine involution is a complex and demanding process with multiple layers contributing to and influencing its goal to reinstate the physiological function of the uterus prior to breeding, a multi-layered approach is required to best analyse and characterise the whole process. In this regard, the approach of this thesis was to complement global transcriptomic analysis with clinical, cellular, microbial, metabolic and systemic immune data in order to better establish what mechanisms are contributing to and influencing the physiological process of uterine involution early postpartum. Developments in next generation sequencing technologies have provided an opportunity to gain unparalleled resolution in the gene expression changes which underpin the immune response in the uterus. The analysis of the entire transcriptome provides a molecular gateway through which the mechanisms associated with physiological uterine involution and dysregulated endometrial inflammation can be understood in greater detail.

In all cows, a pro-inflammatory immune response was elicited early postpartum, evident by the significant elevated expression of immune genes and pathways. In both the pilot and main studies the temporal transcriptomic transition was quite distinct between postpartum time points in healthy cows. Gene enrichment of pathways associated with tissue proliferation and calcium signalling dominated later postpartum compared to earlier postpartum. Therefore, immune activation and inflammation is a transient feature in the healthy postpartum endometrium with a temporal switch toward tissue repair, proliferation and calcium signalling pathways that restore homeostasis and prepare the uterus for a subsequent pregnancy.

However, in SCE cows this resolution or transition to tissue proliferation and calcium signalling does not occur. The influx of neutrophils in the uterine biopsy was underpinned by sustained expression of inflammatory genes. An endometrial cellular profile, predominated by leukocytes and indicative of sustained inflammation 21 DPP, either prevents or delays this temporal switch from inflammation 7 DPP towards calcium signalling in particular 21 DPP. This is especially evident when the transcriptomic profile of sub clinically endometritic (SCE) cows is compared to healthy cows 21 DPP, whereby the expression of genes involved in inflammation is elevated in SCE cows, while simultaneously the expression of genes involved in calcium signalling is elevated in healthy

cows. Inflammation at both cellular and molecular levels was elevated in sub-clinically endometritic cows and the shift in microbial populations may be a contributing factor to the sustained inflammation observed in these cows 21 DPP.

6.1 A Shift in Microbial Populations in the Postpartum Endometrium

Previous studies of the microbial infection in the uterus have relied on culture dependent techniques. In this study using culture independent techniques revealed a shift in microbial populations in the uterus which was significantly different in SCE compared to healthy cows. This result provides a fertile foundation for further research which should be directed towards identifying specific bacterial species that may contribute to the differential sustained inflammation observed in SCE cows. However, these results also highlight the dilemma as to whether sustained endometrial inflammation is the result or cause of a shift in microbial populations. In other words which is the dominant causative factor of sustained endometrial inflammation, the host or microbe or is it a combination of both?

Our results do indicate that not all sub-clinical endometrial inflammation can be attributed directly to the shift in microbial populations, as some SCE samples cluster closely with healthy samples and visa versa. This suggests that in certain instances the host's immune response may be causing and driving sustained endometrial inflammation irrespective of the microbial populations present early postpartum. What is clear is that despite the heterogeneity of microbial populations published by our group (Chapwanya *et al.* 2012), by others (Williams *et al.* 2005) and in this study, enhanced expression of inflammatory genes and potential dysregulation of the immune response is a consistent feature of cows with SCE.

The elevated gene expression of immune genes such as the pattern recognition receptor (PRR) *TLR2* and its accessory recognition molecules *LBP*, *MD2*, *CD14* and *TLR4*, may indicate a greater requirement to recognise gram-positive bacteria in SCE relative to healthy cows 21 DPP and also in healthy cows 7 days relative to 21 days postpartum. Simultaneously in healthy cows there is an elevation of genes encoding phosphoinositide 3-kinases (PI3Ks), *PIK3CA*, *PIK3CB* and *PIK3CD* at 7 days relative to 21 DPP, which are involved in the negatively regulating TLR signalling. The elevated expression of these genes may act to protect the host from over activation of TLRs (Hazeki *et al.* 2007; Li *et al.* 2010). Although genes, involved in recognition of PAMPs on gram-positive bacteria, are up regulated in SCE relative to healthy cows 21 DPP, PI3K genes are not differentially expressed. Therefore, SCE cows may recognise PAMPs in gram-positive bacteria, however the safety mechanism negatively regulating TLR signalling is not occurring which may

result in over stimulation and an excessive inflammatory response. Therefore, this may suggest a host specific factor in SCE cows which may be contributing to sustained inflammation in the presence of gram-positive bacteria.

6.2 S100A Family of Proteins and Endometrial Inflammation Postpartum

The genes *TLR2*, *CD14*, *MD2*, *TNF*, *IL-1 β* , *IL-17C*, *S100A8* and *S100A9* are differentially elevated 7 days relative to 21 DPP in healthy cows and these genes are also elevated in SCE cows relative to healthy cows 21 DPP. This suggests that the elevated expression of these genes is associated both with early inflammation in healthy cows and sustained inflammation in SCE cows. The expression of IL-17C has been shown to be elevated in the presence of agonists for TLR2 (lipopeptides) and also in the presence of TNF and IL-1 β in epithelial cells. Subsequently IL-17C elevates the expression of S100A7, S100A8 and S100A9 which are part of the S100 family proteins involved in cellular processes such as cell growth and differentiation, calcium-dependent cell signalling, cell cycle regulation and antimicrobial defence (Abtin *et al.* 2010; Ramirez-Carrozzi *et al.* 2011). It is interesting in our study that healthy cows appear to require S100A7, S100A8 and S100A9 expression early postpartum and SCE cows have elevated expression of these genes 21 DPP relative to healthy cows.

In bovine endometrial epithelial and stromal cells, gene expression of S100A8, S100A9 and S100A12 was elevated in response to LPS (Swangchan-Uthai *et al.* 2012). However, the major producers of S100A8, S100A9 and S100A12 proteins are activated phagocytic cells such as macrophages and neutrophils (Donato *et al.* 2013; Foell *et al.* 2004; Kessel *et al.* 2013). These S100A proteins have intracellular and extracellular functions, and their excretion from phagocytic cells is either an energy dependent event from activated cells, passive from necrotic cells or during the excretion of neutrophil extracellular traps (NETs) (Urban *et al.* 2009; Vogl *et al.* 2004; Rammes *et al.* 1997). The intracellular functions of S100A8 involve scavenging reactive oxygen species produced by activated neutrophils and in these cells may stabilize nitric oxide, thereby exerting anti-inflammatory effects (Lim *et al.* 2008). S100A9 deficient neutrophils, dendritic cells and macrophages have reduced immune function suggesting an intracellular role for S100A in pro-inflammatory responses (Vogl *et al.* 2007). The intracellular S100A8/S100A9 complex involved in phagocytosis, degranulation and migration of activated neutrophils and monocytes (Foell *et al.* 2007; Vogl *et al.* 2004). Interestingly in S100A8 knockout mice embryos an extracellular immunoregulatory role for S100A8 was observed when maternal cells infiltrated the null embryo causing embryo resorption. This work suggested a non-redundant role for S100A8 preventing

maternal rejection of the implanting embryo (Passey *et al.* 1999). Additional extracellular immune functions of S100A8 include promoting neutrophil chemotaxis, stimulation of TLR4 to induce TNF and IL1 β production in bone marrow cells and it also has oxidant scavenging capacity which may implicate a role for it in the resolution of inflammation. S100A9 also has both pro- and anti-inflammation extracellular functions. Pro-inflammatory activities include enhancing leukocyte chemotaxis, adhesion, bactericidal activity and degranulation of neutrophils and stimulating cytokine production via the TLR4 and Receptor for Advanced Glycation End-products (RAGE). Anti-inflammatory functions include suppressing neutrophil migration, macrophage activation TNF production and scavenging reactive oxygen species. The S100A8/S100A9 complex also has dual pro- and anti-inflammatory actions by stimulating cytokine production and suppressing the activities of pro-inflammatory cytokines (Donato *et al.* 2013).

The intracellular and extracellular functions of S100A8, S100A9 and S100A8/S100A9 are heavily involved in the immune response and there is evidence from our results that these molecules are involved during postpartum endometrial inflammation. However, these molecules also function as extracellular Damage Associated Molecular Patterns (DAMPs) which are produced and released when inflammation is triggered by sterile stimuli in absence of infection, for example following tissue injury or necrotic cell death. The resulting pro-inflammatory mechanisms triggered in response to sterile tissue injury or necrotic cell death, are similar to those during microbial infection, the reason being that the same PRRs recognise both PAMPs and DAMPs. The S100A8/S100A9 complex and S100A8 bind and directly signal through the TLR4, MD2 and CD14 complex stimulating the MYD88-dependent pathway culminating in chemokine and cytokine production (Vogl *et al.* 2007). A DAMP specific recognition receptor known as RAGE has the ability to recognise S100/calgranulin family members, S100A8/S100A9 and S100A12 (Chen & Nuñez 2010; Hofmann *et al.* 1999). Our results revealed the elevated endometrial gene expression of *MD2*, *CD14*, *TLR4*, *S100A8*, *S100A9* and *S100A12* at 7 days relative to 21 DPP in healthy cows and with the exception of *TLR4* the remaining genes are also elevated in SCE cows relative to healthy cows 21 DPP. Therefore, in addition to PAMP associated stimulation of the inflammatory response early postpartum in healthy cows, there is also the potential for elevated DAMP stimulation through the PRR TLR4. The process of parturition and subsequent postpartum uterine involution involves extensive tissue damage, degradation and removal, the superficial layer of the caruncle tissue commences sloughing at 6 and 7 days postpartum, allowing repair and rejuvenation of new endometrial tissue (Archbald *et al.* 1972, Gier & Marion 1968). This is a source of sterile endometrial inflammation postpartum which all postpartum cows are subject to and it resonates with the expression profile of elevated DAMP production 7 days relative to 21

DPP in healthy cows. However, in SCE cows the production of DAMPs appears to be sustained between 7 and 21 DPP and elevated 21 DPP compared to healthy cows.

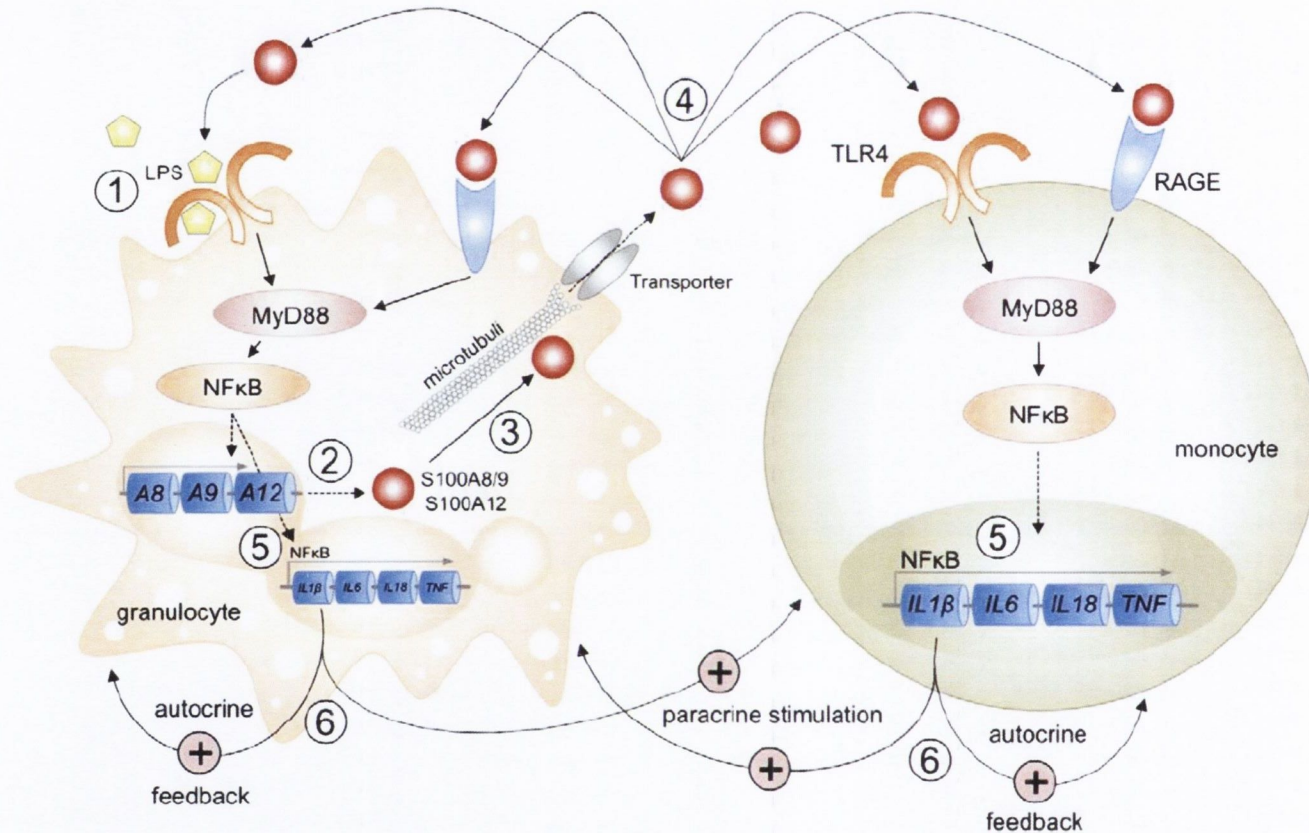


Figure 6.2-1: S100A8/S100A9 and S100A12 enhance inflammatory mechanisms. (1) A pro-inflammatory stimulus triggers NFκB associated expression of (2) S100A/S100A9 and S100A12 through TLR4 on granulocytes. (3) Proteins are excreted from cells through a tubulin-dependent alternative secretory pathway. (4) S100A8/S100A9 and S100A12 bind TLR4 and RAGE in the extracellular environment on target cells. (5) The expression of pro-inflammatory cytokines is stimulated. (6) IL-1β triggers autocrine production of itself from other cells. (picture reference: (Kessel *et al.* 2013)).

It is possible that elevated *S100A8*, *S100A9* and *S100A12* gene expression is simply a reflection of the elevated numbers of immune cell infiltrate in SCE relative to healthy cows 21 DPP. During acute inflammation, elevated levels of systemic *S100A8*, *S100A9* and/or *S100A12* proteins in humans and mice, as a result of activated macrophages and extravasating neutrophils at inflammatory sites, have been identified as non-specific markers of phagocyte activation (Donato *et al.* 2013; Foell *et al.* 2004; Kessel *et al.* 2013). Future work identified as a result of thesis would be to measure protein levels of systemic *S100A8*, *S100A9* and/or *S100A12* in cows with sustained endometrial inflammation 21 DPP to assess if there are elevated levels in these cows compared to healthy cows.

Other members of the *S100* family of proteins are elevated 7 days relative to 21 DPP in healthy cows and also elevated in SCE relative to healthy cows 21 DPP which include *S100A2*, *S100A3*, *S100A5*, and *S100A14* which may also be involved in regulating key cellular processes during sterile and non-sterile endometrial inflammation. A novel finding of this study was the elevated expression of *S100A2*, *S100A3*, *S100A5*, and *S100A14* during postpartum endometrial inflammation and further study would be required to assess the role of these molecules during postpartum involution.

6.3 Interleukin (IL)-17A and IL17-F Biomarkers of Subclinical Endometritis

The differential expression of a number of gene classes has been confirmed by this study. In particular, a role for the cytokine IL-17 in sustained endometrial inflammation postpartum is suggested. This novel research finding may be useful to inform targeted studies of the regulation of immunity in the postpartum dairy cow. It may be the case that tighter regulation of IL-17 in some cows prevents the sustained inflammatory profile that we have associated with the development of sub-clinical endometritis. Subsequent to stringent gene expression analysis and filtering, interleukin (IL)-17A and IL-17F were:

- 1) The most highly significantly elevated genes in SCE relative to healthy cows at both 7 and 21 DPP.
- 2) Have predicted peptide secretory domains, indicating the potential for secretion into the extracellular space.

IL-17 was quantified systemically to assess the influence of the SCE endometrium on the systemic immune profile however IL-17 could not be detected in all samples. Inflammation in the

endometrium did not influence the systemic immune profile. Significant inflammation in the endometrium does not necessarily lead to systemic inflammation. In agreement with our results other studies have shown that significant expression of IL-17 in the endometrium was not found to be significantly different in the serum of dairy goats with mastitis (Jing *et al.* 2012) and either was its gene expression in systemic mononuclear cells differentially expressed systemically in mastitic cows (Whelehan *et al.* 2011). A similar conclusion was drawn from a study in mice which indicate that IL-17A and IL17-F are potential local biomarkers of sub-clinical endometritis in the postpartum endometrium (Mason *et al.* 2013). The specificity of IL-17 to differentiate SCE from healthy cows both 7 and 21 DPP is very exciting and offers a novel biomarker of SCE in postpartum cows.

Locally the effects of IL-17 are initiated once recognised by its receptor IL-17RA which binds to at least three members of the IL-17 family including IL-17A and IL-17F (Gaffen 2009) and is expressed in almost all tissues (Moseley *et al.* 2003). Activation of the IL-17 receptor by its ligands stimulates somatic cells to produce chemokines such as IL-8, CXCL1, CXCL5, CXCL6 and MCP-1, antimicrobial peptides, G-CSF, IL-6, prostaglandin E₂, ICAM-1 and matrix metalloproteinase, accumulatively resulting in an increased pro-inflammatory response during infections and autoimmune disease (Mensikova *et al.* 2013). Multiple autoimmune diseases are associated with the dysregulation of Th17 cells and/or the production of IL-17, including Multiple Sclerosis (Hofstetter *et al.* 2009), Rheumatoid Arthritis (van den Berg & Miossec 2009), Systemic Lupus Erythematosus (Crispín *et al.* 2010), Psoriasis (Lowes *et al.* 2008), Inflammatory Bowel Disease (Sarraf *et al.* 2010) and Type 1 Diabetes (Emamaullee *et al.* 2009). Recently two phase II clinical trials which targeted the IL-17 pathway in psoriasis displayed significant improvement clinically in moderate to severe psoriasis (Ariza *et al.* 2013). The elements of the IL-17 pathway that were targeted included blocking the IL-17 receptor (Papp *et al.* 2012) and blocking the IL-17A cytokine itself (Leonardi *et al.* 2012). In addition to acting as a potential biomarker of sub-clinical endometritis 7 and 21 DPP, blocking the effects of IL-17 by targeting its receptor and IL-17A may have a beneficial role early in postpartum endometritis.

6.4 Calcium Signalling and Fertility Postpartum

During endometrial inflammation in the current study, expression of genes associated with prostaglandin E (PGE) production, *TNF*, *PTGS1* and *PTGES*, and receptor recognition of PGE, *PTGER4*, are elevated 7 days relative to 21 DPP in healthy cows and are also elevated in SCE cows relative to healthy cows 21 DPP. The uterus is the predominant producer of prostaglandin F_{2α}

(PGF) and prostaglandin E (PGE). PGF permits the regression of the corpus luteum (luteolysis) in the absence of an embryo at the end of the estrus cycle thus allowing a subsequent estrus cycle to progress (Asselin *et al.* 1996; Burns *et al.* 1998) having a positive effect on fertility. PGE is a luteotrophic molecule which is associated with prolonged luteal phases contributing to sub-fertility in cows (Opsomer *et al.* 2000; Williams *et al.* 2007). An elevated bacterial presence and production of TNF regulate the production of PGF and PGE in the bovine endometrium (Miyamoto *et al.* 2000; Murakami *et al.* 2001; Herath, Lilly, Fischer, *et al.* 2009). PGE acts through the prostaglandin receptors 2 and 4 (*PTGER2* and *PTGER4*), which are expressed on bovine endometrial cells and are usually associated with the down regulation of inflammation. This provides a feedback loop that may be important in the regulation of the immune response to *E. coli* in the bovine uterus (Herath *et al.* 2009, Sheldon *et al.* 2009, Sheldon *et al.* 2009). Although we cannot definitively confirm or deny that a bacterial presence or a specific microbial population is responsible for the elevation of these genes from the results of this study, by reference to the literature it would be intuitive to suspect that a bacterial presence is inferring a probable influence on the elevated expression of these genes associated with PGE production. This would then support the link between sub-fertility and sustained endometrial inflammation during the presence of a persistent bacterial infection early postpartum.

In the initial hours and days postpartum, calcium is a critical mineral required for the synthesis and secretion of colostrum and milk in dairy cows. It is vital to mobilise calcium stores within the body to ensure an adequate systemic pool of calcium so that homeostasis is maintained during this period (Goff 2008). During the first three days postpartum, reduced serum concentrations of calcium is associated with reduced whole blood neutrophil counts, impaired systemic neutrophil function and an increased risk of metritis (Martinez *et al.* 2012). Although the availability of calcium is associated with postpartum metritis, our results suggest that lacking the infrastructure necessary to facilitate calcium signalling in the endometrium may be closely linked with sustained endometrial inflammation. Evaluation of transcriptomic data revealed enrichment of calcium signalling pathways 21 DPP while immune associated pathways were enriched 7 DPP in healthy cows. In addition to this, the direct comparison between SCE and healthy cows 21 DPP highlighted that immune associated pathways were enriched in SCE cows while in healthy cows the enrichment of calcium signalling pathways was evident. These results raise two interesting questions:

- 1) Regardless of the extent of calcium availability, if the infrastructure to facilitate calcium signalling is not present will this contribute in a similar way to uterine disease?

- 2) Does inflammation somehow reduce the capacity of the endometrium for calcium signalling or does reduced calcium signalling promote endometrial inflammation?

PGF is elevated in the uterus in response to oxytocin receptor activation, by oxytocin produced by the corpus luteum, which elicits a calcium signalling cascade, elevating cytosolic calcium concentrations (Asselin *et al.* 1996; Burns *et al.* 1998). Important components that link this change in calcium concentration and PGF synthesis are phospholipase A2 (PLA₂) and prostaglandin F_{2α} synthase (PGFS) enzymes (Leslie 2004; Madore *et al.* 2003). The genes encoding PLA₂ (*PLA2G4E*, *PLA2G4D*, *PLA2G3* and *PLA2G2F*) and PGFS (*FAM213B*), are elevated in healthy cows 21 days compared to 7 DPP in parallel with the enrichment of calcium signalling pathways. As alluded to earlier, PGF has positive effects on postpartum fertility, therefore due to the prominent role of calcium signalling in PGF production it may have essential implications for postpartum fertility. Interestingly a recent study has suggested targeting the calcium influx of neutrophils to reduce their activation during neutrophil-dependent diseases in cattle (Burgos *et al.* 2011). However from the results presented in this study the implications of using calcium influx blockers to reduce neutrophil activity during sustained inflammation may also impede upon the potential of endometrial PGF production observed in healthy cows and subsequently on postpartum fertility.

6.5 microRNA Regulation of Proliferation During Inflammation

Elevated calcium signalling may also have been essential in eliciting and regulating a number of cellular functions including cell proliferation and differentiation in healthy cows 21 DPP. One of the major findings from the pilot study was the transition towards greater tissue proliferation 30 DPP, subsequent to an elevated inflammatory immune response 15 DPP in healthy cows. A recent study in the bovine postpartum endometrium revealed a role for miRNAs that target and negatively affect genes involved in tissue proliferation during the development and progression of postpartum sub-clinical endometritis (Hailemariam *et al.* 2013). The miR-200 microRNA family members negatively regulate cellular proliferation, migration and/or invasion in the human endometrium (Nothnick 2012). All five members of the mir-200 family, *mir-200a*, *mir-200b*, *mir-200c*, *mir-141* and *mir-429*, were significantly elevated 7 days relative to 21 DPP in SCE cows and not in healthy cows. This would suggest a potential to negatively regulate cellular proliferation at a microRNA level in the endometrium (Panda *et al.* 2012) of sub-clinically endometritic cows which is not present in healthy cows 7 DPP. Preventing the translation of mRNAs associated with proliferation may be a characteristic factor of subclinical endometritis allowing greater energy and focus to be directed towards the resolution of inflammation rather than a temporal switch

towards cellular growth and proliferation. In all tissues, features of wound healing follow a sequential process of (Hart 2002a, Velnar *et al.* 2009):

- 1) coagulation and haemostasis
- 2) inflammation
- 3) proliferation of new tissue
- 4) tissue remodelling

The progress to cellular proliferation from inflammation may be blocked in the case of SCE cows the presence of sustained inflammation which may be due in part to the elevation of the mir-200 family of microRNAs. A novel finding of this study was the differential elevation of three microRNA genes, *bta-mir-205*, *bta-mir-375* and *bta-mir-197*, in SCE relative to healthy cows 7 DPP. Further study would be required before defining the functional relevance of these microRNAs during the early postpartum period in bovine endometrium.

6.6 Conclusion

In conclusion, this thesis represents the first study to use next generation sequencing to characterise the uterine transcriptome. Inflammation at both a cellular and molecular level was elevated in healthy and SCE cows 7 DPP but was resolved in the former group by 21 DPP. In SCE cows, sustained expression of inflammatory genes is likely to contribute to negative fertility outcomes. Culture-independent microbial analysis confirmed divergent bacterial populations between healthy and SCE cows. A result of this research, reduced mRNA expression of genes involved in calcium signalling 21 DPP and increased expression of miRNAs that can regulate genes associated with cellular proliferation 7 DPP, are novel indicators of subclinical endometritis. This study has successfully identified potential local biomarkers of endometritis (IL-17) and also identified some interesting candidates for systemic analysis in additional animals in future work. SAA and HP were both significantly elevated 7 DPP in SCE cows relative to healthy cows which could prove to be an early warning sign of future subclinical endometritis 21 DPP.

Overall, a more comprehensive picture of the immune response has emerged at multiple levels, which represents an important advancement in our understanding of uterine immunity. This research has highlighted a number of research avenues that can now be explored using hypothesis-driven approaches. In the context of the anticipated expansion of the dairy herd in the post-quota era, a greater understanding of the immune response in the postpartum period is a welcome and critical development.

6.7 Future Work

The results of this thesis highlighted that there was a different bacterial population in sub-clinically endometritic compared to healthy cows however not all sub-clinical endometrial inflammation can be attributed directly to this shift in microbial populations, as some SCE samples cluster closely with healthy samples. However further study would have to be carried out to investigate this hypothesis and definitively clarify this. What may be important to consider for future investigations are the variations and outliers at an individual animal level in order to truly comprehend the role of the host versus the role of the microbe during sub-clinical endometrial inflammation.

As concluded from our results and the research of others IL-17A and IL17-F are potential local biomarkers of sub-clinical endometritis in the postpartum endometrium (Mason *et al.* 2013). The specificity of IL-17 to differentiate SCE from healthy cows both 7 and 21 DPP is very exciting and offers a novel biomarker of SCE in postpartum cows. The results here provide the platform from which to base future studies on, in order to develop early methods of SCE detection. Although IL-17A and IL-17F gene expression is elevated in SCE cows both 7 and 21 DPP unless the receptor IL-17RA for the protein products of these genes is adequately expressed on local tissue during inflammation the downstream actions of elevated IL-17 protein production would have limited impact. This is evident in IL-17RA KO mice which were more susceptible to extracellular gram negative bacterial than were IL-17A KO mice (Aujla *et al.* 2008). In the current study healthy cows have elevated gene expression of IL-17RA, 7 days relative to 21 DPP whereas this trend in SCE cows is not repeated and IL-17RA was not significantly different between healthy and SCE cows at either postpartum time point. What would be interesting as an avenue of future study would be to assess if there are polymorphisms in the genetic code underpinning the IL-17RA gene between SCE and healthy cows which may either repress or enhance the ability of endometrial tissue to react to postpartum infection, respectively.

During acute inflammation, elevated levels of systemic S100A8, S100A9 and/or S100A12 proteins in humans and mice, as a result of activated macrophages and extravasating neutrophils at inflammatory sites, have been identified as non-specific markers of phagocyte activation (Donato *et al.* 2013; Foell *et al.* 2004; Kessel *et al.* 2013). It is evident from the results of this thesis that the S100A family of proteins are heavily involved during endometrial inflammation as their gene expression was elevated both in the SCE cows relative to healthy cows 21 DPP and also 7 DPP relative to 21 DPP in healthy cows. If these markers could be detected systemically they have the

potential to differentiate SCE from healthy cows 21 DPP. Future work identified as a result of this thesis would be to measure protein levels of systemic S100A8, S100A9 and/or S100A12 in cows with sustained endometrial inflammation 21 DPP to assess if there are elevated levels in these cows compared to healthy cows. Other members of the S100 family of proteins are elevated 7 days relative to 21 DPP in healthy cows and also elevated in SCE relative to healthy cows 21 DPP which include S100A2, S100A3, S100A5, and S100A14 which may also be involved in regulating key cellular processes during sterile and non-sterile endometrial inflammation. A novel finding of this study was the elevated expression of S100A2, S100A3, S100A5, and S100A14 during postpartum endometrial inflammation and further study would be required to assess the role of these molecules during postpartum involution.

7 Bibliography

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8 Appendices

Table A-1: Primer details for candidate and reference genes used in real-time qRT-PCR

Ensembl ID	Gene Symbol	Primer Sequence	
ENSBTAG00000019234	BMP6	Forward	AACAGCAGCGAGCTGAAGAC
		Reverse	TGGTTGGTAGCGTTCATGTG
ENSBTAG00000017697	CTF1	Forward	CCGCCTCCTACCAAATA
		Reverse	GTTCACTCGCCCTGGAG
ENSBTAG00000007421	CDH5	Forward	TCTGCATCCTCACCTTCACA
		Reverse	TGCACCTGAGCGTAAAGAGA
ENSBTAG00000026278	CLDN4	Forward	GTATCATCCTGGCCGTGTTT
		Reverse	GGTTGTAGAAGTCGCGGATG
ENSBTAG00000007392	SERPINA14	Forward	TCTGCTTCCAAGATTGACC
		Reverse	CTGTGTGAATGGCTGTGTCC
ENSBTAG00000019707	GATA2	Forward	AAACTGTCAGACGACGACCA
		Reverse	GGACCCCTTCTTGCTCTTCT
ENSBTAG00000011082	IGF1	Forward	CATCCTCCTCGCATCTCTTC
		Reverse	CTCCAGCCTCCTCAGATCAC
ENSBTAG00000012503	MGAT3	Forward	ATCAACGCCATCAACATCAA
		Reverse	GGAAGTGGTCCAGAAAGACG
ENSBTAG00000022394	SAA1/2	Forward	AACTATGACGCTGCCCAAAG
		Reverse	AGCAGGTCTGAAGTGGTTGG
ENSBTAG00000039856	DEFB6	Forward	TCTTCTGGTCCTGTCTGCT
		Reverse	TTACCTCCACCTGCAGCATT
ENSBTAG00000011887	CD22	Forward	GGACGACAGGGTCACCTATG
		Reverse	CAGTGCTTGAGGGTCACGTA
ENSBTAG00000001029	RASGRP2	Forward	GTGGTGTGAAGTGCCACAAG
		Reverse	ACCGTCTGAACCTCCTCCTC
ENSBTAG00000026489	TNFRSF13C	Forward	CCAGAGTCCCTGGATGTTGA
		Reverse	CAGCTCAGTGGAAACCCAGTT
ENSBTAG00000016072	SHC2	Forward	CTGGTACCATGGCCAGATG
		Reverse	CCCATTCTGCAGGTGGTAGT
ENSBTAG00000007173	PDGFRA	Forward	GACTCCGACAACGCCTACAT
		Reverse	TGCTGGAACCTGTCTCAATG
ENSBTAG00000004215	RARRES2	Forward	TGCATCAAGCTGGACTCAA
		Reverse	AAACTGTCCGGGGAGGTAGT
ENSBTAG00000020939	PLAC9	Forward	CGAGGAGACTCGGCTCAG
		Reverse	CAAGTTCAGGCCAGCTC
ENSBTAG00000020221	WNT5A	Forward	ATGAACCTGCACAACAACGA
		Reverse	CTTCTCCTTCAGGGCATCAC

ENSBTAG00000020143	RDH10	Forward	CCTCTGAAGCCCGACTACTG
		Reverse	TTTCGCTTCGTTGTTGTTTG
ENSBTAG00000026199	ACTB	Forward	AGATGACCCAGATCATGTTCGA
		Reverse	TGACCCCGTCACCGGAGTCCATCACGAT
ENSBTAG00000014731	GAPDH	Forward	CTCCCAACGTGTCTGTTGTG
		Reverse	TGAGCTTGACAAAGTGGTCG

Table A-2: Total input RNA of 4µg for mRNA-Seq

Cow ID	DPP	RNA Concentration		4 ug Input RNA		
		ng/µl	ug/µl	RNA (µl)	H2O (µl)	Final Vol (µl)
1	7	1158	1.2	3.5	46.5	50
1	21	2119.6	2.1	1.9	48.1	50
2	7	1481.6	1.5	2.7	47.3	50
2	21	2544.5	2.5	1.6	48.4	50
4	7	1074.1	1.1	3.7	46.3	50
4	21	2971.2	3	1.3	48.7	50
5	7	1542.4	1.5	2.6	47.4	50
5	21	1750.3	1.8	2.3	47.7	50
6	7	2413.1	2.4	1.7*	48.3	50
6	21	1980.2	2	2	48	50
7	7	955.9	1	4.2	45.8	50
7	21	3884.4	3.9	1	49	50
8	7	3679.5	3.7	1.1	48.9	50
8	21	1872.8	1.9	2.1	47.9	50
9	7	2719.9	2.7	1.5	48.5	50
9	21	3718.7	3.7	1.1	48.9	50
10	7	2097.1	2.1	1.9	48.1	50
10	21	3836.6	3.8	1	49	50
11	7	1357.6	1.4	2.9	47.1	50
11	21	2619.4	2.6	1.5	48.5	50
12	7	2766.2	2.8	1.4	48.6	50
12	21	3486.1	3.5	1.1	48.9	50
13	7	3886.3	3.9	1	49	50
13	21	1969.1	2	2	48	50
14	7	1817.5	1.8	2.2	47.8	50
14	21	1240.1	1.2	3.2	46.8	50
15	7	794.9	0.8	5	45	50
15	21	2414.1	2.4	1.7	48.3	50
16	7	1591.8	1.6	2.5	47.5	50
16	21	2647.6	2.6	1.5	48.5	50

Table A-3: Adapter indexes and sequences for cDNA sequencing libraries and multiplexed pools.

Cow ID	DPP	Pool	Adapter	Set	Index	Adapter Index Sequence
1 (TRP1)	7	1	AR003	B	TTAGGC(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC TTAGGCAT CTCGTATGCCGTCTTCTGCTTG
1 (TRP1)	21		AR009	B	GATCAG(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GATCAGAT CTCGTATGCCGTCTTCTGCTTG
2	7		AR025	B	ACTGAT(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ACTGATAT ATCTCGTATGCCGTCTTCTGCTTG
6 (TRP1)	21		AR021	B	GTTTCG(G)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GTTTCGGA AATCTCGTATGCCGTCTTCTGCTTG
2	21		AR023	B	GAGTGG(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GAGTGGAT ATCTCGTATGCCGTCTTCTGCTTG
4	7	2	AR022	B	CGTACG(T)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CGTACGTA AATCTCGTATGCCGTCTTCTGCTTG
4	21		AR027	B	ATTCCT(T)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ATTCCTT ATCTCGTATGCCGTCTTCTGCTTG
1 (TRP2)	21		AR009	B	GATCAG(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GATCAGAT CTCGTATGCCGTCTTCTGCTTG
5	7	3	AR001	B	ATCACG(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ATCACGAT CTCGTATGCCGTCTTCTGCTTG
5	21		AR010	B	TAGCTT(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC TAGCTTAT CTCGTATGCCGTCTTCTGCTTG
6	7		AR020	B	GTGGCC(T)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GTGGCCT TATCTCGTATGCCGTCTTCTGCTTG
1 (TRP3)	7		AR003	B	TTAGGC(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC TTAGGCAT CTCGTATGCCGTCTTCTGCTTG
6 (TRP3)	21		AR021	B	GTTTCG(G)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GTTTCGGA AATCTCGTATGCCGTCTTCTGCTTG
7	7	4	AR005	A	ACAGTG(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ACAGTGAT CTCGTATGCCGTCTTCTGCTTG
7	21		AR006	A	GCCAAT(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GCCAATAT CTCGTATGCCGTCTTCTGCTTG
8	7		AR012	A	CTTGTA(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CTTGTAAT CTCGTATGCCGTCTTCTGCTTG
8	21		AR019	A	GTGAAA(C)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GTGAAACGAT CTCGTATGCCGTCTTCTGCTTG
9	7		AR013	A	AGTCAA(C)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC AGTCAACA AATCTCGTATGCCGTCTTCTGCTTG
9	21	5	AR002	A	CGATGT(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CGATGTAT CTCGTATGCCGTCTTCTGCTTG
10	7		AR004	A	TGACCA(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC TGACCAAT CTCGTATGCCGTCTTCTGCTTG
10	21		AR007	A	CAGATC(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CAGATCAT CTCGTATGCCGTCTTCTGCTTG

11	7		AR016	A	CCGTCC(C)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CCGTCCC GATCTCGTATGCCGTCTTCTGCTTG
11	21		AR014	A	AGTTCC(G)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC AGTTCCG TATCTCGTATGCCGTCTTCTGCTTG
12	7	6	AR005	A	ACAGTG(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ACAGTGAT CTCGTATGCCGTCTTCTGCTTG
12	21		AR006	A	GCCAAT(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GCCAATAT CTCGTATGCCGTCTTCTGCTTG
13	7		AR012	A	CTTGTA(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CTTGTAAT CTCGTATGCCGTCTTCTGCTTG
13	21		AR019	A	GTGAAA(C)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GTGAAAC GATCTCGTATGCCGTCTTCTGCTTG
14	7		AR015	A	ATGTCA(G)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC ATGTCA AATCTCGTATGCCGTCTTCTGCTTG
14	21		AR002	A	CGATGT(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CGATGTAT CTCGTATGCCGTCTTCTGCTTG
15	7	7	AR004	A	TGACCA(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC TGACCAAT CTCGTATGCCGTCTTCTGCTTG
15	21		AR007	A	CAGATC(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CAGATCAT CTCGTATGCCGTCTTCTGCTTG
16	7		AR016	A	CCGTCC(C)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC CCGTCCC GATCTCGTATGCCGTCTTCTGCTTG
16	21		AR018	A	GTCCGC(A)	5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC GTCCGCACAT CTCGTATGCCGTCTTCTGCTTG
16	21					

Table A-4: Pooling of multiplexed cDNA sequencing libraries for mRNA-Seq.

Cow ID	DPP	Pool	Stock (μl)	Buffer (μl)	DNA (μl)
1 (TRP1)	7	Pool 1	25	23	2
1 (TRP1)	21		25	22.5	2.5
2	7		25	22.1	2.9
6 (TRP1)	21		25	23.2	1.8
2	21		25	21.8	3.2
4	7	Pool 2	25	21.3	3.7
4	21		25	21.3	3.7
1 (TPR2)	21		25	22.5	2.5
5	7	Pool 3	25	22.5	2.5
5	21		25	22.6	2.4
6	7		25	22.1	2.9
1 (TRP3)	7		25	23	2
6 (TRP3)	21		25	23.2	1.8
7	7	Pool 4	25	21.9	3.1
7	21		25	22.2	2.8
8	7		25	22.7	2.3
8	21		25	22.1	2.9
9	7		25	22.5	2.5
9	21	Pool 5	25	21.3	3.7
10	7		25	22.4	2.6
10	21		25	21.6	3.4
11	7		25	21.8	3.2
11	21		25	21.3	3.7
12	7	Pool 6	25	22.5	2.5
12	21		25	20.4	4.6
13	7		25	22.6	2.4
13	21		25	22.9	2.1
14	7		25	23.2	1.8
14	21	Pool 7	25	22.1	2.9
15	7		25	22.4	2.6
15	21		25	22.9	2.1
16	7		25	22.8	2.2
16	21		25	22.8	2.2

TableA-5: micro-RNA analysis - adapter indexes and sequences for cDNA sequencing libraries and multiplexed pools.

Sample name	DPP	Index	Sequence
1	7	Index 1 (RPI1)	5' CAAGCAGAAGACGGCATAACGAGATCGTGAT GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
1	21	Index 2 (RPI2)	5' CAAGCAGAAGACGGCATAACGAGATACATCG GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
2	7	Index 3 (RPI3)	5' CAAGCAGAAGACGGCATAACGAGATGCCTAA GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
2	21	Index 4 (RPI4)	5' CAAGCAGAAGACGGCATAACGAGATTGGTCA GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
5	7	Index 5 (RPI5)	5' CAAGCAGAAGACGGCATAACGAGATCACTGT GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
5	21	Index 6 (RPI6)	5' CAAGCAGAAGACGGCATAACGAGATATTGGC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
6	7	Index 7 (RPI7)	5' CAAGCAGAAGACGGCATAACGAGATGATCTG GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
6	21	Index 8 (RPI8)	5' CAAGCAGAAGACGGCATAACGAGATTCAAGT GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
9	7	Index 9 (RPI9)	5' CAAGCAGAAGACGGCATAACGAGATCTGATC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
9	21	Index 10 (RPI10)	5' CAAGCAGAAGACGGCATAACGAGATAAGCTA GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
12	7	Index 11 (RPI11)	5' CAAGCAGAAGACGGCATAACGAGATGTAGCC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
12	21	Index 12 (RPI12)	5' CAAGCAGAAGACGGCATAACGAGATTACAAG GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
13	7	Index 13 (RPI13)	5' CAAGCAGAAGACGGCATAACGAGATTTGACT GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
13	21	Index 14 (RPI14)	5' CAAGCAGAAGACGGCATAACGAGATGGAAC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
14	7	Index 15 (RPI15)	5' CAAGCAGAAGACGGCATAACGAGATTGACAT GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
14	21	Index 16 (RPI16)	5' CAAGCAGAAGACGGCATAACGAGATGGACGG GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
15	7	Index 17 (RPI17)	5' CAAGCAGAAGACGGCATAACGAGATCTCTAC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
15	21	Index 18 (RPI18)	5' CAAGCAGAAGACGGCATAACGAGATGCGGAC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
16	7	Index 19 (RPI19)	5' CAAGCAGAAGACGGCATAACGAGATTTTCAC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
16	21	Index 20 (RPI20)	5' CAAGCAGAAGACGGCATAACGAGATGGCCAC GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

Table A-6: Whole blood cell counts in pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP) .

	One Way ANOVA <i>p</i> - value ¹	SCE			Healthy			7DPP			21DPP		
		7 DPP	21 DPP	7 v 21 DPP	7 DPP	21 DPP	7 v 21 DPP	SCE	Healthy	SCE v Healthy	SCE	Healthy	SCE v Healthy
		Average Values		<i>p</i> – value ³	Average Values		<i>p</i> – value ³	Average Values		<i>p</i> – value ²	Average Values		<i>p</i> – value ²
Neutrophils (%)	0.0038	27.82	32.67		40.49	40.48		27.82	40.49	0.036	32.67	40.48	
Lymphocytes (%)	0.0374	60.47	52.50		48.50	43.83	0.020	60.47	48.50		52.50	43.83	
Eosinophils (%)	0.0046	2.00	6.57	0.036	3.58	7.00	0.027	2.00	3.58		6.57	7.00	
Absolute neutrophils (x10E03 cells/μL)	0.0261	1.94	2.66		3.37	3.84		1.94	3.37	0.026	2.66	3.84	
Absolute eosinophils (x10E03 cells/μL)	0.0004	0.10	0.49	0.031	0.28	0.60	0.020	0.10	0.28	0.011	0.49	0.60	
	One Way ANOVA <i>p</i> - value ¹	SCE			Healthy			SCE			Healthy		
		PC	7 DPP	PC v 7 DPP	PC	7 DPP	PC v 7 DPP	PC	21 DPP	PC v 21 DPP	PC	21 DPP	PC v 21 DPP
		Average Values		<i>p</i> – value ²	Average Values		<i>p</i> – value ²	Average Values		<i>p</i> – value ²	Average Values		<i>p</i> – value ²
Neutrophils (%)	0.0038	29.34	27.82		29.34	40.49	0.002	29.34	32.67		29.34	40.48	0.002
Lymphocytes (%)	0.0374	55.88	60.47		55.88	48.50	0.036	55.88	52.50		55.88	43.83	0.024
Eosinophils (%)	0.0046	7.70	2.00	0.005	7.70	3.58	0.024	7.70	6.57		7.70	7.00	
Absolute neutrophils (x10E03 cells/μL)	0.0261	2.37	1.94	0.043	2.37	3.37	0.027	2.37	2.66		2.37	3.84	0.036
Absolute eosinophils (x10E03 cells/μL)	0.0004	0.57	0.10	0.002	0.57	0.28	0.004	0.57	0.49		0.57	0.60	

¹ A one way analysis of variance (ANOVA) compared the means of five groups to determine significance.

² A Mann-Whitney test was used to determine significance between two groups.

³ A Wilcoxon paired test was used to determine significance between two time points within the same cows.

Table A-7: Quantification of acute phase proteins serum amyloid A (SAA) and haptoglobin (HP) in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=5) and healthy (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

	One Way ANOVA p - value ¹	SCE			Healthy			7DPP			21DPP		
		7 DPP Average Values	21 DPP Average Values	7 v 21 DPP p - value ³	7 DPP Average Values	21 DPP Average Values	7 v 21 DPP p - value ³	SCE Average Values	Healthy Average Values	SCE v Healthy p - value ²	SCE Average Values	Healthy Average Values	SCE v Healthy p - value ²
HP (mg/mL)	0.004	1.78	0.54		0.86	0.57		1.78	0.86	0.019	0.54	0.57	
SAA (µg/mL)	0.037	108.49	32.03		51.06	44.62		108.49	51.06	0.029	32.03	44.62	
	One Way ANOVA p - value ¹	SCE			Healthy			SCE			Healthy		
		PC Average Values	7 DPP Average Values	PC v 7 DPP p - value ²	PC Average Values	7 DPP Average Values	PC v 7 DPP p - value ²	PC Average Values	21 DPP Average Values	PC v 21 DPP p - value ²	PC Average Values	21 DPP Average Values	PC v 21 DPP p - value ²
HP (mg/mL)	0.004	0.39	1.78	0.007	0.39	0.86	0.03	0.39	0.54		0.39	0.57	
SAA (µg/mL)	0.037	20.83	108.49	0.002	20.83	51.06		20.83	32.03		20.83	44.62	

¹ A one way analysis of variance (ANOVA) compared the means of five groups to determine significance.

² A Mann-Whitney test was used to determine significance between two groups.

³ A Wilcoxon paired test was used to determine significance between two time points within the same cows.

Table A-8: Quantification of cytokines IL-1 and IL-6 in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=5) and healthy (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

		SCE			Healthy			7DPP			21DPP		
	One Way ANOVA <i>p</i> – value ¹	7 DPP Average Values	21 DPP Average Values	7 v 21 DPP <i>p</i> – value ³	7 DPP Average Values	21 DPP Average Values	7 v 21 DPP <i>p</i> – value ³	SCE Average Values	Healthy Average Values	SCE v Healthy <i>p</i> – value ²	SCE Average Values	Healthy Average Values	SCE v Healthy <i>p</i> – value ²
IL-6 (ng/ml)	0.0049	0.52	0.29		0.53	0.40		0.52	0.53		0.29	0.40	
IL-1	0	0	0		0	0		0	0		0	0	
		SCE			Healthy			SCE			Healthy		
	One Way ANOVA <i>p</i> – value ¹	PC Average Values	7 DPP Average Values	PC v 7 DPP <i>p</i> – value ²	PC Average Values	7 DPP Average Values	PC v 7 DPP <i>p</i> – value ²	PC Average Values	21 DPP Average Values	PC v 21 DPP <i>p</i> – value ²	PC Average Values	21DPP Average Values	PC v 21 DPP <i>p</i> – value ²
IL-6 (ng/ml)	0.0049	0.50	0.52		0.50	0.53		0.50	0.29	0.0062	0.50	0.40	
IL-1	0	0	0		0	0		0	0		0	0	

¹ A one way analysis of variance (ANOVA) compared the means of five groups to determine significance.

² A Mann-Whitney test was used to determine significance between two groups.

³ A Wilcoxon paired test was used to determine significance between two time points within the same cows.

Table A-9: Quantification of blood metabolites beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFAs) and Urea in blood plasma of pre-calving (PC) (n=8), sub-clinically endometritic (SCE) (n=6) and healthy (n=9) cows 7 and 21 days postpartum (DPP). A one way analysis of variance (ANOVA) and subsequent Mann-Whitney and Wilcoxon tests were used to determine significant differences ($p < 0.05$) between 5 groups (PC, SCE 7 DPP, SCE 21 DPP, Healthy 7 DPP and Healthy 21 DPP).

	One Way ANOVA p – value ¹	SCE			Healthy			7DPP			21DPP		
		7 DPP Average Values	21 DPP Average Values	7 v 21 DPP p – value ³	7 DPP Average Values	21 DPP Average Values	7 v 21 DPP p – value ³	SCE Average Values	Healthy Average Values	SCE v Healthy p – value ²	SCE Average Values	Healthy Average Values	SCE v Healthy p – value ²
BHB (mmol/L)	0.0003	1.15	0.93		0.89	0.81		1.15	0.89		0.93	0.81	
NEFA (mmol/L)	<0.0001	0.61	0.42		0.93	0.55		0.61	0.93		0.42	0.55	
UREA (mmol/L)	0.0036	4.17	3.73		4.99	5.04		4.17	4.99		3.73	5.04	

	One Way ANOVA p – value ¹	SCE			Healthy			SCE			Healthy		
		PC Average Values	7 DPP Average Values	PC v 7 DPP p – value ²	PC Average Values	7 DPP Average Values	PC v 7 DPP p – value ²	PC Average Values	21 DPP Average Values	PC v 21 DPP p – value ²	PC Average Values	21DPP Average Values	PC v 21 DPP p – value ²
BHB (mmol/L)	0.0003	0.29	1.15	0.002	0.29	0.89	0.0007	0.29	0.93	0.004	0.29	0.81	0.0004
NEFA (mmol/L)	<0.0001	0.07	0.61	0.002	0.07	0.93	0.0004	0.07	0.42	0.002	0.07	0.55	0.0007
UREA (mmol/L)	0.0036	1.87	4.17	0.021	1.87	4.99	0.0410	1.87	3.73	0.002	1.87	5.04	0.0004

¹ A one way analysis of variance (ANOVA) compared the means of five groups to determine significance.

² A Mann-Whitney test was used to determine significance between two groups.

³ A Wilcoxon paired test was used to determine significance between two time points within the same cows.