A study of circulating neutrophils and exosomes associated with innate immune function in the periparturient grazing dairy cow

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Abstract

Dairy cows are at greatest risk of infectious and metabolic disease during the periparturient period. This period of three weeks either side of calving is also known as the transition period due to the transition into lactation. This thesis had several aims; one was to characterise innate immune function during the transition period in grazing dairy cows by investigating molecular changes in circulating neutrophils and to assess if common on-farm management strategies (pre-calving feeding level and body condition at calving) were able to influence these molecular changes. Next, metabolic stress on neutrophil function was assessed by establishing a model of cows divergent in metabolic health status. This model was further utilised with the aim to investigate nanoparticles (exosomes), which are regulators of innate immune function and indicators of disease state. To address these aims blood was collected from pasture-fed transition dairy cows. Cellular and molecular methods used included cell and exosome isolation, reverse transcriptase (RT)-quantitative PCR, RNA sequencing, cell culture, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results indicated that grazing dairy cows experience a change in innate immune function during the transition period, reflective of reduced functional capacity of the immune system to overcome infectious agents. This altered function was similar to that experienced by housed cows fed a total mixed ration, which adds evidence to support that the dysfunction is a natural part of the transition into lactation at calving. These results also indicated that the functional changes could be influenced by nutrition status, feeding level, and metabolic stress. Analysis of exosomes isolated from the blood of transition cows indicated that these particles carried cargo indicative of metabolic state during the transition period and that they had the ability to alter target cell processes (gene expression, protein expression, and cell proliferation). The conclusions from this thesis increase our understanding of transition cow immune function and how it is influenced by nutrition and cow metabolism. These data are particularly relevant for grazing dairy cows and the findings

will contribute to on-farm recommendations and the improvement in animal health and well-being.

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Preface

I have undertaken this thesis in the form of publishable experimental chapters using a format of thesis by publication. The current status and publication outlet are described in the following list. Published papers do not appear in chronological order.

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Chapter 1A: Biological literature review

Chapter 1B: Technical literature review

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Common Abbreviations

ANOVA	Analysis of variance
AST	Aspartate transaminase
BCS	Body condition score
BHBA	Beta-hydroxybutyrate
$BW^{0.75}$	Metabolisable liveweight
BW	Body weight
CD14	LPS/lipoteichoic acid receptor or cluster of differentiation factor 14
CD18	Cluster of differentiation factor molecule 18
CD62L	L-selectin
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
dsDNA	Double stranded DNA
COG	Cluster of orthologous groups of proteins
COX	Cycloxygenase
Cp	Crossing point
CXCR2	Chemokine (C-X-C motif) receptor 2 or CD182 or IL-8 receptor
CXCR4	Chemokine (C-X-C motif) receptor 4 or fusin or CD184
d	Day(s)
DC	Dendritic cell
DM	Dry matter
DMI	Dry matter intake
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ECV	Extracellular vesicles
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
FBS	Foetal bovine serum
Fc-IgG2	Fragment, crystallisable region of IgG
FL	Feeding level
G-CSF	Granulocyte colony stimulating factor
GDP	Gross domestic product
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
IFC	Integrated fluidic circuit

IFITM	Interferon inducible transmembrane proteins
IFN	Interferon
IL-1	Interleukin 1
IL-17A	Interleukin 17A
IL-23	Interleukin 23
IL-8	Interleukin 8
IPA	Ingenuity pathway analysis
ITGAM/CD11b	Integrin alpha M or cluster of differentiation molecule 11B
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LPS	Lipopolysaccharide
LSM	Least squares mean
Mac-1	Macrophage activating factor 1 (CD11b/CD18 complex)
MDBK	Madin-Darby bovine kidney cells
min	Minute(s)
ME	Metabolisable energy
miRNA	micro RNA
MMP-8	Matrix metalloproteinase 8 or PMNL collagenase
MMP-9	Matrix metalloproteinase 9 or 92 kDa type IV collagenase or 92 kDa gelatinase or gelatinase B
MMP-23	Matrix metalloproteinase 23
MPO	Myeloperoxidase
mRNA	Messenger RNA
MS	Mass spectrometry
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NEV	Number of exosomal vesicles
NGS	Next generation sequencing
NK	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PMN	Polymorphonuclear cell (neutrophils)
RBC	Red blood cells
RNA	Ribonucleic acid
RNAseq	RNA sequencing
rRNA	Ribosomal RNA

ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SD	Standard deviation
SED	Standard error of the difference
SEM	Standard error of the mean
TAC	Total antioxidant capacity
TAG	Triacylglycerol
Th	T helper lymphocyte
TMR	Total mixed ration
TNF	Tumour necrosis factor alpha
WBC	White blood cells
Wk	Week(s)
UPL	Universal Probe Library

Chapter 1

General Introduction

Agriculture contributes significantly to New Zealand's economy and a large part of this contribution is from the dairy industry. A recent report by the New Zealand Institute of Economic Research states that the dairy sector contributes $7.8 billion or 3.5% of New Zealand's total gross domestic product (GDP) (Ballingall and Pambudi, 2017). Furthermore, dairy goods are the biggest earner for New Zealand, contributing $14.4 billion in export revenue over the past five years or over 25% of net export earnings (Ballingall and Pambudi, 2017). Also, the dairy industry employs over 40,000 workers or, approximately, 1.4% of New Zealand's workforce.

Due to the importance of the dairy industry to New Zealand's economy, it is important to consider the sustainability of this sector. Because New Zealand dairy cows are largely pasture-fed, cows in this system are very efficient producers of milk on a greenhouse gas per unit of milk basis (OECD Environmental Performance Reviews: New Zealand, 2017). The unique features of the New Zealand dairy industry include the predominantly pasture-based diet and the seasonality of pasture supply (Roche et al., 2011). The seasonality of pasture requires breeding to be concentrated so that reproduction is synchronised with feed supply. Concentrated breeding means that calving is also concentrated. As a result, 4.5 million dairy cows calve within a three-month period throughout New Zealand. The calving period is the most problematic time for dairy cow health during her lactation cycle. Roughly, 90% of metabolic diseases and 75% of infectious diseases occur during the calving period, with a 3 to 6-fold greater risk of death. Health problems and mortality during the calving period result in an estimated NZ$1.5 billion lossper year, which equates to approximately $300 per cow

(Roche, 2012). The increased risk of disease is a result of the increased metabolic demand required for the onset of lactation as well as a decline in immune function around calving.

The purpose of this thesis is to understand the changes to neutrophil function (reviewed in Appendix A) during the transition between pregnancy and lactation with the aim of understanding the role of nutritional and metabolic states on peripartum immune dysfunction and how this can be altered by intercellular messengers.

Chapter 1A

Biological Literature Review

1.1 The Transition Period

During the World Dairy Congress in 1928, Robert Boutflour identified four factors that undermine the productivity of a dairy cow. One of these factors is "the neglect of the preparation of the cow for her next lactation period" (Boutflour, 1928); thus highlighting the peripartum period as a key time during the lactation cycle for optimised management. This period is also referred to as the transition period because the cow transitions from a pregnant, non-lactating state to a non-pregnant, lactating state.

A successful transition into lactation requires several metabolic, nutritional, physiological, and immunological changes to occur in co-ordination. These changes begin several weeks pre-calving and continue through to several weeks post-calving. Therefore, the transition period is often referred to as a six week period, encompassing three weeks either side of calving (Grummer, 1995). This timeframe allows for the majority of metabolic changes that occur due to the onset of lactation, with 'normal' functionality thought to be restored by one month post-calving. The intense period of change throughout different body systems leaves cows at risk of disease, particularly, metabolic and infectious disease (LeBlanc et al., 2006). Disease prevalence is high; 30 to 50% of cows are affected by some form of infectious or metabolic disease during the transition period (LeBlanc, 2010). This is due to a) an inability to meet the metabolic demands of lactation and b) a decline in immune function during the peripartum period (Detilleux et al., 1995; Mallard et al., 1998; Hammon and Goff, 2006; Heiser et al., 2015). Succumbence of cows to disease during the transition period is termed transition failure or maladaptation.

1.1.1 Transition failure

Individuals that are unable to meet the metabolic demand of lactation are faced with transition failure. During the transition period, it is estimated the demand for glucose by the mammary gland triples, amino acid demand doubles, and fatty acid demand increases fivefold (Bell, 1995). The increased nutrient requirements come at a time when dry matter intake (DMI) drops, causing nutrient supply to fall behind demand (Drackley, 1999). It is, therefore, not surprising that many cows are unable to cope with the metabolic strain, fail to transition effectively, and develop health disorders.

The diseases discussed in this section are considered ‘production diseases’ and are influenced by high production and management (Mulligan and Doherty, 2008). The term ‘production disease’ encompasses nutritional and metabolic diseases as well as infectious diseases (Herdt, 2006). The production diseases are of particular concern for the transition cow and, therefore, of economic importance to the dairy industry (Ingvartsen et al., 2003).

1.1.1.1 Metabolic disease

The increased metabolic strain immediately post-calving means that the majority of metabolic diseases of the dairy cow occur within the two weeks immediately post-calving (Goff and Horst, 1997). Examples of metabolic disorders include hypocalcaemia (milk fever), hyperketonaemia (ketosis), decreased rumen motility, enteritis, left displaced abomasum, right displaced abomasum, and low DMI (Bigras-Poulin et al., 1990; Ingvartsen, 2006). Some common metabolic diseases of the grazing dairy cow during the transition period are reviewed below.

Milk fever

Milk fever usually occurs at the onset of lactation and is associated with low blood calcium concentrations (hypocalcaemia), which accounts for the clinical symptoms e.g., “off-feed”, muscle cramp (tetany), difficulty with urination and defecation, and excessive lying down (Horst et al., 1997). Milk fever is also known as parturient paresis, typically occurring at, or around parturition, particularly in high-producing dairy cows (Horst et al., 1997). The transition into lactation at parturition puts immense strain on the high producing dairy cow to maintain calcium homeostasis, and individuals that fail to do this develop milk fever (Goff, 2008). An estimated 5% of cows in the USA will develop milk fever each year and instances of subclinical hypocalcaemia in older cows are likely to be around 50%, highlighting the severity of the issue (Horst et al., 2003). Rates of milk fever in New Zealand are roughly similar to that in the USA, with an estimated 1-2% of cows becoming recumbent annually due to milk fever and 5% of cows clinically hypocalcaemic (i.e., <1.4 mmol/L total blood calcium); however, rates are dependent on location and seasonal influence (Roche et al., 2013c). Nutritional management strategies to reduce risk of milk fever have included pre-calving dietary supplementation with calcium, magnesium, or phosphorus. Magnesium is required for the production of hormones required for absorption of calcium from the gut and mobilisation of calcium from the bone (DeGaris and Lean, 2008). The use of nutrition management to reduce risk of metabolic disease and improve post-calving cow health is explored in section 1.1.2.1 and experimentally addressed in chapter 4 of this PhD thesis.

Ketosis

Bovine ketosis typically occurs early in lactation and results in clinical symptoms that include a diminished appetite, decreased milk production, weight loss, hypoglycaemia, and hyperketonaemia (Baird, 1982). Increased plasma non-esterified fatty acid (NEFA) concentrations prior to, and at, parturition increase fatty acid uptake and triacylglycerol (TAG) storage in the liver, which is likely linked to clinical spontaneous ketosis (Grummer, 1993; Ospina et al., 2010; Seifi et al., 2011).

Clinical ketosis is diagnosed based on blood beta-hydroxybutyrate (BHBA) concentrations; three consecutive samples between 1.2 mmol/L and 2.9 mmol/L, is considered subclinically ketotic or hyperketonaemic and at least three consecutive samples ≥1.2 mmol/L with at least one ≥2.9 mmol/L, is considered clinically ketotic (Andersson, 1988; Itle et al., 2015). In herds predominantly fed pasture (e.g., New Zealand systems), the estimated incidence of ketosis at 5-12 days post-calving is 16.8% and drops to 3.2% at 33-40 days (Compton et al., 2014). Compton et al. (2014) defined subclinical ketosis as blood BHBA concentrations ≥1.4 mmol/L and determined that prevalence of subclinical ketosis is greater at two weeks post-calving compared with eight weeks post-calving. A limitation of this study was that blood sampling occurred only at two time points during early lactation (Compton et al., 2014). Other studies conducted on herds fed primarily pasture have concluded that BHBA levels peak 2-4 wk post-calving (Verkerk and Guiney, 1999; Pedernera et al., 2008). In another study, concentrations peaked at one week post-calving and declined gradually (Stockdale, 2007). Elevated BHBA concentrations are also indicative of reduced milk yield and health conditions other than ketosis; therefore, concentrations should be monitored over the calving period (Duffield et al., 2009; Compton et al., 2014). BHBA concentrations are also influenced by diet and, therefore, the risk of ketosis may be altered by feed composition (Roche et al., 2010), which is important to consider when offering supplementary feed in pasture-based systems. The varied results in BHBA concentrations at different times and in response to differences in supplementation in these studies highlight the importance of considering the context of the results when measuring plasma BHBA.

Fatty liver

Fatty liver, or hepatic lipidosis, is a metabolic disease characterised by accumulation of lipids stored as TAG in the liver (Grummer, 1993). The aetiology results from excessive NEFA in the blood, mobilised from adipose tissue in response to homeorhetic signals preceding lactation and the associated negative energy balance (NEB) (Goff and Horst, 1997). This results in increased uptake of NEFA by the liver, which is then converted to TAG and stored, as TAG exportation (as very low density lipoproteins) is slow in ruminants (Roche et al., 2009).

The accumulation of TAG leads to decreased metabolic functions of the liver (e.g., lower gluconeogenesis), which causes a decline in health and well-being, lower productivity, and lower reproductive performance (Wensing et al., 1997). Prevalence of fatty liver is highest in the four weeks immediately post-calving, in which time 50% of cows experience some form of TAG accumulation (Grummer, 1993; Jorritsma et al., 2001). Fat storage in hepatic cells around the time of parturition was first published in the 1950's (Ford, 1959). Biopsy samples provided evidence of lipid accumulation in the liver in the early stages of lactation (Ford, 1959), thereby suggesting that fatty infiltration is a normal physiological response to parturition. Cows that are unable to recover from this response or those that have excessive fat accumulation will experience a decline in health associated with hepatic lipidosis.

1.1.1.2 Infectious disease

Like metabolic diseases, infectious disease is most prevalent during the immediate post-partum period than any other time of the lactation cycle. The risk of mastitis, for example, is greatest during the first few weeks immediately post-calving (Østergaard et al., 2005). The same prevalence is evident for cases of uterine infection, which is understandable, due to ubiquitous exposure of the uterus lumen to bacteria post-partum (Sheldon and Dobson, 2004). This section describes the infectious diseases most pertinent to grazing dairy cows during the transition period.

Mastitis

Mastitis is inflammation of the mammary gland and is a global problem that has a severe economic effect (Heikkilä et al., 2012). Halasa et al. (2007) describes it as one of the most frequent and costly diseases in the dairy industry and farmers often underestimate the economic loss of mastitis (Halasa et al., 2007; Huijps et al., 2008). In New Zealand, an estimated 15 in 100 cows per lactation experiences clinical mastitis and of these cases, 85% are in the four weeks immediately post-calving (McDougall et al., 2007).

During the early transition period, leading up to calving, the mammary gland undergoes marked changes to facilitate the onset of lactation, which renders the mammary gland highly susceptible to intramammary infection (Oliver and Sordillo, 1988). Several factors contribute to this high susceptibility, including genetic selection to maximise milk production, thereby increasing metabolic strain in the mammary gland, the use of milking machines that cause teat-end trauma, and changes in the local innate immune response during the periparturient period, as reviewed in Sordillo (2005).

The innate immune defence, as described here in section 1.2.1, is critical for recognition and elimination of many pathogenic, mastitis-causing bacteria (Burton and Erskine, 2003). There is a strong relationship between reduced phagocytic capacity of neutrophils and clinical mastitis (Zecconi et al., 1994). Furthermore, Cai et al. (1994) demonstrated that aberrant neutrophil function is linked to conditions including retained placenta, metritis, and mastitis post-calving. This research provides support to the hypothesis that a change in neutrophil function over the transition period plays a role in the increased incidence of mastitis in the periparturient period.

Uterine infection

Metritis and endometritis are both post-partum diseases of the uterus, often used interchangeably. However, the pathological definition for each is distinct; metritis is inflammation of the deper layers of the uterus and results in systemic signs of illness, whereas, endometritis does not cause systemic illness. Metritis involves the uterine lining (endometrium), the underlying glandular tissue, and the muscle layer; in comparison, endometritis refers to an infection of the endometrium and underlying glandular tissue (Lewis, 1997; Sheldon et al., 2006). These diseases can be caused by bacterial infection and a resultant inflammation due to the recruitment of neutrophils to the uterus. Failure to resolve the inflammation results in fertility-related issues including lower conception rates and higher culling rates due to failure to conceive (Huszenicza et al., 1999; Gilbert et al., 2005; LeBlanc, 2008).

1.1.2 Factors that contribute to transition cow maladaptation

There are several factors that contribute to the increased incidence of disease during the transition period. These factors include energy dysfunction, mineral imbalance, immune dysregulation, chronic inflammation, and NEB. Energy dysfunction, mineral imbalance, chronic inflammation, and NEB are described in this section and immune dysfunction is reviewed in section 1.2. These are interconnecting factors that all influence transition cow health. To avoid maladaptation of the transition cow, all of these factors need to be balanced. There are indicators that help identify when the balance has shifted and the cow is at greater risk of disease (see also section 1.1.1.1). Furthermore, there are management strategies that can be used to reduce the risk of maladaptation, which will be mentioned accordingly.

1.1.2.1 Energy dysfunction

Energy supply must be adequate to meet the demands of lactation. As such, the recommendation was to “steam up” cows by feeding extra rations during the late dry period (Boutflour, 1928). Research supporting this notion indicated a positive correlation between pre-calving DMI and liver TAG, and a negative association with pre-calving DMI and plasma NEFA, and plasma BHBA two wk post-calving (Bertics et al., 1992).

However, a growing body of research indicates that over-consumption of energy pre-calving is detrimental to cow health and liver function post-partum (Rukkwamsuk et al., 1998; Roche et al., 2005, 2007b; Janovick-Guretzky et al., 2007; Douglas et al., 2006; Janovick et al., 2011). There is still no consensus on the appropriate feeding regime pre-partum. Data from grazing systems indicate that a pre-calving restriction (feeding 75% of maintenance requirements) is beneficial for health and liver function as indicated by decreased NEFA, BHBA, liver TAG, and inflammatory mediators post-partum (Roche et al., 2005, 2015; Vailati-Riboni et al., 2016). Therefore, the effects of pre-calving energy restriction on post-partum health parameters needs to be further investigated.

Nutrition

Nutritional requirements change dramatically over the transition period with the onset of lactation. Metabolic adaptations in glucose, fatty acid, and mineral metabolism occur to facilitate lactation and to compensate for the increased metabolic demand that would otherwise lead to metabolic dysfunction (Overton and Waldron, 2004). The objective of nutritional management during the dry period is to ensure nutrient supply is sufficient for post-calving metabolic demand and to minimise over-feeding nutrients that increase the risk of post-calving metabolic disease. For example, feeding nutrient-dense diets can result in ruminal acidosis, which affects feed intake, milk production, rumen microflora, and can cause liver abscesses (Kleen et al., 2003; Stone, 2004; Mohammed et al., 2012).

Over 30 years ago, it was recognised that metabolic disease incidence was related to the diet fed pre-calving; increased protein content in the diet fed during the three weeks pre-calving was associated with decreased incidence of retained placenta and ketosis, and increased energy was associated with decreased incidence of displaced abomasum (Curtis et al., 1985). Since then, several studies have addressed the question of pre-calving feed allowance and composition on blood metabolites and metabolic health in both total mixed ration (TMR) and grazing systems (Martz et al., 1999; Greenfield et al., 2000; Roche et al., 2005, 2010; Douglas et al., 2006; Duske et al., 2009). Furthermore, nutrition can affect immune function, which will alter the risk of disease (Osorio et al., 2013b; Moyes et al., 2014). This makes nutrition management an important strategy to improve health over the transition period, however, the limits of immune sensitivity to nutritional intervention require further investigation. Another nutritional factor utilised to improve transition cow health is supplementation with minerals and micronutrients including calcium, zinc, and methionine. Including these micronutrients into the diet improves immune function and, therefore, reduces risk of disease (Osorio et al., 2013a; Lean et al., 2013; Oetzel, 2013). There is increasing evidence that the mechanism by which pre-calving diet is able to influence post-partum health is associated with modulation of the chronic inflammation that occurs during the peripartum period.

1.1.2.2 Chronic inflammation

An acute, controlled inflammation is essential for several processes post-calving including remodelling of the uterus, the breakdown of muscle for milk production, and the clearance of pathogens, which ensures peripartum infections (e.g., uterine infection) are cleared before they become clinical. Persistent, or chronic, inflammation, however, can be detrimental and lead to transition failure. A typical inflammatory response by the liver is production of acute phase proteins such as haptoglobin. Appearance of haptoglobin in blood is indicative of inflammation (Ceciliani et al., 2012). Serum concentrations of haptoglobin increase in the periparturient period and peak 2-3 days post-calving; however, healthy cows have lower concentrations of serum haptoglobin compared with those that experience transition failure i.e., those that experience retained placenta or other transition diseases (Qu et al., 2014). In contrast, Nightingale et al. (2015) demonstrated that cows with high plasma concentrations of haptoglobin had elevated leukocyte responses (i.e., neutrophil oxidative burst) compared with cows with moderate and low plasma concentrations (Nightingale et al., 2015).

These findings, although somewhat contradictory, indicate that inflammation is an inherent part of the transition from pregnancy to lactation (reviewed by Bradford et al. (2015)). Indeed, dampening this inflammation using non-steroidal anti-inflammatory drugs (NSAIDs) has resulted in mixed outcomes. For example, NSAID treatment to dampen prostaglandin synthesis by targeting cycloxygenase (COX) activity can reduce inflammatory-mediated diseases (Erskine et al., 2003). This could be due to effects on neutrophil function including decreased cell adhesion, degranulation, and oxidative burst production (Neal et al., 1987; Pillinger et al., 1998).

Research into the treatment of transition cows with NSAIDs in grazing systems has not yielded any concrete evidence to suggest that treatment improves health parameters (Priest et al., 2013; Meier et al., 2014). However, treatment does have an effect on liver and adipose gene expression in grazing dairy cows (Vailati-Riboni et al., 2015), which indicates that the effect did not result in any obvious phenotypic changes.

Peripartum inflammation can be influenced by the energy available and the extent of NEB post-calving. Inflammatory events during the periparturient period contribute to increased production of reactive oxygen species, which leads to oxidative stress (Sordillo et al., 2009). Oxidative stress is exacerbated by lipids (i.e., fatty acids and their derivatives), and lipid mediators (e.g., eicosanoids and leukotrienes), which regulate immune and inflammatory responses (Serhan et al., 2008). Therefore, excessive adipose reserves (body condition) contributes to increased inflammation and oxidative stress during the transition period (Sordillo and Mavangira, 2014). The chronic inflammatory state that results from excess body condition perturbs metabolic homeostasis and leads to metabolic stress.

1.1.2.3 Negative energy balance

The early post-partum period is associated with NEB due to the homeorhetic adaptation to lactation from insufficient DMI to meet increased demands. The challenge of NEB results in an increased incidence of metabolic and uterine diseases, and disrupts immune function (Esposito et al., 2014). For example, the inability to adapt to NEB during the transition period can lead to ketosis and fatty liver (Herdt, 2000). An important way to reduce the risk of NEB-associated maladaptation during the transition period is to ensure the cow has adequate, but not excess, body condition at calving to meet the metabolic demands during early lactation.

Body condition

Body condition score (BCS) is a measure of adiposity and, therefore, a measure of body energy reserves and nutrient status. It is well established that the availability of energy reserves pre-calving affects milk production and reproduction, even in subsequent lactation cycles (Roche et al., 2007c, 2009). An optimal BCS at calving is 3.0 to 3.5 (5-point system) or 5.0 to 5.5 (10-point system), which provides sufficient maternal energy reserves to meet energy demands of milk production in early lactation (Wildman et al., 1982; Roche et al., 2004). Cows over- or under-conditioned at calving have an increased risk of health disorders and disease post-calving (Gearhart et al., 1990; Heuer et al., 1999). Over-conditioned cows experience greater oxidative stress, and this likely contributes to the aetiology of several diseases (Bernabucci et al., 2005). This is well-known in humans; obesity-related diseases are an increasing problem, many of which are linked to detrimental effects of over nutrition on the immune system (Samartín and Chandra, 2001). There is some literature describing the effect of body condition on immune function in dairy cows. For example, lymphocyte function is detrimentally affected in over- and under-conditioned cows (Lacetera et al., 2005). Furthermore, NEFA and BHBA, which are greater post-calving in over-conditioned cows compared with adequately conditioned cows (Roche et al., 2013b), inhibit neutrophil respiratory burst activity in vitro (Hoeben et al., 1997). Research into the effect of BCS on health parameters in grazing cows suggests that these cows experience the same detrimental effects on immune function as TMR-fed cows (Roche et al., 2015; Lange et al., 2016).

1.2 Overview of the Immune System

A well-functioning immune system during the transition from pregnancy to lactation is essential in preventing the infectious diseases commonly reported during this period (e.g., mastitis and metritis). The primary purpose of the immune system is to protect the host from the innumerable pathogens that cause infectious disease leading to morbidity and mortality in humans and animals (Day and Schultz, 2014). It can be broadly separated into two branches, the innate and adaptive responses that together provide a coordinated response against infectious challenge. Each branch has distinct cell types that function in a co-ordinated manner.

1.2.1 Innate immune response summary

It is logical to discuss the innate immune response first as it occurs first and is evolutionarily older than the adaptive immune response. The innate immune response is available for 'instant action' in a non-specific manner against pathogens (Day and Schultz, 2014). The first line of defence is provided by epithelial barriers, which often use antimicrobial peptides (e.g., defensins) and enzymes (e.g., lysozyme, phospholipase A) that are able to destroy bacterial cell walls. In the event that a pathogen is able to penetrate through the epithelial barrier, white blood cells (WBC) are rapidly activated and recruited from circulation to the point of 'infection'. These WBC include phagocytic cells (neutrophils and macrophages), dendritic cells (DCs), lymphocytes (B and T cells), and natural killer cells (NKs). The action of the molecules and cells of the innate immune response is to induce inflammation and is initiated by resident cells within tissue such as macrophages, DCs, and mast cells. These cells commence an inflammatory response which stimulates neutrophils to extravasate from blood vessels and enter infected tissue where they locate the invading pathogen via chemotaxis, identify, engulf, and destroy it (Kumar and Sharma, 2010). The action of neutrophils is known as acute inflammation, the second stage of inflammation is known as chronic inflammation and is characterised by the recruitment of monocytes that differentiate into macrophages when in tissue. The final stage of the inflammatory response is tissue repair. Appendix A provides further information on the molecular mechanisms by which neutrophils exit circulation and enter tissue. These processes are essential for neutrophils to perform their anti-microbial function and ensure infectious disease does not ensue. Research over the past decade has revealed that the innate immune system is closely linked to the adaptive immune response and that cells of the innate arm interact with and inform cells of the adaptive immune response (Mantovani et al., 2011).

1.2.2 Adaptive immune response summary

The adaptive immune response is younger in evolutionary terms and differs from the innate immune response in that it takes time to develop (following antigen exposure), it is highly specific and results in immunological memory. Antigen molecules are transported by antigen-presenting cells, including DCs, from the infection site to the lymphoid organs, and presented to antigen-specific T helper (Th) lymphocytes. Th1 cells can initiate cell-mediated immune responses (e.g., by activating macrophages), which result in rapid destruction of the invading pathogens, and Th2 cells secrete cytokines to activate antigen-specific B lymphocytes that differentiate into antibody producing plasma cells. Antibodies can facilitate the removal of pathogenic organisms from the host by opsonization, virus neutralization, and complement activation. Antibody production is referred to as a humoral response. Developing significant T and B lymphocyte responses takes 4-7 days to occur and, therefore, the host is reliant on innate immune defences during the initial phases of an infection.

1.2.3 Unique aspects of leukocyte biology in bovines

The innate immune defence is reliant on the ability of WBCs to recognise foreign material, for example, proteins on the cell-surface of bacteria, and respond in an immediate, non-specific manner. Phagocytic cells (i.e., neutrophils and macrophages) are the first line of defence against many common pathogenic microorganisms. The average leukocyte values in female Jersey cattle at different ages are presented in Table 1. Approximately 20% of adult bovine leukocytes are neutrophils as lymphocyte numbers double rapidly after birth reaching 70-80% of leukocytes within 3 months (Weiss and Wardrop, 2010). In adult humans, the most abundant blood cell type is neutrophils (55-60%) with lymphocytes only comprising 25-30% of leukocytes (Bain, 2011).

Calves are born with an underdeveloped immune system that is progressively developed by exposure. It is essential, therefore, that calves initially acquire immunoglobulins through ingestion of colostrum during the first day post-partum and develop their own immunoglobulins through vaccination and subsequent exposure to environmental microorganisms (Chase et al., 2008). The innate arm of the immune system also has a diminished functional capacity after birth when compared with older cattle, which is also improved by colostrum (Stieler et al., 2012). This progressive development is important as cows are expected to become pregnant as heifers within the first 2 years of life and will experience their first transition period 9 months later. The modern dairy cow has been selected for high fertility and high milk production; her life consists of cycles of pregnancy and parturition with successive lactation. This means that cows must be immunologically equipped to deal with the repeated cycles of parturition and metabolic stress that accompanies the elevated metabolic demand required for high milk production.

Table 1. Absolute leukocyte values for female Jersey cattle[a b]

Age[c]	*N*	Leukocytes	Segmented Neutrophils	Bands	Lymphocytes	Monocytes	Eosinophils	Basophils
1-6 mo	16	8.8 ± 2.5	3.0 ± 1.8	< 0.1	4.7 ± 1.3	0.7 ± 0.4	0.5 ± 0.2	< 0.1
6-12 mo	10	7.8 ± 1.8	0.8 ± 0.5	0	6.3 ± 1.5	0.6 ± 0.2	< 0.1	0
1-2 yr	14	9.0 ± 2.5	2.4 ± 1.4	0	5.9 ± 1.6	0.4 ± 0.2	0.5 ± 0.4	< 0.1
2-3 yr	31	9.4 ± 1.8	2.2 ± 0.9	< 0.1	5.3 ± 1.2	0.5 ± 0.2	1.3 ± 1.0	< 0.1
3-4 yr	28	7.7 ± 1.9	1.9 ± 1.0	0	4.6 ± 1.1	0.3 ± 0.2	0.9 ± 0.7	< 0.1
4-6 yr	29	7.5 ± 1.1	1.8 ± 0.7	< 0.1	4.0 ± 0.9	0.5 ± 0.2	1.2 ± 0.7	< 0.1
>6 yr	21	7.7 ± 2.5	1.8 ± 0.9	< 0.1	4.3 ± 2.1	0.4 ± 0.2	1.3 ± 0.7	< 0.1

[a] Table adapted from Schalm's Veterinary Haematology, 6th Edition (Weiss and Wardrop, 2010)

[b] Leukocyte values ($\times 10^3/\mu L$) are presented as mean ± standard deviation (SD)

[c] mo: months; yr: year

1.2.4 Immune function during the transition period

The innate and acquired host defence mechanisms are lowered during the transition period, which is implicated in the increased incidence of disease in the transition cow. Reduced immune function, often referred to as immunosuppression, is due to altered adaptive and innate host responsiveness. This immune dysfunction occurs during the transition period from 3 weeks pre-calving, is maximal at parturition, and continues through to 3 weeks post-calving (Mallard et al., 1998). This period of immune dysfunction also coincides with the time of the highest incidence of disease in the lactation cycle (discussed in section 1.1.1); for example, incidence of mastitis is highest during the transition period (Overton and Waldron, 2004). The innate immune response to experimentally-induced mastitis has been investigated during the transition period (Burvenich et al., 1994; Moyes et al., 2010a; Quesnell et al., 2012). These studies reported that cows are most susceptible to intramammary mastitis during the early post-partum period due to a dampened immune function and that neutrophils are key mediators of controlling acute mastitis. This confirmed the very early work on the importance of neutrophils during a mastitis infection (Schalm et al., 1971) and highlights the importance of neutrophil function for combating disease during the transition period. Neutrophils are key mediators during mastitis infection and are also important in other infections such as post-partum uterine disease. Neutrophil infiltration to the uterus occurs in response to bacterial invasion of the uterine environment and impaired neutrophil function is linked to post-partum endometritis and retained placenta (Kimura et al., 2002; Kim et al., 2005). This demonstrates the importance of immune cell function for the outcome of disease during the transition period.

Research conducted using Holstein cattle during the 1980's and early 1990's established that there is impaired function of immune cells around parturition (Kashiwazaki, 1984; Kashiwazaki et al., 1985; Kehrli and Goff, 1989; Kehrli et al., 1989a, 1989b; Detilleux et al., 1995). This research identified a change in the function of lymphocytes and neutrophils over the calving period. Several aspects of neutrophil function including random migration, phagocytosis of bacteria, and neutrophil cytotoxicity or oxidative burst were demonstrated to be affected (Kehrli et al., 1989b; Detilleux et al., 1995). Not only do neutrophils undergo a change in function around parturition, they also increase in number pre-calving and at parturition and rapidly decline immediately post-parturition (Detilleux et al., 1995; Burton et al., 2005). Detilleux et al. (1995) also made the observation that the decline in neutrophil numbers shortly after calving was followed by an increase in immature neutrophils at the band cell stage and this increase likely coincided with the observed altered neutrophil function, particularly the capacity for oxidative burst. Despite early work on neutrophil function with the use of functional assays at the cellular level, there has been little research exploring this change in function at the molecular level. Madsen et al. (2002) investigated gene expression changes at 12 time points over the transition period with a number of technologies; differential-display reverse transcription- polymerase chain reaction (ddRT-PCR), cDNA dot plot hybridisation, Northern blot hybridisation, and quantitative slot blot analysis. This study provided an interesting approach to quantitative gene analysis with a good trial design; however the sample numbers were too small (n = 5 periparturient cows and n = 2 mid-gestation (control) cows) for meaningful statistical analyses using these technologies (Madsen et al., 2002).

1.3 Uniqueness of a grazing system

The majority of research on immune dysfunction during the transition period has been conducted in TMR systems with conventional housing. This means that cows are housed predominantly indoors in free-stall or tie-stall barns. In grazing systems, cows are kept outdoors with their diet comprised of predominantly fresh pasture. Therefore, cows in grazing systems experience different nutritional and environmental pressures compared with those in conventional housing. For example, supplementation in TMR systems increases the

DMI of dairy cows and can explain more than 60% of the greater milk production difference in these cows compared with grazing cows (Kolver and Muller, 1998). In grazing systems, there is a temporal pattern of pasture supply, which must be managed accordingly to ensure requirements meet demand (Roche et al., 2011). Dairy cows in New Zealand have been bred for a predominantly pasture-based diet and, therefore, have genetic differences that enable them to adapt to the lower DMI in grazing systems compared with TMR systems (Roche et al., 2002).

Despite the lower milk production in grazing cows, they still experience greater risk of metabolic disease during the transition period (Compton et al., 2014), as discussed in section 1.1.1.1. Likewise, infectious disease is still most prevalent during the transition period in grazing systems, despite constant exposure to different environmental pathogens (Compton et al., 2007). Also, cows housed in free-stall barns are kept in close quarters with increased population density, therefore, contagious mastitis-causing bacteria can spread with greater ease (Fox and Gay, 1993). It is interesting that grazing dairy cows demonstrate the same epidemiological pattern of increased metabolic and infectious disease during the transition period, considering the lower milk production and, seemingly, lower metabolic pressure. However, it is not known whether the same immunological maladaptations occur in grazing dairy cows as with cows in a TMR system.

Hypotheses

The working hypothesis for this thesis is that grazing cows undergo changes in immunological function over the transition period similar to those reported in cows in a TMR system, and that these would be evident in changes in neutrophil gene expression. It is proposed that these perturbations in neutrophil function could be influenced by nutritional state (BCS and feeding level) and that the metabolic state (during the transition period) would affect the neutrophil transcriptome. As a consequence of these changes, it is also proposed that the protein contents of circulating exosomes would also be affected and that these modified circulating exosomes have the potential to alter gene and protein expression of target cells.

A range of molecular techniques and technologies used for this study are reviewed in Chapter 1B: technical literature review.

Chapter 1B

Technical Literature Review

1.4 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Reverse transcription (RT) of RNA to complementary cDNA and subsequent quantification using real-time PCR is a common method for analysing gene expression. The quantification end-point of RT-qPCR is the crossing point (C_P) or threshold cycle (C_T). This is the point at which the reporter dye crosses an arbitrary fluorescence threshold and ensures PCR is in the exponential phase of amplification (Schmittgen and Livak, 2008). The C_P and C_T are the same point; however, the different notation is dependent on the company that designed the RT-qPCR software. For ease of writing, this shall, henceforth, be termed the C_P.

The C_P is inversely correlated with the amount of template cDNA present in the sample (i.e., the higher the amount of starting transcript, the lower the C_P value). Expression data can be presented in many different ways and may be calculated using either absolute or relative quantification.

- Absolute quantification is calculated using a calibration- or standard-curve whereby serial dilutions of a ‘known’ sample are used to quantify concentrations of expression in “unknown” samples (Ginzinger, 2002). This method has been used to calculate the exact copy number per cell (Chen et al., 2005) and may be useful in determining viral load, for example, by quantifying the precise quantity of amplicon present (Niesters, 2001). However, this quantification method, despite being termed ‘absolute’, relies on relative concentrations calculated in comparison to another sample of which there is no way of knowing the exact starting number of transcripts. Therefore, for the current study design, absolute quantification is not considered to give an indication of the precise transcript copies present within a sample and is deemed a ‘relative’ method.

- In relative quantification, changes in target gene expression are calculated by comparison with expression of a reference gene or genes. This is then used to calculate the change in expression between two treatment groups or between a control and treatment and indicates an expression fold-change between the two groups. This calculation is also known as the comparative C_P method or the $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001). The expanded mathematical equation to calculate fold change (fold change = $2^{-\Delta\Delta Cp}$) is shown below:

$$2^{-\Delta\Delta Cp} = [(C_P \text{ gene of interest} - C_P \text{ internal control gene})\text{sample A} - (C_P \text{ gene of interest} - C_P \text{ internal control gene})\text{sample B})]$$

Using the $2^{-\Delta\Delta Cp}$ equation assumes the efficiency of the target and reference reactions to be similar and that the amplification efficiency is close to 100%. The PCR efficiency can be calculated using the equation $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2001). A different mathematical model used to calculate relative gene expression was presented by Pfaffl (2001), whereby the relative ratio of a target gene in comparison to a reference gene is based on the efficiency (*E*) and the C_P deviation of an unknown sample verses a control sample.

$$\text{fold change} = \frac{(E_{\text{target}})^{\Delta Cp \text{ target}(control - sample)}}{(E_{\text{ref}})^{\Delta Cp \text{ ref}(control - sample)}}$$

If the C_P of the chosen endogenous gene(s) is the same for the two samples being compared, in this case the 'control' and 'sample', the denominator should be stable ($\Delta C_P = 0$, $E_{ref} = 1$). Therefore, the above equation can be simplified as below.

$$\text{fold change} = (E_{\text{target}})^{\Delta \text{Cp target}(control - sample)}$$

The above equation represents a real-time PCR reaction under constant reference gene expression. Normalisation of the target gene with endogenous control gene(s) compensates for inter-PCR variations (Pfaffl, 2001).

Multiple endogenous control genes can be used to reliably calculate a normalisation factor (Vandesompele et al., 2002). The basic calculation software geNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004) can be used to calculate whether there is improved stability with the use of multiple endogenous control genes. The endogenous control gene(s) should be selected on endogenous control gene validation with the aforementioned calculation software based on an experiment by experiment basis.

There are different fluorescent chemistries for gene expression quantification with qRT-PCR that may intercalate with double stranded DNA (dsDNA), such as SYBR Green I, or probe-based like TaqMan (Roche Molecular Diagnostics) or Roche Universal ProbeLibrary (UPL). SYBR Green I is a nonspecific dsDNA-binding fluorophore. It exhibits fluorescence when it binds to dsDNA therefore the fluorescent signal is proportional to the amount of amplicon present and will increase as the target amplicon is amplified. Gene expression analysis has been successfully completed on whole blood neutrophils using SYBR Green I (Hugejiletu et al., 2013). Use of SYBR Green I has advantages such as the need for only two primers and no probe design, as probes are expensive. The major drawback of dsDNA dyes for qRT-PCR is the lack of specificity and, therefore, the dye will bind to all double stranded products and affect the quantification accuracy. The use of a fluorescent probe has the advantage of specific detection of the target sequence and does not influence quantification. The chemistry of the probe involves a fluorescent reporter in close proximity to a quencher which inhibits fluorescence. During annealing, the reporter is cleaved and is no longer quenched; the fluorescence is therefore relative to the amount of target in a reaction. An example of the use of bovine-specific TaqMan probes in an immunological study is described by Leutenegger et al. (2000). They support the argument for using a probe-based system similar to the one employed in the current thesis, over conventional methods. These assays use the Roche UPL system chemistry with fluorescein (FAM) at the 3' end and dark quencher at the 5', similar to TaqMan, but with custom designed primers combined with a finite array (165 8- and 9-mers) of fluorescent probes. This system has been validated on the LightCycler480 platform for bovine samples (Grala et al., 2010; Walker et al., 2009, 2010).

1.4.1 Microfluidics qRT-PCR

Microfluidics allows RT-qPCR quantification for greater numbers of samples and gene targets using less reagents than standard RT-qPCR methods due to the nL reaction volumes. This technology utilises microfluidic matrices that allow quantification of (semi) large-scale gene expression. These microfluidic devices allow all possible combinations of target assays to be mixed with all samples concurrently loaded on a chip and, therefore, greatly reduce pipetting steps and reagent requirements (Liu et al., 2003; Spurgeon et al., 2008). The performance of Fluidigm® integrated fluidic circuits (IFCs; 48.48 dymanic array) has been compared with a standard qRT-PCR platform, the ABI 79000HT (Devonshire et al., 2011). Devonshire et al. (2011) used TaqMan® assays to compare the impact of pre-amplifcation on the range and precision of results between the ABI and Fluidigm® platforms with agreement of results between the two. Fluidigm dynamic arrays have been used successfully for the study of gene expression (Guo et al., 2010; Viturro et al., 2014). This provides evidence for microfluidics as a reliable platform for gene expression analysis.

1.5 Transcriptomics

Large-scale transcriptomics analysis using Next-Generation Sequencing (NGS) provides unparalleled data quality and quantity for gene expression quantification. This technology has improved and evolved in recent years with the newest technology, RNA sequencing (RNAseq), able to accurately survey the RNA content of cells providing accurate quantification of gene expression.

1.5.1 RNAseq

The advantages of RNAseq are discussed in Wang et al. (2009). Taking into account all of the benefits discussed, RNAseq is the first method that allows high throughput, quantitative analysis of the whole transcriptome (Wang et al., 2009). RNAseq is highly accurate for quantification of gene transcripts as determined by RT-qPCR (Nagalakshmi et al., 2008). Methods have been developed for sample preparation prior to sequencing to ensure good quality sequencing data are generated (Wilhelm and Landry, 2009). Bioinformatics tools including programs for differential gene expression analysis, provide a straightforward workflow for RNAseq data analysis (Trapnell et al., 2012). Accuracy of differential gene expression is reliant on good quality library preparation and when achieved, can provide an accurate, quantitative measure of gene expression (Wilhelm and Landry, 2009).

1.6 Proteomics

Mass spectrometry (MS) allows the investigation of the proteome in a biological sample. The coupling of liquid chromatography (LC) with MS enables high-throughput proteomics analysis that can measure hundreds or thousands of proteins in a sample (Savaryn et al., 2016). This makes proteomics a powerful tool, which has been used to predict biomarkers of disease states (Duijvesz et al., 2013). For example, comparative proteomic profiling between urine from control patients and those with glomerular disease has been used to investigate biomarkers of kidney disease (Pisitkun et al., 2006; Spahr et al., 2001). This highlights how useful this technology can be for potential clinical diagnosis. Furthermore, protein profiles can be integrated with other '-omics' data (e.g., genomics or transcriptomics) to provide systems information at multiple levels (Günther et al., 2014). The review by Pisitkun et al. (2006), not only discusses the different MS technologies, but also emphasises that the protein origin should be considered when investigating body fluids for markers indicative of health status, i.e., urine-derived proteins, whole cells, or exosomes (Pisitkun et al., 2006). This highlights that the need for selection of appropriate body fluids for proteomic investigation, when exploring the health status of transition dairy cows.

1.6.1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The use of Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for disease diagnostics and prognostics in dairy cows is a growing area of research. LC-MS/MS has been employed to investigate predictive biomarkers of disease primarily using metabolomics, e.g., for diagnosis of ketosis before the appearance of clinical symptoms (Li et al., 2014; Humer et al., 2016; Zhang et al., 2017). Although a promising area of research for dairy cow health diagnostics, there is very little research that focuses on identifying transition failure using proteomics. An emerging area for disease diagnostics in humans is proteomic investigation of exosomes (Lee et al., 2016a; Raimondo et al., 2016; Vella et al., 2016). LC-MS/MS of exosomes can, therefore, be used for biomarker discovery or as indicators of disease state. There is also a potential for the use of exosomes to treat diseases by targeted delivery of drugs or silencing RNA to affected tissue types (Lee et al., 2016b; Shahabipour et al., 2017). However, the mechanisms by which exosomes target specific cell types are still unclear.

1.7 Exosomal Technologies

Exosomes are a new and emerging technology that could be utilised to identify cows at risk of transition failure. Exosomes are membrane-bound nano-vesicles ranging from 30-120 nm in size. They are formed by inward budding of endosomes in the cytoplasm of a cell of origin (Valadi et al., 2007). After fusion with the plasma membrane, they are released as exosomes into the extracellular space and/or into circulation. Exosomes are capable of influencing the function of distant cells (e.g., membrane trafficking, cell proliferation and angiogenesis) by releasing their cargo (e.g., proteins, mRNA, and miRNA), making them important cell-to-cell messengers (Valadi et al., 2007). Due to the nature in which they are formed, exosomes provide important functional information of the propagating cell. As such, they have the potential to be used as indicators of disease state and as biomarkers of health status (Lee et al., 2016a; Vella et al., 2016). Furthermore, there is potential for exosomes to be used to treat diseases by utilising them for targeted delivery of drugs or silencing RNA (microRNA) to affected tissue types (Lee et al., 2016b; Shahabipour et al., 2017). The mechanisms by which exosomes target specific cell types are still unclear but further research will expose their prognostic and diagnostic potential.

1.8 Summary of Chapter 1

The biological literature review provides evidence that there are several factors contributing to the high rates of transition failure in periparturient dairy cows. Of particular interest, however, is the dysfunction in the innate immune system during this period. Existing research results indicate compromised function of neutrophils over the transition period in conventionally housed, TMR-fed cows (i.e., in non-grazing systems). Peripartum dysfunction has yet to be investigated in the neutrophils of cows from grazing systems. An integral part of this study is the use of gene expression as an indicator of cell function. The research reviewed in Appendix A provides a comprehensive overview of the genes involved in neutrophil function and provides a rationale for changes in these genes through the transition period.

The main objectives of this PhD thesis were to:

(1) Develop reverse transcriptase-quantitative PCR assays for use with microfluidics gene expression arrays and use them to investigate gene expression in bovine neutrophils from grazing transition dairy cows.

(2) Investigate the role of nutritional state in neutrophil function through the transition into lactation.

(3) Investigate the effects of metabolic health on the transcriptome of circulating neutrophils during the transition period.

(4) Investigate if circulating exosomal proteins from transition dairy cows are informative biomarkers of pre-pathological disease.

(5) Investigate if the metabolic health of transition dairy cattle influences exosomal messaging.

Chapter 2

Evaluation of endogenous control gene expression in bovine neutrophils by reverse transcriptase-quantitative PCR using microfluidics gene expression arrays

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2.1 Abstract

Reverse-transcriptase quantitative-PCR (RT-qPCR) is commonly used for assessing the cellular response to changes in physiologic and pathologic conditions. The selection of stable endogenous control genes is an important step of any RT-qPCR study, as expression can vary depending on the experimental environment. Our objective was to identify endogenous control genes in circulating neutrophils isolated from cows during the peripartum period. To do this, we used microfluidics gene expression arrays (Fluidigm) for RT-qPCR analysis. Selection of the endogenous control genes was based on previous research investigating gene expression in neutrophils. The selected genes included *ACTB*, *B2M*, *G6PD*, *GAPDH*, *GCH1*, *GOLGA5*, *OSBPL2*, *PGK1*, *RPL13A*, *RPL19*, *RPS9*, *SDHA*, *SMUG1*, *SNRPA*, *TBP*, *UXT*, and *YWHAZ*. Four genes (*GAPDH*, *GOLGA5*, *PGK1*, and *UXT*) did not provide satisfactory quantification results using the selected method and were therefore excluded from the analyses. The suitability of the remaining 13 genes for use as endogenous control genes was assessed using geNorm and Normfinder. The gene pair with the greatest stability using geNorm was *RPL13A* and *RPL19*, whereas Normfinder ranked *RPL19* and *YWHAZ* as the most stable pair. The two genes deemed most suitable for the experimental design were *RPL19* and *YWHAZ*, which were selected for subsequent gene expression analysis. This study highlights that genes used as endogenous controls for relative quantification should be assessed on an experimental basis, even if the genes have been used in previous research.

2.2 Introduction

The immune system is dysregulated during the periparturient period in the transition dairy cow (Mallard et al., 1998; Heiser et al., 2015; Lange et al., 2016). Consequently, there is a high incidence of infectious disease during this time (Drackley et al., 2005; LeBlanc, 2010). Neutrophils are of particular interest due to their important role in defending against infectious disease (Cai et al., 1994). Neutrophil function is altered during the peripartum period in dairy cows (Detilleux et al., 1995); however, there are limited studies characterizing the extent of this alteration at the level of gene expression. Furthermore, the selection of endogenous control genes for quantitative gene expression analysis requires further investigation.

Reverse transcriptase-quantitative PCR (RT- qPCR) is a technique commonly used to analyse the expression of genes in a biological experiment (Bustin, 2002). Quantification of genes can inform changes in protein expression and enable changes in cell function to be inferred. Quantification is normally achieved by comparing abundance of target genes relative to the expression of known endogenous control genes, also known as housekeeping or reference genes (Vandesompele et al., 2002). The criteria for endogenous control genes include stable expression in the cell or tissue type under investigation and that they do not differ between treatment groups. The selection of stably expressed genes for target gene normalization is essential for accurate gene expression results (García-Vallejo et al., 2004; Janovick-Guretzky et al., 2007). Endogenous control gene expression can vary depending on cell or tissue type, as well as experimental conditions. Therefore, in order to accurately validate RT-qPCR experiments it is important to explore several possible endogenous control genes prior to relative quantification analysis.

One of the platforms available for RT-qPCR analysis is the BioMark HD™ (Fluidigm, San Francisco, CA), which utilizes microfluidics technology. In contrast to standard RT-qPCR using 384-well plates, the integrated fluidic circuits (IFC) used in the current study (96.96 Dynamic Array™ IFC; Fluidigm) generate 24 times the number of data points. Furthermore, the 96.96 IFCs allow quantification of 96 genes and 96 samples per chip using less reagents and starting material than standard RT-qPCR reactions of 10-20 µL. For example, a 96.96 IFC requires 360 µL of PCR master mix to generate 9,216 gene expression results, compared with 1.92 mL to generate 384 data points in a standard 384-well plate (using 10 µL reaction volumes). The microfluidics technology provides intermediary throughput compared with gene expression throughput using standard RT-qPCR or microarray technology. Fluidigm IFC arrays have been used successfully for large-scale gene expression analysis (Guo et al., 2010; Viturro et al., 2014). The RT-qPCR data from Fluidigm IFC arrays have been validated by comparison with those from an ABI 7900 HT platform using TaqMan® assay technology (Devonshire et al., 2011). Here, we describe the use of microfluidics technology in combination with a different probe-based assay (Roche Diagnostics, Switzerland) using RT-qPCR for the selection of endogenous control genes that are suitable for gene expression analysis of circulating bovine neutrophils isolated during the transition period.

2.3 Materials and Methods

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46’S 175°18’E) between July and October 2013.

A subset of cows of mixed age and breed (Holstein-Friesian, Jersey, Holstein-Friesian × Jersey) were selected from a parent transition cow experiment; described in detail by Roche et al. (2015). Cows were one of two BCS categories (4.0 or 5.0; based on a 10-point scale, equivalent to approximately 2.75 and 3.25, respectively, on a 5-point scale; Roche et al., 2004) and one of two levels of energy intake preceding calving (75 or 125% of estimated requirements). Blood was sampled by coccygeal venipuncture at five time points over the transition period; one wk before estimated calving date (-1 wk; n = 46), day of calving (d 0; n = 46), and days 7 (1 wk; n = 46), 14 (2 wk; n = 45) and 28 (4 wk; n = 43) post-calving.

Blood for neutrophil isolation (total of ~50 mL/cow at each time point) was collected in six evacuated blood tubes (Vacutainer; BD Bioscience, Plymouth, UK) containing acid citrate dextrose (solution A; trisodium citrate 22.0 g/L, citric acid 8.0 g/L, dextrose 24.5 g/L). Blood tubes were inverted eight times and placed immediately on ice awaiting neutrophil isolation by differential centrifugation based on protocols described elsewhere (Osorio et al., 2013b; Moyes et al., 2014). Total RNA was extracted from neutrophils using TRIzol® reagent (Life Technologies, New Zealand) as per the manufacturers' instructions. All RNA samples were DNase treated using the Ambion DNA-free kit (Life Technologies). The quantity and purity of RNA was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, DE, USA), which measures the optical density (OD) at the absorption ratio OD_{260nm}:OD_{280nm}. Quantity of total RNA per sample ranged from 2.7 µg to 112.1 µg with an average (± SD) of 29.6 ± 19.1 µg. RNA was considered of sufficient purity for cDNA synthesis if the OD_{260nm}:OD_{280nm} was >1.80. RNA integrity was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, CA, USA). Samples had an average RNA integrity number (± SD) of 8.0 ± 0.77. Samples were stored at -80°C until cDNA synthesis.

Total RNA (2 µg) from each sample was reverse transcribed using a Superscript III Supermix kit (Life Technologies) with random pentadecamer primers (Integrated DNA Technologies [IDT], Singapore) at a final concentration of 27 µM. Reverse-transcriptase negative controls were also generated by excluding the enzyme, and cDNA samples were stored at -20°C.

A diagram of the RT-qPCR workflow is displayed in Figure 1. Seventeen endogenous control genes were selected based on current literature (Table 2). Primer design for these genes for use in RT-qPCR was undertaken using publicly available bovine gene sequences from the National Centre for Biotechnology Information (NCBI) gene database (NCBI; http://www.ncbi.nlm.nih.gov). Assays were designed to span an intron-exon junction where possible, using Roche Universal Probe Library (UPL) design software (Table 3). Primer sequences were verified by BLAST and PrimerBLAST analysis (NCBI) to ensure specificity.

Table 2. Endogenous control gene information.

Gene symbol, gene name, biological function, and evidence of use in previous studies.

Gene symbol	Full name	Function	Reference(s)
ACTB	Actin, cytoplasmic 1	Cytoskeleton	Madsen et al., 2004; Vorachek et al., 2013
B2M	Beta-2-microglobulin precursor	MHC class 1	Vorachek et al., 2013
G6PD	Glucose-6-phosphate 1-dehydrogenase	Pentose phosphate pathway	Vorachek et al., 2013
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	Madsen et al., 2004; Zhang et al., 2005; Vorachek et al., 2013
GCH1	GTP cyclohydrolase 1	Folate and biopterin biosynthesis pathway	Wright et al., 2013
GOLGA5	Golgin subfamily A member 5	Golgi protein	Moyes et al., 2010a; Seo et al., 2013
OSBPL2	Oxysterol-binding protein-related protein 2	Golgi protein	Moyes et al., 2010a; Seo et al., 2013
PGK1	Phosphoglycerate kinase 1	Glycolysis	Vorachek et al., 2013
RPL13A	60S ribosomal protein L13a isoform 1	Ribosomal protein	Vandesompele et al., 2002
RPL19	60S ribosomal protein L19	Ribosomal protein	Vorachek et al., 2013; Madsen et al., 2004
RPS9	40S ribosomal protein S9	Ribosomal protein	Walker et al., 2009; Ji et al., 2012
SDHA	Succinate dehydrogenase complex flavoprotein subunit A	Citric acid cycle	Vorachek et al., 2013
SMUG1	Single-strand selective monofunctional uracil DNA glycosylase	Base excision repair	Moyes et al., 2010a; Seo et al., 2013
SNRPA	TATA-box-binding protein	Spliceosomal protein	Heiser et al., 2015
TBP	U1 small nuclear ribonucleoprotein A	Transcription factor	Zhang et al., 2005
UXT	Protein UXT	Transcription activator	Walker et al., 2009
YWHAZ	14-3-3 protein zeta/delta	Signal transduction	Vorachek et al., 2013

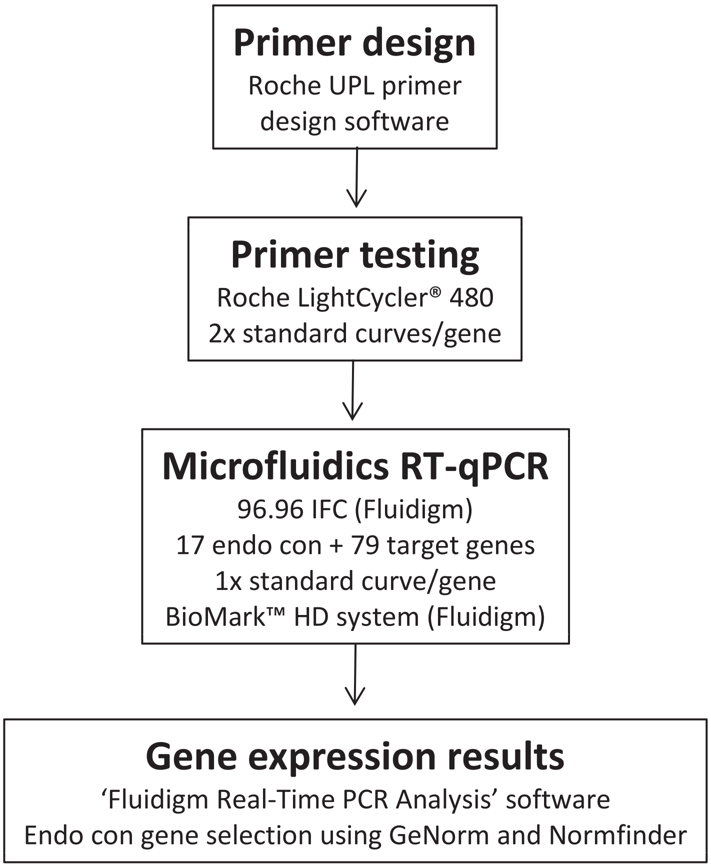

Figure 1. The workflow employed for RT-qPCR analysis including primer design through to gene expression results.

UPL = universal probe library; IFC = integrated fluidic circuit; endo con = endogenous control gene.

Table 3. Primer information for endogenous control genes investigated in neutrophils.

Gene symbol, mRNA accession number, gene ID, universal probe library (UPL) number and sequence (Roche, Germany), forward and reverse primers, the intron/exon boundary (if no boundary, only a single exon location is presented in brackets), amplicon size (number of nucleotides; nt), primer length, and the RT-qPCR efficiency using the Fluidigm Real-Time PCR Analysis software.

Gene symbol	Accession number	GeneID	UPL #	Probe Sequence	Forward	Reverse	Exon Boundary	Size (nt)	Fluidigm Efficiency
ACTB	NM_173979.3	280979	122	TGCCCTGA	CATCACCATCGGCAATGA	TGATGGAATTGAAGGTAGTTTCG	Exon 3/4	107	1.88
B2M	NM_173893	280729	7	CTTCTCCC	CCACCCCAGATTGAAATTGAT	CAGGTCTGACTGCTCCGATT	None (2)	66	1.89
G6PD	NM_001076124.2	538710	96	ACAGGCAG	AAAAGCCAACCTACAGATTTCG	TGAGCAGCAAGGTAGATTCG	Exon 3/4	103	1.91
GAPDH	NM_001034034.2	281181	9	CATCACCA	GAAGCTCGTCATCAATGGAAA	CCACTTGATGTTGGCAGGAT	Exon 4/5	67	-
GCH1	XM_010800681.1	286815	67	UGCUGGAG	AGCAATCATAGAGGCCTTGC	TTCTGCACTCCTCGCATTAC	Exon 3/4	87	1.87
GOLGA5	NM_001098007	510412	157	ACCACCAU	CCAGCGTCAATTCTGTGAAC	TTTCATGGCCAGAATCTGAA	Exon 2/3	111	-
OSBPL2	NM_001035020	505328	125	CUUGGAGC	GCGTGTGGAGCATATTGAAG	GCTATGGGCATCGTGATCTT	Exon 4/5	64	1.90
PGK1	NM_001034299.1	507476	71	GCAGCCAG	TGTCCCCATGCCTGATAAGT	CCACACAGTCCTTCAAGAACAA	Exon 3/4	95	-
RPL13A	NM_001076998.2	767925	157	ATGGTGGT	TCCCACCACCCTATGACAAG	CTTCAGACGCACAACCTTGA	Exon 5/6	68	1.89
RPL19	NM_001040516	510615	8	GAAGGCAG	GCGGATCCTCATGGAACAT	CGGGCTTCCTTGGTCTTAG	None (1)	105	1.89
RPS9	NM_001101152.2	533892	138	ATCCACCA	TGAGGATTTCTTGGAGAGACG	ATGTTCACCACCTGCTTGC	Exon 4/5	126	1.85
SDHA	NM_174178.2	281480	60	CTTCCCCA	CTGAAGCAGGTTTCAACACG	GTTGTCCTCCTCCATGTTCC	Exon 3/4	113	1.90
SMUG1	NM_001014958.1	539771	105	TGGGCATC	AATGATGAGCTGAGGCAACTG	GCGTATTCCACAGGGTTGTAG	None (2)	68	1.99
TBP	NM_001075742.1	516578	106	AGCCAGAG	TTGTGCTTACCCACCAACAG	GGAGAACAATTCTCGGTTTGA	Exon 7/8	90	1.84
SNRPA	NM_001046039.2	509802	24	GGGAGCTG	TTTTGTGGTCGGCACTCA	GCCATTGACAAAGTTGCTGA	Exon 1/2	87	2.08
UXT	NM_001037471.2	525680	29	GGCAGAAG	GGCCCTTGGATATGGTTTTT	AGTCCTTGGTGAGGTTGTCG	Exon 5/6	110	-
YWHAZ	NM_174814.2	287022	30	GGCTGAGG	TGAAAATGAAAGGAGACTACTACCG	GCTGTGACTGGTCCACAATC	Exon 3/4	84	1.90

Assays were tested using two standard curves on a LightCycler® 480 instrument (Roche, Basel, Switzerland). To do this, cDNA from two samples (from two divergent treatment groups) were serially diluted to make a 6-point 10-fold standard curve starting at 1× dilution to 1×10^{-6} dilution. Each 10 µL RT-qPCR reaction contained custom designed forward and reverse primers (200 nM, IDT, Singapore), UPL unique probe (50 nM, Roche), LightCycler® 480 Probes Master (2× conc) and RNAse/DNase-free distilled water. Standard cycling conditions were used (95°C for 10 min, [95°C for 10 s, 60°C for 30 s] × 50 cycles, 40°C for 40 s). Positive and negative samples were verified with gel electrophoresis to ensure a single product free of genomic DNA contamination and to verify the amplicon size.

Quantification of the endogenous control genes by RT-qPCR was achieved using the BioMark HD™ real-time PCR system (Ramaciotti Centre, NSW, Australia). Three microfluidics 96.96 Dynamic Array™ IFC chips (Fluidigm, San Francisco, CA) combined with Roche UPL chemistry were used to generate expression results for 17 endogenous control genes for all neutrophil samples (n = 226). Controls containing no template as well as reverse transcriptase negative controls were included on each IFC. All samples were run individually, as per manufacturers' recommendations. Four samples were run in duplicate across each chip to calculate well-to-well and chip-to-chip coefficient of variation (data not presented). A pre-amplification of all sample cDNA (1.25 µL of 1:4 diluted cDNA per chip) was undertaken (as per recommended instructions due to the nanoscale volumes utilized by the IFCs) to create specific target amplified reactions. This was achieved by multiplexing forward and reverse primers (200 nM) of the 96 assays with 5× concentration PreAmp Master Mix (Fluidigm) and the following cycling protocol; 95°C for 10 min, followed by 14 cycles × (95°C for 15 s and 60°C for 4 min). Assay mixtures, including forward and reverse primers (800 nM), Roche UPL probes (100 nM), and 2× concentration assay loading reagent (Fluidigm) were combined with 2× concentration Roche LightCycler® 480 Probes Master (Roche) and 20× gene expression sample loading reagent (Fluidigm) on a 96.96 IFC (Fluidigm) for use in the BioMark HD™ instrument. The gene expression cycling protocol

'GE 96×96 UPL v1.pcl' (BioMark HD Data Collection software v3.0.2); 70°C for 30 min, 25°C for 10 min, 95°C for 1 min followed by 35 cycles of 96°C for 5 s and 60°C for 20 s, was used to generate crossing point (Cp) values (Figure 2).

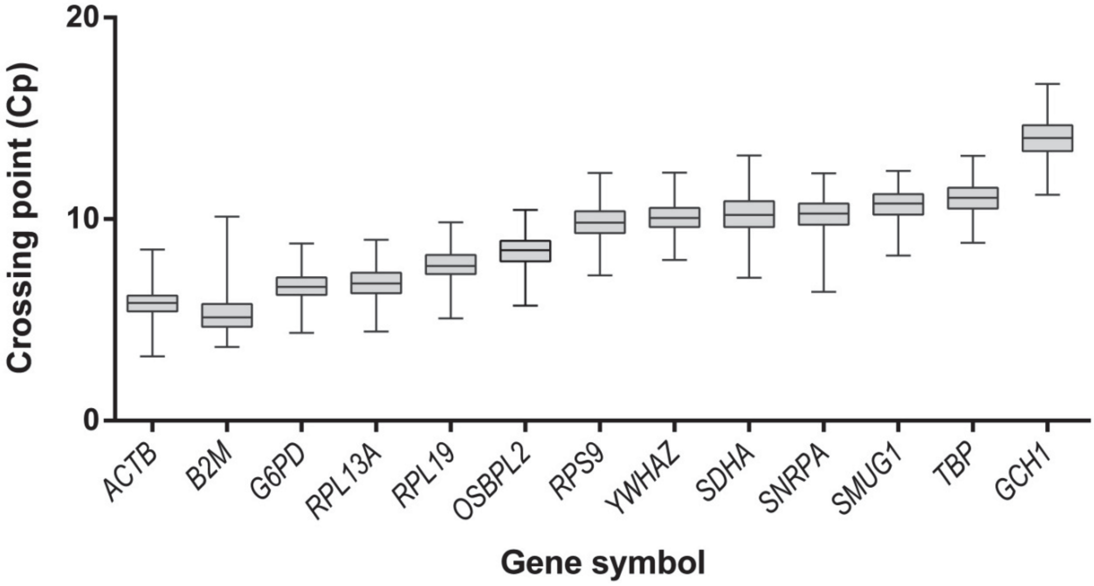

Figure 2. Expression graph of candidate endogenous control genes in bovine neutrophil samples using Microfluidics 96.96 gene expression arrays (Fluidigm).

Values are presented as the crossing point (Cp)/cycle threshold and are inversely proportional to the amount of template. Expression levels are demonstrated as the 25th and 75th quartiles (horizontal lines), median (central horizontal line) and whiskers. Whiskers represent the minimum to maximum Cp value. Genes are ordered from the most abundant (lower Cp, on the left) to least abundantly expressed (higher Cp, on the right).

A 5-point standard curve was included (3-fold serial dilutions from 3.33×10^{-1} to a final dilution of 4.12×10^{-3}) from one pre-amplified sample to calculate the RT-qPCR efficiency of all gene assays. The assay efficiency (Table 3) was calculated using the slope of the standard curve (Yuan et al., 2006). Endogenous control gene stability was tested across all samples using geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) software (Figure 3 and Figure 4, respectively).

2.4 Results and Discussion

This study is the first to investigate potential endogenous control genes in bovine neutrophils using microfluidics technology. The genes *YWHAZ* and *RPL19* were selected as the most suitable for gene expression analysis using relative quantification, despite having the best stability in only one of the endogenous control gene analyses. Although, *RPL19* expression was categorized as the most stable in both software packages, the expression of *YWHAZ* was ranked the most stable in combination with *RPL19* using the NormFinder software (stability of 0.072), but not the geNorm software (Table 4). The final selection was based on the following rationale: 1) more weighting was placed on the NormFinder analysis because this software considers gene expression variation attributed to treatment group (Andersen et al., 2004; De Spiegelaere et al., 2015), and 2) the use of two ribosomal genes as endogenous control genes was avoided to avert potential bias (Vandesompele et al., 2002). There were three treatment groups included in the gene expression data in the current study and the inter- and intra-variation among groups considered using NormFinder. In comparison, the geNorm software utilizes pairwise stability to find two optimal genes that have an equal expression ratio in all samples.

Table 4. Ranking of the candidate endogenous control genes according to their stability using geNorm and NormFinder analyses.

	NormFinder		geNorm	
Rank	**Gene symbol**	**Stability**	**Gene symbol**	**Stability**
1	*RPL19/YWHAZ*	0.072	*RPL13A/RPL19*	0.337
2	*RPS9*	0.139	*RPS9*	0.361
3	*RPL13A*	0.142	*SDHA*	0.412
4	*ACTB*	0.144	*YWHAZ*	0.485
5	*SDHA*	0.151	*ACTB*	0.514
6	*G6PD*	0.163	*GCH1*	0.534
7	*OSBPL2*	0.169	*G6PD*	0.551
8	*GCH1*	0.172	*OSBPL2*	0.562
9	*TBP*	0.218	*TBP*	0.581
10	*SMUG1*	0.221	*SNRPA*	0.600
11	*B2M*	0.222	*SMUG1*	0.623
12	*SNRPA*	0.252	*B2M*	0.661

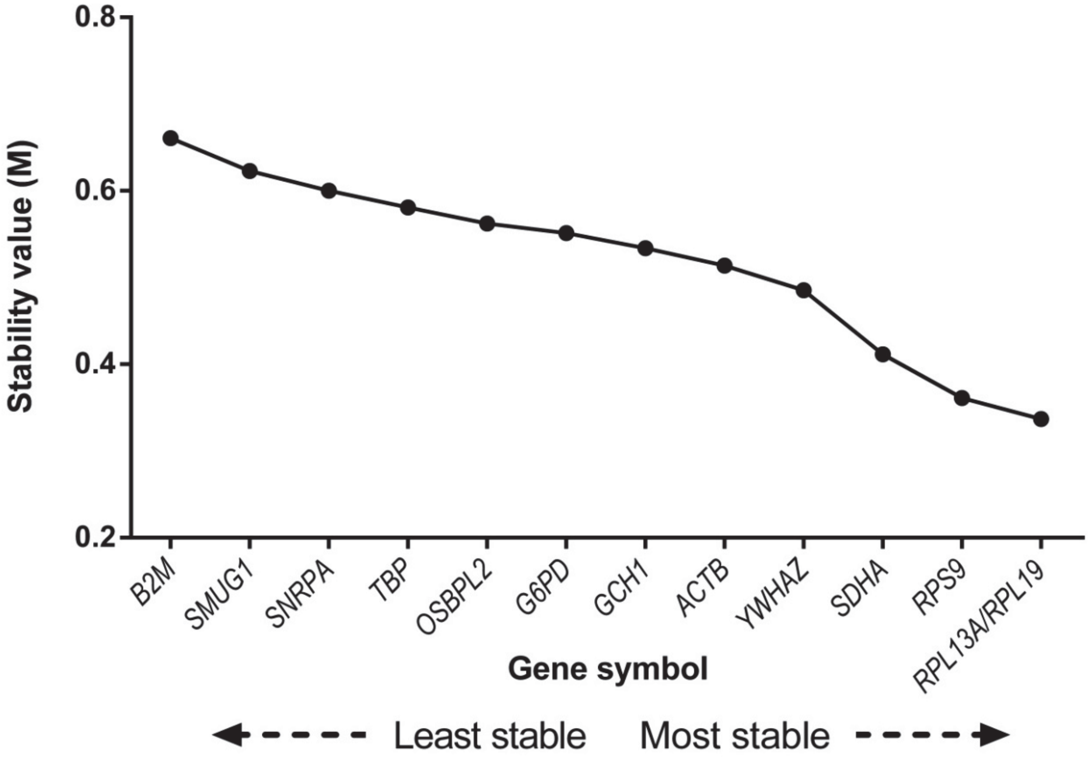

Figure 3. Expression stability of endogenous control genes analysed by geNorm. Average expression stability values (M) following stepwise exclusion of the least stable reference genes across all treatment groups. A lower M value indicates more stable gene expression.

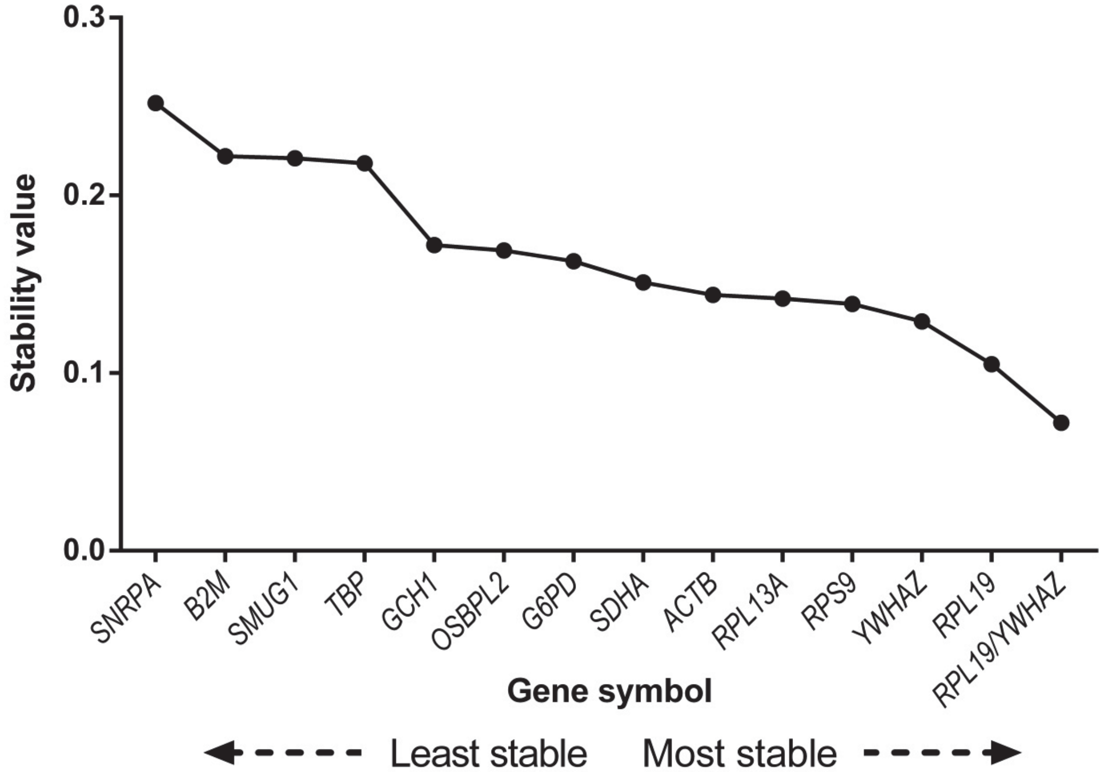

Figure 4. Expression stability of the candidate reference genes analysed by NormFinder.

Includes the combination of the two genes most stable (*RPL19* and *YWHAZ*).

This provides information on how many endogenous control genes should be used to improve stability (Figure 5). However, the most stable genes using the geNorm software often have similar expression patterns (e.g., ribosomal genes; rRNA). Vandesompele et al. (2002) advise caution when selecting rRNA molecules as endogenous control genes because rRNA expression may not be a good representation of the total mRNA pool. The final selection of endogenous control genes should consider experimental design and gene regulation to avoid selecting genes that are co-regulated. The use of rRNA genes as the chosen endogenous control genes was, therefore, disregarded, despite the rRNA pair of *RPL13A* and *RPL19* having the best stability in geNorm.

It is likely that some assays were inhibited during the RT-qPCR protocol using microfluidic IFC arrays and would require further optimization for use with a microfluidics platform. The expression data of four genes (*GAPDH*, *GOLGA5*, *PGK1*, and *UXT*) was below the fluorescence threshold for quantification analysis and were, therefore, excluded from endogenous control gene data analysis. These genes were expected to be expressed by circulating neutrophils based on primer optimization and previous studies (Holzer et al., 2005; Bai et al., 2014; Seo et al., 2013; Vorachek et al., 2013). A possible reason for inhibition is that the starting cDNA was too concentrated. In support of this, expression curves that passed the fluorescence threshold were evident for genes *GOLGA5*, *PGK1*, and *UXT* at lower concentrations of the standard curve. RT-qPCR reactions can fail when concentrations of primers are too high, or in the presence of amplification inhibitors (Cone et al., 1992; Gallup and Ackermann, 2006). An advantage of the microfluidics chip is that small amounts of cDNA are required to generate expression data for 96 genes.

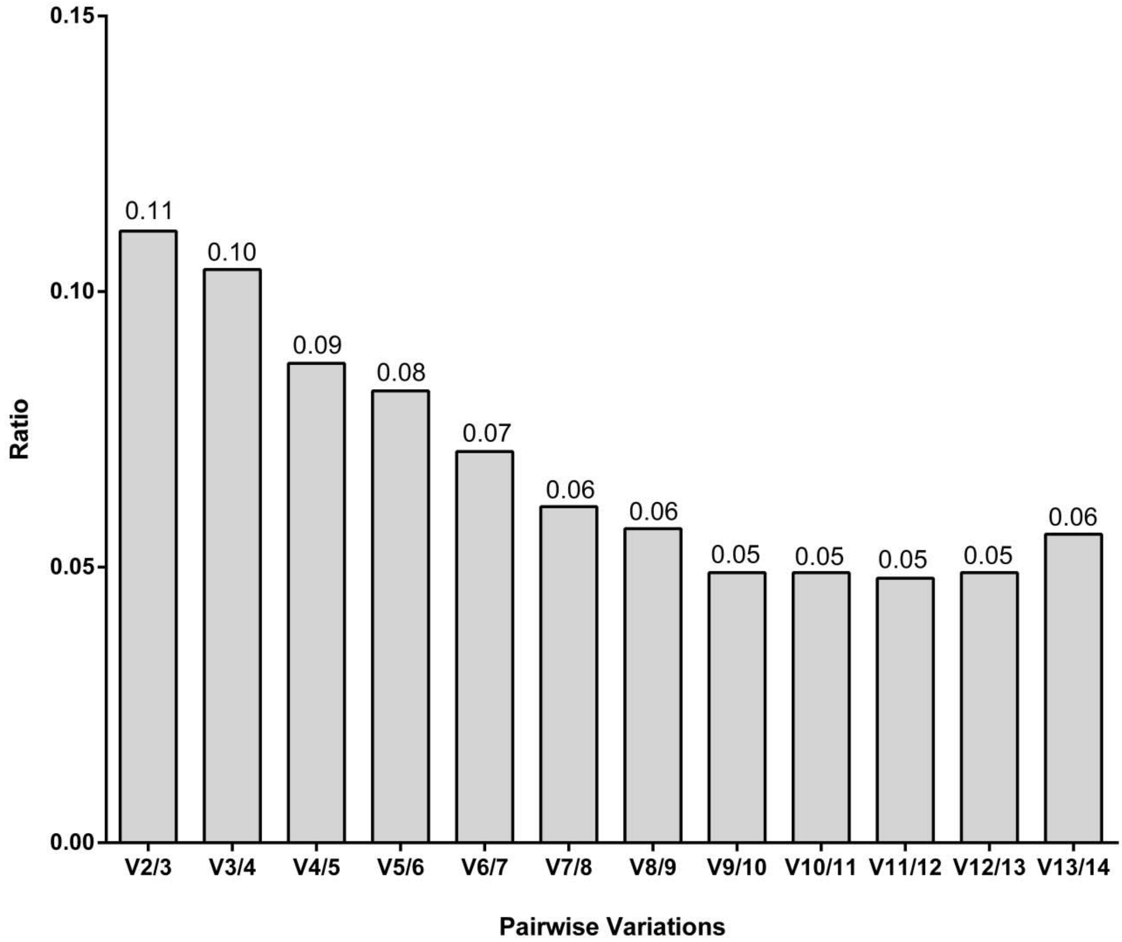

Figure 5. Pairwise variation (Vn/Vn +1) between the normalisation factors NF_n and NF_{n+1}.

Pairwise variation ratios help determine the optimal number of reference genes for normalisation using geNorm. A recommended ratio is <0.15.

This is because the structural design of the chip means that all 96 assays are added to a reaction mix containing the individual sample cDNA (White et al., 2011). However, starting concentrations are amplified when the reagents are injected into the microfluidic circuit. This may cause inhibition in some cases due to the starting concentrations being too high. This inhibition is normally overcome by using different dilutions of cDNA, which are calculated based on cycle thresholds generated during primer optimization. A drawback to the IFC array is that it does not support running samples at different concentrations as all the gene expression data are generated in one reaction vessel of 6.7 nL volume (White et al., 2011). Furthermore, one aspect of endogenous control gene expression that is often not considered is that control genes should have similar expression levels as target genes. The selection of genes for use with microfluidics technology should consider the relative expression to ensure the dynamic range is not outside the limits of the technology.

The 17 genes selected for investigation have been used as endogenous control genes in previous RT-qPCR studies (Table 2), highlighting the importance of validating reference genes appropriate for the experimental design. Furthermore, Figure 6 demonstrates the relative expression (ΔCt) of one target gene (*SELL*) over the transition period normalised to different endogenous control gene pairs. All normalisations demonstrated significant ($P < 0.05$) changes over time with similar expression curves, with the exception of the normalisation with *B2M* and *SMUG1*. Tukey comparisons between weeks indicated that significance changed depending on the genes used for normalisation, highlighting the importance of a descriptive selection process. The stability results presented here are consistent with rankings in previous studies; however, several of these commonly-used endogenous control genes were not considered appropriate for gene expression analysis of bovine neutrophils. De Ketelaere et al. (2006) identified *YWHAZ*, *SDHA*, and *18S* rRNA as the most stable genes in bovine neutrophils, which is consistent with our results. In comparison, Bai et al. (2014) identified *UXT* and *RPS9* to be among the most stable reference genes; however, their study used neutrophils isolated from yak (*Bos grunniens*) milk. The most extensive endogenous control gene study in circulating neutrophils from ruminant animals was conducted on cells isolated from sheep (*Ovis aries*) under different disease states (Vorachek et al., 2013). In agreement with the results of our study, genes *PGK1*, *ACTB*, and *B2M* were considered unreliable endogenous control genes. However, Vorachek et al. (2013) concluded that two different sets of endogenous controls were suitable, depending on the disease state; *SDHA*/*G6PD* in healthy sheep and *GAPDH*/*YWHAZ* in diseased sheep. Our study used healthy cattle (*Bos taurus*); the results suggest that there are species-specific differences that also must be considered.

Furthermore, previous studies investigating endogenous control gene selection in neutrophils have not used microfluidics technology for RT-qPCR analysis (Zhang et al., 2005; De Ketelaere et al., 2006; Vorachek et al., 2013; Bai et al., 2014). There is added variation introduced with the preamplification required for RT-qPCR using IFC arrays, which is evident when comparing well-to-well and plate-to-plate variation in samples that have undergone preamplification independently (Spurgeon et al., 2008). Future investigations will compare the results from Fluidigm microfluidics IFC arrays and the Roche LightCycler480 for which the assay chemistry was designed. In summary, multiple factors highlighted in the current study must be considered when selecting endogenous control genes: the technology employed, the cell (or tissue) type, and the species of origin.

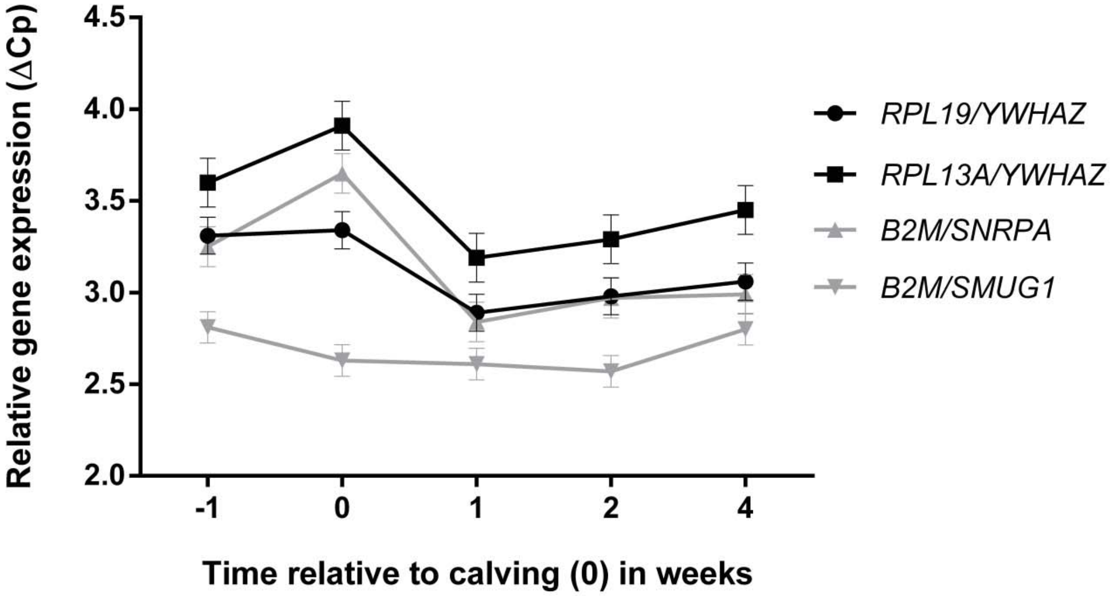

Figure 6. **Relative gene expression (ΔCp) of L-selectin (*SELL*) during the transition period using different pairs of genes for normalisation.**

Pairs were chosen based on GeNorm and Normfinder results (Table 4) by selecting the most stable genes (black lines) and the least stable genes (grey lines). A repeated measures analysis of variance (ANOVA; SAS 9.3) was used to determine the effect of time (week) on gene expression. All comparisons demonstrated significant ($P < 0.05$) changes in *SELL* gene expression over time. However, Tukey comparisons between time points differed between normalisation calculations.

2.5 Conclusion

Microfluidics technology is a powerful tool for gene expression analysis. The cost per gene is less compared with conventional RT-qPCR methods due to the reduced volume of expensive reagents required. We have demonstrated that the use of Roche UPL chemistry is compatible with the Fluidigm gene expression arrays. Furthermore, we recommend using the geometric mean of the two commonly-used endogenous control genes *RPL19* and *YWHAZ* for gene expression analysis in bovine neutrophils. This study emphasizes that it is essential to validate endogenous control genes in a RT-qPCR experiment by comparing the stability of multiple genes. We have used this study as the basis for the relative gene expression quantification in the subsequent experiments described in Chapters 3 and 4.

2.6 Acknowledgements

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Chapter 3

Parturition in dairy cows temporarily alters the expression of genes in circulating neutrophils

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3.1 Abstract

Extensive metabolic and physiologic changes occur during the peripartum, concurrent with a high incidence of infectious disease. Immune dysfunction is a likely contributor to the increased risk of disease at this time. Studies using high yielding, total mixed ration (TMR)-fed cows have indicated that neutrophil function is perturbed over the transition period; however, this reported dysfunction has yet to be investigated in moderate-yielding, grazing dairy cows. Therefore, we investigated changes in the expression of genes involved in neutrophil function. Blood was collected from cows at five time points over the transition period: pre-calving (-1 wk; n = 46), day of calving (d 0; n = 46) and post-calving at wk 1 (n = 46), wk 2 (n = 45), and wk 4 (n = 43). Neutrophils were isolated by differential centrifugation and gene expression was investigated. Quantitative reverse transcriptase PCR with custom designed primer pairs and Roche Universal Probe Library chemistry, combined with microfluidics integrated fluidic circuit chips (96.96 Dynamic Array) were used to investigate the expression of 78 genes involved in neutrophil function and 18 endogenous control genes. Statistical significance between time points was determined using a repeated measures ANOVA. Genes that were differentially expressed over the transition period included those involved in neutrophil adhesion (*SELL*, *ITGB2*, and *ITGBX*), mediation of the immune response (*TLR4*, *HLA-DRA*, and *CXCR2*), maturation, cell cycle progression, apoptosis (*MCL1*, *BCL2*, *FASLG*, and *RIPK1*), and control of gene expression (*PPARG*, *PPARD*, and *STAT3*). There was reduced gene expression of pro-inflammatory cytokines (*IFNG*, *TNF*, *IL12*, and *CCL2*) on the day of calving, whereas anti-inflammatory cytokine gene expression (*IL10*) was up-regulated. Increased gene expression of antimicrobial peptides (*BNBD4*, *DEFB10*, and *DEFB1*) occurred on the day of calving. Collectively, transcription profiles are indicative of functional changes in neutrophils of grazing dairy cows over the transition period and align with studies in cows of conventional TMR systems. This altered function may predispose cows to disease over the transition period and is likely to be a natural change in function due to parturition.

3.2 Introduction

The transition period in a dairy cow is a critical period of metabolic and physiologic change encompassing parturition and the onset of lactogenesis (Grummer, 1995). A considerable alteration in nutrient and metabolic demand is associated with an increased susceptibility to metabolic and infectious disease conditions during the peripartum period (Overton and Waldron, 2004; Ingvartsen, 2006). For example, the incidence of intramammary infections is increased in the four weeks immediately post-calving both in TMR (Østergaard et al., 2005) and pasture-based dairy systems (Compton et al., 2007). An estimated 30 to 50% of cows are reported to experience health disorders during the transition period, and the majority (75%) of infectious disease and metabolic disorders in dairy cows occur during this time (Ingvartsen, 2006; LeBlanc et al., 2006). In pasture-based systems an estimated NZ$300 per cow is lost annually due to costs associated with transition failure (i.e., cows that experience health disorders; Compton et al., 2007; Roche, 2013c).

Previous studies have indicated that the high incidence of disease during the transition period is linked to a state of reduced immune function (Detilleux et al., 1995; Goff and Horst, 1997; Heiser et al., 2015). In these investigations, neutrophils have received much attention due to their essential role in defence against pathogens responsible for mastitis and uterine infections (Madsen et al., 2004; Hammon and Goff, 2006; Graugnard et al., 2012a). Functional studies at the cellular level have indicated neutrophil dysfunction during the peripartum period; for example, studies have shown reduced superoxide production, phagocytosis, expression of cell surface adhesion molecules, and capacity for migration (Kehrli et al., 1989b, 1995; Burton et al., 2005). Consequently, there is reduced efficiency of the neutrophil inflammatory response, thereby contributing to greater incidence of infection. The extent of this peripartum neutrophil dysfunction has not been fully elucidated.

Furthermore, the studies referred to were all undertaken using high-yielding housed cows. However, milk production is 30 to 50% less in pasture-based systems (Kolver and Muller, 1998), the population density on farm is lower, different management systems may alter pathogen load, and the infectious disease-causing pathogens may differ. Therefore, we hypothesized that the changes in peripartum neutrophil function will be different in grazing dairy cows in the absence of stimulation, i.e., without an in vitro or in vivo challenge. To investigate this, neutrophils were extracted over the transition period from moderate-yielding grazing dairy cows and the expression of 78 genes involved in neutrophil function were investigated.

3.3 Materials and Methods

Animal Handling

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46'S 175°18'E) between July and October 2013.

Experimental Design and Treatments

A subset of cows of mixed age and breed (Holstein-Friesian, Jersey, Holstein-Friesian × Jersey) were selected from a parent experiment; described in detail by Roche et al. (2015). Cows were one of two pre-calving BCS categories (4.0 or 5.0; based on a 10-point scale; Roche et al., 2004) and one of two levels of energy intake preceding calving (75 or 125% of estimated requirements). Blood was sampled by coccygeal venipuncture at five time points over the transition period; one wk before estimated calving date (-1 wk; n = 46), day of calving (d 0; n = 46), day 7 (1 wk; n = 46), day 14 (2 wk; n = 45) and day 28 (4 wk; n = 43) post-calving. Blood for neutrophil isolation (total of 36 mL per cow at each time point) was collected in six evacuated blood tubes (Vacutainer; BD Bioscience, Plymouth, UK) containing acid citrate dextrose (solution A; trisodium citrate 22.0 g/L, citric acid 8.0 g/L, dextrose 24.5 g/L). Blood tubes were inverted eight times and placed immediately on ice awaiting neutrophil isolation by differential centrifugation based on protocols (Osorio et al., 2013b; Moyes et al., 2014) with modifications described below. Blood for metabolite analysis was collected in evacuated blood tubes containing a lithium heparin anti-coagulant, inverted and placed immediately on ice; within 30 min, blood tubes were centrifuged at 1,500 × *g* for 12 min at 4°C, plasma aspirated and stored at -20°C until assayed.

Liver Sampling for Triacylglycerol Analysis

Liver samples were collected from all cows by biopsy the wk before calving (wk -1), and subsequently on wk 1, 2, and 4 post-calving. Briefly, the skin was shaved and disinfected and the area anaesthetised with 7 mL of 2% lignocaine (Lopaine 2%, lignocaine hydrochloride 20 mg/mL, Ethical Agents, South Auckland, New Zealand). An incision was made through the skin in the right 11th intercostal space at the level of the greater trochanter, through which a 12G × 20 cm biopsy needle was inserted into the liver and approximately 1 g (wet weight) of liver tissue was collected. Liver samples were snap-frozen in liquid nitrogen and stored at -80°C.

Neutrophil Isolation

Neutrophils were isolated according to procedures previously described (Moyes et al., 2010a; Osorio et al., 2013b) with modifications. Blood collected in the six acid citrate dextrose evacuated blood tubes were inverted and pooled by transfer into a 50 mL conical tube. Tubes were centrifuged for 30 min at 850 × *g* at 4°C. The plasma, buffy coat, and up to 1/3 of the red blood cells were aspirated by Pasteur pipette and discarded, leaving 2/3 of remaining red blood cells and neutrophils. Water (milliQ, Millipore, New Zealand) was added (25 mL) and mixed for 10 - 30 s to lyse the red blood cells (RBC) and 5 mL of 5× concentrated PBS (pH 7.4, Life Technologies, New Zealand) was added to each tube to restore isotonicity. Tubes were spun at 330 × *g* for 10 min at 4°C and supernatant decanted to isolate the white cell pellet. The pellet was washed with 10 mL of 1× concentrated PBS (pH 7.4, Life Technologies), vortexed and centrifuged for 5 min at 660 × *g* at 4°C. If RBC remained, the water lysing step was repeated. Cells were re-suspended in 5 mL of 1× phosphate buffered saline (PBS; pH 7.4) and 100 µL transferred to a 1.5 mL microcentrifuge tube (Raylab, New Zealand) and placed on ice for subsequent viability and staining assays. The 50 mL tubes were centrifuged again (5 min at 660 × *g* at 4°C), liquid decanted, and the cell pellet resuspended in 1 mL TRIzol® reagent (Life Technologies). The 1 mL cell suspension was transferred to a 2 mL microcentrifuge tube (Eppendorf, Germany), placed immediately on ice, and stored at -80°C for subsequent RNA extraction.

Flow Cytometry Analysis

A subpopulation of isolated cells from randomly selected samples (n = 41) was used to determine cell viability and neutrophil population by flow cytometry analysis (Figure 7) using the BD FACS Aria II cell sorter (BD Biosciences, USA). Viability was measured using LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (L23105, Life Technologies) and neutrophil populations were determined with the bovine granulocyte specific primary monoclonal antibody CH138A (WS0608B-100, Kingfisher Biotech Inc., MN, USA) and phycoerythrin-conjugated secondary antibody (SB102009S, Southern Biotech, AL, USA). Of total granulocytes isolated (78.6% ± 14.4% median ± SD) with this procedure, 93.4% ± 7.9% (mean ± SD) were neutrophils and of these, 93.3% ± 10.2% (mean ± SD) were live cells.

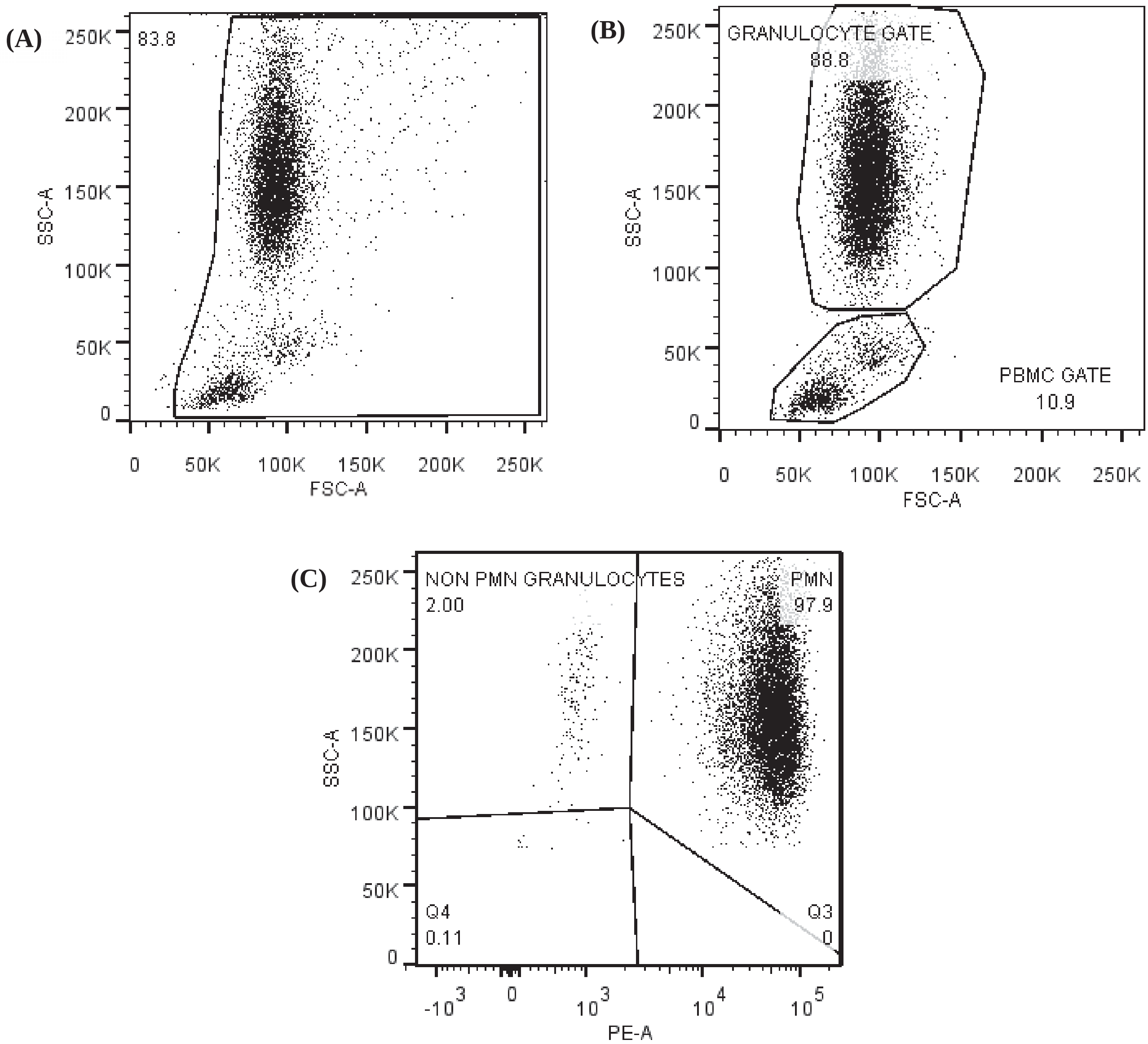

Figure 7. Flow cytometric pictures of representative sample demonstrating gating strategy.

(A) Initial gating removes counts that are sitting on the edges of the flow cytometry parameters. Counts were plotted by forward scatter-area (FSC-A) and FSC-height (FSC-H) to remove doublets (data not shown). **(B)** Gating strategy to identify population of granulocytes from peripheral blood mononuclear cells (PBMC) based on side scatter-area (SSC-A) and FSC-A. **(C)** Of the total granulocytes isolated, neutrophils (quadrant 2 **(C)**; PMN) could be distinguished from non-neutrophil granulocytes (quadrant 1 **(C)**; non PMN granulocytes) based on CH138A-phycoerythrin (PE) fluorescence.

RNA Extraction

Total RNA was extracted from neutrophils (n = 226) using TRIzol® reagent as per the manufacturers' instructions. Briefly, cell suspensions in TRIzol® were homogenized using a PRO200 homogenizer (Pro Scientific, CT, USA); suspensions underwent two 20 s bursts of the homogenizer at full speed (500 Hz) and placed on ice after every homogenization. The homogenizer tool was rinsed twice with 75% ethanol and water, and once with RNAseZap® (Life Technologies) between each sample. Chloroform was added to the homogenized samples, and after centrifugation the aqueous phase was carefully removed. Precipitation of RNA was achieved with the addition of isopropanol (Merk Millipore, Germany) and the subsequent RNA pellet was washed (with 75% ethanol), dried, and re-suspended in UltraPure™ RNAse/DNase-free distilled water (Life Technoligies). All RNA samples were DNase treated using the Ambion DNA-free kit (Life Technologies). The quantity of RNA was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, DE, USA) and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, CA, USA). Samples had an average RNA integrity number (± SD) of 8.0 ± 0.77. Samples were stored at -80°C until cDNA synthesis.

cDNA Synthesis and Primer Design

Total RNA (2 μg) from each sample was reverse transcribed using a Superscript III Supermix kit (Life Technologies) with random pentadecamer primers (Integrated DNA Technologies [IDT], Singapore) at a final concentration of 27 μM. Reverse-transcriptase negative controls were also generated by excluding the enzyme, and cDNA samples were stored at -20°C.

Assay design for 96 gene targets (78 genes involved in neutrophil function and 18 possible endogenous control genes identified using current literature, Supplementary Table 1) for use in reverse transcription-quantitative PCR (RT-qPCR) was undertaken using publicly available bovine gene sequences from the National Center for Biotechnology Information (NCBI) gene database (NCBI; http://www.ncbi.nlm.nih.gov). Assays were designed to span an intron-exon boundary, where possible, using Roche Universal Probe Library (UPL) design software. Primer sequences were verified by BLAST and PrimerBLAST analysis (NCBI) to ensure specificity. Assays were tested on a LightCycler® 480 instrument (Roche, Basel, Switzerland). Each RT-qPCR reaction contained custom designed forward and reverse primers (200 nM, IDT, Singapore), UPL unique probe (50 nM, Roche), LightCycler® 480 Probes Master (2× conc) and RNAse/DNase-free distilled water. Standard cycling conditions were used (95°C for 10 min, [95°C for 10 s, 60°C for 30 s] × 50 cycles, 40°C for 40 s). To test assays, a 6-point 10-fold standard curve was generated starting at 1× dilution (1:4 dilution of raw cDNA) to 1×10^{-6} dilution. Positive and negative samples were verified with gel electrophoresis to ensure a single product free of genomic DNA contamination and to verify the amplicon size.

Gene Expression

Expression of genes involved in neutrophil function was assayed using RT-qPCR using the BioMark HD™ real-time PCR system (Ramaciotti Centre, NSW, Australia). Microfluidics 96.96 Dynamic Array™ integrated fluidic circuit chips combined with Roche UPL chemistry were used to generate expression results for 96 gene targets for all samples (n = 226). A pre-amplification of cDNA was undertaken to create specific target amplified reactions by multiplexing forward and reverse primers (200 nM) of the 96 assays with 5× concentration PreAmp Master Mix (Fluidigm, PN, USA) and the following cycling protocol; 95°C for 10 min, (95°C for 15 s, and 60°C for 4 min) × 14 cycles. Assay mixtures, including forward and reverse primers (800 nM), Roche UPL probes (100 nM), and 2× concentration assay loading reagent (Fluidigm) were combined with 2× concentration Roche LightCycler® 480 Probes Master (Roche) and 20× GE sample loading reagent (Fluidigm) on a 96.96 gene expression chip (Fluidigm) for use in the BioMark HD™ instrument. The cycling protocol GE 96×96 UPL v1.pcl; 70°C for 30 min, 25°C for 10 min, 95°C for 1 min followed by 35 cycles of 96°C for 5 s and 60°C for 20 s was used to generate crossing point (Cp) values.

Possible endogenous control genes were tested across all samples using GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) software (Chapter 2). A 5-point standard curve was included (3-fold serial dilutions from 3.33×10^{-1} to a final dilution of 4.12×10^{-3}) to calculate RT-qPCR efficiency of all 96 genes. There were 18 potential endogenous control genes included in the experiment. Of the 18 endogenous control genes tested, *RPL19* and *YWHAZ* were chosen as the two endogenous control genes for this neutrophil study. The NormFinder stability of the combination of these two genes was 0.072 and the GeNorm stability values (M) were < 0.58 for both genes, with no improvement in stability with the addition of a third endogenous control gene.

Relative quantification was achieved using the delta Cp method; the geometric mean of the Cp values of the endogenous control genes was calculated and the target assay Cp values were made relative to the geometric mean of endogenous controls (ΔCp). The fold change relative to the day of calving (ΔΔCp; data not shown) can be calculated as shown in Equation [1].

$$\Delta\Delta Cp = E_{\text{gene of interest}} \wedge {}^{-(\Delta Cp\ \text{time point} - \Delta Cp\ D\ 0)} \qquad [1]$$

Where E is the efficiency of the RT-qPCR reaction (Supplementary Table 1) calculated using the slope of the standard curve generated by RT-qPCR and the Fluidigm Real-Time PCR Analysis software.

Metabolite Analysis

Blood metabolite and the liver triacylglycerol (TAG) assays were undertaken by Gribbles Veterinary Pathology Ltd (Hamilton, New Zealand). Full details of blood metabolite analysis are described in Roche et al. (2015). Briefly, blood metabolites were assayed using colorimetric techniques at 37°C with a Hitachi Modular P800 analyser (Roche Diagnostics). Roche reagent kits were used to measure plasma concentrations of albumin (g/L), BHBA (mmol/L), Ca (mmol/L), Mg (mmol/L), and total protein (g/L). Plasma globulin was calculated as the difference between total protein and albumin. Plasma NEFA concentrations (mmol/L) were measured using Wako Chemicals (Osaka, Japan) kit. Interleukins (IL-1β and IL-6) and haptoglobin were analysed by commercial bovine ELISA kits (Pierce, Thermo Scientific, IL, USA, and GenWay Biotech Inc., CA, USA. and Life Diagnostics Inc., PA, USA). Cholesterol and total antioxidant capacity (TAC) were also measured through commercially available kits (10007640 and 709001 respectively, Cayman Chemical Company, MI, USA). Reactive oxygen species (ROS) were measured through a commercially available fluorimetric kit (STA-342, Cell Biolabs Inc., CA, USA).

Liver TAG content was analysed in biopsy samples (wk 1, 2, and 4 post-calving) using the standard procedure provided in the Wako LabAssay TM Triglyceride Kit (290-63701, Wako Chemicals USA Inc., VA, USA).

Statistics

All variables (gene expression, metabolites, and production) were subjected to repeated measures ANOVA to assess the effect of time, using a mixed models approach (Proc Mixed, SAS 9.3) that included the fixed effects of treatment in the parent experiment, wk, and their interaction, and cow as a random effect. The model for gene expression also included the Fluidigm chip as a blocking factor, while the models for metabolites and production, where possible, included covariate data from the previous lactation. Metabolite data including BHBA, NEFA, aspartate transaminase (AST), haptoglobin, IL-1β, IL-6, and ROS were $\log_{10}$ transformed to achieve homogeneity of variance. If the effect of time was significant ($P < 0.05$), Tukey's t-test was used for pairwise comparisons among wk. Statistical significance was considered $P \leq 0.05$ and a trend was considered when $P \leq 0.10$.

Results are presented as least-squares means (LSM) with standard error of the difference (SED).

3.4 Results

The expression of genes involved in neutrophil function across the transition period is presented in Table 5. Genes are grouped into functional pathways and presented in alphabetical order within those groups. Relative expression (ΔCp) at each time point is presented, along with SED and significance of time effect (*P* value). Genes that were not significantly ($P \geq 0.05$) altered by time were *CCL3*, *CXCR4*, *IL8*, *TRAF6*, *CD14*, *FCGR1A*, *TAP*, *CPNE3*, *IAP*, *CDC42*, *DAXX*, *NFKB*, *PPARA*, and *ABCA1* (data not shown).

Expression of Genes Involved in the Inflammatory Response

Gene expression of pro-inflammatory cytokines *IFNG*, *TNF*, and *IL23A* and the chemokine *CCL2* was down-regulated ($P \leq 0.05$) on the day of calving compared with all other time points, and *IL12* was down-regulated at calving compared with 2 wk and 4 wk post-calving. Consistent with this, there was increased gene expression of cytokines *IL10*, an anti-inflammatory cytokine (Saraiva and O'Garra, 2010) and *IL6*, which can act as an anti-inflammatory cytokine during acute inflammation (Xing et al., 1998). Gene expression of *IL8* in neutrophils was not altered over the transition period (data not shown). Transcript abundance of IL8 receptors however (*CXCR1* and *CXCR2*), were down-regulated post-calving compared with -1 wk and the day of calving. Expression of the gene encoding MHC class II molecule *BOLA-DRA* decreased on the day of calving compared with all other time points. There were no significant ($P = 0.18$) changes in the expression of *CXCR4* over the transition period.

Toll-like Receptor Signalling. Expression of genes involved in Toll-like receptor (TLR) signalling, including *TLR2*, *TLR4*, *XBP1*, *TRAF6*, and *MYD88*, declined after calving relative to pre-calving expression levels. For example, gene expression of *XBP1* and *TLR4* was greater before calving than on the day of calving and 1 wk post-calving, respectively ($P < 0.05$). Similarly, *TLR2* and *MYD88* gene expression was greater on the day of calving compared with post-calving time points. However, TLR signalling genes *TRAF6* and *CD14* were not differentially expressed over the transition period.

Expression of Genes Involved in Antimicrobial Function of Neutrophils

Expression of antimicrobial peptide genes *BNBD4*, *DEFB1*, and *DEFB10* was up-regulated ($P < 0.05$) on the day of calving, compared with pre- and post-calving time points. Likewise, *LAP* gene expression was greater ($P < 0.01$) on the day of calving compared with pre-calving and 4 wk post-calving. There was a tendency ($P = 0.06$) for the gene expression of *TAP* to change with time, with a 1.5-fold increase at 1 wk post-calving compared with the day of calving. Expression of other microbicidal genes, *MPO* and *SOD1*, were also differentially expressed over the transition period; *SOD1* gene expression was lowest at -1 wk, increased on the day of calving, and remained elevated across the 4 wk measurement period. In comparison, expression of *MPO* was 2.0-fold less on the day of calving ($P < 0.05$) before returning to pre-calving expression levels at 4 wk.

Expression of matrix metalloproteinase genes *MMP8* and *MMP9* was up-regulated on the day of calving compared with all other time points. Expression of both transcripts dropped immediately post-calving; however, transcript abundance of *MMP9* remained above pre-calving levels at 1 wk (1.6-fold) and 2 wk (1.5-fold) post-calving ($P < 0.05$).

Table 5. Expression of genes involved in neutrophil function over the transition period. Genes that demonstrated significant ($P < 0.05$) expression changes over the transition period are presented. Expression is presented as means over time in arbitrary units normalised to two endogenous control genes (*RPL19* and *YWHAZ*; Chapter 2, ΔCp). Results of Tukey's t-test are also presented in superscript for pairwise comparisons between wk; where any two time points, for each gene, with at least one common letter do not differ significantly ($P >$ 0.05) and those without at least one common letter differ significantly ($P < 0.05$).

		Time relative to parturition (D 0)						
Function	**Gene**	**-1 wk**	**D 0**	**1 wk**	**2 wk**	**4 wk**	**SED**	***P* value**
Inflammation								
	CCL2	13.57^{a}	8.75^{b}	13.24^{a}	13.38^{a}	13.89^{a}	0.73	< 0.01
	IFNG	21.25^{a}	14.48^{b}	21.48^{a}	21.03^{a}	23.78^{a}	1.76	< 0.01
	IL1R2	5.22^{bc}	6.34^{a}	5.53^{b}	5.15^{bc}	4.87^{c}	0.21	< 0.01
	IL10	3.71^{b}	4.62^{a}	3.64^{b}	3.72^{b}	3.53^{b}	0.13	< 0.01
	IL12	17.00^{ab}	16.26^{b}	17.09^{ab}	17.55^{a}	17.74^{a}	0.48	< 0.01
	IL1RN	2.72^{a}	2.59^{ab}	2.37^{b}	2.46^{b}	2.48^{ab}	0.09	< 0.01
	IL23A	3.61^{b}	2.63^{c}	4.14^{a}	3.90^{ab}	4.23^{a}	0.16	< 0.01
	IL6	7.33^{bc}	8.50^{a}	8.08^{ab}	7.61^{abc}	7.25^{c}	0.35	< 0.01
	TNF	5.64^{b}	5.10^{c}	5.99^{a}	6.03^{a}	6.03^{a}	0.14	< 0.01
Immune mediators								
	BOLA-DRA	3.20^{a}	2.50^{c}	3.15^{ab}	2.89^{b}	3.09^{ab}	0.10	< 0.01
	CXCR1	3.26^{ab}	3.31^{a}	2.82^{c}	2.95^{bc}	2.80^{c}	0.10	< 0.01
	CXCR1&2	30.62^{ab}	30.70^{a}	30.07^{c}	30.20^{bc}	30.21^{bc}	0.18	< 0.01
	CXCR2	4.04^{ab}	4.28^{a}	3.48^{c}	3.66^{bc}	3.68^{bc}	0.14	< 0.01
	FCER1G	6.56^{ab}	6.68^{a}	6.23^{c}	6.30^{bc}	6.47^{abc}	0.10	< 0.01
	MYD88	3.44^{b}	3.70^{a}	2.99^{c}	3.10^{c}	3.11^{c}	0.11	< 0.01
	PTPRC	2.46^{ab}	2.41^{ab}	2.31^{ab}	2.30^{b}	2.54^{a}	0.08	< 0.05
	SYK	2.08^{b}	2.17^{ab}	2.16^{ab}	2.17^{ab}	2.26^{a}	0.07	< 0.05
	TLR2	2.60^{b}	3.06^{a}	2.42^{bc}	2.43^{bc}	2.24^{c}	0.10	< 0.01
	TLR4	4.46^{a}	4.28^{ab}	4.06^{b}	4.18^{ab}	4.30^{ab}	0.13	< 0.05
Antimicrobial								
	BNBD4	10.42^{c}	11.76^{a}	11.13^{b}	10.63^{bc}	10.36^{c}	0.24	< 0.01
	DEFB1	5.42^{b}	6.76^{a}	5.90^{b}	5.62^{b}	5.42^{b}	0.24	< 0.01
	DEFB10	6.20^{b}	7.56^{a}	6.86^{b}	6.44^{b}	6.04^{b}	0.27	< 0.01
	LAP	15.91^{bc}	16.65^{a}	16.21^{ab}	16.05^{abc}	15.63^{c}	0.19	< 0.01
	MMP8	8.63^{b}	9.74^{a}	8.65^{b}	8.64^{ab}	8.46^{b}	0.37	< 0.01
	MMP9	3.16^{c}	4.59^{a}	3.85^{b}	3.73^{b}	3.57^{bc}	0.21	< 0.01
	MPO	8.60^{ab}	7.84^{bc}	7.45^{c}	7.33^{c}	9.09^{a}	0.38	< 0.01
	PRKCB1	2.24^{b}	2.42^{ab}	2.32^{ab}	2.43^{ab}	2.53^{a}	0.08	< 0.01

	SOD1	5.98^{b}	6.57^{a}	6.60^{a}	6.48^{a}	6.41^{a}	0.19	< 0.01
	TAP	7.77^{a}	7.53^{a}	8.11^{a}	7.15^{a}	6.85^{a}	0.53	0.06
	XBP1	17.64^{a}	16.46^{b}	17.45^{ab}	17.30^{ab}	17.75^{ab}	0.34	< 0.05
Adhesion								
	CD44	2.27^{b}	2.59^{a}	2.35^{b}	2.21^{b}	2.30^{b}	0.08	< 0.01
	ITGA4	4.60^{b}	3.70^{c}	4.59^{b}	4.54^{b}	4.97^{a}	0.13	< 0.01
	ITGAL	2.45^{b}	2.44^{b}	2.50^{ab}	2.64^{ab}	2.78^{a}	0.09	< 0.01
	ITGAM	2.51^{b}	2.49^{b}	2.45^{b}	2.45^{b}	2.82^{a}	0.09	< 0.01
	ITGAX	2.52^{b}	3.13^{a}	2.65^{b}	2.51^{b}	2.59^{b}	0.10	< 0.01
	ITGB2	1.89^{b}	2.23^{a}	2.03^{ab}	2.01^{ab}	2.10^{a}	0.08	< 0.01
	LGALS8	3.10^{b}	2.68^{c}	3.36^{ab}	3.31^{ab}	3.52^{a}	0.11	< 0.01
	SELL	3.24^{a}	3.27^{a}	2.82^{b}	2.89^{b}	2.99^{ab}	0.10	< 0.01
	TLN2	15.02^{a}	13.74^{b}	15.78^{a}	15.67^{a}	15.84^{a}	0.35	< 0.01
Maturation								
	ANXA3	2.30^{a}	1.96^{b}	2.15^{ab}	2.13^{ab}	2.21^{a}	0.08	< 0.01
	BCL2	3.27^{b}	2.39^{c}	3.67^{a}	3.57^{ab}	3.80^{a}	0.11	< 0.01
	CASP8	2.26^{a}	1.98^{b}	2.13^{ab}	2.16^{ab}	2.32^{a}	0.07	< 0.01
	FADD	7.91^{a}	7.89^{a}	4.95^{a}	5.03^{a}	4.93^{a}	1.27	< 0.05
	FASLG	3.96^{bc}	3.48^{c}	4.02^{b}	3.93^{b}	4.65^{a}	0.13	< 0.01
	FCGR3A	6.02^{a}	4.54^{c}	5.37^{b}	5.60^{ab}	5.68^{ab}	0.16	< 0.01
	KIT	4.67^{b}	3.37^{c}	5.53^{a}	5.21^{a}	5.55^{a}	0.16	< 0.01
	MCL1	3.23^{ab}	3.40^{a}	2.89^{c}	3.04^{bc}	3.15^{abc}	0.12	< 0.01
	PTPRC	2.46^{ab}	2.41^{ab}	2.31^{ab}	2.30^{b}	2.54^{a}	0.08	< 0.05
	RIPK1	2.91^{a}	2.50^{ab}	2.45^{c}	2.64^{bc}	2.71^{b}	0.08	< 0.01
	S100A9	2.91^{b}	4.14^{a}	3.01^{b}	3.01^{b}	2.71^{b}	0.17	< 0.01
	THY1	4.08^{c}	5.93^{a}	4.76^{b}	4.61^{bc}	4.26^{bc}	0.24	< 0.01
Transcription factors								
	NCOR1	1.79^{ab}	1.77^{b}	1.89^{ab}	1.85^{ab}	1.98^{a}	0.06	< 0.01
	NCR3	3.00^{b}	2.35^{c}	2.82^{b}	3.03^{b}	3.48^{a}	0.12	< 0.01
	PPARD	3.40^{b}	3.72^{a}	3.11^{b}	3.21^{b}	3.13^{b}	0.13	< 0.01
	PPARG	5.78^{ab}	6.21^{a}	5.89^{ab}	5.40^{b}	5.55^{b}	0.17	< 0.01
	RXRA	5.07^{b}	5.64^{a}	4.99^{b}	5.02^{b}	4.85^{b}	0.18	< 0.01
	STAT3	9.01^{a}	8.88^{a}	7.75^{b}	8.41^{ab}	8.39^{ab}	0.42	< 0.01
Leukotriene pathway								
	ALOX5	5.28^{a}	5.08^{ab}	4.35^{b}	4.21^{b}	5.65^{a}	0.24	< 0.01
	ALOX5AP	2.58^{b}	3.56^{a}	2.62^{b}	2.65^{b}	2.69^{b}	0.14	< 0.01
	PLA2G4A	2.94^{b}	3.46^{a}	2.75^{b}	2.77^{b}	2.93^{b}	0.12	< 0.01
Cellular metabolism								
	SLC2A1	5.11^{b}	4.23^{c}	4.81^{b}	4.74^{bc}	5.71^{a}	0.17	< 0.01
	LDHA	3.27^{b}	3.84^{a}	3.30^{b}	3.11^{b}	3.12^{b}	0.11	< 0.01

Expression of Genes Involved in Attachment and Extravasation

Expression of the *SELL* gene was down-regulated ($P < 0.05$) at all time points post-calving relative to pre-calving and the day of calving. Relative gene expression of the integrins across the transition period was dependent on the integrin considered; *ITGAL* and *ITGAM* remained stable throughout the immediate calving period, but expression was greater ($P < 0.01$) at wk 4 post-calving. The abundance of transcripts for the *ITGAX* and *ITGB2* integrins and *CD44* was greater ($P < 0.05$) on the day of calving, compared with most other time points, while *ITGA4* integrin and extracellular matrix adhesion proteins *LGALS8* and *TLN2* were down-regulated on the day of calving, and exhibited greatest expression at wk 4 post-calving. There was no change in the gene expression levels of *IAP* and *CPNE3* across the time points considered.

Neutrophil Maturation and Cell Cycle

Several genes involved in the regulation of apoptosis were differentially expressed over the transition period. Gene expression of *FASLG*, *BCL2*, and *KIT* was decreased on the day of calving compared with all other time points. Furthermore, *CASP8* gene expression was also lower on the day of calving compared with 1 wk pre-calving and 4 wk post-calving. Gene expression of *RIPK1*, *FCGR3A*, and *ANXA3* was lower on the day of calving compared with pre-calving levels. *RIPK1* gene expression remained low for all post-calving time points ($P < 0.05$) whereas gene expression of both *FCGR3A* and *ANXA3* recovered within the first wk post-calving. Gene expression of *MCL1* was greater on the day of calving, compared with wk 1 and 2 post-calving. Other genes involved in cell differentiation, including *S100A9* and *THY1*, had increased gene expression on the day of calving compared with all other time points.

Expression of Transcription Factors

The gene expression of the transcription factors *PPARD* and *RXRA* was greater on the day of calving compared with all other time points ($P < 0.05$). Gene expression of the other transcription factors investigated (*NCOR1*, *NCR3*, *PPARG*, and *STAT3*) changed with time but did not have any consistent expression changes. There were no significant effects of wk on gene expression of *NFKB1* and *PPARA*.

Cholesterol Transport, Leukotrienes, Glucose Transport, and Glycolysis

Expression of genes involved in cholesterol transport and leukotriene synthesis was generally lower post-calving compared with pre-calving or the day of calving. For example, transcript abundance of *PLA2G4A* and *ALOX5AP* was greater on the day of calving relative to all other time points, while pre-calving *ALOX5* exceeded that at 1 wk (1.8-fold) and 2 wk (2.1-fold) post-calving and returned to pre-calving levels at 4 wk. There was no significant alteration in *ABCA1* gene expression over the calving period.

Gene expression of *SLC2A1* (encoding the glucose transporter GLUT1) was decreased on the day of calving compared with most other time points, and the expression was greatest at 4 wk post-calving (2.8 fold-greater than d 0). Abundance of *LDHA* was greater on the day of calving compared with all other time points ($P < 0.05$).

Milk Production and Metabolic Data

Milk yield increased from wk 1 to 4, but fat and protein % declined (Table 6). As a result, fat and protein yields were not affected by time. The profile of changes in metabolite data over the transition period is presented in Table 7. All metabolites changed with time ($P < 0.05$). Total protein was greater ($P < 0.05$) on the day of calving and at 4 wk post-calving compared with other time points. Albumin also increased on the day of calving, and globulin concentrations varied over the transition period; however, an increase in plasma globulin at 4 wk post-calving resulted in a decreased albumin:globulin ratio (AGR) this time point. Liver TAG, AST, and plasma IL-6 concentrations were lowest pre-calving and increased post-calving. Haptoglobin increased at calving, with variable concentrations post-calving. Blood cholesterol concentrations were elevated at 4 wk post-calving compared with other post-calving time points.

Table 6. Production results over transition period.

Results are presented as least squared means (LSM) with standard error of the difference (SED) and significance of wk (*P* value). Results of Tukey's t-test are presented in superscript for pairwise comparisons between wk; where any two wk, for each parameter, with at least one common letter do not differ significantly ($P > 0.05$) and those without at least one common letter differ significantly ($P < 0.05$).

	Time relative to parturition (D 0)				
	Day 0-7	Day 8-14	Day 21-28		***P* value**
Parameter	**1 wk**	**2 wk**	**4 wk**	**SED**	**Wk**
Milk weight, kg	21.15^{c}	22.31^{b}	24.16^{a}	0.28	< 0.01
Composition, %					
Fat	5.13^{a}	4.7^{b}	4.22^{c}	0.11	< 0.01
Protein	4.15^{a}	3.62^{b}	3.33^{c}	0.05	< 0.01
Lactose	4.69^{b}	4.82^{a}	4.83^{a}	0.09	< 0.01
Yield, kg/d					
Fat	1.1	1.06	1.02	0.03	0.08
Protein	0.89^{a}	0.81^{b}	0.80^{b}	0.02	< 0.01
Log_{10} SCC	5.09^{a}	4.91^{a}	4.67^{b}	0.08	< 0.01

Table 7. Metabolite results over transition period.

Results are presented as least squared means (LSM) with standard error of the difference (SED) and significance of wk (*P* value). Superscript letters denote results of Tukey's t-test for pairwise comparisons between wk; time points with at least one common letter, for each parameter, do not differ significantly (*P* > 0.05) and those without at least one common letter differ significantly (*P* < 0.05).

		Time relative to parturition (D 0)						***P* value**
Parameter	**Units**	**-1 wk**	**D 0**	**1 wk**	**2 wk**	**4 wk**	**SED**	**Wk**
Metabolites								
Total protein	g/L	68.58^{c}	70.67^{b}	68.31^{c}	70.39^{bc}	73.44^{a}	0.77	< 0.01
Albumin	g/L	35.54^{b}	36.84^{a}	35.26^{b}	35.14^{b}	35.57^{b}	0.42	< 0.01
Globulin	g/L	33.03^{c}	33.82^{bc}	33.11^{c}	35.24^{b}	37.87^{a}	0.56	< 0.01
Alb:Glob		1.10^{a}	1.11^{a}	1.09^{a}	1.02^{b}	0.95^{c}	0.02	< 0.01
BHBA	mmol/L	0.51^{b}	0.80^{a}	0.72^{ab}	0.73^{ab}	0.65^{b}	0.06	< 0.01
AST	U/L	61.1^{b}	82.08^{a}	78.22^{a}	85.34^{a}	82.73^{a}	3.26	< 0.01
NEFA	mmol/L	0.66^{bc}	0.55^{c}	1.07^{a}	0.76^{b}	0.56^{c}	0.07	< 0.01
Haptoglobin	ng/mL	1629^{c}	29712^{a}	13543^{b}	10317^{bc}	3631^{c}	5928	< 0.01
Cholesterol	µM	2202^{ab}	2086^{b}	1881^{b}	2206^{b}	2625^{a}	219	< 0.01
Liver TAG	%	1.48^{b}	.	4.02^{a}	4.69^{a}	4.62^{a}	0.40	< 0.01
Cytokines								
IL-1β	pg/mL	20.65^{b}	21.18^{b}	31.37^{a}	27.75^{a}	16.53^{c}	9.83	< 0.01
IL-6	pg/mL	468.45^{c}	3700.97^{a}	1682.8^{b}	420.3^{c}	271.9^{d}	428.6	< 0.01
Inflammatory mediators								
ROS	µM	0.05^{d}	0.30^{c}	0.81^{a}	0.56^{b}	0.33^{c}	0.043	< 0.01
TAC	mM	1.77^{b}	0.29^{c}	3.47^{a}	1.22^{b}	2.96^{a}	0.32	< 0.01
Minerals								
Mg	mmol/L	0.62^{b}	0.70^{a}	0.63^{ab}	0.60^{b}	.	0.03	0.02
Ca	mmol/L	2.13^{b}	2.03^{c}	2.26^{a}	2.30^{a}	.	0.04	< 0.01

3.5 Discussion

The presented results provide evidence of altered gene expression signatures in the neutrophils from moderate-yielding dairy cows grazing fresh pasture immediately prior to and after parturition. Results are consistent with reported changes in cellular function in higher yielding cows fed TMR (Kehrli et al., 1989b; Kehrli and Goff, 1989; Detilleux et al., 1995). The current interpretation of the transcription results supports the hypothesis of a change in neutrophil function during the transition period that is likely a normal adaptation to calving. Collectively, the data indicate changes to the inflammatory response, the antimicrobial capacity, cellular adhesion and metabolism, and the developmental maturity of the circulating population of neutrophils during the peripartum period. For example, gene expression of cytokines and cytokine receptors, adhesion proteins, and genes involved in apoptosis were differentially expressed over the transition period. Furthermore, the transcription profiles indicate changes in the maturation state of neutrophils around the time of calving. It is conceivable that these changes may be associated with peripartum neutrophil dysfunction and the associated increase in incidence of infectious disease during this period (Mallard et al., 1998).

Inflammation and the Inflammatory Response

The inflammatory state of neutrophils was altered at calving; a response similar to that observed during acute inflammation. Genes involved in inflammation and mediation of the inflammatory response, for example those for cytokines, TLR, and the MHC class II molecule BOLA-DRA, were differentially expressed over the transition period. Consistent with an inflammatory state around calving is the increase in plasma IL-6 and haptoglobin, indicating activation of the acute phase response at calving and in the early post-partum (Sordillo and Aitken, 2009; Bossaert et al., 2012). At calving, gene expression of *TLR2* and *IL6* was increased and *BOLA-DRA* decreased, comparable to the neutrophil response to an intramammary infection challenge (Moyes et al., 2010b). This gene expression profile is also exhibited in leukocytes isolated from post-partum grazing cows during an in-vitro challenge with staphylococcal enterotoxin B and lipopolysaccharide (LPS) (Heiser et al., 2015) and in response to post-partum subclinical endometritis (Walker et al., 2015). Parturition allows entry of pathogens into the uterus, thereby activating the immune response (Sheldon and Dobson, 2004). Neutrophils have a dual role in this response; destroying invading pathogens and maintaining an appropriate level of inflammation (Kehrli and Goff, 1989). This dual response must be balanced to ensure neutrophils retain the ability to destroy invading pathogens while avoiding detrimental inflammation. The extent of this response varies considerably between individual cows (Heiser et al., 2015), suggesting that some individuals are more susceptible to infectious disease than others.

Decreased expression of *BOLA-DRA* on the day of calving could infer a change in the interaction of neutrophils with the adaptive immune response. This is consistent with the reported decrease in MHC-II populations immediately post-calving in whole blood leukocytes (Van Kampen and Mallard, 1997). Gene expression profiles from circulating neutrophils over the transition period are indicative of a change in their inflammatory phenotype on the day of calving. This change is likely a normal adaptation to parturition that can impact the inflammatory response of neutrophils and, in some individuals, increase susceptibility to infection.

Expression of Antimicrobial Peptides

Expression of genes encoding antimicrobial peptides (*DEFB1*, *DEFB10*, *BNBD4*, and *LAP*) in circulating neutrophils is up-regulated on the day of calving, indicating an increase in the antimicrobial capability of neutrophils in response to parturition. Antimicrobial peptides, such as β-defensins, are cytotoxic to pathogenic microorganisms and are expressed in bovine neutrophils (Savoini et al., 1984; Lai and Gallo, 2009; Meade et al., 2014). Expression of defensins occurs during myelopoiesis and mature peptides are packaged into neutrophilic granules (Yount et al., 1995). Defensins are constitutively expressed in neutrophils. However, expression can be induced in a variety of epithelial tissues during infection; for example, tracheal antimicrobial peptide (encoded by *TAP*) in ciliated respiratory epithelium, lingual antimicrobial peptide (encoded by the *LAP* gene) in epithelium of mammary gland alveoli (Diamond et al., 1996, 2000; Russell et al., 1996) and β-defensins in uterine tissue (Walker et al., 2015). Furthermore, β-defensin expression can be stimulated with LPS in bovine monocytes (Merriman et al., 2015). Defensins play an important role in innate immune responses and the reported transcription profile during the calving period in this study likely reflects the host response to damaged tissue inflammatory signals (DAMPS) and the inevitable increased exposure of internal epithelial surfaces to potential pathogenic and non-pathogenic microorganisms.

Adhesion and Extravasation

The ability of neutrophils to exit the blood and enter infected tissue via chemotaxis, a property crucial for clearance of an infection, appears to be reduced during the weeks immediately following parturition. Parturition altered expression of integrins, extracellular matrix adhesion proteins, and matrix metalloproteinases and expression remained inconsistent post-calving in the current study. Experimental models of infection have demonstrated that the efficient influx of leukocytes into tissues is related to outcome of the infection (Hill, 1981; Burvenich et al., 1994; Van Werven et al., 1997). Initial attachment and rolling of neutrophils along blood vessel endothelium is mediated by selectin molecules (L-selectin; *SELL*) and the ability of neutrophils to migrate out of blood vessels is reliant on integrin molecules and extracellular matrix binding proteins. Inhibition of integrin function highlights the importance of these proteins for successful trans-endothelial migration (Furie et al., 1991; Ding et al., 1999); in fact, recurrent bacterial infections is a common feature of mutations in genes of the adhesion cascade that result in leukocyte adhesion deficiency syndromes (Schmidt et al., 2013). The evident change in *SELL* expression in the grazing cows described here is consistent with previous experiments in TMR-fed cows (Lee and Kehrli, 1998). These authors used flow cytometry and demonstrated decreased L-selectin and CD18 (encoded by the *ITGB2* gene) expression on neutrophils 24 hours post-parturition relative to day of calving. The differential expression patterns of the integrin genes presented here indicate possible changes to the adhesion specificity of neutrophils (Loike et al., 1991) and provides evidence for a change in the population of neutrophils at different maturation states over the transition period (Ingalls and Golenbock, 1995). There is evidence that sub-populations of neutrophils differ in the expression of adhesion molecules, thereby affecting their response to inflammatory stimuli (chemotaxis and extravasation; Kamp et al., 2012). The changes in expression of adhesion proteins and altered adhesion specificity of neutrophils demonstrated in our study is likely to have a detrimental effect on the neutrophil response to inflammatory stimuli and, as a result, may increase susceptibility to infection in the weeks immediately post-calving.

Maturation State

The transcription profiles from our study are indicative of an increase in immature neutrophils on the day of calving, likely due to the inflammatory nature of parturition. Expression results indicate that there is likely to be an increase in the number of immature cells at the band or promyelocyte stage of maturation on the day of calving; decreased expression of *FCGR3A*, changes in expression of adhesion molecules, and an increase in neutrophil collagenase (*MMP8*) and gelatinase (*MMP9*). Decreased protein expression of FcγRIII (*FCGR3A*) is associated with immature neutrophils at the band stage, as well as reduced cellular function (Pillay et al., 2010b). Furthermore, there is differential expression of adhesion molecules depending on the maturation state of neutrophils; for example, mature neutrophils express the Mac-1 complex (CD11b/CD18; *ITGAM/ITGB2*) over CD11c/CD18; *ITGAX*/*ITGB2* (Ingalls and Golenbock, 1995). According to the study by Pillay et al. (2010b), distinct neutrophil subsets exist within circulating populations with unique cytokine and chemokine production. Shifts in the neutrophil population over the transition period could provide the mechanism for altered neutrophil function and provides an explanation for the unique cytokine expression signature on the day of calving. Systemic inflammation or periods of stress causes neutrophil egress from the bone marrow and results in increased immature neutrophils in circulation (Van Merris et al., 2002); this has been confirmed by flow cytometric and morphological investigation following LPS treatment in humans (Brown et al., 1989) and cows (Prin-Mathieu et al., 2002). Induction of parturition is an inflammatory event (Van Engelen et al., 2009) and neutrophil numbers increase up to parturition in dairy cows, followed by an increase in immature (band) cells in circulation (Detilleux et al., 1995). Additional supporting evidence of an increase in immature neutrophils in circulation comes from the increased expression of *MMP8* and *MMP9* on the day of calving in the current study. These peptides are usually packaged in granules in an inactive form to be activated after exocytosis (Faurschou and Borregaard, 2003). Therefore, an increase in mRNA transcripts may indicate that fewer transcripts are in a tertiary protein structure packaged within granules in early lactation in the current experimental model; however this would need to be confirmed by investigating granule formation and expression of MMP8 and -9 at the protein level. Data supports an increase in immature neutrophils in circulation on the day of calving, which may compromise neutrophil function systemically and the ability of the neutrophil to neutralize invading pathogens.

Further evidence for a shift in the maturation state of circulating neutrophils around calving is the differential expression of genes involved in apoptosis. We propose from our results that the decreased expression of pro-apoptotic genes (e.g. *CASP8*, *KIT*, and *FASLG*; Theilgaard-Mönch et al., 2004; Croker et al., 2011), with concomitant increase in expression of anti-apoptotic gene *MCL1* (Dzhagalov et al., 2007), are indicative of an increased neutrophil lifespan post-calving compared with the pre-calving state. Neutrophil apoptosis is tightly regulated (Luo and Loison, 2008) and a delay in apoptosis may be advantageous at the beginning of an infection, as the onset of apoptosis leads to functional impairment

(Whyte et al., 1993). Supporting evidence of a change in neutrophil apoptosis during the periparturient period was also reported by Chang et al. (2004). Their results indicate that the peripartum rise in glucocorticoids reduces Fas-induced apoptosis. Similarly, our results demonstrate decreased expression of *FASLG* on the day of calving, lending support to the conclusion that decreased apoptosis in response to glucocorticoids (e.g., cortisol) is a mechanism for increasing lifespan of neutrophils around parturition (Chang et al., 2004). Investigation of additional Bcl-2-related targets (e.g., *BAD*, *BAK*, and *BAX*) involved in apoptosis may provide further insight into neutrophil apoptotic regulation over the transition period. The data presented here does not conclusively confirm increased lifespan of neutrophils on the day of calving; however, the transcription profile of genes involved in apoptosis is altered during the transition period, which may support a reprogramming of key apoptotic regulators in response to high cortisol concentrations at parturition.

Cellular Metabolism

Altered expression of transcription factors indicates changes to the control of inflammatory networks over the transition period. The expression of *PPARD* and *RXRA* are decreased one week post-calving compared with on the day of calving, which is in agreement with results demonstrated by Osorio et al. (2013b). Additional evidence of activation of the PPAR network on the day of calving is 1) the increased expression of genes involved in leukotriene synthesis (*ALOX5AP* and *PLA2G4A*) and 2) decreased expression of the gene encoding the glucose transporter (*SLC2A1*) with increased expression of the gene encoding lactate dehydrogenase A (*LDHA*), an enzyme involved in glycolysis. These results are consistent with a change in the PPAR-RXRA inflammatory network and likely influenced by a change in the utilization of arachidonic acid for prostaglandin and eicosanoid production, which can activate the PPAR network (Osorio et al., 2013b; Zhou et al., 2015b). Arachidonic acid, the precursor of leukotrienes, increases in neutrophils isolated under inflammatory conditions (Klock and Pieprzyk, 1979). Leukotrienes derived from oxidation of arachidonic acid (e.g., leukotriene B_4) induce PPAR expression; this stimulates production of the anti-inflammatory cytokine IL-10, which ensures chronic inflammation does not occur (Kuehl and Egan, 1980; Buckley et al., 2014). The reduced expression of *SLC2A1* (GLUT1) may be a reflection of increased glucose requirements by the mammary gland (Bell, 1995) or a response to foetal cortisol release at parturition (Preisler et al., 1999). In this study we did not measure blood glucose concentrations; however, previous work suggests an increase on the day of calving (Roche, 2007). Neutrophils rely on glucose uptake (via GLUT1) and glycolysis for anti-bactericidal and phagocytosis; therefore, if cellular metabolism is perturbed, neutrophil function will be affected (Borregaard and Herlin, 1982; Weisdorf et al., 1982). It is likely that the altered cellular metabolism in response to parturition dampens the inflammatory response of neutrophils during the transition period and may prevent unnecessary excessive inflammation.

3.6 Conclusion

Our results indicate an altered inflammatory state in neutrophils during the transition period, providing additional evidence of changes in neutrophil function in response to the calving event and the homeorhetic adaptation to lactation. The gene expression profiles in grazing dairy cows align with current research indicating neutrophil dysfunction in conventional housing systems. The changes in the inflammatory response likely increase the susceptibility of dairy cows to infection during the peripartum period. The reported gene expression changes may be associated with altered neutrophil populations favouring an immature phenotype; however, further investigation will provide greater comprehension and possible mechanisms to shift populations potentially alleviating immunological strain.

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3.8 Summary

The research described here in Chapter 3 is the most extensive study to-date on gene expression changes in neutrophils over the transition period in grazing cows. Importantly, this research highlights that altered neutrophil gene expression, and therefore function, in grazing cows is consistent with that in conventionally-housed, TMR-fed cows. Furthermore, now that these changes have been characterised, it is possible to investigate whether management strategies e.g., body condition and feeding management, can alter these changes. If so, these management practises may be utilised to alleviate metabolic strain and improve dairy cow health during the transition period. Chapter 4 describes experiments to establish if body condition at calving or prepartum pasture allowance (feeding level) can alter the expression of genes involved in neutrophil function.

Chapter 4

Effects of pre-calving body condition and prepartum feeding level on gene expression in circulating neutrophils

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4.1 Abstract

Extensive metabolic, physiological, and immunological changes are associated with calving and the onset of lactation. As a result, cows transitioning between pregnancy and lactation are at a greater risk of metabolic and infectious diseases. The ability of neutrophils to mount an effective immune response to an infection is critical for its resolution and increasing evidence indicates that pre-calving nutrition affects post-partum neutrophil function. The objectives of the current study were to investigate the effect of two pre-calving body condition scores (BCS 4 vs. 5 on a 10-point scale) and two levels of feeding (75 vs. 125% of estimated maintenance requirements) on gene expression in circulating neutrophils. RNA was isolated from the neutrophils of cows (n = 45), at five time points over the transition period; pre-calving (-1 wk), day of calving (d 0) and post-calving at wk 1, wk 2, and wk 4. Quantitative reverse transcriptase PCR with custom designed primer pairs and Roche Universal Probe Library chemistry, combined with microfluidics integrated fluidic circuit chips (96.96 Dynamic Array) were used to quantify the expression of 78 genes involved in neutrophil function and 18 endogenous control genes. Statistical significance between time points was determined using repeated measures ANOVA with Tukey-Kramer multiple-testing correction to determine treatment effects among weeks. Pre-calving BCS altered the inflammatory state of neutrophils, with significant increases in overall gene expression of antimicrobial peptides (*BNBD4* and *DEFB10*) and the anti-inflammatory cytokine *IL10*, and significant decreased expression of pro-inflammatory cytokine *IL23A* in thinner cows (BCS 4) compared with cows calving at BCS 5. Feeding level had a time-dependent effect on gene expression; for example, increased expression of genes involved in leukotriene synthesis (*PLA2G4A* and *ALOX5AP*) occurred only at 1 wk post-calving in cows overfed (125% of requirements) pre-calving compared with those offered 75% of maintenance requirements. Results indicate that pre-calving body condition and changes in prepartum energy, lead to altered gene expression of circulating neutrophils, highlighting the importance of transition cow nutrition for peripartum health.

4.2 Introduction

A dairy cow undergoes several homeorhetic adaptations to facilitate the challenges associated with calving and the onset of lactation (Bauman and Currie, 1980; Goff and Horst, 1997). Failure to successfully adapt to this transition leads to reduced productivity and increased incidence of disease (Ingvartsen et al., 2003). Ultimately, this leads to reduced longevity and has a considerable impact on the dairy industry. For example, Godden et al. (2003) reported that 20% of cows culled annually left the herd in the first three wk post-calving. Furthermore, an estimated 30-50% of dairy cows are affected by metabolic or infectious disease during the transition period (LeBlanc, 2010).

Immune function is altered during the transition period, which may contribute to the increased risk of health disorders (Detilleux et al., 1995; Mallard et al., 1998; Chapter 3). It is increasingly evident that prepartum nutrition and nutrient availability play an important role in the successful transition of dairy cows; for example, BCS and level of feeding affect indicators of health and metabolic function during the transition period (Loor et al., 2006; Douglas et al., 2006; Roche et al., 2005, 2013b, 2015). Roche et al. (2013b) reported that cows calving at a BCS 4.5-5.0 (10-point scale) had a superior health profile to those calving at either 3.5 or 5.5. Similarly, improvements in markers of health (e.g., plasma concentrations of NEFA, BHBA, percentage of liver TAG, and markers of inflammatory state) have been reported when cows were fed 75-90% of their metabolisable energy (ME) requirements before calving compared with cows consuming energy in excess of requirements (Douglas et al., 2006; Loor et al., 2006; Roche et al., 2015). There is little information on the effect of pre-calving BCS and feeding level on the state of the immune system, despite a link between nutrition and immune function (Sordillo and Aitken, 2009; Sordillo and Mavangira, 2014). But, it is conceivable that pre-calving nutrition could affect gene expression of immune cells during the transition period. Many studies investigating immune function during the peripartum period have focused on neutrophils, primarily because of their role in pathogen destruction during infection (Nagahata et al., 1988; Mallard et al., 1998; Kimura et al., 2014). The ability of neutrophils to function effectively has been related to the incidence of peripartum-related diseases, including mastitis, displaced abomasum, and retained placenta (Goff and Horst, 1997; Kimura et al., 2002; Burton et al., 2001; LeBlanc, 2008, 2010; Cardoso et al., 2013). There is increasing evidence to support the hypothesis that prepartum nutrition state affects neutrophil function (Graugnard et al., 2012b; Moyes et al., 2014; Zhou et al., 2015b). For example, the severity of NEB during early lactation contributes to the degree of immune 'suppression' and susceptibility to disease during the peripartum period (Goff, 2006). The expression of genes involved in the immune response of neutrophils to an intramammary infection is also affected by NEB (Moyes et al., 2010a). Furthermore, dietary energy during the prepartum period affects the expression of neutrophil inflammatory mediators (Zhou et al., 2015b). Therefore, identifying a nutritional regime that will positively influence neutrophil function may reduce susceptibility of cows to disease during the transition period. The objectives of this study were to compare 1) the effect of pre-calving BCS, and 2) the effect of under- and overfeeding of cows pre-calving on the transcriptional profile of neutrophils during the transition period.

4.3 Materials and Methods

Animal Handling and Experimental Design

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46'S 175°18'E) between January and October 2013 using New Zealand Holstein-Friesian and Holstein-Friesian × Jersey cross dairy cows.

Comprehensive details of the experiment and animal management are described in Roche et al. (2015). Briefly, a total of 150 cows were allocated to 1 of 6 treatment groups (25 cows per group) matched for age, expected calving date, and breed in a 2 × 3 factorial arrangement. Cows were managed during mid- and late-lactation and from dry-off to 21 d prior to predicted calving date to achieve the two pre-calving BCS treatment groups: BCS 4.0 and BCS 5.0 on a 10-point scale, equivalent to approximately 2.75 and 3.25 respectively, on a 5-point scale (Roche et al., 2004). From -23 ± 5.6 d relative to expected calving until parturition, cows received fresh pasture at 3 levels of energy intakes (75, 100, or 125%) based on estimated ME requirements (1.05 MJ/kg $BW^{0.75}$; Roche et al., 2005). Post-calving, all cows grazed together and were offered a diet of fresh pasture (average DMI = 12.4 ± 0.8 kg dry matter (DM)/d), corn silage (average DMI = 0.8 ± 0.8 kg DM/d), and pasture silage (average DMI = 1.1 ± 1.0 kg DM/d) as supplementary feeds. Three treatment groups from the parent experiment were selected to investigate the effect of pre-calving BCS and prepartum feeding level on neutrophil gene expression: BCS 4 offered 75% (BCS 4; n = 15) and BCS 5 offered 75% (BCS 5 and underfed; n = 15), and BCS 5 offered 125% (overfed; n = 15).

Blood Sampling

Blood (~50 mL/cow) was sampled for neutrophil isolation and metabolite analysis (~10 mL/cow) at five time points over the transition period; pre-calving (-1 wk), day of calving (d 0), and post-calving at wk one (1 wk), wk two (2 wk), and wk four (4 wk). Blood for neutrophil isolation was collected in six evacuated blood tubes (BD, Auckland, New Zealand) containing acid citrate dextrose (solution A; trisodium citrate 22.0 g/L, citric acid 8.0 g/L, dextrose 24.5 g/L). Tubes were inverted eight times and placed immediately on ice until neutrophil isolation, which occurred within 2 h of blood collection. Blood for metabolite analysis was collected in evacuated blood tubes containing a lithium heparin anti-coagulant, inverted and placed immediately on ice; within 30 min, blood tubes were centrifuged at 1,500 × *g* for 12 min at 4°C, plasma aspirated and stored at -20°C until assayed.

Liver Sampling for Triacylglycerol Analysis

Liver tissue samples were collected from all cows by biopsy the wk before calving (wk -1) and on wk 1, 2, and 4 post-calving. Briefly, the skin was shaved and disinfected and the area anaesthetised with 7 mL of 2% lignocaine (Lopaine 2%, lignocaine hydrochloride 20 mg/mL, Ethical Agents, South Auckland, New Zealand). An incision was made through the skin in the right 11th intercostal space at the level of the greater trochanter, through which a 12G × 20 cm biopsy needle was inserted into the liver and approximately 1 g (wet weight) of liver tissue was collected. Liver samples were snap-frozen in liquid nitrogen and stored at -80°C.

Neutrophil Isolation

Neutrophils were isolated from all cows according to procedures previously described (Chapter 3). Briefly, Blood collected in the acid citrate dextrose evacuated blood tubes was inverted and pooled (for each cow at each time point) by transfer into a 50 mL conical tube. Tubes were centrifuged for 30 min at 850 × *g* at 4°C. The plasma, buffy coat, and up to 1/3 of the red blood cells (RBC) were aspirated by Pasteur pipette and discarded, leaving 2/3 of remaining RBC and neutrophils. Water (milliQ, Millipore, New Zealand) was added (25 mL) and mixed for 10 - 30 s to lyse the RBC and 5 mL of 5× concentrated PBS (pH 7.4, Life Technologies, New Zealand) was added to each tube to restore isotonicity. Tubes were spun at 330 × *g* for 10 min at 4°C and supernatant decanted to isolate the white cell pellet. The pellet was washed with 10 mL of 1× concentrated PBS (pH 7.4, Life Technologies), vortexed and centrifuged for 5 min at 660 × *g* at 4°C. If RBC remained, the water lysing step was repeated. Cells were re-suspended in 5 mL of 1× PBS (pH 7.4) and 100 μL was transferred into a 1.5 mL microcentrifuge tube (Raylab, New Zealand) and placed on ice for subsequent viability and staining assays. The 50 mL tubes were centrifuged again (5 min at 660 × *g* at 4°C), liquid decanted, and the cell pellet re-suspended in 1 mL TRIzol® reagent (Life Technologies). The 1 mL cell suspension was transferred into a 2 mL microcentrifuge tube (Eppendorf, Germany), placed immediately on ice, and stored at -80°C for subsequent RNA extraction.

Cell Differentials and Viability

A subpopulation of isolated cells from randomly selected samples (n = 41) was used to determine cell viability and neutrophil population by flow cytometry analysis using the BD FACS Aria II cell sorter (BD Biosciences, USA). Viability was measured using LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (L23105, Life Technologies) and neutrophil populations were determined with the bovine granulocyte specific primary monoclonal antibody CH138A (WS0608B-100, Kingfisher Biotech Inc., MN, USA) and phycoerythrin-conjugated secondary antibody (SB102009S, Southern Biotech, AL, USA). Neutrophils were gated based on CH138A-phycoerythrin fluorescence (Figure 7). Non-neutrophil granulocytes could be distinguished from neutrophils based on auto-fluorescence. Of total granulocytes isolated (78.6% ± 14.4% median ± SD) with this procedure, 93.4% ± 7.9% (mean ± SD) were neutrophils and of these, 93.3% ± 10.2% (mean ± SD) were live cells.

RNA Extraction

Total RNA was extracted from all neutrophil samples using TRIzol® reagent as per the manufacturers' instructions. Briefly, cell suspensions in TRIzol® were homogenized using a PRO200 homogenizer (Pro Scientific, CT, USA); suspensions underwent two 20 s bursts of the homogenizer at full speed (500 Hz) and were placed on ice after every homogenization. The homogenizer tool was rinsed between each sample twice with 75% ethanol and water, and once with RNaseZap® (Life Technologies). Chloroform was added to the homogenized samples, and after centrifugation (12,000 × *g* for 15 min at 4°C) the aqueous phase was carefully removed. Precipitation of RNA was achieved with the addition of isopropanol (Merk Millipore, Germany) and the subsequent RNA pellet was washed (with 75% ethanol), dried, and re-suspended in UltraPure™ RNase/DNase-free distilled water (Life Technologies). All RNA samples were DNase treated using the Ambion DNA-free kit (Life Technologies). The quantity and purity of RNA was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, DE, USA) and the RNA integrity number (RIN) was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, CA, USA). Final sample sets (n = 225; 15 cows per treatment over 5 time points) based on whether samples from all 5 time points for each cow had sufficient quantity (>2 μg) and quality (RIN >7) of RNA. Final neutrophil RNA samples had an average RIN (mean ± SD) of 8.0 ± 0.77. Samples were stored at -80°C until cDNA synthesis.

Complementary DNA Synthesis and Primer Design

Total RNA (2 μg) from each sample was reverse transcribed using a Superscript III Supermix kit (Life Technologies), with random pentadecamer primers (Integrated DNA Technologies [IDT], Singapore) at a final concentration of 27 μM. Reverse-transcriptase negative controls were also generated by excluding the enzyme. Complementary DNA samples were stored at -20°C.

Assay design for 96 gene targets (78 genes involved in neutrophil function and 18 possible endogenous control genes chosen based on current literature; Supplementary Table 1) for use in quantitative reverse transcription-PCR (RT-qPCR) was undertaken using publicly available bovine gene sequences from the National Center for Biotechnology Information (NCBI) gene database (NCBI; http://www.ncbi.nlm.nih.gov). Assays were designed to span an intron-exon boundary, where possible, using Roche UPL design software. Primer sequences were verified by BLAST and PrimerBLAST analysis (NCBI) to ensure specificity. Assays were tested on a LightCycler 480 instrument (Roche, Germany). Each RT-qPCR reaction contained custom designed forward and reverse primers (200 nM, IDT, Singapore), UPL unique probe (50 nM, Roche), LightCycler480® Probes Master (1× conc) and RNAse/DNase-free distilled water. Standard cycling conditions were used (95°C for 10 min, [95°C for 10 s, 60°C for 30 s] × 50 cycles, 40°C for 40 s). To test assays, a 6-point 10-fold standard curve was generated from 1× dilution (1:4 dilution of raw cDNA) to 1×10^{-6} dilution. To ensure a single product free of genomic DNA contamination and to verify the amplicon size, reactions positive for gene expression as well as H_2O negative controls were verified with gel electrophoresis.

Gene Expression

Expression of genes involved in neutrophil function was quantified using RT-qPCR using the BioMark HD™ real-time PCR system (Ramaciotti Centre, NSW, Australia). Microfluidics 96.96 Dynamic Array™ (Fluidigm, San Francisco, CA) integrated fluidic circuit chips combined with Roche UPL chemistry were used to generate expression results for 96 gene targets for all samples (n = 225). A pre-amplification of cDNA was undertaken to create target-specific amplified reactions by multiplexing forward and reverse primers (200 nM) of the 96 assays with the following cycling protocol; 95°C for 10 min, (95°C for 15 s, and 60°C for 4 min) × 14 cycles. Assay mixtures, including forward and reverse primers (800 nM), Roche UPL probes (100 nM), and 2× concentration assay loading reagent (Fluidigm) were combined with 1× concentration Roche LightCycler480® Probes Master (Roche Diagnostics, Basel, Switzerland) and 20× gene expression sample loading reagent (Fluidigm) on a 96.96 gene expression chip (Fluidigm) for use in the BioMark HD™ instrument. The gene expression cycling protocol, "GE 96×96 UPL v1.pcl" (BioMark HD Data Collection software v3.0.2), was used to generate crossing point (Cp) values: 70°C for 30 min, 25°C for 10 min, 95°C for 1 min followed by 35 cycles of 96°C for 5 s and 60°C for 20 s.

Possible endogenous control genes were tested across all samples using GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) software (Chapter 2).

Briefly, a 5-point standard curve was included (3-fold serial dilutions from 3.33×10^{-1} to a final dilution of 4.12×10^{-3}) to calculate the RT-qPCR efficiency for all 96 genes. Of the 18 endogenous control genes tested, *RPL19* and *YWHAZ* were chosen as the two endogenous control genes for this neutrophil study. The NormFinder stability of the combination of these two genes was 0.072 and the GeNorm stability values (M) were < 0.58 for both genes, with little improvement in stability with the addition of a third endogenous control gene (Chapter 2).

Relative quantification was achieved using the delta Cp method; the geometric mean of the Cp values of the endogenous control genes was calculated and target assay Cp values were made relative to the geometric mean of endogenous controls (ΔCp). Fold change (ΔΔCp) relative to BCS 5 was calculated as:

$$\text{Fold change} = \text{qPCR efficiency}_{\text{gene of interest}} \wedge ^{(\Delta\text{Cp BCS 5} - \Delta\text{Cp BCS 4})}$$

The fold change calculation for the effect of overfeeding (125% of estimated ME requirements) relative to feeding 75% of estimated ME requirements was calculated as:

$$\text{Fold change} = \text{qPCR efficiency}_{\text{gene of interest}} \wedge ^{(\Delta\text{Cp 75\%} - \Delta\text{Cp 125\%})}$$

Metabolite Analysis

Blood metabolite and liver TAG assays were undertaken by Gribbles Veterinary Pathology Ltd (Hamilton, New Zealand). Full details of blood metabolite analysis are described in Roche et al. (2015). Briefly, blood metabolites were assayed using colorimetric techniques at 37°C with a Hitachi Modular P800 analyser (Roche Diagnostics). Roche reagent kits were used to measure plasma concentrations of albumin (g/L), BHBA (mmol/L), Ca (mmol/L), Mg (mmol/L), and total protein (g/L). Plasma globulin was calculated as the difference between total protein and albumin. Plasma NEFA concentrations (mmol/L) were measured using Wako Chemicals kit (Osaka, Japan). Interleukins (IL-1β and IL-6) and haptoglobin were analysed by commercial bovine ELISA kits (Pierce, Thermo Scientific, IL, USA, and GenWay Biotech Inc., CA, USA. and Life Diagnostics Inc., PA, USA). Cholesterol and total antioxidant capacity (TAC) were also measured using commercially available kits (10007640 and 709001 respectively, Cayman Chemical Company, MI, USA). Reactive oxygen species (ROS) were measured using a commercially available fluorometric kit (STA-342, Cell Biolabs Inc., CA, USA). Liver TAG content was analysed in biopsy samples (wk 1, 2, and 4 post-calving) using the standard procedure provided in the Wako LabAssay TM Triglyceride Kit (290-63701, Wako Chemicals USA Inc., VA, USA).

Statistics

All variables (gene expression and metabolites) were subjected to repeated measures ANOVA to assess the effect of treatment and treatment effects over time, using a mixed models approach (PROC Mixed, SAS 9.3) that included the fixed effects of treatment (pre-calving BCS and feeding level), wk, and their interaction, and cow as a random effect. The model for gene expression also included the Microfluidics 96.96 Dynamic Array™ (Fluidigm) chip as a blocking factor, while the models for metabolites and production, where possible, included covariate data from the end of the previous lactation. Metabolite data including BHBA, NEFA, AST, haptoglobin, IL-1β, IL-6, and ROS were $\log_{10}$ transformed to achieve homogeneity of variance. Positively skewed variables were $\log_{10}$ transformed, variables with negatively skewed distribution were reflected and $\log_{10}$ transformed. If the effect of time was significant ($P < 0.05$), Tukey's t-test was used for pairwise comparisons among weeks. Statistical significance was considered at $P \leq 0.05$ and a trend was considered when $P \leq 0.10$. Results are presented as LSM with standard error of the difference (SED).

4.4 Results and Discussion

Gene expression results from 96 genes were generated (Supplementary Table 2); however, results presented are limited to those that tended ($P < 0.10$) to be or were significantly ($P \leq$

0.05) altered by either BCS (Figure 8) or feeding level (Figure 9). Genes that indicated an interaction between feeding level and time relative to calving (wk) are also presented. With the exception of *ABCA1*, there were no significant interactions between BCS and wk. We have previously reported temporal changes in the neutrophil transcriptional phenotype in grazing dairy cows across the calving period (Chapter 3). Briefly, the expression of genes involved in neutrophil adhesion and migration, mediation of the immune response, neutrophil life-span, and antimicrobial action, were differentially expressed over the transition period. These changes were consistent with previous reports of peripartum changes in neutrophil function, wherein oxidative burst metabolism, random migration, and phagocytosis were perturbed over the calving period (Kehrli et al., 1989b; Detilleux et al., 1995). In the current study, both pre-calving BCS and pre-calving feeding level affected the transcriptional regulation of the neutrophil genes previously investigated (Chapter 3). However, the effect of pre-calving feeding level on the neutrophil transcriptional phenotype was time dependent. These results indicate a potential role for pre-calving diet to modify the peripartum changes in neutrophil function, and the timing at which these cells return to 'normal' function after calving.

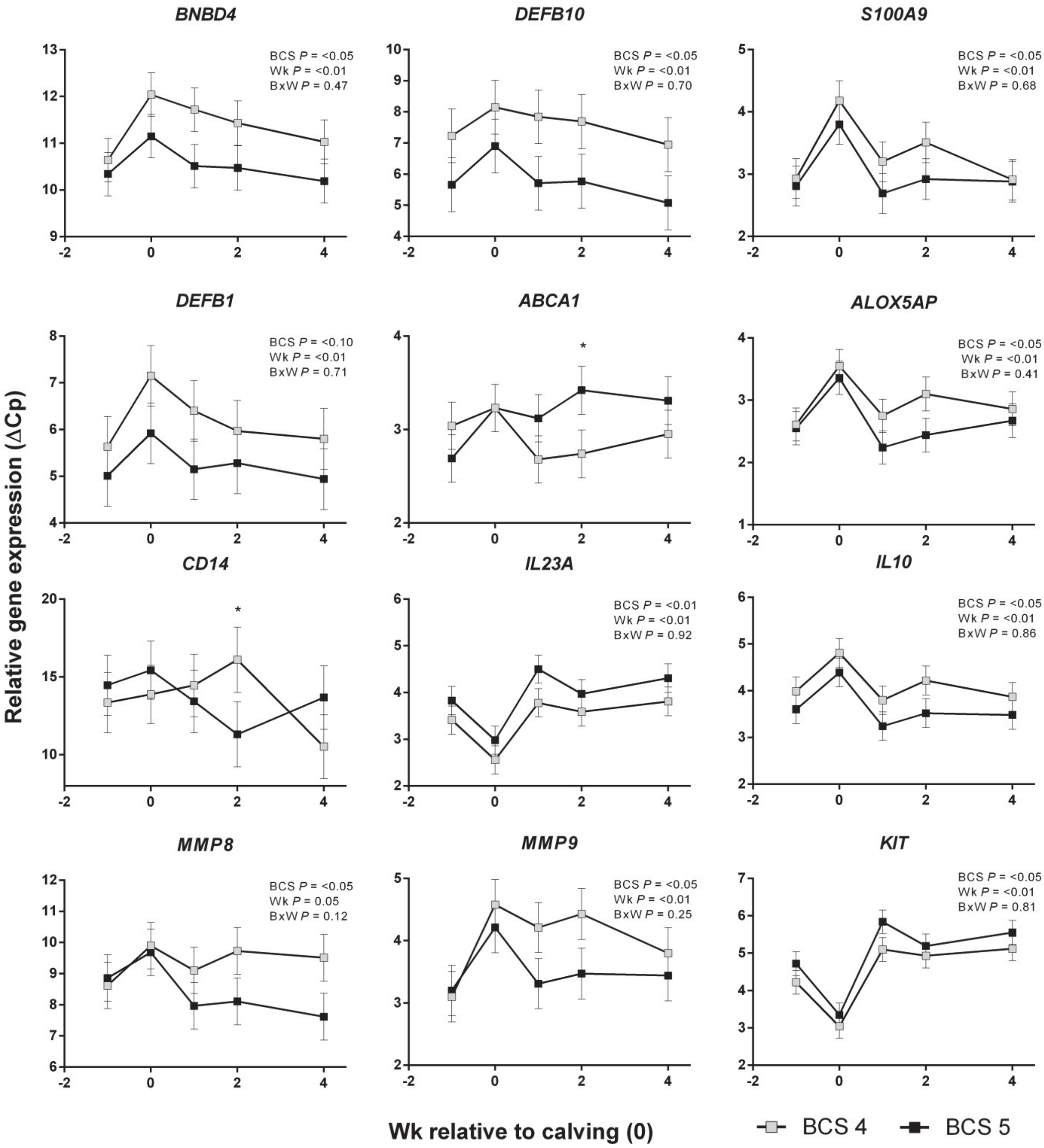

Figure 8. **Effect of body condition score (BCS) on gene expression in circulating neutrophils over the transition period.** Figure legend over page.

Figure 8. Effect of BCS on gene expression in circulating neutrophils over the transition period (previous page). Relative gene expression is presented as delta Cp. Cows calved at BCS 4 or 5 (10-point scale). BCS 4 = under-conditioned, (n = 15; grey squares) and BCS 5 = adequately conditioned (n = 15; black squares). Values are expressed as LSM ± standard error of the difference. Statistical significance was calculated using repeated measures ANOVA. The *P* value for treatment (BCS) and time point (Wk) and the interaction of treatment and time point (B × W) are only displayed on the graphs where there was no significant interaction ($P > 0.05$). For genes with a significant interaction ($P < 0.05$), significant differences between means are denoted with an asterisk (*). Abbreviations: *ABCA1* = ATP binding cassette subfamily A member 1; *ALOX5AP* = arachidonate 5-lipoxygenase activating protein; *BNBD4* = defensin, beta 4A; *DEFB1* = beta-defensin 1; *DEFB10* = beta-defensin 10; *CD14* = CD14 molecule; *IL10* = interleukin 10; *IL23A* = interleukin 23 subunit alpha; *KIT* = KIT proto-oncogene receptor tyrosine kinase; *MMP8* = matrix metallopeptidase 8; *MMP9* = matrix metallopeptidase 9; *S100A9* = S100 calcium binding protein A9.

Effect of Pre-calving BCS on the Transcript Abundance of Genes Involved in Neutrophil Function

The presented genomic phenotype indicates an increase in the bactericidal capacity and a reduction in the inflammatory state of circulating neutrophils in BCS 4 cows. Cows with a BCS 4 compared with BCS 5, demonstrated overall increased ($P < 0.05$) expression of the defensins, *BNBD4* (1.9-fold), *DEFB10* (2.6-fold), and *S100A9* (1.2-fold), and a trend ($P = 0.09$) for increased *DEFB1* (2.3-fold) expression, over the five wk period that blood samples were collected (Figure 8). Defensins are small cationic polypeptides with antimicrobial and immunomodulatory activity that have been identified in a wide variety of cell types and in many different species, including bovine neutrophils (Ganz, 2003; Selsted et al., 1993). The increased expression of these antimicrobial peptides in BCS 4 cows relative to their fatter counterparts probably indicates an increased capacity of neutrophils from these animals to destroy pathogens (Kaiser and Diamond, 2000). An association between defensin concentration in plasma and adiposity has also been reported in humans, although the direction of the association is not consistent and is likely due to the differences in the tissue of origin of the circulating defensins. For example, in a study investigating cardiovascular disease risk factors, López-Bermejo et al. (2007) presented results consistent with those reported here; individuals in the upper quartile of plasma defensin concentrations were leaner and experienced a greater sensitivity to insulin. The authors claimed that this association reflected superior metabolic health. Conversely, another study demonstrated that alpha defensin gene expression in human plasma was greater in obese patients, with lower expression after bariatric or weight loss surgery (Manco et al., 2010). Plasma defensins are pathologically different from those in immune cells and human alpha defensins are distinct from bovine beta defensins (Luenser and Ludwig, 2005). However, this indicates that these peptides may be indicative of immunological alterations to metabolic change, and reflect changes in expression in cases of unbalanced inflammation (e.g., cardiovascular disease or with changes in adiposity). Furthermore, the greater expression of *S100A9* in neutrophils from BCS 4 cows indicates increased antimicrobial capacity, as the S100A9 enzyme is involved in the production of H_2O_2, a respiratory burst product for the destruction of pathogens (Bréchard et al., 2013). The gene expression changes in response to pre-calving BCS indicate an association between adiposity and inflammation, and suggest a reduced inflammatory state in thinner animals; however, further work is required to determine if this relationship is quadratic and, if so, to define the turning point indicative of optimum immunological function.

Pre-calving BCS also altered the expression of several genes involved in mediating the immune response in neutrophils, indicating a shift to a less pro-inflammatory state in BCS4 cows. For example, expression of *IL10*, *MMP8*, and *MMP9* was greater in BCS 4 cows, on average, while expression of *IL23A* and *KIT* was less overall in BCS 4 cows compared with BCS 5 cows (Figure 8). Interleukin-10 is an anti-inflammatory cytokine required for the resolution of the inflammatory response (Couper et al., 2008). In comparison, interleukin-23 has an important role in regulating the production of inflammatory cytokines, and is involved in maintaining neutrophil homeostasis and granulopoiesis (Stark et al., 2005). Matrix metalloproteinases MMP-8 and MMP-9 (neutrophil collagenase and gelatinase, respectively) are required for neutrophil migration and their activity and regulation are critical for recruitment to tissue (e.g., mammary gland tissue in cases of mastitis (Matrisian, 1990; Li et al., 1999)). The increased expression of enzymes that are critical for neutrophil migration may indicate a greater capacity for these cells to be recruited into tissue in BCS 4 cows.

The changes in the inflammatory phenotype in BCS 4 cows may be a result of the reduced adiposity in these cows, as adipose tissue can be pro-inflammatory in nature (Vailati-Riboni et al., 2016). The current study demonstrates evidence of altered cholesterol utilization in response to BCS; compared with BCS 5 cows, there was an increase (1.5-fold, $P < 0.05$) in *ALOX5AP* gene expression in thinner cows. The protein product of *ALOX5AP* associates with 5-lipoxygenase for the synthesis of leukotrienes. Furthermore, there was an interaction between BCS and wk on expression of *ABCA1*, due to a significant decrease in expression in

BCS 4 cows 2 wk post-calving. The ABCA1 cholesterol efflux transporter is regulated by the concentration of cholesterol in the cell; decreased expression, therefore, suggests an increase in cellular cholesterol, which could be utilized for subsequent cellular processes such as cell membrane maintenance or prostaglandin and leukotriene synthesis. Combined with an increased expression of *ALOX5AP*, the lower expression of *ABCA1* may indicate an increase in pro-inflammatory leukotrienes (Funk, 2001) at wk 2 post-calving in BCS 4 cows. Concomitant increased expression of *PLA2G4A* and *ALOX5* did not occur, however, which would have been expected if there was activation of the leukotriene synthesis pathway. The expression results, therefore, cannot confirm increased prostaglandin or leukotriene synthesis in neutrophils of BCS 4 cows, but indicate altered utilization of intracellular cholesterol that is consistent with a reduction in peripheral adipose tissue.

Body condition score-associated changes in indicators of metabolic function were consistent with changes in neutrophil gene expression. Concentrations of NEFA, BHBA, and albumin in plasma, and TAG in liver were significantly ($P < 0.01$) less in BCS 4 cows after calving and plasma globulin concentrations were significantly ($P < 0.01$) greater; albumin to globulin ratio was also less (Table 8). Furthermore, milk production was less in BCS 4 cows compared with BCS 5 cows (Table 9). These changes in metabolic factors and production are consistent with previous reports on the effect of pre-calving BCS differences on indicators of health (Roche et al., 2013b) and increased concentrations of NEFA (in vitro) enhance neutrophil oxidative burst and reduce cell viability (Scalia et al., 2006). In comparison, Hoeben et al. (2000) described a negative association between BHBA and NEFA concentrations and neutrophil function during the peripartum period, therein suggesting that these metabolic markers may be characteristic of impaired neutrophil function. Collectively, the results support a relationship between adiposity and an effect on immune cell function, which may be exploited to regulate peripartal neutrophil function. However, the direct link between neutrophil function and blood metabolites was not explored in the current study.

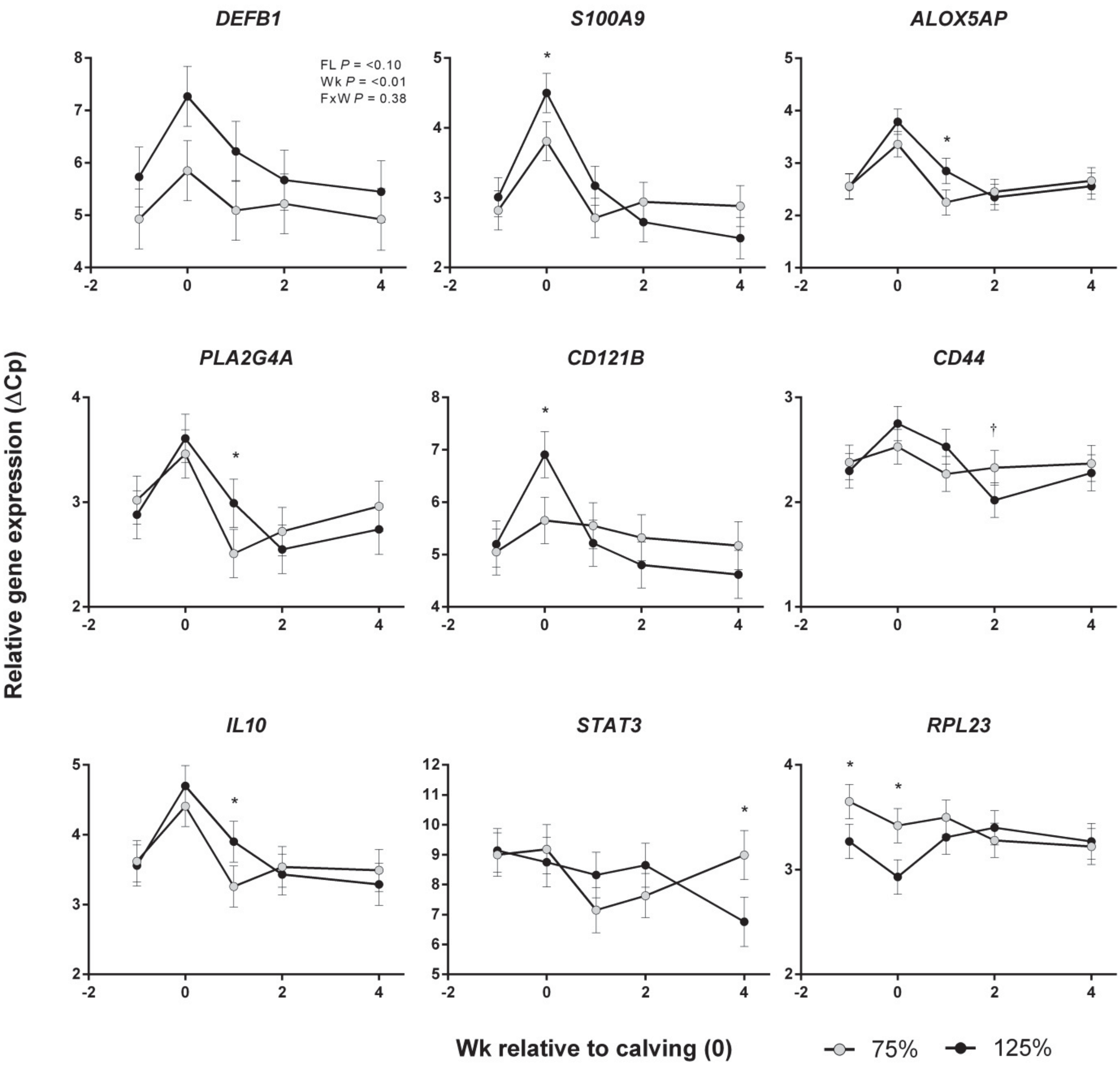

Figure 9. Effect of pre-calving feeding level on gene expression in circulating neutrophils over the transition period. Figure legend over page.

Figure 9. Effect of pre-calving feeding level on gene expression in circulating neutrophils over the transition period (previous page).

Relative gene expression (delta Cp) of neutrophils isolated from cows underfed (75% of maintenance requirements, n = 15; grey circles) and overfed (125% of maintenance requirements, n = 15; black circles) pre-calving. Values are expressed as LSM ± standard error of the difference. Statistical significance was calculated using repeated measures ANOVA. The *P* value for treatment (FL) and time point (Wk) and the interaction of treatment and time point (F × W) are only displayed on the graphs where there was no significant interaction ($P > 0.05$). For genes with a significant interaction ($P < 0.05$), significant differences between means are denoted with an asterisk (*) and a trend ($P < 0.10$) is denoted with a cross (†). Abbreviations: *ALOX5AP* = arachidonate 5-lipoxygenase activating protein; *CD121B* = interleukin 1 receptor, type I; *CD44* = CD44 molecule; *DEFB1* = beta-defensin 1; *IL10* = interleukin 10; *PLA2G4A* = phospholipase A2 group IVA; *RPL23* = ribosomal protein L23; *S100A9* = S100 calcium binding protein A9; *STAT3* = signal transducer and activator of transcription 3.

Effect of Feeding Level on the Transcript Abundance of Genes Involved in Neutrophil Function

Pre-calving feeding level affected neutrophil gene expression; but, the effect was time dependent. For example, the expression of genes involved in leukotriene synthesis *PLA2G4A* and *ALOX5AP*, was significantly ($P < 0.05$) greater in overfed cows compared with underfed cows (BCS 5 offered 75% compared with BCS 5 offered 125%), but only at 1 wk post-calving (Figure 9). This possibly indicates increased leukotriene synthesis in the neutrophils of overfed cows and, therefore, a greater degree of inflammation than in cows underfed during the pre-calving period. Similarly, the effect of pre-calving feeding level on the expression of other genes involved in inflammatory pathways (e.g., *CD121B*, *CD44*, *IL10*, and *STAT3*) was dependent on the time point during the transition period. Overfed cows, fed 125% of maintenance requirements, had greater expression of *CD121B* on the day of calving and increased expression of *IL10* at 1 wk post-calving, when compared with underfed cows consuming 75% of ME requirements. The enzyme phospholipase A2, encoded by *PLA2G4A*, hydrolyses phospholipids for the production of arachidonic acid. Arachidonic acid is subsequently metabolized into eicosanoids, which are pro-inflammatory lipid-signalling leukotriene molecules, e.g., leukotriene B_4, by the enzyme 5-lipoxygenase (encoded by the *ALOX* gene) and 5-lipoxygenase-activating protein (FLAP, encoded by *ALOX5AP*) (Funk, 2001). Consistent with our results, there was also an increased expression of *ALOX5AP* and *PLA2G4A* in the neutrophils of cows with increased level of prepartum feeding in grain-fed cows (Zhou et al., 2015b). These cows also demonstrated altered neutrophil function that indicated a state of chronic inflammation (Zhou et al., 2015b). Further evidence of increased inflammation in overfed cows is the increased expression of *S100A9*; however, this effect was only evident on the day of calving in our study. Moyes et al. (2014) also noted an increase in *S100A9* expression in the neutrophils of cows overfed TMR during the dry period. These results indicate that the effects of prepartum overfeeding in pasture-fed cows in the current study are similar to the immunological effects of overfeeding in conventionally housed, TMR-fed cows, and support findings of an increased inflammatory state of neutrophils in overfed cows.

This effect of feeding level on neutrophil activation early post-partum may be affected by greater circulating NEFA and plasma BHBA concentration. A feeding level of 125% of maintenance requirements did not affect plasma NEFA or BHBA compared with cows offered 75% of maintenance requirements ($P > 0.1$); however, there was a numerical difference, with overfed animals having greater FA and BHBA (Table 8), which is consistent with other studies that have investigated the effect of pre-calving energy intake on post-calving metabolism (Bertics et al., 1992; Roche et al., 2005; Douglas et al., 2006; Roche, 2007). In vitro investigation into the link between plasma NEFA concentrations and neutrophil function has indicated that high concentrations of NEFA (>2 m*M*) increased generation of ROS, reduced neutrophil viability, and increased neutrophil necrosis (Scalia et al., 2006). The average concentrations of NEFA across all time points in the current study were 0.75 and 0.86 mmol/L in the 75% and 125% groups, respectively. Therefore, circulating NEFA concentrations were likely not high enough to considerably alter neutrophil gene expression. Collectively, the gene expression profiles demonstrate that pre-calving feeding level affects the neutrophil inflammatory state early post-partum and could potentially be used as a tool for immunomodulation in the transition cow.

Table 8. **Effect of body condition (BCS) at calving and pre-calving feeding level (FL) on plasma metabolite and cytokine concentrations and liver triacylgycerol.**

Results are presented as LSM with standard error of the difference (SED). Statistical significance calculated using repeated measures ANOVA for effect of BCS and FL their interaction with time (wk).

		BCS Overall			*P* value			FL Overall			*P* value		
	Units	**4**	**5**	**SED**	**BCS**	**Wk**	**BCS*wk**	**75%**	**125%**	**SED**	**FL**	**Wk**	**FL*wk**
Blood metabolite													
Total Protein	g/L	70.30	70.50	1.01	0.84	< 0.01	0.20	71.17	71.37	1.08	0.85	< 0.01	0.25
Albumin	g/L	34.59	37.00	0.75	< 0.01	< 0.01	0.06	37.09	36.02	0.53	0.06	< 0.01	0.61
Globulin	g/L	35.36	33.85	0.72	< 0.05	< 0.01	0.17	34.22	35.24	1.00	0.32	< 0.01	0.21
Alb:Glob ratio		1.01	1.10	0.03	< 0.05	< 0.01	0.12	1.10	1.04	0.04	0.13	< 0.01	0.53
BHBA	mmol/L	0.60	0.80	0.06	< 0.01	< 0.01	0.39	0.77	0.86	0.09	0.41	0.13	0.62
NEFA	mmol/L	0.50	0.76	0.08	< 0.01	< 0.01	0.13	0.75	0.86	0.09	0.15	< 0.01	0.68
AST	U/L	81.5	81.3	3.9	0.93	< 0.05	0.47	81.3	81.8	4.4	0.92	0.57	0.32
Haptoglobin	ng/mL	3331.9	9741.4	5527.3	< 0.05	0.12	0.87	7983.4	5598.7	5525.9	0.91	0.27	0.49
Cholesterol	µM	1994.7	2146.3	286.6	0.60	< 0.01	0.73	2100.5	2641.8	332.3	0.06	0.02	0.74
ROS	µM	0.64	0.54	0.09	0.32	< 0.01	0.96	0.55	0.61	0.06	0.29	< 0.01	0.50
TAC	mM	3.48	2.36	1.21	0.06	< 0.01	0.18	3.25	3.69	1.02	0.50	< 0.01	< 0.05
TAG	% w/w	2.03	6.04	1.07	< 0.01	0.16	0.07	6.02	5.48	1.40	0.67	0.03	0.89
Blood cytokines													
IL-1β	pg/mL	27.8	22.6	1.6	< 0.01	< 0.01	< 0.01	27.6	26.6	1.0	0.09	< 0.01	0.94
IL-6	ng/mL	309.4	688.1	133.6	< 0.01	< 0.01	0.22	673.4	470.5	126.9	0.76	< 0.01	0.007
Blood minerals													
Mg	mmol/L	0.61	0.67	0.04	0.17	0.17	0.36	0.68	0.71	0.04	0.38	0.01	0.77
Ca	mmol/L	2.21	2.18	0.06	0.66	< 0.01	0.41	2.18	2.11	0.05	0.21	< 0.01	0.87

Table 9. **Effect of calving body condition (BCS) and pre-calving feeding level (FL) on the average production parameters post-calving.**

Results are presented as LSM with standard error of the difference (SED) and overall significance of treatment (*P* value) as there were no interactions ($P > 0.05$) between BCS*wk and FL*wk.

		BCS Overall			***P* value**	**FL Overall**			***P* value**
Parameter	**Units**	**4**	**5**	**SED**	**BCS**	**75%**	**125%**	**SED**	**FL**
Milk weight	kg	20.36	23.39	1.05	0.01	23.52	23.91	1.02	0.71
Composition	%								
Fat		4.43	4.64	0.16	0.21	4.77	4.95	0.18	0.33
Protein		3.74	3.65	0.09	0.33	3.67	3.70	0.09	0.65
Lactose		8.14	8.27	0.20	0.51	8.42	8.66	0.24	0.32
Yield	kg/d								
Fat		0.91	1.09	0.06	0.01	1.13	1.17	0.06	0.54
Protein		0.77	0.85	0.04	0.06	0.86	0.88	0.04	0.62
Log_{10} SCC		5.01	4.80	0.14	0.14	4.76	4.86	0.15	0.53

4.5 Limitations of the Experimental Design

For the majority of genes investigated (61 of 78), gene expression was not different between the two pre-calving BCS groups and two feeding level treatments imposed (Supplementary Table 2). Rather than a lack of effect of either BCS or pre-calving feeding level, it is possible that the imposed treatments were not extreme enough to elicit changes in these physiological pathways. In extreme cases of adipose deposition, (e.g., obesity), increased oxidative stress and elevated systemic inflammation interact with leukocytes and affect cell function (Chan et al., 2002; Fantuzzi, 2005; Manna and Jain, 2015). Obesity-related inflammation occurs because of changes in adipocyte metabolism as a result of increased adipose deposition (Gregor and Hotamisligil, 2011). Sub-acute inflammation during the transition period can be likened to obesity-related metabolic changes, as reviewed by Bradford et al. (2015). Deviation from the optimal calving BCS (5.0; 10-point scale; Roche et al., 2009) increases the risk of clinical pathologies; for example, over- and under-conditioned cows are at greater risk of mastitis (Kaneene and Miller, 1995). Cows with a calving BCS >5.5 (>4 on a 5-point scale) experience a more severe NEB during the peripartal period and are at greater risk of metabolic and infectious diseases (Treacher et al., 1986; Stockdale, 2001; Ingvartsen, 2006; Roche et al., 2009, 2013a), which is likely associated with a concurrent reduction in leukocyte function (Lacetera et al., 2005). Regarding the differential gene expression between the two feeding levels investigated here, research by Osorio et al. (2013b) investigated the effect of overfeeding dams (120% of control diet) for 21 d pre-calving on calf neutrophils, demonstrating an increase in neutrophil oxidative burst and changes in neutrophil gene expression in calves born to overfed dams. However, the majority of gene expression changes attributed to treatment were at 2 d post-calving (Osorio et al., 2013b). It is, therefore, possible that greater expression changes may have been evident in our study if we had collected post-calving samples closer to calving i.e., 2 d vs. 1 wk. In cases of severe fasting or starvation in humans, there are negative effects on lymphocyte and neutrophil function, including decreased chemotaxis and microbial destruction (McMurray et al., 1990). In our current study, the low BCS and low DMI treatments indicated possible improvements in neutrophil function. The natural variation in pre-calving BCS and feeding level that is common in pasture-based dairy herds will not likely adversely affect neutrophil function. However, more extreme states were not investigated, likely contributing to the small number of genes that were differentially expressed. This highlights that the points at which a low BCS and low DMI negatively affect immune function require further investigation.

4.6 Conclusion

The pre-calving BCS and pre-calving feeding levels evaluated demonstrated few differences in transcript abundance of the selected genes involved in neutrophil function. Nevertheless, results indicate a reduced inflammatory state of neutrophils and an enhanced capacity for microbial destruction in thinner cows, probably reflecting a negative effect of added body condition rather than support for under-conditioning transition cows. Furthermore, prepartum feeding level altered the inflammatory state of neutrophils during the peripartum period; however, this was dependent on the time relative to calving. Treatment groups were selected to represent the norm within optimally managed pasture-based herds and did not reflect extreme BCS and feeding level treatments. This may provide the reason for the limited number of observed differences in expression of neutrophil genes between treatments. Additional research is required to further elucidate the relationship between pre-calving BCS and prepartum energy intake and immune function throughout the transition period.

4.7 Acknowledgements

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4.8 Summary

The previous two chapters have characterised gene expression changes over the transition period and investigated whether on-farm management strategies can alter cell function. The aim of Chapter 5 was to investigate the link between metabolic stress during the transition period and neutrophil gene expression. To do this, two groups of cows divergent in their metabolic health status at week 1 and week 2 post-calving were selected. RNA sequencing was then used to investigate the changes in the transcriptome of neutrophils in these two groups of cows at three post-calving time points; day 0, week 1, and week 4.

Chapter 5

Transcriptomic analysis of circulating neutrophils in metabolically-stressed peripartal grazing dairy cows

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5.1 Abstract

The high metabolic demand during the transition into lactation means that cows are at greater risk of succumbing to metabolic and infectious disease than at any other time in the lactation cycle. Additionally, there is a change in the innate immune response during this period, which contributes to the increased risk of disease. In the current study, we have compared the transcriptomes of neutrophils from dairy cows divergent in their metabolic health post-calving i.e., cows at low or high risk of disease during the transition period. Cows (n = 5 per Risk group) were selected based on plasma concentrations of non-esterified fatty acids (NEFA), plasma β-hydroxybutyrate (BHBA), and liver triacylglycerol (TAG) at weeks 1 and 2 post-calving from a parent experiment (n = 45 cows). Those with high concentrations of NEFA, BHBA, and TAG were deemed 'High Risk' of developing metabolic or infectious disease. Circulating neutrophils were isolated at three time points during the transition period (day 0 and weeks 1, 4 post-calving) and gene expression analysed using RNA sequencing. Differential gene expression between the risk groups was determined using EdgeR and pathway analysis was conducted using Ingenuity Pathway Analysis (IPA). Statistical analysis indicated no interaction between risk and week. Therefore, the overall effect of 'Risk' was analysed across all time points. In total, there were 3,500 genes that were differentially expressed between the 'High Risk' and 'Low Risk' cows (false discovery rate < 0.05). Of them, 2,897 were identified as 'analysis-ready' and were used for pathway analysis using IPA. Of the relevant pathways identified, neutrophils isolated from 'High Risk' cows demonstrated down-regulation of genes involved in the recruitment of granulocytes, interferon signalling, and apoptosis, and an up-regulation of genes involved in cell survival. The differential expression of genes in the 'High Risk' (metabolically-stressed) cows compared with the 'Low Risk' cows indicated that these cows had reduced neutrophil function during the peripartum period. This indicates that subclinical metabolic disease has an effect on innate immune function and is likely contributing to the state of 'immunosuppression' occurring during the peripartum period. The metabolic stress

in the ‘High Risk’ cows may affect neutrophil function in ways that affect their ability to combat infection.

5.2 Introduction

A successful transition between pregnancy and lactation in dairy cows requires several complex metabolic and physiological changes to occur in concert. If these changes are not sufficiently co-ordinated, the animals experience metabolic stress and health dysfunction (subclinical or clinical) (Sordillo et al., 2009; LeBlanc, 2010). The incidence of health dysfunction during the transition period is very high; 30-50% of cows will experience metabolic or infectious disease during the transition period, and the incidence of subclinical disease is grossly underestimated (LeBlanc, 2010; Compton et al., 2014), highlighting how precarious a successful transition can be. Increasing evidence indicates that a balanced inflammation during the transition period is required for a successful transition that is free of complications (Challis et al., 2009; Farney et al., 2013a; Vailati-Riboni et al., 2015).

Parturition is an inflammatory event, which modifies local and systemic immune responses (Molloy et al., 2004; Christiaens et al., 2008). The ability to restore homeostasis after parturition is essential for a successful transition. Dysfunctional inflammation likely increases the risk of metabolic and infectious disease during the transition period. We, and others, have demonstrated altered gene and protein expression in peripheral blood leukocytes during the transition period (Kehrli et al., 1995; Lee and Kehrli, 1998; Heiser et al., 2015; Chapter 3). Neutrophils are part of the first line of defence against pathogens and are important regulators of the innate immune response. During the transition period their number and performance regulate the incidence of infectious disease.

Metabolic changes during the transition period cause a state of negative energy balance in early lactation. This is accompanied by the mobilization of non-esterified fatty acids (NEFA) from adipose tissue reserves and increased production of beta-hydroxybutyrate (BHBA) by the liver. Severe negative energy balance that causes extended adipose mobilization leads to NEFA accumulation in the liver as triacylglycerol (TAG), which impedes liver function (Vazquez-Añon et al., 1994). Uncontrolled lipid mobilization is implicated in the pathogenesis of health disorders during the transition period (Drackley, 1999). In extreme cases, the metabolic condition (high NEFA, BHBA, and liver TAG) can be likened to cardiovascular disease and type 2 diabetes in humans (Blaak, 2003; Desideri-Vaillant et al., 2004; Pilz and März, 2008). Furthermore, an interaction between metabolic stress, inflammation, and immune function exists during the transition period (Sordillo et al., 2009; Sordillo and Aitken, 2009). Therefore, these are suitable proxies for metabolic health status during the transition period.

These metabolic factors also affect the innate immune system. For example, high concentrations of NEFA have negative effects on neutrophil function in vitro (Scalia et al., 2006), severe fatty liver can lower production of reactive oxygen species (ROS) and expression of cell surface molecules in neutrophils in vivo (Zerbe et al., 2000) and high BHBA concentrations (in vitro) have been demonstrated to inhibit chemotaxis in ovine neutrophils (Sartorelli et al., 1999). These changes in neutrophil responses are associated with gene expression changes involved in cellular function that reportedly occur over the transition period (Burton et al., 2001; Chapter 3). Therefore, we hypothesised that metabolic stress would affect the neutrophil transcriptome. To test this hypothesis, we selected two groups of cows divergent in metabolic health (assessed via plasma NEFA, BHBA, and liver TAG) and used RNA sequencing (RNAseq) to investigate changes in the transcriptome of circulating neutrophils in these cows at three time points over the transition period (post-calving on the day of parturition, week 1, and week 4). The objective, therefore, was to investigate the effect of metabolic stress, as indicated by subclinical health dysfunction, on the transcriptome of circulating neutrophils in periparturient grazing dairy cows.

5.3 Materials and Methods

Experimental Design and Sample Collection

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (RAEC#12799) in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The parent experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46’S 175°18’E) between July and October 2013.

A subset of cows of mixed age and breed (Holstein-Friesian and Holstein-Friesian × Jersey) were selected from the cows described in Chapter 3. Cows (n = 5/group) were separated into ‘High Risk’ or ‘Low Risk’ based on the indicators of metabolic health at wk 1 and wk 2 post-calving: plasma concentrations of NEFA and BHBA as well as liver TAG (Figure 10). All cows displayed a body condition score of 4 or 5 at calving (based on a 10-point scale, equivalent to approximately 2.75 and 3.25, respectively, on a 5-point scale) and were offered a diet of fresh pasture (average dry matter intake [DMI] = 12.4 ± 0.8 kg/d), supplemented with corn silage (average DMI = 0.8 ± 0.8 kg DM/d), and pasture silage (average DMI = 1.1 ± 1.0 kg DM/d) post-calving. None of the cows displayed any signs of clinical disease or were treated for any medical conditions during the trial period.

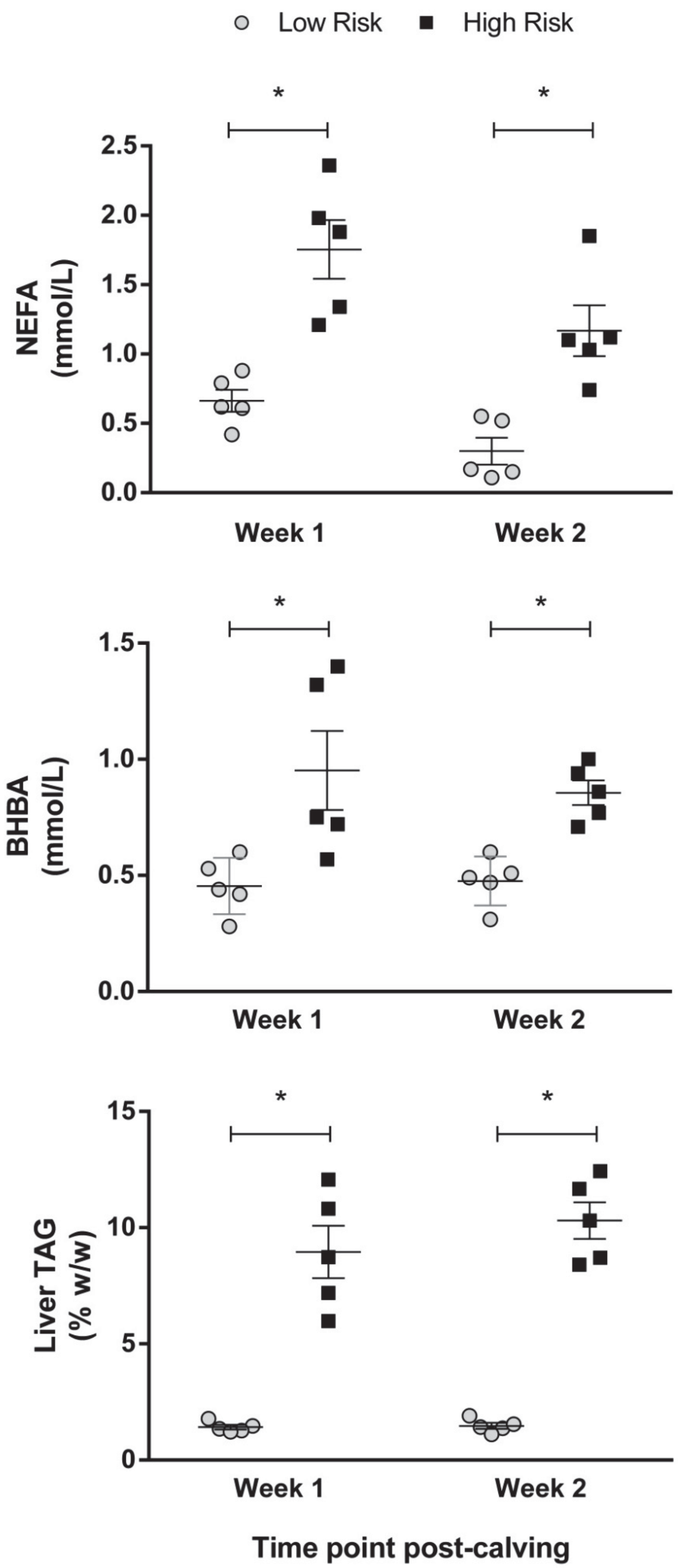

Figure 10. Indicators of metabolic health status for determining 'Risk' category. Figure legend over page.

Figure 10. Indicators of metabolic health status for determining 'Risk' category (previous page). Concentrations of plasma non-esterified fatty acids (NEFA), plasma beta-hydroxybutyrate (BHBA), and liver triacylglycerol (TAG). Data include plots of individual cows within 'Risk' groups to indicate distribution. Horizontal lines indicate the arithmetic mean and error bars denote standard error of the mean (SEM). Significance between groups is indicated by an asterisk (*), determined by one-way ANOVA at each time point (SAS 9.3). Significance was considered at $P < 0.05$.

Liver Sampling for Triacylglycerol Analysis

Liver tissue samples were collected from all cows by biopsy the wk before calving (wk -1) and on weeks 1, 2, and 4 after calving. Briefly, the skin in the right 11th intercostal space at the level of the greater trochanter was shaved, disinfected, and the area anaesthetised with 7 mL of 2% lignocaine (Lopaine 2%, lignocaine hydrochloride 20 mg/mL, Ethical Agents, South Auckland, New Zealand). An incision was made through the skin, a 12G × 20 cm biopsy needle was inserted into the liver and approximately 1 g (wet weight) of liver tissue was collected. Liver samples were snap-frozen in liquid nitrogen and stored at -80°C.

Neutrophil Isolation

Neutrophils were isolated and checked for viability and differentials from all cows according to procedures previously described in Chapter 3. Briefly, blood collected in the acid citrate dextrose evacuated tubes was inverted and pooled (for each cow at each time point) by transfer into a 50 mL conical tube. Tubes were centrifuged for 30 min at 850 × *g* at 4°C. The plasma, buffy coat, and up to 1/3 of the red blood cells (RBC) were aspirated by Pasteur pipette and discarded, leaving 2/3 of remaining RBC and neutrophils. Water (milliQ, Millipore, New Zealand) was added (25 mL) and mixed for 10 - 30 seconds to lyse the RBC and 5 mL of 5× concentrated PBS (pH 7.4, Life Technologies, New Zealand) was added to each tube to restore isotonicity. Tubes were spun at 330 × *g* for 10 min at 4°C and supernatant decanted to isolate the white cell pellet. The pellet was washed with 10 mL of 1× concentrated PBS (pH 7.4, Life Technologies), vortexed and centrifuged for 5 min at 660 × *g* at 4°C. If RBC remained, the water lysing step was repeated. Cells were re-suspended in 5 mL of 1× PBS (pH 7.4) and 100 µL was transferred into a 1.5 mL microcentrifuge tube (Raylab, New Zealand) and placed on ice for subsequent viability and staining assays. The 50 mL tubes were centrifuged again (5 min at 660 × *g* at 4°C), liquid decanted, and the cell pellet re-suspended in 1 mL TRIzol® reagent (Life Technologies). The 1 mL cell suspension was transferred into a 2 mL microcentrifuge tube (Eppendorf, Germany), placed immediately on ice, and stored at -80°C for subsequent RNA extraction.

RNA Extraction and Sequencing

Total RNA from neutrophil lysates in TRIzol® reagent was extracted according to the manufacturer's protocol with details described previously in Chapter 3. RNA quantity and purity was assessed using spectrophotometry (Nanodrop, ThermoFisher Scientific, New Zealand). All RNA samples had a 260/280 ratio >1.90 and a 260/230 ratio >1.75 (Nanodrop, ThermoFisher Scientific). RNA quality was determined by capillary gel electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, CA, USA). Samples had an average RNA integrity number (± SD) of 8.1 ± 0.71 and were stored at -80°C until sequencing analysis.

Paired-end cDNA libraries were constructed using TruSeq stranded total RNA Library prep with RiboZero ribodepletion and ERCC spike-in controls according to manufacturer's recommendations (Illumina, CA, USA). Libraries were sequenced on an Illumina HiSeq 2500 platform using 4 lanes; 2 × 125 bp paired-end reads using V4 chemistry (Illumina). Sequencing resulted in 1.65B total paired reads, with a mean percentage of bases >=Q30 of 91.6%, and a mean average quality score per base of 35.2. Average library size (± SD) was 339 ± 17 bp, with the exception of a 4 wk sample, 200 (268 bp library). Library construction and sequencing was completed by New Zealand Genomics Ltd., Dunedin, New Zealand.

Data Processing and Analysis

Raw read quality was analysed with FastQC (v. 0.11.2 (Andrews, 2010)), followed by removal of sequencing adaptors and PCR primers with cutadapt (v. 1.9.1 (Martin, 2011)). Reads were trimmed to contiguous sequences with minimum Phred scores of 25 and a minimum final length of 50 nucleotides using SolexaQA (v. 3.1.4 (Cox et al., 2010)). Ignoring sample 200, 27.5 million trimmed (±2.66 million) reads per sample were available for mapping; 15.7 million trimmed reads were available for sample 200. Reads were mapped to the umd3.1 bovine genome reference assembly using STAR aligner (v. 2.5.0b (Dobin et al., 2013)) in two-pass mode. Unannotated splice sites discovered in the first pass were filtered to reject junctions covered by less than four uniquely mapped reads, less than 5 nucleotide overlap with flanking exons, and observed in less than four samples of any given treatment group. Curated junctions were combined with the splice junctions annotated in the reference assembly for the second read mapping pass, where discovery of novel junctions was disabled. Ignoring sample 200, 73.9% (± 4.3%) of the reads were mapped to unique locations, while 21.6% (± 3.7%) were too short to be mapped successfully. Only 18.5% of reads from sample 200 were uniquely mapped, and 77.5% were too short for mapping. For each sample, the counts of uniquely mapped, reads mapped per gene, and gene length was determined with featureCounts (v. 1.5.0 (Liao et al., 2014)) and custom scripts.

Differential expression of genes was analysed by both DESeq2 (Love et al., 2014) and EdgeR (Chen et al., 2014) using the statistical computing software 'R' (version 3.2.2) to determine the most appropriate statistical package, a 2-way ANOVA model was used with DESeq2 and the model used with EdgeR allowed comparisons both between and within subject. Low read counts were filtered (≥ 5 reads per million in ≥ 3 samples per risk group), which retained 34% of genes for analysis (n = 9,813 for EdgeR and n = 9,800 for DESeq2 of 28,538). The interaction between Risk and week, the effect of Risk at each time point (d0, wk 1, and wk 4 post-calving), and the overall effect of Risk across all weeks was determined using both packages. There was no interaction between Risk and week with either package. Therefore, the overall effect of Risk was analysed across all time points. Significant differential expression was defined as FDR < 5% and |logFC| > 1 using EdgeR and FDR < 10% and |logFC| = 0.4 using DESeq2. The number of genes differentially expressed was n = 1,823 using EdgeR and n = 627 using DESeq2.

The final dispersion estimate was 0.065 for EdgeR and 0.175 for DESeq2 and a biological co-efficient of variation (CV) of 26% and 42%, respectively. Therefore, EdgeR was deemed most suitable for the differential gene expression analysis compared with DESeq2. This was due to the lower CV and the ability to cope with the repeated measures aspect of the data.

Ingenuity Pathway Analysis (IPA)

Network and pathway analyses were generated using IPA (Ingenuity Systems, Qiagen; www.ingenuity.com), which groups differentially expressed genes into known functional pathways and networks based on current knowledge, primarily from rodent and human studies (Krämer et al., 2014). Significantly differentiated genes (FDR < 0.05) from the EdgeR Risk comparison were uploaded to IPA containing Ensembl Gene ID, *P*-value, FDR, and expression fold-change of 'High Risk' compared with 'Low Risk'. This retained 2,897 analysis-ready molecules for network and function analysis by IPA using default analysis settings. Both up- and down-regulated genes were analysed simultaneously. Analyses focused on pathways involved in haematological system development and function, and networks involved in immunological or metabolic disease.

5.4 Results and Discussion

Elevated concentrations of plasma NEFA, BHBA, and liver TAG during the peripartum period are associated with an increased risk of metabolic and infectious disease (McArt et al., 2013; Ospina et al., 2013). Furthermore, altered neutrophil function during the transition period also contributes to the increased risk of periparturient disease (Cai et al., 1994). We have previously established that neutrophil gene expression changes during transition from pregnancy to lactation in Chapter 3. The effects of high circulating NEFA, BHBA, and liver TAG post-calving on neutrophil gene expression in vivo are unknown. Here we aim to establish the molecular effects of metabolic stress caused by subclinical health dysfunction on the transcriptome of circulating neutrophils during the transition period.

The principal findings from the transcriptomic analysis indicated that the neutrophils from 'High Risk' cows demonstrate transcriptional changes consistent with metabolic stress, which likely increases their risk of infectious disease during the peripartum period. Genes regulating neutrophil recruitment and migration, as well as those involved in interferon signalling, were down-regulated in the 'High Risk' cows, suggesting the cells will not respond as efficiently as those in 'Low Risk' cows. Circulating neutrophils from the 'High Risk' cows demonstrated an increased life span due to a down-regulation of genes involved in apoptosis and an increased expression of genes that regulate cell survival, which is also evident during infection and inflammation and can lead to a detrimental chronic inflammatory state. Furthermore, analysis of disease networks identified genes up-regulated in the instance of metabolic disease.

Recruitment of Neutrophils

Physiological stress in the 'High Risk' animals resulted in the down-regulation of genes involved in the recruitment of neutrophils (Table 10). The process of granulocyte recruitment within the system of haematological development was decreased in 'High Risk' cows (Figure 11). Rapid recruitment of neutrophils to the site of tissue injury is essential for phagocytosis and destruction of pathogens. For example, a reduced capacity for neutrophil recruitment during the transition period has been associated with an increased risk of mastitis (Burton and Erskine, 2003). In fact, there is an inverse relationship between the capacity for neutrophils to be recruited to the udder and the outcome of a mastitis infection (Shuster et al., 1996). Further supporting a reduced capacity for attachment and migration of neutrophils in the 'High Risk' cows and an increased risk of infectious disease is lower expression of *C5AR1*, *CXCR2*, and *SELL* in the 'High Risk' cows than in 'Low Risk' (Figure 11). *C5AR1* (encodes complement C5a receptor 1), which was 2.6-fold down-regulated in the 'High Risk' cows compared with the 'Low Risk'. Differential expression of *C5AR1* has been demonstrated in several studies of mastitis in cattle, confirming that down-regulation likely increases susceptibility to mastitis (Ogorevc et al., 2009). Furthermore, Seo et al. (2013)

reported a decrease in *C5AR1*, *CXCR2*, and *SELL* in neutrophils soon after calving, indicating a reduced capacity for attachment and migration during the peripartum period. Overall, our results indicate that neutrophil recruitment is down-regulated in 'High Risk' cows, implying that neutrophil function is impaired in these cows and, probably, that they are at an increased risk of peripartum infectious diseases.

Apoptosis and Cell Survival

The gene expression signatures in neutrophils isolated from the 'High Risk' cows are indicative of changes in neutrophil lifespan suggesting a disruption of cell homeostasis caused by metabolic stress. The molecular and cellular functions of differentially expressed genes identified by IPA, included altered cellular growth and proliferation and cell death and survival (Figure 12): apoptosis (-2.19 z-score) and cell death (-3.84 z-score) were decreased in 'High Risk' cows; conversely, cell survival (4.25 z-score) and cell viability (4.30 z-score) were increased (Table 10). Furthermore, expression of *BCL2*, which is a potent regulator of cell survival, was greater in 'High Risk' cows (Figure 12). Neutrophil apoptosis is delayed in response to factors such as pro-inflammatory cytokines (TNF), bacterial proteins (LPS), hypoxia, and in disease conditions, like chronic granulomatous disease (Wright et al., 2013; Luo and Loison, 2008; Hsieh et al., 1997). Therefore, neutrophils encounter these regulatory factors in times of stress and inflammation. The delay in apoptosis is controlled, at least in part, by the down-regulation of apoptosis-inducing genes and the up-regulation of genes involved in cell survival, e.g., *BCL2* (McCracken and Allen, 2014). Other regulators of apoptosis were also affected by cow risk category. For example, gene expression of heat shock proteins *HSP90* and *HSP90AA1* was down-regulated in the 'High Risk' cows (Figure 12). Both proteins are stress-induced chaperones that maintain cellular activity; therefore, perturbed expression results in detrimental changes to normal cell function. For example, inhibition of Hsp90 inhibits recruitment and activation of neutrophils (Samavedam et al., 2015; Tukaj et al., 2015). Circulating neutrophils from the 'High Risk' cows demonstrate an overall increase in cell survival and decreased apoptosis, which is likely to exacerbate inflammatory responses as regulation of neutrophil survival/apoptosis is critical for controlling and resolving inflammation.

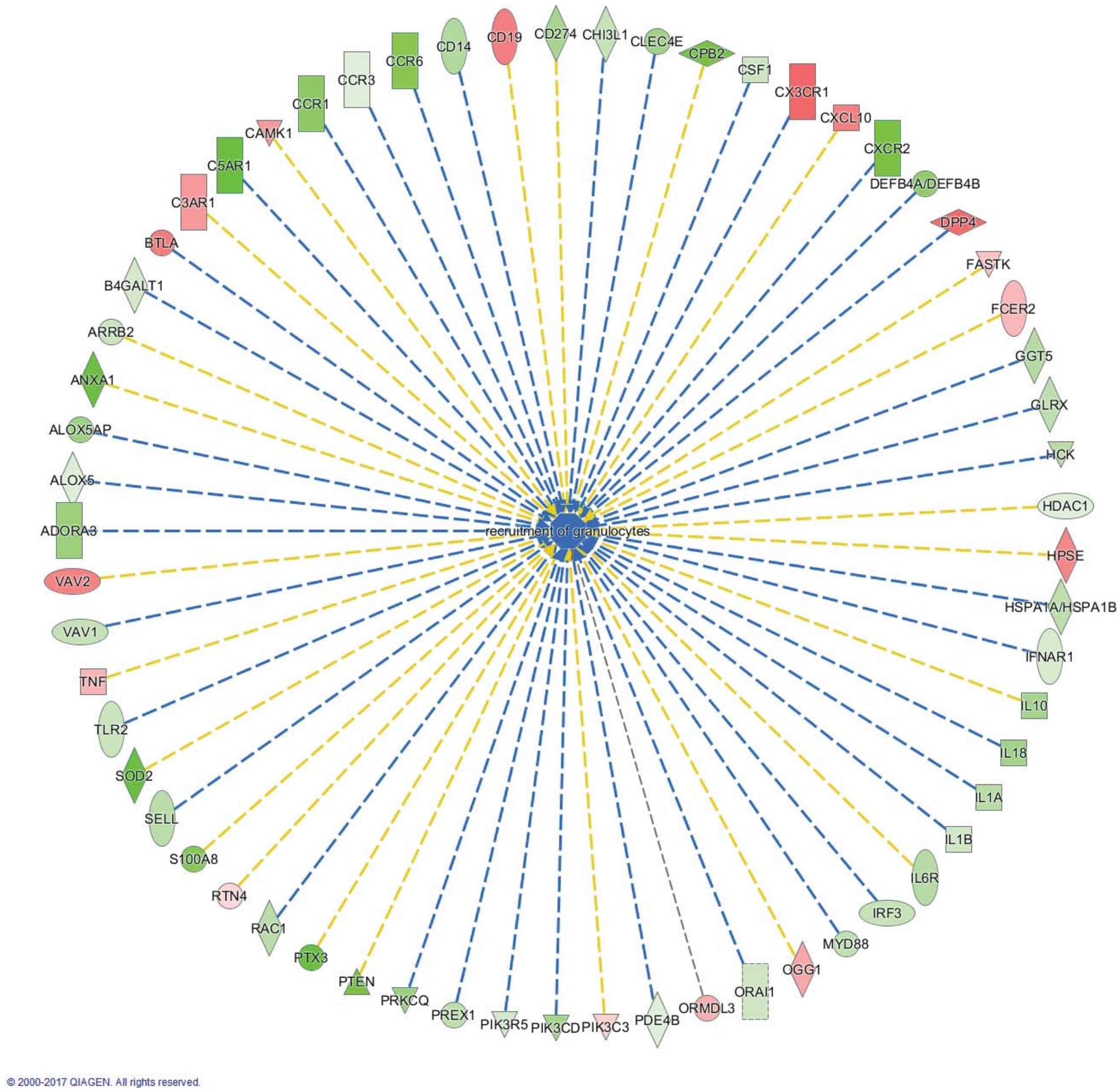

Figure 11. Differential expression of genes involved in the recruitment of granulocytes. Genes involved in the recruitment of granulocytes that are differentially expressed in neutrophils of 'High Risk' cows compared with 'Low Risk' cows. Colour intensity indicates the degree of increased (red) or decreased (green) relative expression. Arrows indicate predicted relationships: blue arrows indicate the molecule leads to activation of granulocyte recruitment and a yellow arrow indicates that the findings are inconsistent in understanding the molecules' relationship with granulocyte recruitment.

Table 10. Cellular functions perturbed in the 'High Risk' cows compared with 'Low Risk' cows.

Relevant functions within the categories of haematological system development and function, cell death and survival, and canonical pathways that were differentially regulated between the risk groups. *P*-values calculated using Fisher's Exact Test and are considered significant at $P < 0.01$. The activation score (z-score) indicates predicted 'direction' of the function (negative = inhibited, positive = activated) based on known relationships between molecules.

Categories	Function	*P*-value	Z-score
Functions decreased in 'High Risk' cows			
Haematological System Development and Function	Recruitment of granulocytes	6.80E-06	-2.281
Cell Death and Survival	Cell death	2.17E-14	-3.842
Cell Death and Survival	Apoptosis	1.36E-13	-2.194
Cell Death and Survival	Cell death of myeloid cells	1.17E-06	-2.497
Cell Death and Survival	Apoptosis of myeloid cells	1.56E-06	-2.518
Canonical pathway	Interferon signalling	4.78E-04	-3.464
Functions increased in 'High Risk' cows			
Cell Death and Survival	Cell survival	3.80E-12	4.252
Cell Death and Survival	Cell viability	7.65E-12	4.304

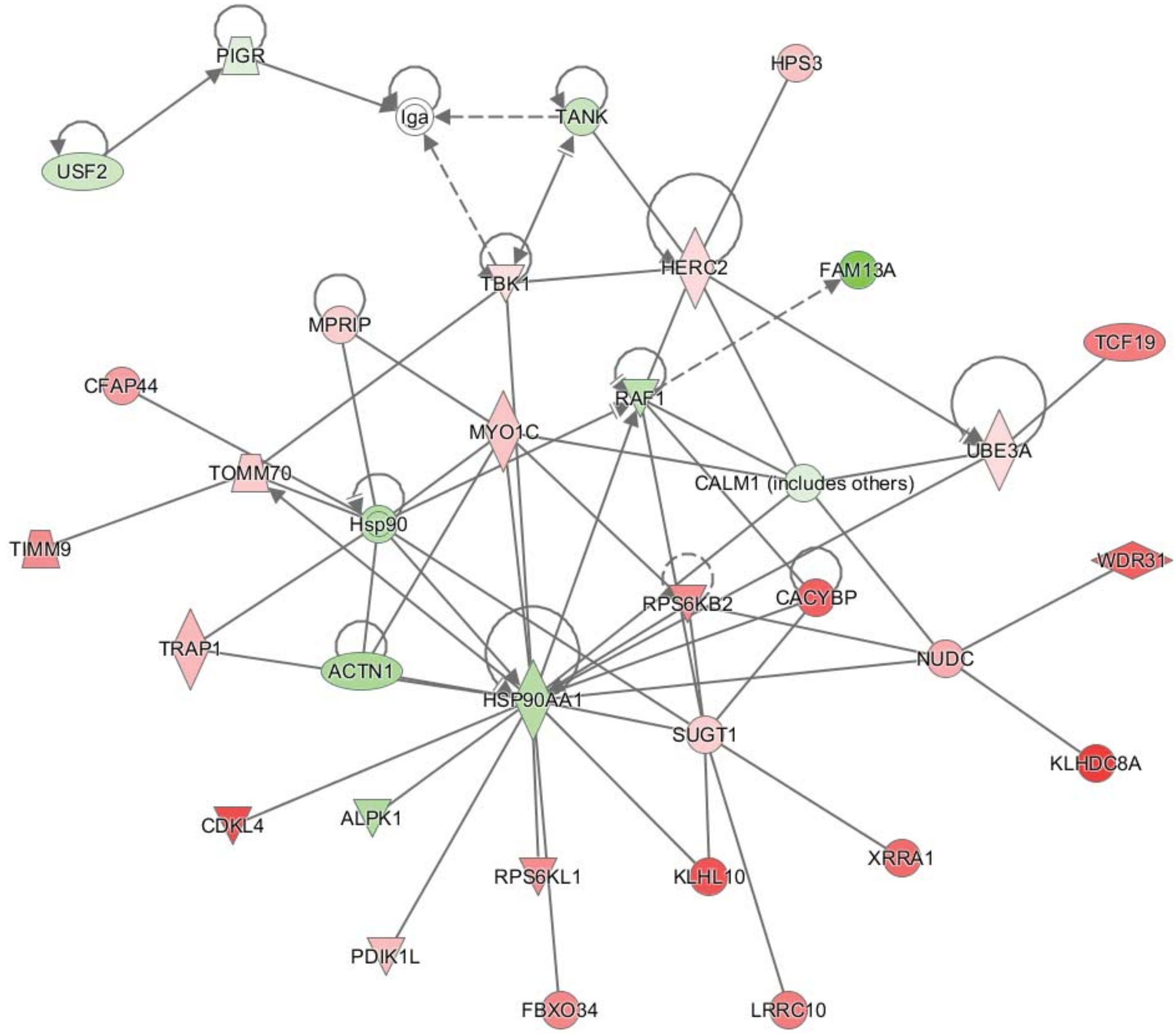

Figure 12. Differential expression of genes involved in cell signalling, cell death, and cell survival.

Genes involved in cell signalling, cell death, and cell survival that are differentially expressed in 'High Risk' cows compared with 'Low Risk' cows. Colour intensity indicates the degree of increased (red) or decreased (green) relative expression. A white colour indicates genes that did not demonstrate expression differences.

Interferon Signalling

Expression of genes involved in interferon (IFN) signalling was lower in the 'High Risk' cows compared with the 'Low Risk' cows, which also suggests a reduced capacity to respond to IFN-γ as an attempt to attenuate inflammation. Expression of the genes encoding the gamma IFN receptor (*IFNGR1* and *IFNGR2*) as well as signalling molecule *STAT1* and IFN-signalling target genes (*IFITM2*, *IFITM*, *IFIT1*, *MX1*, *G1P2*, and *IRF9*) was lower in 'High Risk' cows compared with 'Low Risk' animals (Figure 13). The IFNα signalling pathway confers viral resistance and has roles in innate immune regulation. Aberrant activation of the IFN-signalling pathway in neutrophils occurs in cases of inflammatory disease, for example, Rheumatoid Arthritis, and neuro-inflammatory diseases, such as Parkinson's Disease and Multiple Sclerosis (Wright et al., 2015; Yan et al., 2016). As such, therapies to regulate interferon signalling are of interest to ameliorate disease symptoms, demonstrating its importance in the pathogenesis of pro-inflammatory disease (Shuai and Liu, 2003). The ability of a cell to respond to IFN is dependent on the expression of its receptor on the cell surface (Kumaran et al., 2007; De Weerd and Nguyen, 2012). Therefore, gene expression of the receptors and subsequent expression on the neutrophil cell membrane is essential for a fast response to pathogen invasion. Subsequent expression of target genes (IFN response genes) is required for an innate immune response that is essential for clearance of a pathogen. For example, the IFN-inducible transmembrane proteins (IFITM) 1, 2, and 3, are critical for inhibiting replication of several virus strains (Brass et al., 2009). Furthermore, neutrophils require *MX1* (encoding myxovirus resistance protein 1) to contribute to influenza-related lethality as demonstrated in an IFN signalling-knockout mouse model (Pillai et al., 2016). Therefore, it is conceivable that down-regulation of genes involved in the IFN-signalling pathway in neutrophils from 'High Risk' cows may result in increased susceptibility to infection due to an impaired response to IFN signalling. An investigation of protein expression profiles will be required to confirm this hypothesis.

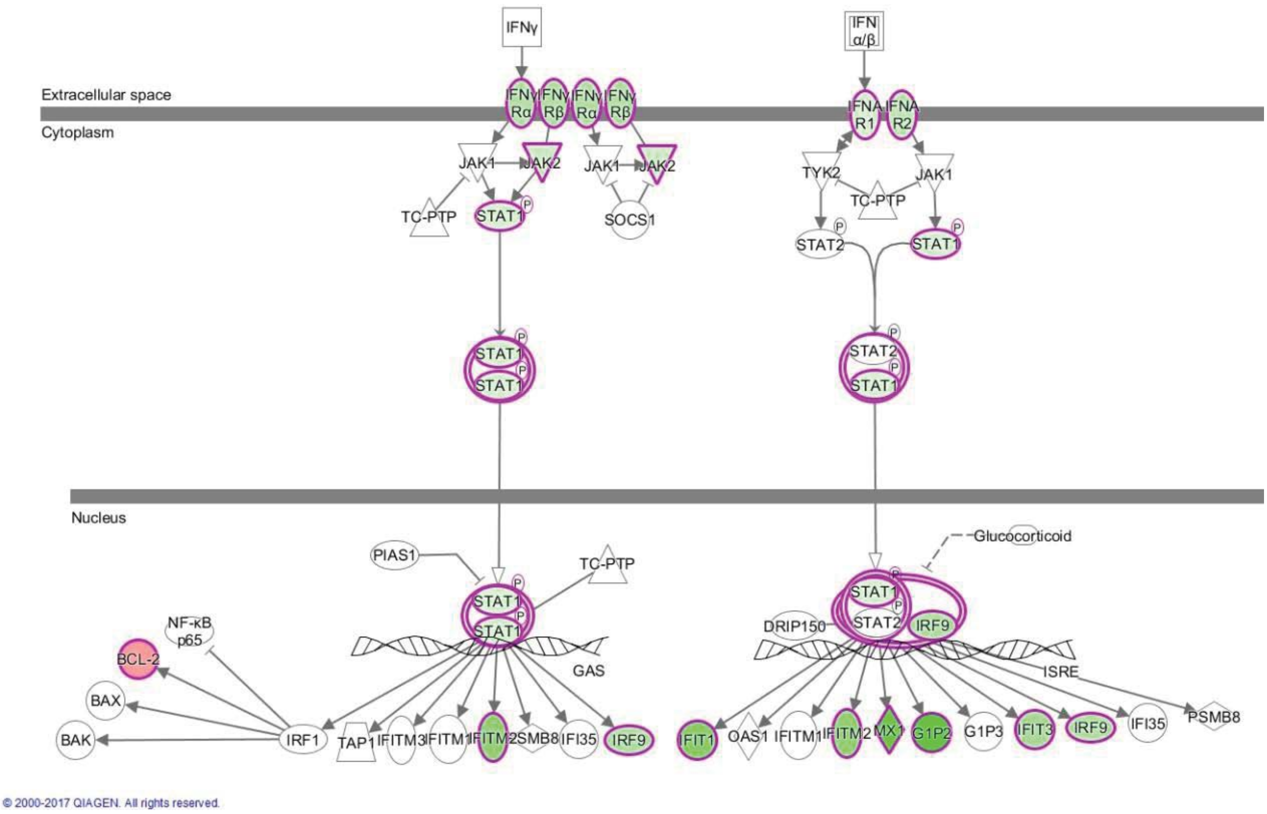

Figure 13. Differential gene expression in the interferon signalling pathway.

Genes within the interferon signalling canonical pathway of IPA demonstrating differential gene expression in neutrophils of ‘High Risk’ cows compared with ‘Low Risk’ cows. Colour intensity indicated the degree of increased (red) or decreased (green) relative expression in the ‘High Risk’ group. A white colour indicates the genes did not demonstrate differential expression.

Evidence that Metabolic Stress contributes to Neutrophil Dysfunction

Comparison of the transcriptome profiles of neutrophils from 'High Risk' cows and 'Low Risk' cows indicates effects of metabolic state on neutrophil gene expression. One of the top 25 networks identified included genes involved in the 'Diseases and Functions' of metabolic disease, organismal injury and abnormalities, and hereditary disease (Figure 14). Differential expression of these genes indicates perturbation in the neutrophil inflammatory response e.g., increased expression of *ABR*, *DOCK10*, *ELMO3*, and COG genes, and decreased expression of *RAC1* and *ELMO2*. The active breakpoint cluster-related protein (encoded by *ABR*) has roles in negatively regulating the neutrophil inflammatory response, neutrophil degranulation, and neutrophil extravasation (Cunnick et al., 2009; Deronic et al., 2014). Similarly, the protein products of *RAC1*, *DOCK10*, and *ELMO*, *ELMO2*, and *ELMO3* (engulfment and motility proteins) are involved in regulating cell motility and adhesion (Gadea and Blangy, 2014; Schnoor et al., 2017). *COG2*, *COG5*, and *COG6* gene products are involved in glycosylation of cell surface receptors required for neutrophil attachment, migration, and cell-cell interactions (Freeze and Schachter, 2009). For example, neutrophil interaction with dendritic cells requires glycosylation of Mac-1 (adhesion molecule CD11b/CD18) (van Gisbergen et al., 2005). Several of the differentially expressed genes in this network function in adhesion and migration, which likely indicates a perturbed neutrophil inflammatory response in 'High Risk' cows.

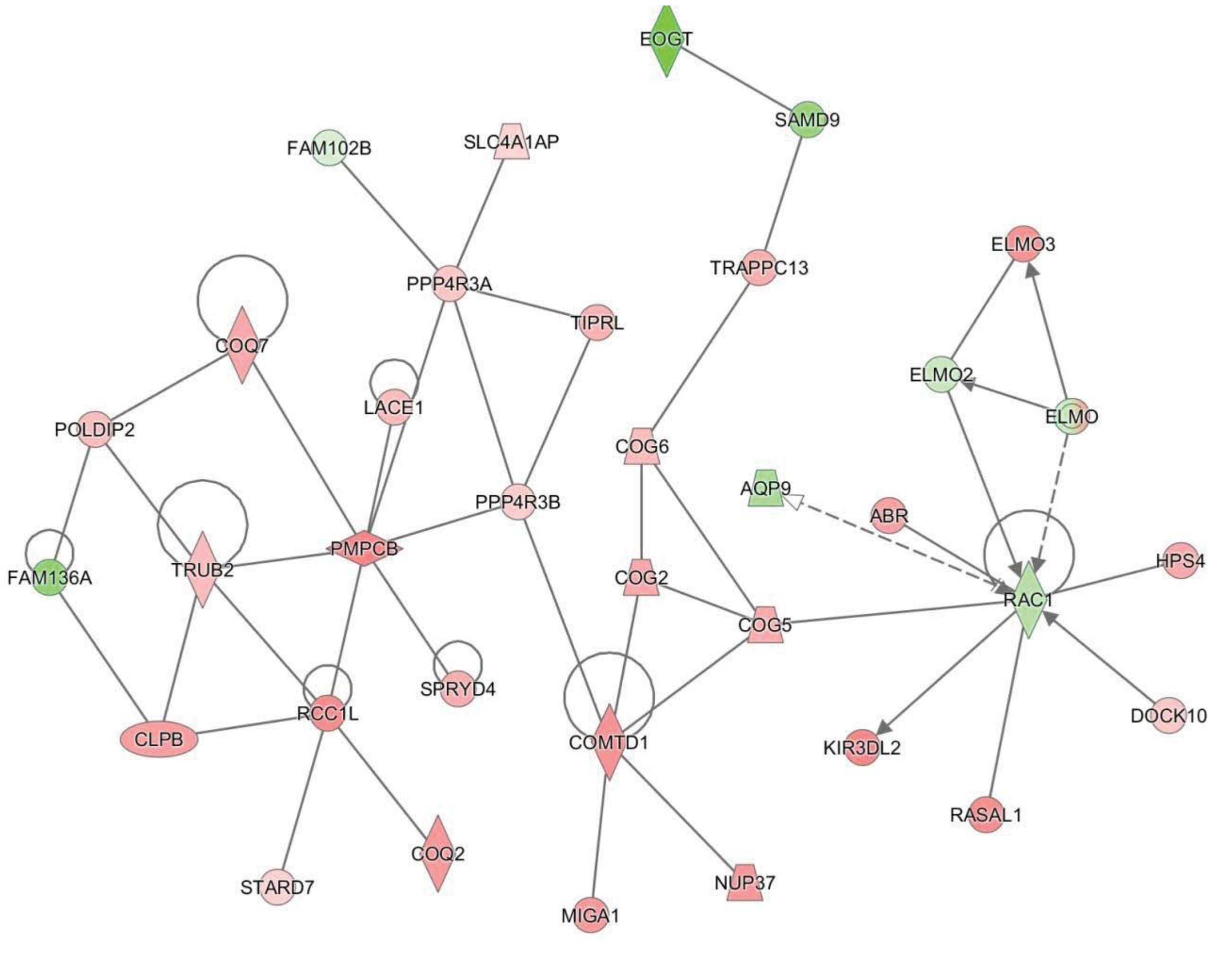

Figure 14. Network involved in metabolic disease, organismal injury and abnormalities, and hereditary disease.

Network of genes involved in metabolic disease that are differentially expressed in neutrophils of 'High Risk' cows compared with 'Low Risk' cows. This network was given an Ingenuity score of 32 and is comprised of 34 Focus Molecules. Colour intensity indicates the degree of increased (red) or decreased (green) relative expression. Connections between molecules indicate known interactions.

Furthermore, several genes up-regulated in this network are important for mitochondrial function (e.g., *CLPB*, *LACE1*, *MIGA1*, and *PMPCB*; Figure 14). The dysregulation of these genes is indicative of metabolic stress. Mitochondria are required for several important processes of neutrophil function including chemotaxis, phagocytosis, production of reactive oxygen species, and regulation of apoptosis (Fossati et al., 2003). The 'High Risk' cows were selected for their high concentrations of BHBA, NEFA, and liver TAG, all of which have inhibitory effects on neutrophil function. For example, Hoeben et al. (1997) demonstrated that BHBA had a small inhibitory effect on oxidative burst in neutrophils from hyperketonemic periparturient cows in vitro. They concluded that the high BHBA concentrations post-calving might contribute, at least in part, to the increased susceptibility to infectious disease during this time (Hoeben et al., 1997). Furthermore, NEB, the severity of which is indicated by concentrations of circulating NEFA, is negatively correlated with neutrophil function (Hammon et al., 2006). Also, increased liver TAG is associated with a decrease in neutrophil function including the down-regulation of cell surface adhesion proteins, during the peripartum period (Zerbe et al., 2000). It is likely that the model of metabolic stress is affecting neutrophil function and there is evidence to indicate that high concentrations of NEFA, BHBA, and liver TAG do have an effect on neutrophil function.

5.5 Conclusion

The results presented here support the hypothesis that non-clinical metabolic stress might negatively affect the innate immune system during the periparturient period. We have established that the cows with metabolic stress have perturbed transcription of neutrophil genes that have a key role in the innate immune response. These changes indicate increased immunological stress as a result of metabolic stress, which is adversely affecting neutrophil function and likely increasing susceptibility to infectious disease during the transition period.

5.6 Acknowledgements

The authors wish to acknowledge farm staff (Scott Farm, DairyNZ) for animal handing and blood sampling. New Zealand Genomics Ltd. completed the RNA sequencing. Computation for this project was supported by New Zealand eScience Infrastructure (NeSI). This work was supported by funding from New Zealand dairy farmers through DairyNZ Inc. (RD1403) and the Ministry of Business, Innovation and Employment (DRCX1201).

5.7 Summary

Chapter 5 characterised the neutrophil gene expression changes in metabolically-divergent cows. Chapter 6 describes experiments to further investigate how the divergent metabolic phenotype affects peripheral immune function. To do this, the proteome of plasma-derived exosomes isolated from these cows was characterised. Exosomes are important cell-cell signalling molecules and therefore, have an integral role in regulating immune function. Furthermore, these microvesicles could be used as potential biomarkers of metabolic disease and a change in immune state.

Chapter 6

Proteins from circulating exosomes represent metabolic state in transition dairy cows

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6.1 Abstract

Biomarkers that identify pre-pathological disease could enhance preventative management, improve animal health and productivity, and reduce costs. Circulating extracellular vesicles, in particular, exosomes are considered to be long-distance, intercellular communication systems in human medicine. Exosomes provide tissue-specific messages of functional state and can alter the cellular activity of recipient tissues through their protein and micro RNA content. We hypothesised that exosomes circulating in the blood of cows during early lactation would contain proteins representative of the metabolic state of important tissues, such as liver, which play integral roles in regulating the physiology of cows post-partum. From a total of 150 cows of known metabolic phenotype, 10 cows were selected with high (n = 5; 'High Risk') and low (n = 5; 'Low Risk') concentrations of non-esterified fatty acids, β-hydroxybutyrate, and liver triacylglycerol during wk 1 and 2 post-calving. Exosomes were extracted from blood on the day of calving (d 0) and post-calving at wk 1 and wk 4, and their protein composition determined by mass spectroscopy. Extracellular vesicle protein concentration and the number of exosome vesicles were not affected by risk category; however, the exosome protein cargo was different between the groups, with proteins at each time-point identified as unique to the high and low risk groups. The proteins alpha-2 macroglobulin, fibrinogen, and oncoprotein-induced transcript 3, are unique to the 'High Risk' cows on d 0, and have been associated with metabolic syndrome and liver function in humans. Their presence in the 'High Risk' cows may indicate a more severe inflammatory state and a greater degree of liver dysfunction than 'Low Risk' cows, consistent with their greater plasma β-hydroxybutyrate and liver triacylglycerol concentrations. The commonly shared proteins and those unique to the 'Low Risk' category indicate a role for exosomes in immune function. The data provide preliminary evidence of a potential role for exosomes in the immune function in transition dairy cows and exosomal protein cargo as biomarkers of metabolic state.

6.2 Introduction

During the peripartum period, cows are at greater risk of metabolic and infectious disease. This is evident by an increase in the incidence of health events; an estimated 50% of cows experience at least one disease during the transition period (LeBlanc, 2010). Identification of individuals at risk of metabolic dysfunction could provide potential for preventative intervention (Ingvartsen et al., 2003; Halasa et al., 2007).

Exosomes are small, membrane-bound extracellular vesicles (ECV; 40-100 nm in diameter) exocytosed by cells. They contain components from the propagating cell (Simpson et al., 2009; Mathivanan et al., 2010), including proteins (Looze et al., 2009), lipids (Subra et al., 2007), and various nucleic acids (Gallo et al., 2012), and are hypothesized to regulate the function of target cells throughout the body (Denzer et al., 2000). Aberrant cellular function (particularly pathologic conditions) can alter the rate of release of ECV (Baran et al., 2010; Kim et al., 2003) as well as their protein composition, such that the contents reflects the physiological state of the originating cell (Mathivanan et al., 2010). Furthermore, as ECV are transported in blood as long-distance, intercellular messengers, they are potential biomarkers of disease state (Distler et al., 2006; Taylor and Gercel-Taylor, 2008). For example, tumor-derived exosomes contain different protein contents compared with those secreted from non-malignant cells (Andre et al., 2002). Furthermore, proteomic investigation of bovine milk exosomes has revealed that these nanoparticles are important for cellular communication in bovines during lactation, particularly for lactation physiology and mammary immune function (Reinhardt et al., 2012, 2013).

Due to the reported link between disease pathogenesis and circulating ECV proteins, we hypothesized that exosomes isolated from cows at risk of metabolic dysfunction during the transition period will exhibit an altered protein composition and, if so, could be useful biomarkers of health. In the current study, exosomes were isolated from the plasma of two cohorts of cows identified as being either a 'Low Risk' or 'High Risk' for metabolic dysfunction post-calving, and their protein content characterized using mass spectroscopy.

6.3 Materials and Methods

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (RAEC#12799) in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46'S 175°18'E) between July and October 2013.

Cows used in this study were selected as described in Chapter 5. Briefly, 10 cows were identified as either 'High Risk' (n = 5) or 'Low Risk' (n = 5) of metabolic dysfunction on the basis of blood plasma non-esterified fatty acid (NEFA; mmol/L) and BHB (mmol/L), and liver triacylglycerol (TAG; % liver wet weight) after calving. The 'High Risk' group cows exhibited higher ($P < 0.01$) mean (± SD) values for the three variables than those in the 'Low Risk' group during wk 1 (day 4 ± 2) and wk 2 (day 11 ± 2) post-calving: 1.80 vs 0.66 ± 0.32, 1.00 vs 0.45 ± 0.25, and 9.0 vs 1.8 ± 1.59, for NEFA, BHB, and TAG respectively, during wk 1 post-calving and 1.20 vs 0.30 ± 0.33, 0.86 vs 0.48 ± 0.11, and 10.3 vs 1.4 ± 1.59, for NEFA, BHB, and TAG respectively, during wk 2. Average milk yield ($P < 0.05$), fat percent ($P = 0.2$) and protein percent ($P < 0.05$) during wk 1 and wk 2 of lactation for the 'High Risk' and 'Low Risk' groups were 23.6 kg, 5.0%, and 3.9%, and 18.4 L, 4.2%, and 4.2%, respectively.

Metabolite Analysis and Liver Triacylglycerol

Blood samples for metabolite analysis (wk 1 and wk 2) and exosome extraction (d 0, wk 1, and wk 4) were collected by coccygeal venipuncture into evacuated blood tubes containing either a heparin or ethylenediaminetetraacetic acid (EDTA) anticoagulant, respectively. Blood was placed immediately on ice, centrifuged at 1,500 × *g* for 12 min at

4°C, and the plasma aspirated and stored at -20°C until assayed. Approximately 1 g (wet weight) of liver tissue was collected (wk 1 and wk 2) for TAG analysis by as outlined by Lucy et al. (2009). Collected liver samples were snap-frozen in liquid nitrogen and stored at -80°C.

Metabolite analysis and the liver TAG assays were undertaken by Gribbles Veterinary Pathology Ltd (Hamilton, New Zealand). Plasma BHB (mmol/L) was assayed with a Roche reagent kit using colorimetric techniques on a Hitachi Modular P800 analyzer at 37°C (Roche Diagnostics). Plasma NEFA concentrations (mmol/L) were measured using Wako Chemicals kit (Osaka, Japan). Liver TAG content was analysed using the Wako LabAssay™ Triglyceride Kit (290-63701, Wako Chemicals USA Inc., VA, USA). For detailed analysis, refer to Roche et al. (2015).

Exosome Isolation and Validation

Exosomes were isolated from plasma (1 mL) at d 0, wk 1, and wk 4 by ultracentrifugation as previously described (Salomon et al., 2014a; Sarker et al., 2014). In brief, plasma was diluted with an equal volume of PBS (pH 7.4) and centrifuged at 2,000 × *g* for 30 min at 4°C, supernatant was removed and centrifuged at 12,000 × *g* for 45 min at 4°C (Sorvall®, high speed microcentrifuge, fixed rotor angle: 900, Thermo Fisher Scientific Ins., Asheville, NC, USA,). The resulting supernatant was transferred to an ultracentrifuge tube, centrifuged at 100,000 × *g* for 75 min, the pellet re-suspended in PBS (30 mL) and filtered through a 0.22 μM filter, and then centrifuged at 100,000 × *g* for 75 min. The resulting pellet was re-suspended in PBS (500 μL), layered on a discontinuous iodixanol gradient and 12 fractions were collected, diluted with PBS and centrifuged at 100,000 × *g* for 2 h at 4°C. Finally, the pellet containing the enriched exosome population was re-suspended in 50 μL PBS. After isolation, exosomes were characterized by the size distribution, presence of endocytic marker (i.e., TSG101 and CD63) and morphology by nanoparticle tracking analysis, Western blot, and electron microscopy, respectively. Concentration of ECVs was determined by the DC™ Protein Assay (BIO-RAD, Hercules, CA, USA). To ascertain the number of exosomal vesicles (NEV) per mL of plasma, a commercially available kit was used following manufacturer's instructions (EXOEL-CD63A-1; ExoELISA™ kit, System Biosciences, Mountain View, CA). Briefly, ECV concentration was determined as total immunoreactive CD63, and NEV (per mL) was calculated using the calculated ECV concentration.

Liquid Chromatography-Mass Spectroscopy and Proteomics

To determine protein composition within exosomes, a modified version of the methodology described by Salomon et al. (2014b) was used. Isolated exosomes from plasma were sonicated for 15 min and samples were diluted in 50 mM ammonium bicarbonate, pH 8.5, and reduced with dithiothreitol for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid for 1h in the dark. The sample was diluted to 1:10 with 50 mM ammonium bicarbonate and digested with trypsin (1 µg trypsin/ 20 µg protein; Promega, Madison, WI, USA) at 37 °C for 18 h. The samples were then desalted by solid phase extraction using a STAGE tip protocol (stop and go extraction tips for matrix-assisted laser desorption/ionization, nano-electrospray, and liquid chromatography/mass spectrometry [LC/MS] sample pre-treatment in proteomics). The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and re-dissolved in Solution A (0.1% formic acid in water). The resulting peptide mixture was analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Triple TOF® 5600 System mass spectrometer (AB Sciex, Framingham, USA) equipped with an Eksigent Nanoflow binary gradient HPLC system and a nanospray III ion source. Digested peptides were separated using a ChromXP C18-CL TRAP cHiPLC (200 µm × 6 mm, 3 µm, 120 Å) and analytical cHiPLC columns (200 µm × 15 cm; 3 µm, 120 Å; Eksigent, Redwood City, CA) using a linear gradient of 5% to 10% Solvent B (acetonitrile/0.1% formic acid) for 2 min, 10 - 40% Solvent B (58 min), 40-50%

Solvent B (10 min), 50-95% (10 min) with a flow rate of 500 nL/min. The column was flushed at 95% Solvent B for 15 min and re-equilibrated with 5% Solvent B for 6 min. MS/MS spectra were collected using Information Dependent Acquisition using a survey scan (m/z 300–2,000 with an accumulation time of 250 ms) followed by 12 data-dependent product ion scans of the 12 most intense precursor ions (m/z 100-2000 with an accumulation time of 150ms). The precursor selection criteria included a minimum intensity of 50 counts per second and a charge state greater than 2+. Mass spectra were analysed using the Mascot and Protein Pilot search engines against the Swissprot-database with the species set as bovine. Positive identifications were ascribed where Mascot scores were greater than 30 (Supplementary Table 4). False discovery rate was estimated using a reversed sequence database.

Statistics

To test the effects of Risk category, wk, and their interaction on protein concentration and NEV, a mixed model approach with repeated measures ANOVA was used (PROC MIXED; SAS/STAT 12.1, SAS Institute Inc. Cary, NC, USA). This was followed by Tukey's t-test for pairwise comparisons. Exosome number data were $\log_{10}$-transformed to achieve homogeneity of variance. Significance is declared if $P < 0.05$. Results are presented as LSM ± SED.

6.4 Results and Discussion

The plasma concentration of ECV protein and the NEV in cows categorized as having either a 'High Risk' or 'Low Risk' of metabolic dysfunction during the transition period are presented in Figure 15. On average, cows had 740 ± 279.7 µg/mL of ECV protein in blood and $6.7 \pm 3.74 \times 10^{10}$ NEV/mL plasma; these concentrations are similar to those measured in other species (from 2×10^{9} to 9×10^{12} in humans, depending on stage of gestation; (Salomon et al., 2014a). There was no relationship (R^2 = 0.001) between ECV protein concentration and NEV (P = 0.91).

Neither protein concentration nor $\log_{10}$ NEV were affected by risk category (P = 0.50 and 0.91, respectively) or wk post-calving (P = 0.28 and 0.44, respectively), and there was also no interaction between risk category and wk post-calving (P = 0.42 and 0.79, respectively). Both ECV protein concentration and NEV reportedly rise in situations of hepatic steatosis and non-alcoholic steatohepatitis (Povero et al., 2014). The lack of a difference in either variable in our results was, therefore, somewhat surprising, considering the large differences in circulating NEFA and liver TAG between the risk categories. This is possibly due to limited statistical power, with only five animals per treatment; future investigations should involve more animals. However, Povero et al. (2014) highlighted that the effect of hepatic steatosis (fatty liver disease) on ECV was progressive, with evident differences in steatosis after 4 wk in a mouse model of diet-induced non-alcoholic fatty liver disease. The diet, high in fat and deficient in choline L-amino acid, failed to result in differences in ECV protein concentration, but further exposure to the diet produced both a greater degree of steatosis and a linear increase in ECV protein with the degenerative liver condition. These results indicate that non-symptomatic fatty liver in early lactation dairy cows may not be sufficiently severe or of sufficient duration to elevate the concentration of ECV protein or NEV.

Although there was no evident difference in either ECV protein concentration or NEV between risk categories or days post-calving (Figure 15), there were exosomal protein signatures unique to each risk category (Figure 16), suggesting that the exosomal cargo may vary with metabolic state during the transition period. Between 19 and 25 distinct proteins (depending on post-calving time point) were identified from between 44 and 72 individual accession numbers. Details of the exosomal proteins unique to risk categories, as well as those in common are presented in Table 11. Of the proteins identified, eight were unique to the blood exosomes extracted from ‘High Risk’ cows on d 0, 10 were unique to cows in the ‘Low Risk’ category, and five were common to both categories (Figure 16). Unique proteins identified in each risk category at each post-calving time point over the transition period are presented in Figure 17. At wk 1 post-calving, five proteins were identified as unique to cows in the ‘High Risk’ category, 15 to cows in the ‘Low Risk’ category, and five proteins were common to both groups. By wk 4 post-calving, only four proteins were unique to cows in the ‘High Risk’ category, 12 to the ‘Low Risk’ category, and three proteins were commonly shared.

Several of the exosomal proteins on d 0 and wk 1 post-calving that were unique to cows categorized as being at a ‘High Risk’ of metabolic dysfunction are associated with metabolic syndrome in humans. For example, Feng et al. (2015) identified increased concentrations of alpha-2-macroglobulin in saliva in patients with Type II diabetes. Similarly, elevated concentrations of fibrinogen in blood have been extensively associated with metabolic syndrome; cardiovascular disease and Type II diabetes (reviewed by Mertens and Van Gaal, 2002) and as a marker of inflammation in dairy cows (McSherry et al., 1970). A number of parallels have been drawn between metabolic syndrome in humans and the biology of the post-partum dairy cow (Lucy et al., 2004). For example, chronic inflammation, the presence of insulin resistance, and the resultant increased concentration of NEFA (Chagas et al., 2007; Farney et al., 2013b) create a natural link between the endocrine physiology of post-partum cows and the physiology of Type II diabetic states presented in humans (Lewis et al., 2002; Harris, 2005). It is not surprising that the exosome phenotype of the ‘High Risk’ cows signifies a degree of metabolic dysfunction. Cows in the ‘High Risk’ category were chosen because their metabolic profile reflected an extreme tendency to mobilize adipose reserves (i.e., high NEFA), an inability of the liver to oxidize mobilized NEFA completely (i.e., high BHB), and a failure of the liver to export the resultant very low density lipoprotein to peripheral tissues (i.e., high liver TAG).

In addition to these markers of metabolic state, both envoplakin and oncoprotein-induced transcript-3 were also elevated in the cows categorized by their metabolic profile as at increased risk of disease. These proteins have been identified as being important in liver function, with envoplakin isolated from plasma in a human patient with hepatocellular carcinoma (Yokokura et al., 2006) and oncoprotein-induced transcript-3 identified as a protein specific to liver (Shen et al., 2009). It is unclear whether their presence in the exosomes of 'High Risk' cows is a reflection of metabolic dysfunction or merely a signature of more metabolically-active liver cells indicated by the greater uptake of NEFA and the accumulation of TAG. We propose that these proteins reflect the hepatic steatosis used to characterize these cows and, as such, may be useful biomarkers of a more severe pathological condition before clinical signs are evident.

Although identified by their metabolic profile as being at risk of disease, it is noteworthy that none of these animals succumbed to a clinical condition. This lack of progression to a disease state may also be reflected in their exosome profile. By wk 4, the proteins peculiar to the 'High Risk' cows are no longer reflective of the metabolic syndrome phenotype, which is consistent with the timing of a return to normal physiological function; for example, the maximum rate of lipolysis has ended (McNamara and Hillers, 1986b; Roche et al., 2007a, 2009), the process of lipogenesis has been reinstated (McNamara and Hillers, 1986a), the somatotropic axis has re-coupled (Lucy et al., 2009), and most measures of metabolic state and immune function have returned to near pre-calving baseline concentrations (Bauman and Currie, 1980; Heiser et al., 2015; Roche et al., 2015). Our results provide hints that exosomes could carry a signature of metabolic dysfunction, which, with appropriate characterization, could for example be used in lieu of a liver biopsy. Further work is required to determine the sensitivity of the exosome protein cargo to the metabolic state of the periparturient cow and whether the signature could be evaluated from non-invasive samples, such as urine, milk, or saliva.

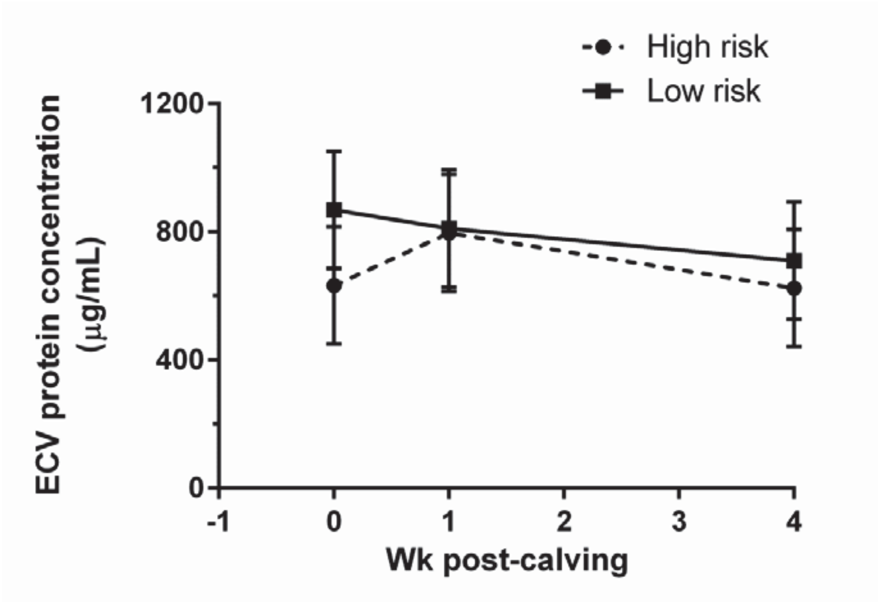
Table 11. Unique proteins identified in exosomes isolated from 'High Risk' and 'Low Risk' cows (over page). List of unique proteins identified in plasma exosomes isolated from cows characterised as being at either a 'High Risk' (n = 5) or 'Low Risk' (n = 5) of metabolic dysfunction on the basis of circulating concentrations of NEFA and BHB, and liver triacylglycerol during wk 1 and 2 post-calving. Blood for exosome extraction was sampled on the day of calving (d 0) and at wk 1 and 4 post-calving.

Table 11. **Unique proteins identified in exosomes isolated from 'High Risk' and 'Low Risk' cows.** Figure legend on previous page.

d 0		wk 1		wk 4	
Accession	**Protein name**	**Accession**	**Protein name**	**Accession**	**Protein name**
Unique to 'High Risk' cows					
R9QSM8	Alpha-2-macroglobulin	F6PMQ1	Coiled-coil domain containing 84	F1N2J5	Hyaluronan and proteoglycan link protein 4
B8Y9T0	Fibronectin	E1B9N6	Envoplakin	G5E5H2	Immunoglobulin
A5PJE3	Fibrinogen alpha chain	F1MB40	Zinc finger protein 320	G3N0V2	Keratin
F1MAV0	Fibrinogen beta chain	E1BMR0	NACC family member 2, BEN and BTB (POZ) domain containing	E1BMR0	NACC family member 2, BEN and BTB (POZ) domain containing
F1MGU7	Fibrinogen gamma-B chain	F1MM36	Nuclear receptor coactivator 5		
Q3SYR8	Immunoglobulin J chain				
G3X690	Kinesin-like protein				
F1MH22	Oncoprotein-induced transcript 3				
Unique to 'Low Risk' cows					
F1MRD0	Actin, cytoplasmic 1	Q3SZG7	60S ribosomal protein L13	B0JYQ0	Albumin
B0JYQ0	Albumin	B0JYQ0	Albumin	Q5GN72	Alpha-1-acid glycoprotein
B0JYN6	Alpha-2-HS-glycoprotein	G3MZX1	C6 orf 132	R9QSM8	Alpha-2-macroglobulin
F1N6F5	Cytochrome P450, family 27, subfamily C, polypeptide 1	F1N4V6	ELAV-like protein	F1MS24	Choroideraemia (Rab escort protein 1)
G3MYD7	Histone H3	F1MIY4	Growth arrest-specific 2 like 3	F1N2I0	Choroideraemia-like (Rab escort protein 2)
A5D792	Histone H4	E1BLD1	HECT domain containing E3 ubiquitin ligase 1	F1N6F5	Cytochrome P450, family 27, subfamily C, polypeptide 1

G3N0V2	Keratin	G3MYD7	Histone H3	A5PJE3	Fibrinogen alpha chain
E1BMR0	NACC family member 2, BEN and BTB (POZ) domain containing	Q05FF0	Interferon gamma receptor 2	F1MAV0	Fibrinogen beta chain
G3MXX8	Transcriptional repressor scratch 1	F1MZT1	Leucine-rich repeat LGI family, member 2	G3MYD7	Histone H3
E1BHT5	E3 ubiquitin-protein ligase UBR4	A6QNR4	Myotubularin related protein 4	A6QNR4	Myotubularin related protein 4
		G5E560	Pleckstrin and Sec7 domain containing 1	E1B7S1	Poly (ADP-ribose) polymerase family, member 12
		G3MZN6	Pleckstrin and Sec7 domain containing 2	E1BNF9	Signaling lymphocytic activation molecule family member 9
		E1BNF9	Signaling lymphocytic activation molecule family member 9		
		E1B793	Serine/threonine kinase 36		
		E1B7F5	WD repeat and HMG-box DNA binding protein 1		
Common to both risk categories					
A5D7N2	Histone H2B	G5E5H2	Immunoglobulin	A5D7N2	Histone H2B
G5E513	Immunoglobulin heavy constant mu	F1N6F5	Cytochrome P450, family 27, subfamily C, polypeptide 1	G5E5T5	Immunoglobulin heavy constant mu
Q3T101	Immunoglobulin lambda	A5D7N2	Histone H2B	Q3T101	Immunoglobulin lambda
E1BNF9	Signaling lymphocytic activation molecule family member 9	G5E5T5	Immunoglobulin heavy constant mu		
G5E5H2	Immunoglobulin	Q3T101	Immunoglobulin lambda		

(A)



(B)

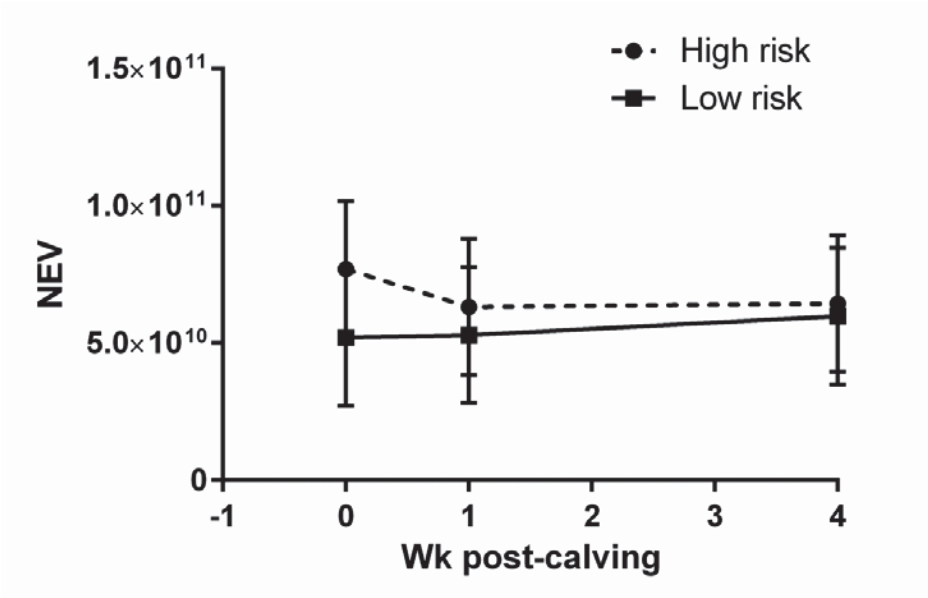


Figure 15. Plasma concentration of extracellular vesicles (ECV) and number of exosomal vesicles (NEV) from 'High Risk' and 'Low Risk' cows.

(A) Plasma concentration of total protein (μg/mL) extracted from extracellular vesicles (ECV) and **(B)** number of exosomal vesicles (NEV) per mL of plasma from cows characterized as being at either a 'High Risk' (n = 5) or 'Low Risk' (n = 5) of metabolic dysfunction during the transition period. Exosome number data were $\log_{10}$-transformed before statistical analysis; back-transformed data are presented for ease of interpretation and error bars denote ± SEM.

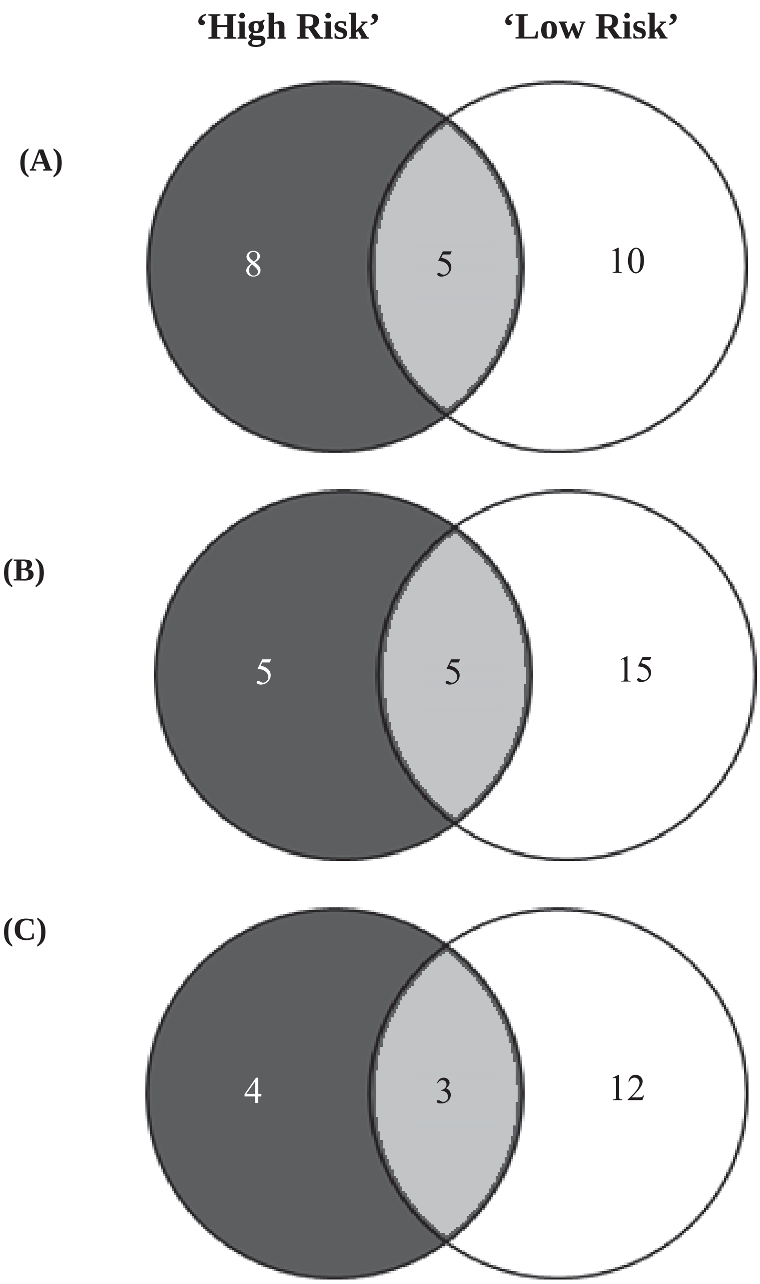


Figure 16. Comparison of exosomal proteins identified between risk groups over time. Graphical representation of unique proteins identified in plasma exosomes isolated from cows characterized as 'High Risk' or 'Low Risk' of metabolic dysfunction during the transition period on the basis of circulating concentrations of NEFA and BHB and liver TAG at wk 1 and 2 post-calving. **(A)** blood sampled on the day of calving (d 0) **(B)** blood sampled wk 1 post-calving, and **(C)** blood sampled wk 4 post-calving. Elements unique to 'High Risk' cows are dark grey, those unique to 'Low Risk' cows are white, and common elements are light grey.

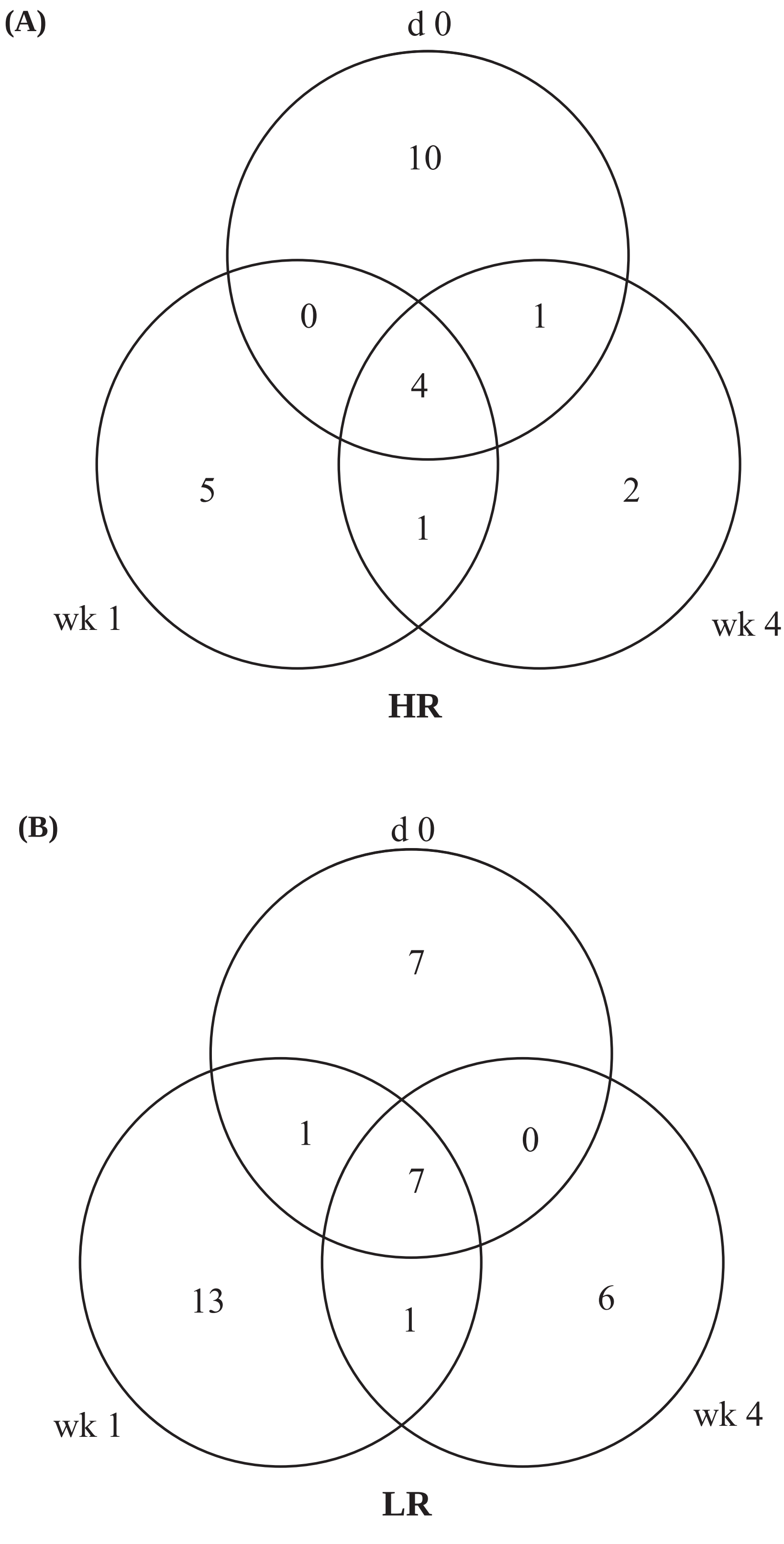


Figure 17. Comparison of exosomal proteins identified between time points within risk groups.

Graphical representation of unique proteins at three time points (day of calving [d 0] and wk 1 and wk 4 post-calving) in (**A**) 'High Risk' (HR) and (**B**) 'Low Risk' (LR) cows.

The proteins unique to the ‘Low Risk’ dairy cows and those in common between groups indicate a role for exosomes in immune function regulation. For example, signalling lymphocytic activation molecule 9 is a protein involved in regulation of immune cell interactions and is required for normal development, homeostasis, and immune cell function (Volkova et al., 2014). Similarly, the commonality of immunoglobulin proteins is consistent with a role for exosomes in transporting important immune molecules between tissues. Furthermore, alpha-1-glycoprotein is an acute phase protein produced in response to inflammation (Hochepied et al., 2003). It has several immunoregulatory functions including inhibition of inflammatory responses of several immune cells, such as lymphocytes and neutrophils, indicating a possible role for exosomes in regulating immune function in early lactation (Chiu et al., 1977; Theilgaard-Mönch et al., 2005).

6.5 Conclusion

Although ECV protein concentration in blood and NEV were not different between risk categories in the current experiment and therefore, would not prove suitable for identifying at risk cows, the exosome protein cargo was different between cows categorized as either a high or low risk of metabolic dysfunction during the transition period. The proteins identified as unique to the ‘High Risk’ cows are biologically consistent with the metabolic state on which cows were categorized. Further research will endeavour to improve the sensitivity of the LC-MS/MS protocol; however, these preliminary results indicate that exosomal protein cargo may have potential as a biomarker of subclinical disease. Furthermore, additional research is needed to determine if exosome signatures are sufficiently sensitive to identify ‘at risk’ cows likely to recover or those likely to suffer clinical disease.

6.6 Acknowledgements

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6.7 Summary

Chapter 6 determined that exosomal content differs in cows with divergent metabolic health status. This lead to the hypothesis that exosomes isolated from the divergent cows will differentially affect the function of target cells because of the differing exosomal content. This is explored in Chapter 7 using a co-culture model, whereby exosomes isolated from the ‘Low Risk’ and ‘High Risk’ cows were co-cultured with a bovine epithelial-like cell line. Protein and gene expression in the co-culture models were compared for the effect of ‘Risk’ as an indication of differences in cell function between risk groups.

Chapter 7

Effect of circulating exosomes from transition cows on Madin-Darby bovine kidney cell function

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7.1 Abstract

The greatest risk of metabolic and infectious disease in dairy cows is during the transition from pregnancy to lactating (the transition period). The objective of this experiment was to determine the effects of extracellular vesicles (microvesicles involved in cell-to-cell signalling) isolated from transition cows on target-cell function. We have previously identified differences in the protein profiles of exosomes isolated from cows divergent in metabolic health status. Therefore, we hypothesized that these exosomes would affect target tissues differently. To investigate this, two groups of cows (n = 5/group) were selected based on the concentration of β-hydroxybutyrate (BHB) and fatty acids (FA) in plasma, and triacylglycerol (TAG) concentration in liver at wk 1 and wk 2 post-calving. Cows with high concentrations of BHB, FA, and TAG were considered at increased risk of clinical disease during the transition period ('High Risk'; n = 5) and were compared with cows that had low concentrations of the selected health indicators ('Low Risk'; n = 5). At two time points during the transition period (post-calving at wk 1 and wk 4) blood was sampled and plasma exosomes were isolated from the 'High Risk' and 'Low Risk' cows. The exosomes were applied at 10 μg/mL and 1 μg/mL concentrations (total exosomal protein) to 5×10^3 Madin-Darby bovine kidney (MDBK) cells grown to 50% confluence in 96-well plates. Results indicate a numerical ($P > 0.10$) increase in cell proliferation when exosomes from 'High Risk' cows were applied compared with those from 'Low Risk' cows. Consistent with an effect on cell proliferation, quantitative reverse transcriptase-PCR indicated a trend ($P < 0.10$) for up-regulation of three pro-inflammatory genes (*CSF3*, *CNTF*, and *CD70*) with the application of 'High Risk' exosomes, which are involved in cellular growth and survival. Proteomic analysis indicated two proteins in the 'Low Risk' group not identified in the 'High Risk' (endoplasmin and catalase), which may also be indicative of the metabolic state of origin. It is likely that the metabolic state of the transition cow affects cellular function through exosomal messaging; however, more in-depth research

into crosstalk between exosomes and target cells is required to determine whether exosomes influence MDBK cells in this manner.

7.2 Introduction

Exosomes are extracellular vesicles that contain proteins, mRNA, and micro-RNA (miRNA) and are transported in blood as long-distance, intercellular messengers. They are biomarkers of metabolic or disease state (Distler et al., 2006; Taylor and Gercel-Taylor, 2008; Chapter 6), with their effect on target tissues influenced by the physiological state of the secreting cell (Théry et al., 2009; Mathivanan et al., 2010). For example, tumour-derived exosomes contain a different protein cargo to exosomes secreted from non-malignant cells (Andre et al., 2002). Furthermore, proteomic investigation of bovine exosomes has suggested that these nanoparticles are important for lactation physiology and mammary immune function, and as indicators of metabolic health (Reinhardt et al., 2013).

The transition to lactation in dairy cows is associated with metabolic and physiological stress as well as altered immune function (Drackley, 1999; Sordillo and Raphael, 2013; Chapter 6). During this period there is a high incidence of inflammatory disorders, such as mastitis and endometritis, which negatively affect milk production and reproductive function (LeBlanc et al., 2002a; b; Halasa et al., 2007). But, the degree of physiological and immunological stress in early lactation varies among cows (Heiser et al., 2015). We have previously identified differences in the protein cargo of exosomes from dairy cows divergent in fertility (Mitchell et al., 2016) and metabolic health status over the transition period. We further hypothesized that differences in exosomal cargo evident in cows divergent in metabolic health have integral roles in regulating the physiology of important tissues, such as liver, for example, via intercellular cross-talk.

To investigate this hypothesis, exosomes were isolated from the plasma of cows with divergent indicators of metabolic health during the transition period. To assess the action of these exosomes on target cells, they were co-cultured in vitro with Madin-Darby Bovine Kidney (MDBK) cells, which have been established as a model system for aspects of bovine liver cell metabolism (Bionaz et al., 2008; Thering et al., 2009; White et al., 2012; Zhou et al., 2015a).

7.3 Materials and Methods

Animals

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (RAEC#12799) in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The parent experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46'S 175°18'E) between July and October 2013.

Cows previously identified as either 'High Risk' (n = 5) or 'Low Risk' (n = 5) of metabolic dysfunction, based on blood plasma FA (mmol/L), BHB (mmol/L), and liver TAG (% liver wet weight) during the two weeks immediately after calving, were utilized for this study. The classification of these cows has been described previously in Chapter 5 and Chapter 6; briefly, the 'High Risk' cows had greater ($P < 0.01$) values for the three variables compared with cows in the 'Low Risk' category during wk 1 (day 4 ± 2) and wk 2 (day 11 ± 2) post-calving; 1.80 vs 0.66 ± 0.32, 1.00 vs 0.45 ± 0.25, and 9.0 vs 1.8 ± 1.59, for FA, BHB, and TAG, respectively, during wk 1 post-calving and 1.20 vs 0.30 ± 0.33, 0.86 vs 0.48 ± 0.11, and 10.3 vs 1.4 ± 1.59 during wk 2 (high risk vs low risk mean ± SE of the difference). Average milk yield ($P < 0.05$), fat % ($P = 0.2$) and protein % ($P < 0.05$) during wk 1 and wk 2 of lactation for the 'High Risk' and 'Low Risk' groups were 23.6 kg, 5.0%, and 3.9%, and 18.4 kg, 4.2%, and 4.2%, respectively.

Blood samples for exosome isolation (wk 1, and wk 4) were collected by coccygeal venipuncture into evacuated blood tubes containing EDTA anticoagulant. Blood was placed immediately on ice, centrifuged at 1,500 × *g* for 12 min at 4°C, and the plasma aspirated and stored at -20°C until assayed.

Exosome Isolation

The workflow employed for the isolation of exosomes is presented in Figure 18. Exosomes were isolated from plasma (2 mL) at wk 1 and wk 4 by ultracentrifugation as previously described (Salomon et al., 2014a; Sarker et al., 2014). Briefly, plasma was diluted 1:1 with PBS (pH 7.4) and centrifuged at 12,000 × *g* for 30 min at 4°C (Sorvall®, high speed microcentrifuge, fixed rotor angle: 900, Thermo Fisher Scientific Ins., Asheville, NC, USA). The resulting supernatant (2 mL) was filtered (0.2 µm), 8 mL of PBS (pH 7.4) was added, and the solution centrifuged at 100,000 × *g* for 120 min at 4°C (Sorvall®, T-8100, fixed angle ultracentrifuge rotor). The supernatant was then carefully removed, 300 µL of PBS was added, and the pellet (ECVs) was re-suspended. A discontinuous OptiPrep™ (Sigma Aldrich, Castle Hill, NSW, Australia) gradient was used to isolate exosomes from total ECVs. The gradient was made by diluting a stock solution of OptiPrep (60% W/V) with 0.25 M sucrose/10 nM Tris, pH 7.5 to make 40% (W/V), 20% (W/V), 10% (W/V), and 5% (W/V) solutions that were carefully layered and the sample placed on top. Tubes were centrifuged in an ultracentrifuge swinging bucket rotor (SW40 Ti Rotor [Beckman Coulter, Australia Pty Ltd]) at 100,000 × *g* for 20 h at 4ºC. Fractions from each sample gradient (12 × 1 mL) were removed and washed in ultracentrifuge tubes (Sorvall®) by adding 9 mL of PBS and centrifuging at 100,000 × *g* for 2 h at 4ºC. The resulting supernatant was discarded and the exosome pellets from each of the 12 fractions were re-suspended in 50 µL of PBS and stored at -20°C until further analysis.

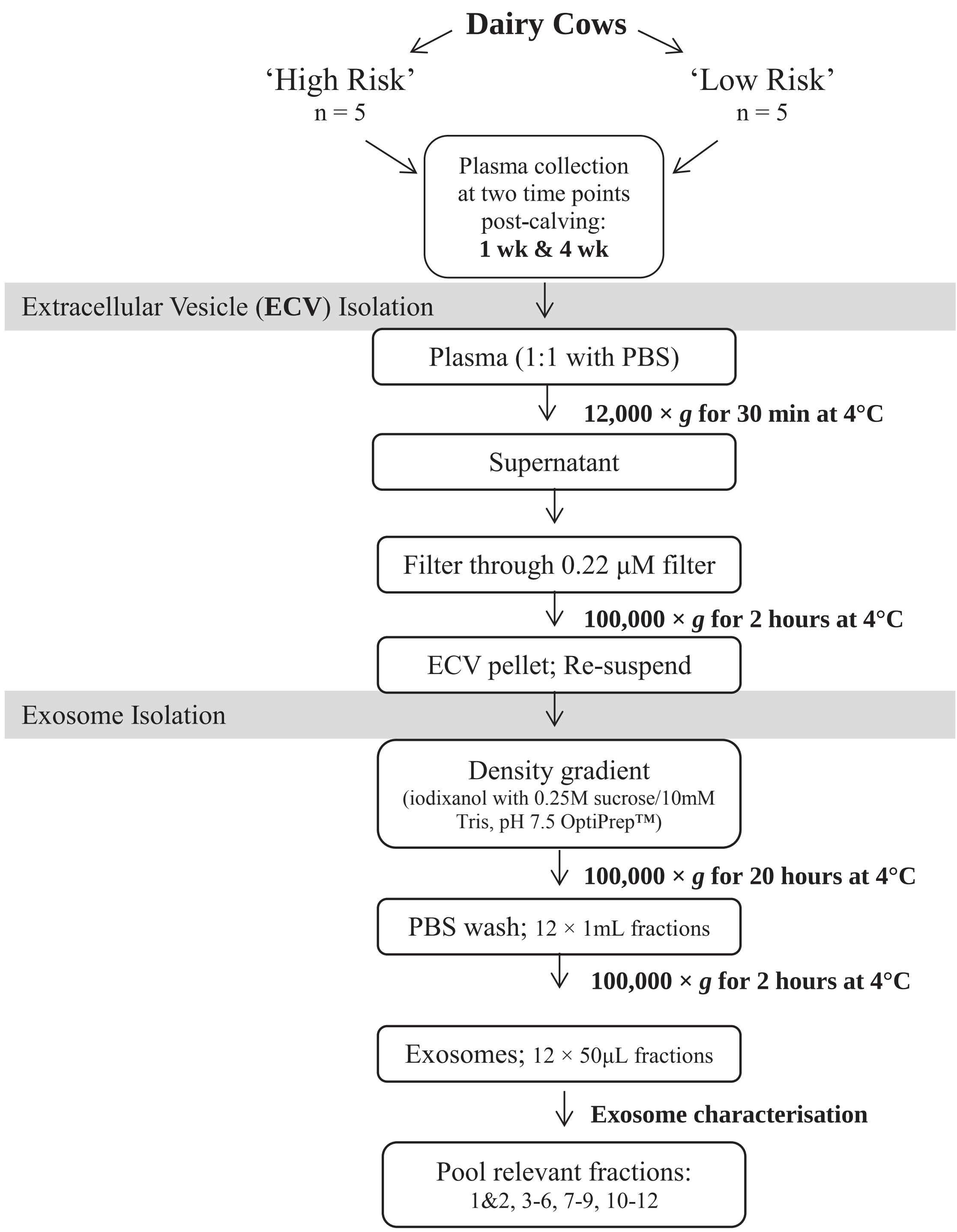


Figure 18. Workflow for the isolation of exosomes from bovine plasma in 'High Risk' and 'Low Risk' cows.

Validation of Exosome Isolation

The density of each fraction was measured in a control OptiPrep™ gradient tube by determining the absorbance at 244 nm (SPECTROstar Nano microplate reader, BMG Labtech, Mornington, VIC, Australia). The density of each fraction was calculated from absorbance readings and exosomes were expected at 1.12 to 1.19 g/mL (Schröder et al., 1997; Tauro et al., 2012). Exosomes were characterised by size distribution, using a NanoSight NS500 instrument (NanoSight NTA version 2.3 Nanoparticle Tracking and Analysis Release Version Build 0033 [NanoSight Ltd, United Kingdom]) as previously described (Salomon et al., 2014a; Sarker et al., 2014; Supplementary Figure 1). Western blot was used to verify the presence of endocytic markers TSG101 (1:500; sc-6037, Santa Cruz Biotechnology, TX) and CD63 (1:1000; EXOAB-CD63A-1, System Biosciences, CA) (Théry et al., 2006; Koh et al., 2016).

Exosome Co-Culture

Epithelial (MDBK) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as described previously (Bionaz et al., 2008; Thering et al., 2009). Five thousand cells per well were plated on 96-well plates and grown at 37°C and 5% CO_2 for 24 h in 100 µL/well of Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific) supplemented with 5% foetal bovine serum (FBS) (Bovogen, Interpath Services Pty Ltd, Australia) and 1% antibiotic, antimycotic solution (10,000 units penicillin, 10,000 g streptomycin, and 25 g amphotericin B per mL; Gibco, ThermoFisher Scientific) to achieve 50% confluency prior to treatment exposure. Upon 50% confluence, cells were incubated for 40 h in DMEM supplemented with 5% exosome-depleted FBS plus exosome treatments or controls. Exosome-depleted FBS was prepared using ultracentrifugation (Sorvall®, ThermoFisher Scientific); 100,000 × *g* for 20 h at 4°C. All treatments, either 10 µg/mL or 1 µg/mL of exosomal protein from fractions 3-6 and 7-9, were applied in triplicate in three preparations of MDBK cells. Negative controls included

DMEM media supplemented with PBS (10 μL), FBS (10 μL), and exosome-depleted FBS (10 μL). Cell proliferation and morphology were analysed using the IncuCyte® Live-Cell Imaging system (Essen BioScience), which uses high density phase contrast real-time cell imaging as a proliferation parameter (Salomon et al., 2013; Koh et al., 2016). Images of cell proliferation were acquired every 2 h for the duration of the experiment (Figure 19 and Figure 20).

After 40 h of exosome treatment, the cell culture media were collected and technical triplicates were pooled and stored at -20°C for ELISA analysis. The cells and exosome co-culture from each well were collected using 200 μL of 'RLT' lysis buffer (Qiagen, Australia) with 1% β-Mercaptoethanol per well. Technical triplicates were pooled and stored at -80°C for subsequent RNA and protein extraction.

RNA Extraction

Cell lysates were homogenized by passing through a 25-gauge needle attached to a 1 mL sterile syringe at least 15 times. Total RNA was extracted from cell homogenates using a Qiagen RNeasy Mini kit according to manufacturer's instructions (Qiagen GmbH). An on-column DNA digest was used to remove genomic DNA (Qiagen, Australia). RNA was eluted in 20 μL of DNase/RNase free water by completing two elutions of 10 μL. The quantity and purity of RNA was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and stored at -80°C until further analysis.

Complementary DNA synthesis and Gene Expression Analysis

Total RNA (400 ng) was reverse transcribed using the RT^2 HT First Strand Kit (Qiagen, Australia). Reverse-transcriptase negative controls were also generated by excluding the enzyme, and cDNA samples were stored at –20°C. The bovine RT^2 Profiler™ PCR Array was used to quantify the gene expression of chemokines and cytokines by RT-qPCR (PABT-150ZC-24; Qiagen). The PCR arrays were cycled using the following parameters: 1 × (95°C for 10 min) followed by 40 × (95°C for 15 s and 60°C for 1 min) cycles using the ABI StepOne™ machine (Applied Biosystems, Foster City, CA). The endogenous control genes included on the array were beta-actin, *ACTB*; TATA box binding protein, *TBP*; glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*; and hypoxanthine phophoribosyltransferase 1, *HPRT1*. Gene expression was normalised to the endogenous control gene *HPRT1* using the ΔΔCp method as this gene was the most stably expressed across all treatments. Gene expression results were generated for wk 1 co-culture samples. The data for co-cultures of both fractions (3-6 and 7-9) were combined for gene expression analysis to determine the difference between 'High Risk' and 'Low Risk' exosome co-cultures.

Protein Extraction

Total protein from cells was extracted using acetone precipitation from the protein fraction that flows through the column during the RNA binding step of the RNA extraction. Four volumes (4.8 mL) of ice-cold acetone was added to the 'flow-through' in 15 mL corning tubes and incubated overnight at -20°C. Samples were centrifuged for 10 min at 13,000 × *g*, air-dried, and re-suspended in 100 µL of 50 mM ammonium bicarbonate, pH 8.5. Protein was stored at -20°C pending mass spectrometry analysis.

Mass Spectrometry Analysis

Co-culture samples chosen for liquid chromatography (LC)-mass spectrometry (MS) (LC-MS/MS) were based on IncuCyte® and gene expression results; MDBK co-cultured with exosomes isolated from wk 1 plasma using fractions 3-6 were chosen for proteomic analysis. These co-culture samples were considered the most physiologically relevant, as the cows were at their most metabolically-divergent at this time point. Total protein from these samples was sonicated for 15 min and reduced with dithiothreitol for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid for 1 h in the dark. The samples were diluted to 1:10 with 50 mM ammonium bicarbonate and digested with trypsin (20 μg) at 37°C for 18 h. The samples were then desalted by solid phase extraction using a STAGE tip protocol (stop and go extraction tips for matrix-assisted laser desorption/ionization, nano-electrospray, and LC/MS sample pre-treatment in proteomics, [Rappsilber et al., 2003]). The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and re-dissolved in Solvent A (0.1% formic acid in water). The resulting peptide mixture was analysed by LC-MS/MS on a 5600 Triple TOF mass spectrometer (AB Sciex, Framingham, MA) equipped with an Eksigent Nanoflow binary gradient HPLC system and a nanospray III ion source. MS/MS spectra were collected using Information Dependent Acquisition with a survey scan (m/z 350–1,500) followed by 12 data-dependent product ion scans of the 12 most intense precursor ions. All mass spectra were analysed using the MASCOT search engine (www.matrixscience.com) and ProteinPilot (AB Sciex, MA) against the Swissprot database with the species set as bovine (http://www.ebi.ac.uk/uniprot/download-center). Positive identifications were ascribed where Mascot scores were greater than 30. False discovery rate was estimated using a reversed sequence database.

Peptide information from ProteinPilot™ (version 4.5; AB Sciex, MA) was used to calculate relative abundance of proteins within the dataset. Data were quality trimmed by removing peptides with an unused score (Unused) of <0.5, a contribution score (Contrib) of 0, and a confidence score (Conf) of <50. Mass accuracy was calculated (ppm error) using the equation:

$$\text{ppm error} = (\text{dMass} / \text{Prec MW}) \times 1{,}000{,}000$$

where dMass = observed value from mass spectrometer and Prec MW = theoretical value calculated from periodical table. Peptides were retained if ppm error was between -20 to 20. The relative abundance of proteins within a sample was calculated by summing the peptide intensity (PrecursorSignal) for a given protein and dividing by the total peptide intensity of that sample as per the equation:

Relative abundance (%) = (“sum of PrecursorSignal of a given protein”/ “sum of total PrecursorSignal”) × 100

Statistics

To test the effect of Risk category and dose of exosomes on MDBK cell proliferation during the course of the co-culture experiment, both an area under the curve analysis for cellular kinetics and a linear mixed model analysis were used (SAS 9.3). The linear mixed model included the fixed effects of risk category, dose, and time of acquisition, their interactions, and random effects of animals. The covariance error structure for repeated measures over time-points, within animals in each risk category was determined using Akaike’s information criterion. A first-order autoregressive model was found to be the most appropriate error structure. The distribution of proliferation data was tested for normality using Shapiro-Wilk, Kolmogorov-Smirnov, and Anderson-Darling tests and the residuals of data were found to be normally distributed. An unpaired t-test was used to determine the effect of Risk (‘Low Risk’ and ‘High Risk’ co-culture samples) on expression of genes as quantified by RT-qPCR. The arithmetic mean and standard deviation (SD) of relative protein abundance was calculated for the Control, ‘Low Risk’, and ‘High Risk’ co-culture samples. If a peptide signal was not detected a numerical placeholder of 0.01% was entered. Relative protein abundance was analysed using two-sample t-test (PROC Mixed, SAS 9.3) and significance was declared if $P < 0.05$ (Supplementary Table 5).

7.4 Results and Discussion

The aim of this experiment was to investigate whether exosomes isolated from cows different in their metabolic state during the peripartum period affected the proliferation and function of MDBK cells. Proliferation data of MDBK cells treated with exosomes are presented in Figure 19 and Figure 20. Gene expression data are presented in Figure 21 and LC-MS/MS proteomics results are presented in Figure 22 and Figure 23 and Supplementary

Table 5. The results indicate that there may be protein changes associated with cow risk category; 40 and 15 unique proteins were identified in the 'Low Risk' and 'High Risk' samples, respectively (Figure 22). There tended to be an effect of exosome concentration on proliferation in the 'Low Risk' cows at wk 1 whereby cells treated with 1 µg of exosomes exhibited faster growth at 24 – 40 h compared with cells treated with 10 µg of exosomes (Figure 19). There was no effect ($P > 0.05$) of risk category on MDBK cell proliferation (Figure 20), cytokine gene expression (Figure 21), and protein abundance (Figure 23). However, there are sufficient differences to indicate an effect of exosome source on the cellular physiology of MDBK cells (Figure 22) and that further research is required with greater numbers of cows to determine the effect of metabolic state on exosome messaging of target tissues.

IncuCyte® Results

The 'High Risk' cows were selected based on indicators of liver dysfunction: excessive fatty acid (FA) mobilisation and a failure to fully oxidize FA, which led to increased BHB production and the accumulation of FA in liver. Such a metabolic profile would be expected to generate systemic and tissue-specific inflammation (Ohtsuka et al., 2001; Sordillo and Mavangira, 2014); Chapter 6, which investigated the protein cargo of the exosomes used in the current study, indicated differences between these treatment groups supporting such an effect.

Initial analyses support the hypothesis that the metabolic state of the cow (i.e., from 'High Risk' or 'Low Risk' cows) may differentially alter cell proliferation. MDBK cells treated with 'Low Risk' exosomes at wk 1 (Figure 19) tended to demonstrate lower proliferation ($P > 0.05$) when exosomes were applied at the largest dose (10 µg compared with 1 µg). Furthermore, there was a numerical ($P > 0.05$) increase in cell proliferation in the 'High Risk' cows compared with the 'Low Risk' when applied at 10 µg concentration (Figure 20).

The lower rate of proliferation with the application of more 'Low Risk' exosomes (Figure 19) is consistent with Aucher et al. (2013), who demonstrated that exosome-derived miRNA is capable of inhibiting target-cell proliferation. The 'Low Risk' exosomes were isolated from cows with a reduced inflammatory phenotype and may carry miRNA specific to this. Recent research suggests that exosomal miRNA could be used to treat metabolic disease (Thomou et al., 2017). Labelling of exosomal contents to track transfer of exosomal contents into target-cells should be considered in future experiments (Lai et al., 2015). Although limited by the low numbers of animals in the current study, results indicate that the peripartum metabolic state of the dairy cow likely affects the influence that exosomes have on cell function.

Furthermore, the numerical differences in proliferation between the 'High Risk' and 'Low Risk' co-cultures are consistent with published studies that indicate pro-inflammatory conditions increase cellular proliferation. The conditions of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis are characterized by hepatic inflammation and fatty acid accumulation in the liver (Hardy et al., 2016), characteristics similar to those reported in 'High Risk' cows in the experiment described here and in Chapter 6. Our previous study reported that the exosomal protein cargo was influenced by the metabolic state of the 'High Risk' cows, which was pro-inflammatory in nature (Chapter 6). Consistent with this, exosomes isolated from a primary hepatocyte culture model of non-alcoholic steatohepatitis were pro-inflammatory and could induce inhibition of cell apoptosis (Hirsova et al., 2016). Therefore, it was hypothesized that exosomes isolated from divergent risk groups would result in differences in MDBK cell proliferation. It is possible that the exosomes isolated from the 'High Risk' and 'Low Risk' cows are not programmed to target MDBK cells due to their kidney origin or that the non-clinical fatty liver disease in early lactation dairy cows may not alter proliferation of kidney cells. Additionally, the exosomes isolated from blood represent a composite of release from all tissues which may serve to dilute any liver-specific changes in exosome released for the 'High Risk' and 'Low Risk' cows tested here.

Nevertheless, the results presented indicate no significant effect of the metabolic state of the exosome-donor cows on the proliferation of MDBK cells, despite a numerical difference in cellular proliferation in favor of the 'High Risk' treatment.

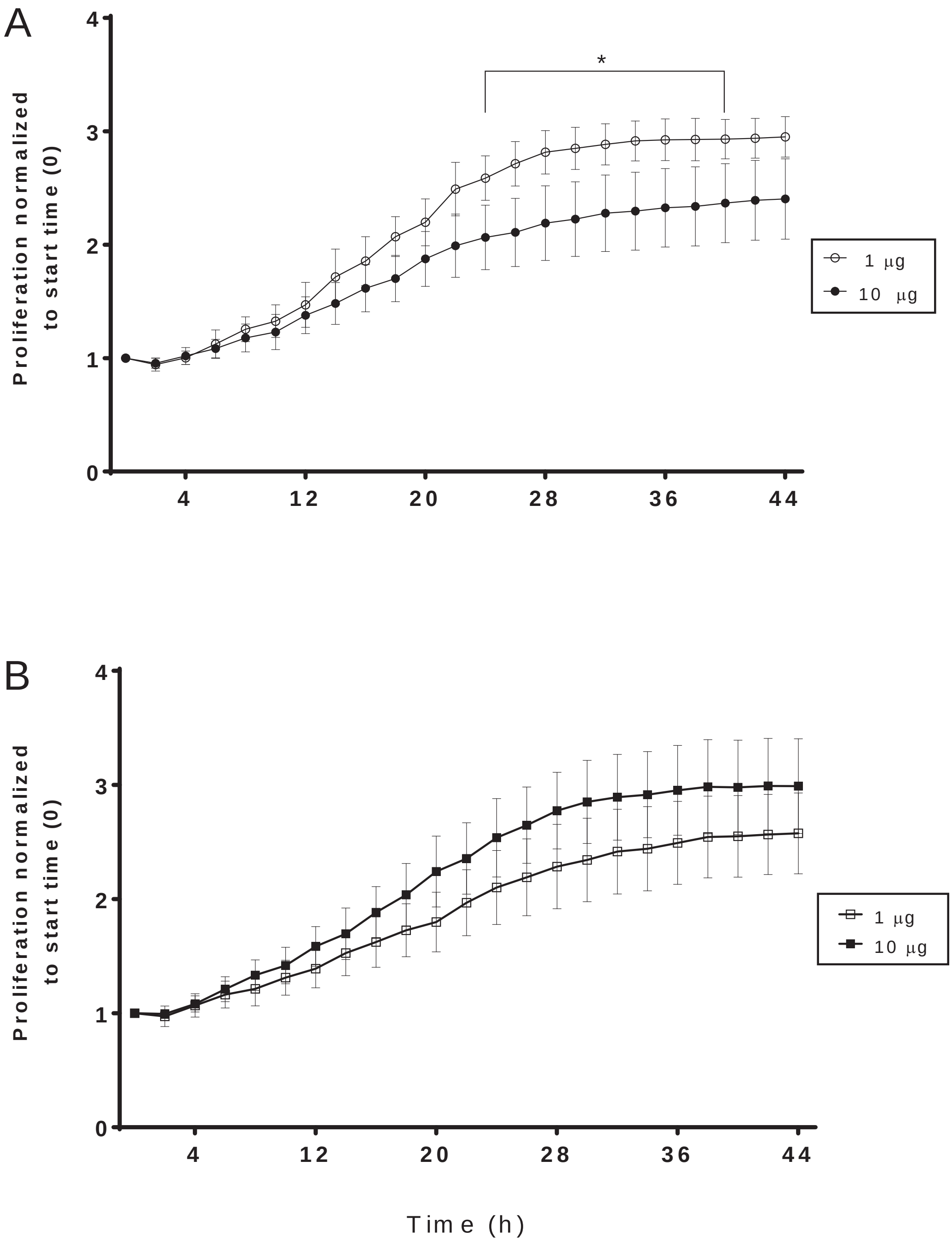


Figure 19. Proliferation of MDBK cells with application of exosomes from 'Low Risk' and 'High Risk' cows – comparison of dose. Figure legend over page.

Figure 19. IncuCyte® proliferation data of MDBK co-cultures with exosome fractions 3-6 from 'High Risk' and 'Low Risk' cows – comparison of dose (previous page). (**A**) exosomes isolated at wk 1 from 'Low Risk' cows at 1 μg/mL concentration (open circles) and 10 μg/mL concentration (filled circles); (**B**) exosomes isolated at wk 1 from 'High Risk' cows at 1 μg/mL concentration (open squares) and 10 μg/mL concentration (filled squares); Results are presented as means at each time point ± SEM, n = 5. There was no interaction between Dose, Time, and Risk Group ($P > 0.05$). However, the effect of dose was most evident at 24 – 40 h ($P < 0.05$) in the 'Low Risk' group (*). Dose of exosomes isolated at wk 4 did not affect proliferation ($P > 0.05$; data not shown).

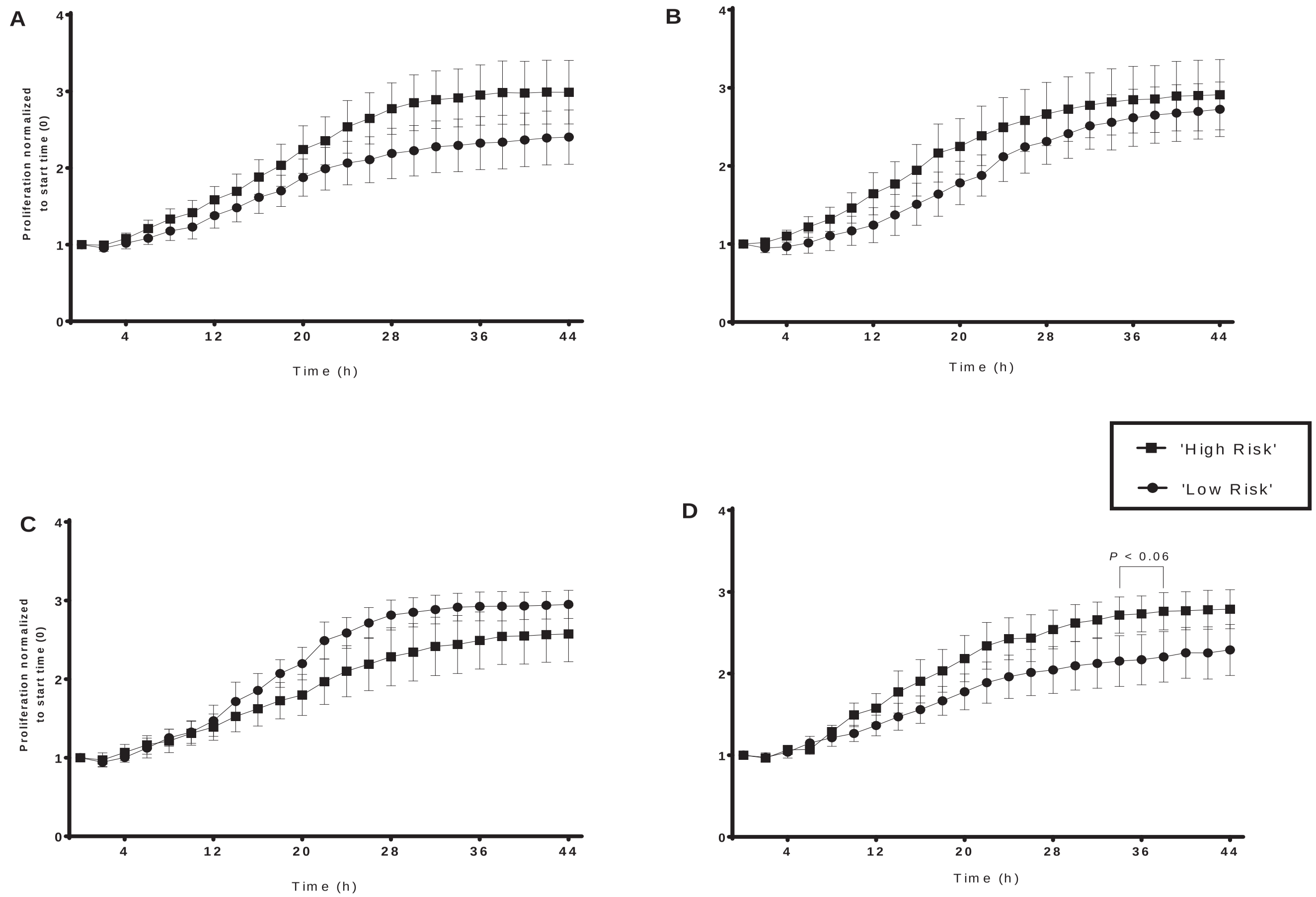


Figure 20. Proliferation of MDBK cells with application of exosomes from ‘Low Risk’ and ‘High Risk’ cows – comparison of ‘Risk’.

(**A**) exosomes isolated at wk 1, 10 µg concentration; (**B**) exosomes isolated at wk 4, 10 µg concentration; (**C**) exosomes isolated at wk 1, 1 µg concentration; (**D**) exosomes isolated at wk 4, 1 µg concentration. Results are presented as means at each time point ± SEM, n = 5.

Gene Expression Results

The cytokine and chemokine PCR array identified 32 genes that meet cycle threshold/crossing point (Cp) cut offs (Cp < 30) for gene expression quantification. Of these, there were no significant differences in gene expression between 'High Risk' and 'Low Risk' exosome co-culture samples. However, there were three genes that tended to be increased in the 'High Risk' co-culture samples compared with the 'Low Risk' samples (Figure 21). These were the genes encoding granulocyte-colony stimulating factor (*CSF3*; $P < 0.10$), ciliary neurotrophic factor (*CNTF*; $P < 0.10$), and CD27 ligand (*CD70*; $P = 0.14$), all of which were up-regulated in the 'High Risk' cows relative to 'Low Risk' cows.

Granulocyte-colony stimulating factor (G-CSF) is an important survival and proliferation factor of neutrophils (Basu et al., 2002). G-CSF can act on neuronal cells as a neurotrophic factor to induce neurogenesis and inhibit apoptosis (Schneider et al., 2005). Interestingly, this is the same function as the protein product of the gene *CNTF*, the expression of which also tended to be increased in 'High Risk' co-culture samples. The similar functions of these two genes in cellular proliferation support numerical differences presented in Figure 20. MDBK cells are an epithelial-like cell line. The three genes that tended to be up-regulated in the 'High Risk' co-culture samples are all expressed in thymic epithelium (Haynes et al., 2000; Coquet et al., 2013). The epithelial cells of the thymus express several cytokines for T lymphocyte (T cell) development and confer immunotolerance to self-antigens. Other epithelial cells have a capacity for immunomodulation as they provide the first barrier against foreign invaders (Rescigno et al., 2008; Malvisi et al., 2016); therefore, MDBK cells may have some capacity for immunomodulation. Conversely, these genes may be indicative of the exosomal tissue of origin and could potentially reflect mRNA from the thymus. This is a limitation of the co-culture model, and one that requires further investigation. The tendency for differences in gene expression between the 'High Risk' and 'Low Risk' cultures potentially indicate altered immune function and support for the numerical differences in proliferation (Figure 20) by exosomes from cows in the 'High Risk' category for metabolic dysfunction; however, further investigation is required to confirm this.

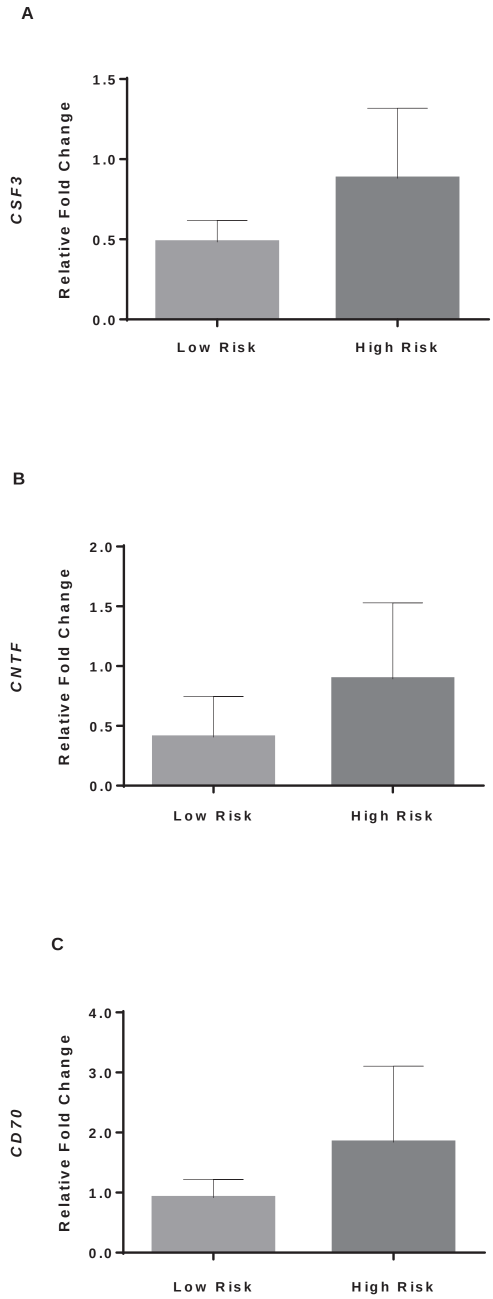


Figure 21. **Gene expression results of 'High Risk' and 'Low Risk' co-culture models.** Data from co-cultures of pooled wk 1 exosome fractions [3-6 and 7-9] are combined. (**A**) *CSF3* (*P* = 0.10), (**B**) *CNTF* (*P* = 0.09), and (**C**) *CD70* (*P* = 0.14). Gene expression is normalised to endogenous control *HPRT1* using the ΔΔCp method. Results are presented as mean ± SEM, n = 5.

Proteomics Results

The results of the protein analysis are presented in Table 12, Figure 22 and Figure 23 and Supplementary Table 5. Unique proteins were identified between cells treated with plasma exosomes isolated from 'High Risk' and 'Low Risk' cows. These differences may be indicative of the metabolic state of the cows from which they were derived; however, extensive changes were not evident. Two of the unique proteins identified in the 'Low Risk' group (≥2 cows), and not identified in the 'High Risk' cows were endoplasmin (gp96) and catalase. Endoplasmin is a chaperone protein with a key role in regulating folding of immune proteins and is, therefore, critical to immune function (Schild and Rammensee, 2000). This result supports previously-discussed changes in gene expression related to immune regulation. Furthermore, recombinase-mediated loss of endoplasmin in hepatocytes leads to decreased cellular activity and compromises liver function (Rachidi et al., 2015), highlighting an important role in metabolic health. Catalase activity has been described in microvesicles where it acts as a scavenger of reactive oxygen species (ROS) and protects against oxidative damage (Hulsmans and Holvoet, 2013). These results may indicate enhanced cellular function in MDBK cells treated with exosomes from 'Low Risk' cows. However, there are no substantial proteomic changes that can be related to the divergent metabolic states from where the exosomes originated.

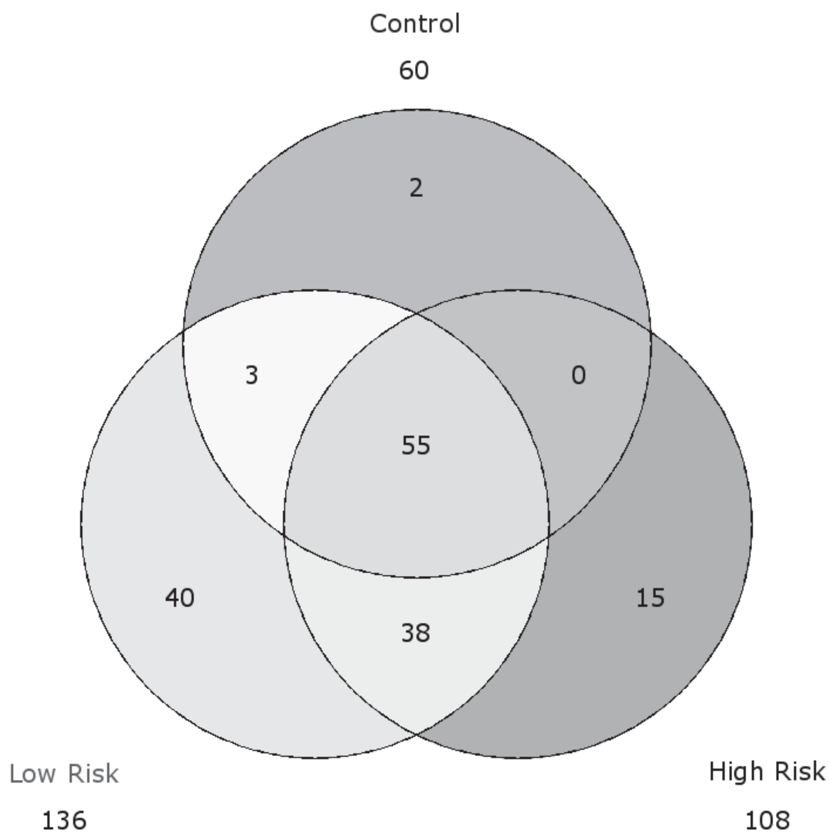


Figure 22. **Venn diagram of unique proteins in the co-culture models.**

Comparison of number of unique proteins in Control, 'High Risk', and 'Low Risk' co-cultures using liquid chromatography–mass spectrometry (LC-MS)/MS demonstrating proteins shared or unique to each group. Proteins were counted if they were identified in ≥1 sample in each group.

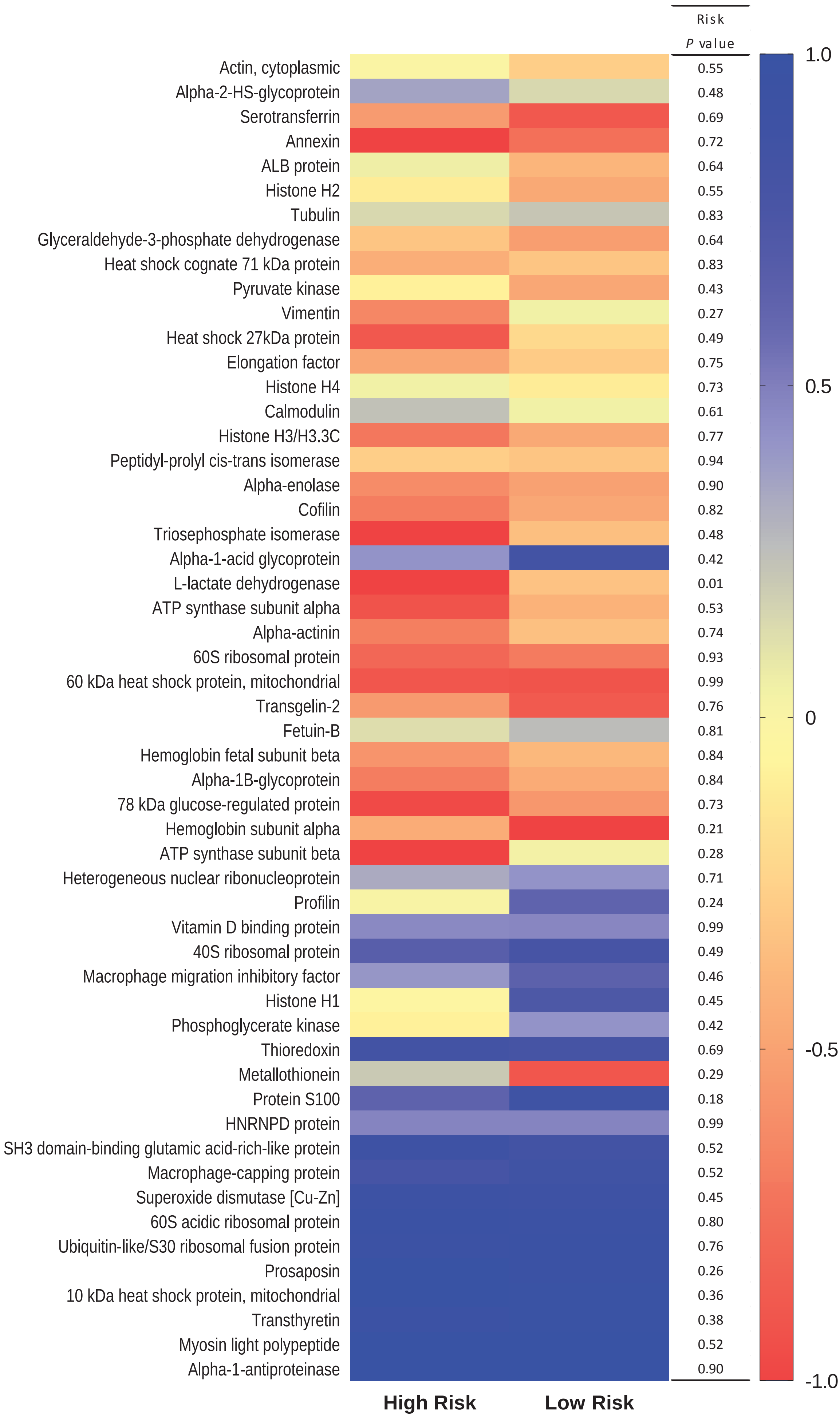
Table 12. Proteins identified in the co-culture models compared with controls (over page).

List of unique proteins and representative accession number of proteins identified in Control, ‘High Risk’, and ‘Low Risk’ groups using liquid chromatography–mass spectrometry (LC-MS)/MS. Proteins were counted if they were identified in ≥1 sample in each group. Accession numbers in bold refer to the proteins identified in ≥50% of cows in a particular risk group and 0 in the other risk group.

Common to all groups		Common to exosome treatment		Common to 'Low Risk' and Control		Unique to 'Low Risk'		Unique to 'High Risk'		Unique to Control	
Accession	Protein name	Accession	Protein name	Accession	Protein name	Accession	Protein name	Accession	Protein name	Accession	Protein name
A4ZZF8	Alpha-actinin	A6QPM9	PTMS protein	A5D7E8	Protein disulfide-isomerase	A0JNP8	LOC516032 protein	Q5E962	Aldo-keto reductase family 1, member B1	E1BKE1	FRYL
A5PJ79	Transketolase	E1B9K1	Polyubiquitin-C	G8JL04	Thymosin beta 4 X-linked	A4IFI0	IGLL1 protein	**S5G966**	Ribosomal protein L23a Y-linked protein	G3N131	G3N131
A6H6Y0	HNRNPD protein	E1BCL5	60S acidic ribosomal protein P1	**Q95M18**	Endoplasmin	A5PJE3	Fibrinogen alpha chain	P48427	Tubulin-specific chaperone A		
B0JYL8	Cofilin	E1BF20	HNRNPH1			A6QLL8	Fructose-bisphosphate aldolase	**P63048**	Ubiquitin-60S ribosomal protein L40		
B0JYN3	L-lactate dehydrogenase	F1MCT8	SYNCRIP			A6QNL5	PDIA6 protein	**A1L5B7**	SERPINE1 mRNA binding protein		
B0JYN6	Alpha-2-HS-glycoprotein	F1MMK9	Protein AMBP			A8E641	DPYSL5 protein	Q2TBR3	SET nuclear oncogene		
B0JYQ0	ALB protein	F1MXP8	Prosaposin			B0JYP6	IGK protein	P62992	Ubiquitin-40S ribosomal protein S27a		
E1BH22	Histone H2A	F1N169	FLNA			E1B925	ARID1A	G3MXG3	CCDC88A		
E1BHA5	40S ribosomal protein S19	F1N3A1	Thrombospondin-1			E1BNF9	SLAMF9	P63243	Guanine nucleotide-binding protein		
E1BHJ0	Profilin	F1N647	Fatty acid synthase			E1BQ37	SFPQ	B9VPZ5	Lactoferrin		
E9RHW1	Heat shock 27kDa protein	G3X6S5	SH3 domain-binding glutamic acid-rich-like protein			F1MAV0	Fibrinogen beta chain	P61283	Barrier-to-autointegration factor		
F1MB08	Alpha-enolase	O46375	Transthyretin			F1MDH3	TLN1	Q861P7	HLA-B-associated transcript		
F1MLB8	ATP synthase subunit alpha	P00442	Superoxide dismutase [Cu-Zn]			F1MY44	HNRNPM	G5E5F7	MAGEB4		
F1MPU0	Clathrin heavy chain	P13696	Phosphatidylethanolamine-binding protein			F1N301	RPL22	F1MTV9	Nucleophosmin		
F1MUZ9	60 kDa heat shock protein, mitochondrial	P34955	Alpha-1-antiproteinase			F1N6D5	116 kDa U5 small nuclear ribonucleoprotein	A6H767	Nucleosome assembly protein 1-like		
F1N6C0	Calmodulin	P60661	Myosin light polypeptide 6			G3X7A5	Complement C3				
G3MZ21	Hemoglobin fetal subunit beta	P61603	10 kDa heat shock protein, mitochondrial			G3X8D6	Iodothyronine deiodinase				
G3N2P2	Histone H3.3C	P62866	Ubiquitin-like/S30 ribosomal fusion protein			**P00432**	Catalase				
G3X6N3	Serotransferrin	P68002	Voltage-dependent anion-selective channel protein			P07589	Fibronectin				
G3X807	Histone H4	Q2HJH9	Programmed cell death protein			P12234	Phosphate carrier protein, mitochondrial				
G8JKZ8	Thioredoxin	Q3B7M5	LIM and SH3 domain protein			P16116	Aldose reductase				
I6YIV1	Annexin A2	Q3SYU2	Elongation factor			P28801	Glutathione S-transferase P				
I7CT57	Vitamin D binding protein	Q3SZ57	Alpha-fetoprotein			P35705	Thioredoxin-dependent peroxide reductase, mitochondrial				
O18787	Elongation factor 1 alpha	Q3SZ65	Eukaryotic initiation factor 4A-II			P81644	Apolipoprotein A-II				
P01966	Hemoglobin subunit alpha	Q3T0Q4	Nucleoside diphosphate kinase B			Q0IIL9	Receptor protein-tyrosine kinase				
P02253	Histone H1.2	Q3T0Q6	Cellular nucleic acid-binding protein			Q0QF29	Malate dehydrogenase				
P10096	Glyceraldehyde-3-phosphate dehydrogenase	Q3T165	Prohibitin			Q1RMN8	Immunoglobulin light chain, lambda gene cluster				
P19120	Heat shock cognate 71 kDa protein	Q3ZBA4	RNA binding motif (RNP1, RRM) protein			Q2HJH4	HNRPDL protein				
P21752	Thymosin beta-10	Q3ZBH0	T-complex protein 1 subunit beta			Q2KJH6	Serpin H1				
P48616	Vimentin	Q3ZBS7	VTN			Q3MHY1	Cysteine and glycine-rich protein				
P60712	Actin, cytoplasmic	Q3ZBT1	Transitional endoplasmic reticulum ATPase			Q3SZ20	Serine hydroxymethyltransferase, mitochondrial				
P62935	Peptidyl-prolyl cis-trans isomerase A	Q3ZBZ8	Stress-induced-phosphoprotein			Q3SZ62	Phosphoglycerate mutase				
P63103	14-3-3 protein	Q5E9E2	Myosin regulatory light polypeptide			Q3SZZ9	FGG protein				
P81947	Tubulin alpha-1B chain	Q5EAD2	D-3-phosphoglycerate dehydrogenase			Q3T0B6	Complement component 1 Q subcomponent-binding protein				
Q0QEM9	ATP synthase subunit beta	Q7SIH1	Alpha-2-macroglobulin			Q3T0E0	Copper transport protein ATOX1				
Q0QEV5	Ribosomal protein L18	Q862P4	Similar to elongation factor-1-gamma			Q3T101	IGL@ protein				
Q0VCX2	78 kDa glucose-regulated protein	Q865V6	Macrophage-capping protein			Q3ZBF7	Prostaglandin E synthase				
Q2KJF1	Alpha-1B-glycoprotein	V6F9A2	Apolipoprotein A-I preproprotein			Q5E9B7	Chloride intracellular channel protein				
Q2VYC3	Eukaryotic translation elongation factor 1 beta					Q8SQH5	ADP/ATP translocase				
Q3SYT9	Poly(RC) binding protein 2					V6F832	Alpha-crystallin B chain				
Q3SZN8	Ribonuclease/angiogenin inhibitor										
Q3T087	60S ribosomal protein										
Q3T0P6	Phosphoglycerate kinase										
Q3ZC44	Heterogeneous nuclear ribonucleoprotein A/B										
Q3ZC87	Pyruvate kinase										
Q3ZCH0	Stress-70 protein, mitochondrial										
Q58D62	Fetuin-B										
Q5E956	Triosephosphate isomerase										
Q5E9F5	Transgelin-2										
Q5GN72	Alpha-1-acid glycoprotein										
Q6PVY3	Macrophage migration inhibitory factor										
Q862H7	Protein S100										
Q862Q2	Similar to ribosomal protein S3										
Q8MII4	Metallothionein										
Q9BGI4	Peroxiredoxin										

Figure 23. **Heat map of proteins identified in co-culture samples (over page).**

'High Risk' and 'Low Risk' by liquid chromatography–mass spectrometry (LC-MS)/MS. Relative abundance of proteins is the sum of peptide signals for a given protein as a percentage of total peptide signals for that sample. Proteins presented are those identified in ≥50% of cows in both risk groups. Order of proteins is based on abundance of protein within control sample with most abundant at the top. Results are presented as a ratio relative to the protein abundance in the control sample whereby a ratio of 0 is the same abundance as the control, >0 (blue) is protein abundance greater than the control, and >0 (red) is lower abundance compared with the control. The *P* value for the effect of Risk, analysed using a two sample t-test (proc mixed, SAS 9.3), is included next to the heat map.


Risk
P value
Actin, cytoplasmic 0.55
Alpha-2-HS-glycoprotein 0.48
Serotransferrin 0.69
Annexin 0.72
ALB protein 0.64
Histone H2 0.55
Tubulin 0.83
Glyceraldehyde-3-phosphate dehydrogenase 0.64
Heat shock cognate 71 kDa protein 0.83
Pyruvate kinase 0.43
Vimentin 0.27
Heat shock 27kDa protein 0.49
Elongation factor 0.75
Histone H4 0.73
Calmodulin 0.61
Histone H3/H3.3C 0.77
Peptidyl-prolyl cis-trans isomerase 0.94
Alpha-enolase 0.90
Cofilin 0.82
Triosephosphate isomerase 0.48
Alpha-1-acid glycoprotein 0.42
L-lactate dehydrogenase 0.01
ATP synthase subunit alpha 0.53
Alpha-actinin 0.74
60S ribosomal protein 0.93
60 kDa heat shock protein, mitochondrial 0.99
Transgelin-2 0.76
Fetuin-B 0.81
Hemoglobin fetal subunit beta 0.84
Alpha-1B-glycoprotein 0.84
78 kDa glucose-regulated protein 0.73
Hemoglobin subunit alpha 0.21
ATP synthase subunit beta 0.28
Heterogeneous nuclear ribonucleoprotein 0.71
Profilin 0.24
Vitamin D binding protein 0.99
40S ribosomal protein 0.49
Macrophage migration inhibitory factor 0.46
Histone H1 0.45
Phosphoglycerate kinase 0.42
Thioredoxin 0.69
Metallothionein 0.29
Protein S100 0.18
HNRNPD protein 0.99
SH3 domain-binding glutamic acid-rich-like protein 0.52
Macrophage-capping protein 0.52
Superoxide dismutase [Cu-Zn] 0.45
60S acidic ribosomal protein 0.80
Ubiquitin-like/S30 ribosomal fusion protein 0.76
Prosaposin 0.26
10 kDa heat shock protein, mitochondrial 0.36
Transthyretin 0.38
Myosin light polypeptide 0.52
Alpha-1-antiproteinase 0.90
1.0
0.5
0
-0.5
-1.0
High Risk
Low Risk

The proteomic data presented here are consistent with previous studies investigating exosomes. Relative abundance between risk groups did not reveal significant changes associated with metabolic risk category. Lactate dehydrogenase was the only protein greater ($P < 0.05$; Supplementary Table 5) in 'Low Risk' co-cultures compared with 'High Risk' co-cultures, calculated using relative protein abundance. Lactate dehydrogenase catalyses the conversion of pyruvate to lactate with the regeneration of nicotinamide adenine dinucleotide (NADH) to NAD^+, which is essential in hypoxic and anaerobic conditions and as a result, is up-regulated in such conditions (Alegre et al., 2015). Several other proteins involved in cellular metabolism were identified by proteomic analysis in the current study that are frequently identified in exosomes; e.g., histone proteins (H2B, H4, and H3.3), annexin proteins (A2), cofilin1, heat shock proteins (60 kDa, 70 kDa), 14-3-3 proteins (sigma, zeta/delta), phosphoglycerate kinase, and adenosine triphosphate (ATP) synthase (Mathivanan et al., 2010; Welton et al., 2010). The proteins identified in the current study are consistent with exosome-related changes in target cell metabolism; however, there is no clear evidence that the changes are related to metabolic state of exosomal origin.

Limitations of the study design

We acknowledge that the number of animals included in each risk category is low and, therefore, results need to be considered with caution. The number of animals in each risk group (n = 5) was limited by the number of animals in the parent experiment (n = 45, described in Chapter 3). There were only 10 cows with significantly divergent metabolic profiles at both wk 1 and wk 2 post-calving. Furthermore, there was limited exosomal protein isolated from the plasma samples. Finally, only two concentrations of exosomes were used as a dose comparison (1 μg/mL and 10 μg/mL). Based on the results of the dose response proliferation curves (Figure 19), it is possible that comparisons including more dose rates would reveal further changes.

7.5 Conclusion

The current study investigated the effect of plasma exosomes from two groups of metabolically-divergent cows on MDBK cell function. We conclude that only limited, non-significant changes in gene expression and protein expression of MDBK cells were evident when exposed to exosomes derived from cows with ‘high’ or ‘low’ risk of metabolic dysfunction. The functional changes were inconclusive in determining the risk category of origin but demonstrated differences that would suggest exosomal cargo from differing metabolic states likely affects target cell function. This study provides a foundation for future studies investigating crosstalk between exosomes and target cells in cows with subclinical health dysfunction during the transition period.

7.6 Acknowledgements

The authors wish to acknowledge the help of DairyNZ farm staff (Scott Farm, DairyNZ) and the statistical analysis and advice of Barbara Kuhn-Sherlock, DairyNZ. We also acknowledge the advice of Martin Middleditch (Mass Spectroscopy Centre, University of Auckland). This work was supported by funding from New Zealand dairy farmers through DairyNZ Inc. (RD1403) and the Ministry of Business, Innovation and Employment (DRCX1201).

Chapter 8

General Discussion

The objective of this thesis was to study factors of immune function of grazing dairy cows during the transition period and investigate how immune dysfunction may increase risk of disease around calving (Figure 24). Specifically, the aims for this thesis were to firstly, establish microfluidics gene expression chips as a suitable platform for RT-qPCR gene expression analysis (Chapter 2). This technology was then used to characterise innate immune function during the transition period in grazing dairy cows by investigating changes in gene expression in circulating neutrophils (Chapter 3). Furthermore, this technology was used to investigate the effect of common on-farm management strategies (body condition at calving and pre-calving feeding level) on neutrophil gene expression and, therefore, function (Chapter 4). The effect of metabolic stress on neutrophil function was then investigated using RNAseq by establishing a model of cows divergent in metabolic health status (Chapter 5). This model was further utilised with the aims of investigating exosomal cargo between health status (Chapter 6) and the effect of these exosomes on epithelial cell function (Chapter 7).

8.1 Originality and overview

The neutrophil gene expression results in Chapter 3 demonstrated functional changes in moderate milk producing grazing dairy cows similar to those reported in high-yielding cows housed in confinement. The results indicated that the altered immune function during the transition period is a natural part of the calving process. These functional changes can be influenced by nutrition status and feeding level (Chapter 4) and by metabolic health and the extent of metabolic stress during the transition period (Chapter 5). Molecular signatures of metabolic stress that are packaged in circulating intercellular messengers are distinctive in cows differing in the degree of metabolic stress (exosomes; Chapter 6), which have the capacity to influence function, including immune responses, of target cells/tissues (Chapter 7). Overall, this thesis has improved our understanding of molecular changes in innate immune function and regulation over the transition period and explored how these changes are influenced by extrinsic (i.e., management strategies) and intrinsic (i.e., metabolic stress) factors.

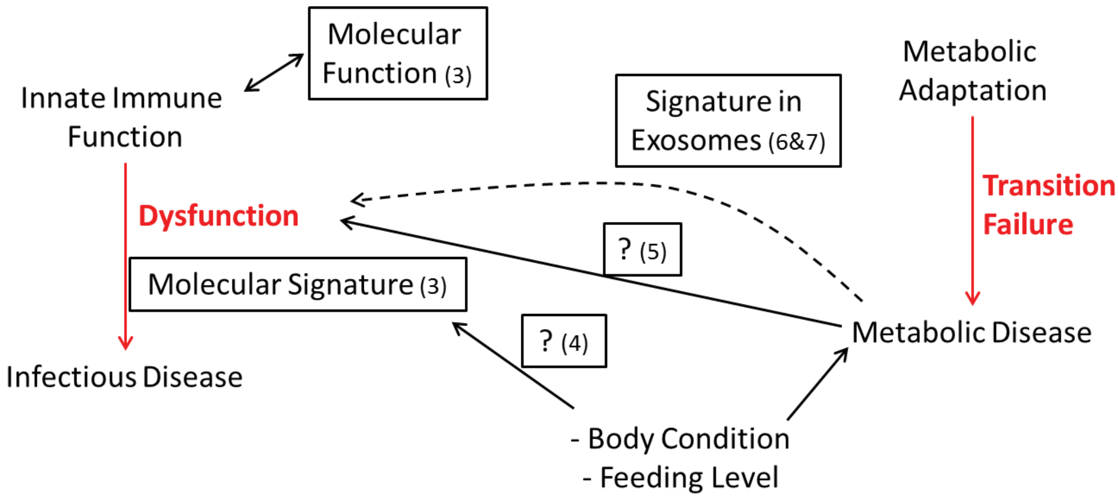


Figure 24. Flow diagram depicting overall aims of thesis.

Items in boxes indicate questions addressed in this PhD thesis with associated chapter numbers in brackets. Solid arrows indicate known interactions and dotted arrows indicate hypothesised interactions. Red arrows indicate negative associations.

The work undertaken in this thesis is the first to describe temporal changes in innate immune function in the transition between pregnancy and lactation in low- to moderate-yielding grazing dairy cows. The results are consistent with and expanding upon changes in immune function that have been reported in high-yielding cows housed in confinement systems (which increases the risk of infectious diseases, such as metritis and mastitis). The molecular approaches used in this thesis, to elucidate neutrophil function are novel in the field of dairy science and indicate that the approach is representative of function, e.g., changes in adhesion molecule gene expression like L-selectin and Mac-1. Furthermore, the characterisation of the exosome cargo in cows exhibiting different states of metabolic health, and how these exosomes affect target cells, have not previously been investigated.

8.2 Temporal characterisation of neutrophil function in peripartum grazing dairy cows

The temporal changes in gene expression described in Chapters 3 and 4 are indicative of a dysregulation of innate immune function in moderate-yielding grazing dairy cows during the transition period. Such a change in immune function is consistent with reported functional measurements from blood samples in high-yielding dairy cows. Previous research results have indicated that high-yielding cows in confinement systems experience a 75% reduction in neutrophil function, which implies they have impaired capability when they reach the site of inflammation (Kehrli et al., 1989b). In moderate-yielding cows, the same molecular signature of dysfunction is evident and indicates that neutrophil functionality has been reduced (Chapter 3). The data presented from unchallenged, circulating neutrophils indicate that the ability of neutrophils to reach the site of infection is also impeded. For example, expression of *SELL* (L-selectin), a protein involved in initial attachment of neutrophils to the endothelial wall of blood vessels, is down-regulated after calving. Down-regulation of cell-surface L-selectin after calving was previously demonstrated using flow-cytometry in high yielding cows (Lee and Kehrli, 1998). Appropriate expression of L-selection is essential for neutrophils to respond to an inflammatory stimulus. For example, inhibition of L-selectin in mice reduced recruitment of neutrophils to an inflamed peritoneum by ~60% (Tedder et al., 1995). Collectively, these results reflect an altered state of function in circulating neutrophils in peripartum cows, irrespective of milk production, and that the dampened innate immune response measured using stimulated neutrophils is also evident in gene expression signatures in circulating, unstimulated cells. These results indicated that transcriptomic assays on circulating neutrophils accurately represent changes identified using functional assays.

There are plausible evolutionary reasons for the down-regulation of the innate immune response during the calving period. For example, parturition exposes the uterus to the presence of a ‘foreign’ body (i.e., the calf) and the outside environment; a dampening of the innate immune response would avoid an overt detrimental inflammation in response to invading environmental pathogens. Therefore, it is in the best interest of the animal to reduce the capacity of neutrophils to reach the site of inflammatory signalling and its functional capacity for a short time around the calving period. However, extended periods of reduced functionality do not have a functional benefit and, likely, expose the cow to infectious diseases, particularly of the uterus and mammary gland.

8.3 Peripartum nutritional state and neutrophil function

The temporal changes in neutrophil function are moderately affected by pre-calving feeding level, but not by calving body condition (Chapter 4). The limited effect of body condition on neutrophil gene expression probably indicates that the range of BCS investigated (4.0-5.0 on a 10-point scale) is within a physiological range to avoid adverse effects on neutrophil function. In support of this, neutrophil numbers are within an optimal range when at the BCS investigated in Chapter 4, which is an indicator of normal haematopoiesis (Roche et al., 2013b). Therefore, it is not surprising that changes in the expression of large numbers of genes were not detected in the two BCS categories investigated; extreme differences in BCS (e.g., severely under-conditioned compared with severely over-conditioned) would likely elicit a greater effect, similar to the changes in neutrophil function in patients suffering from anorexia nervosa and obesity in human studies (Lambert et al., 1997; Nijhuis et al., 2009).

Expression of a number of genes was affected by BCS and feeding level, demonstrating that pre-calving nutrition has a role in regulating the immune system around calving. For example, expression of antimicrobial genes *BNBD4* and *DEFB10* and the anti-inflammatory cytokine *IL10* was increased in neutrophils from BCS 5 compared with BCS 4 cows. Furthermore, expression of genes involved in leukotriene synthesis (*PLA2G4A* and *ALOX5AP*) increased at 1 wk post-calving in cows that were overfed prior to calving, compared with animals that were underfed. Nutrient availability can alter metabolic state, which interacts with the immune system, as indicated by the important role that inflammation plays in the pathogenesis of metabolic disease (Hotamisligil, 2006).

Despite the small effects of adiposity and feeding level, it is known that these management strategies have an impact on metabolic health during the transition period and that there is a relationship between metabolic stress and immune function (Sordillo and Mavangira, 2014). The importance of nutrition in maintaining metabolic and, to an extent, innate immune health was further explored in Chapter 5.

8.4 Peripartum metabolic stress and neutrophil function

Metabolic state affected the neutrophil transcriptome in a way that indicates metabolic stress, and is detrimental to neutrophil function even without clinical symptoms of metabolic disease. Pathway analysis indicated that the process of neutrophil recruitment is down-regulated in cows experiencing metabolic stress ('High Risk'). As with the temporal changes across the transition period (Chapter 3), there was a down-regulation of genes involved in attachment and migration of neutrophils (e.g., L-selectin; Chapter 5) in cows with metabolic stress. The ability of neutrophils to reach the site of infection is critical for containment and clearance of infectious agents. Furthermore, apoptosis was down-regulated and cell survival up-regulated in cows with metabolic health dysfunction (Chapter 5). Neutrophil cell survival can be extended in response to pro-inflammatory cytokines or bacterial products (Hsieh et al., 1997) and in these cases it is advantageous to extend cell lifespan in order to combat infection. It is interesting that the gene expression patterns identified in the model of subclinical health dysfunction is consistent with an increased state of inflammation, which is also known as metabolic inflammation (Gerner et al., 2013; Lyons et al., 2016). It could be a result of increased inflammation in particular tissues (e.g., liver), or alternatively, a consequence of increased stress during the calving period brought about by increased glucocorticoids (e.g., cortisol), which affects the regulation of genes involved in apoptosis, adhesion, migration, and tissue remodelling in neutrophils (Kehrli et al., 1995; Buckham Sporer et al., 2007). The cows from the model of health dysfunction will have experienced a dampening of the innate immune response due to calving (Chapter 3) and the results presented in Chapter 5 indicate that such an effect is exacerbated by metabolic stress, further increasing the risk of infectious disease. These studies support the concept that metabolic stress has a detrimental effect on neutrophil function.

8.5 Intercellular messengers, inflammation, and innate immune function

The protein cargo in circulating extracellular vesicles from cows divergent in metabolic and immune health was different and, to some degree, reflective of metabolic health status (Chapter 6). Exosome cargo is capable of regulating the immune system (Bobrie et al., 2011) and immune cell-derived exosomes (e.g., neutrophils) are capable of regulating target cell function (Vargas et al., 2016). Exosomal cargo has been reported to vary with metabolic state in humans; miRNAs differ between individuals with metabolic syndrome and those presenting with risk factors (Karolina et al., 2012). Therefore, it is plausible that the same occurs in cows, whereby, circulating factors are indicative of the risk of clinical manifestation of metabolic disease. Inflammatory markers in exosomes from the 'High Risk' cows led me to postulate that these messengers may be key regulators of inflammation during the transition period. Therefore, an investigation of the effect of exosomes on an epithelial cell line using a co-culture model was initiated (Chapter 7). The exosome protein was in limited supply, which restricted the scope of the experiments, but despite this, evidence of regulatory activity was obtained. The application of higher concentrations of exosomes from cows with a low risk of disease decreased cell proliferation and tended to exhibit decreased expression of pro-inflammatory genes, while those from the 'High Risk' cows increased cell proliferation. The numerical change was consistent with pro-inflammatory markers in the protein cargo of the extracted exosomes. These results are consistent with those obtained in human studies. For example, it has now been established that exosomes are regulators of chronic inflammation in the microenvironment of cancerous tumours (Benito-Martin et al., 2015). It is conceivable that exosomes from metabolically-stressed cows will exhibit a similar inflammatory phenotype that is detrimental to both metabolic and immunological health. A better understanding of this regulation could guide immunomodulatory approaches to improve dairy cow health over the transition period.

8.6 Use of 'omics' techniques to investigate neutrophil function and intercellular messaging

The approaches used to investigate the molecular mechanisms of neutrophil function and key regulators of innate immune function (exosomes) over the transition period are novel in the field of bovine molecular immunology. The use of Fluidigm microfluidics technology (Chapter 2) enabled greater numbers of samples and specific gene targets to be investigated at a similar cost to fewer gene targets using standard RT-qPCR methods (White et al., 2011). RNA sequencing (Chapter 5) allowed very high-throughput data generation at a greater cost per sample, but at a reduced cost per gene. Furthermore, high-throughput generation of protein expression data using LC-MS/MS (Chapters 6 and 7) is a powerful tool for proteomic investigation similar to transcriptomic analysis using RNAseq. The advantage of these technologies is their ability to connect molecular regulation to changes in cell function. Also, these '–omic' technologies inform us of aberrant pathways, which is essential for understanding disease pathogenesis (Jiang et al., 2015; Raimondo et al., 2011). The molecular techniques and experimental approaches employed in this thesis are pioneering in transition cow biology. Overall, the molecular approaches indicated that endogenous control gene selection should be explored for every experiment, microfluidics RT-qPCR can be used as a substitute for standard RT-qPCR methods and very high-throughput '–omics' technologies allow unprecedented data generation to address questions regarding metabolic health and immune function over the transition period.

8.7 Limitations of the experimental approaches used

The limitations of the work undertaken include limited sample numbers and protein concentrations for the exosome work (Chapters 6 and 7) and the potential contamination of cell types in the preparation of neutrophil samples used in Chapters 2-5. The decision to isolate exosomes was retrospective to the completion of the field experimentation; therefore, there was a limited volume of plasma available for isolation. The molecular approaches (LC-MS/MS, RT-qPCR, and cell culture) provided as much data as could be generated from the limited exosomal protein available. In future work, a greater volume of starting material could be used for exosome isolation to ensure exosome protein concentrations are adequate for downstream applications. Furthermore, the limited sample numbers (five cows in each treatment group) were insufficient to detect statistically significant results in what could be biologically relevant changes. With greater numbers and exosomal concentrations, statistical power would be sufficient to reduce the likelihood of Type II error. A further limitation was the potential contamination of other immune cells in the neutrophil samples. A method for isolation of neutrophil populations with improved purity would have enhanced the robustness of the results. The important reason why the method employed was successful was that it was very fast; the number of samples could not have been processed in the same amount of time using other methods. It was assumed that because all of the samples were processed in the same manner, there was a similar level of contamination across all samples collected. Furthermore, an improvement to the RNAseq experiment would have been the inclusion of a neutrophil sample from a non-pregnant control (e.g., from a bull or non-cycling heifer). This would have provided a contrasting comparison for gene expression analysis that would have provided additional depth to the experiment but was not necessary to answer the specific research question.

8.8 Implications and future work

The research undertaken in this thesis has implications for the prevention and treatment of infectious and metabolic disease during the transition period. Our improved understanding of the temporal 'dampening' of innate immune responses around calving creates an opportunity to improve innate immune function during the transition period. Treatment methods that could 'boost' innate immune function (e.g., G-CSF) during the time when cows are at greatest risk of infectious disease might be beneficial in a grazing system. Furthermore, the research presented in Chapter 4 reiterates that the current BCS targets and nutritional guidelines for managing transition cows in a grazing system (Roche et al., 2013a; b) are optimal for innate immune health. The differences in exosomal cargo in animals divergent in their metabolic health phenotype support the possibility of identifying specific circulating markers of metabolic disease. Metabolic stress negatively influences tissue function (e.g., ovarian, uterine, brain, leukocytes, etc.) due to oxidative damage (Sordillo and Aitken, 2009). If exosomes regulate this metabolic stress-related damage, a cure may lie in exosomes. Future implications include the use of exosomes to treat metabolic and/or infectious disease by alleviating metabolic stress through immunomodulatory mechanisms. A subsequent investigation may include the co-culture of exosomes from metabolically-divergent cows with white blood cells or whole blood and compare responses to an immunogenic challenge. This would help elucidate the extent of the immunomodulatory capacity of exosomes in dairy cows for the development of potential treatments. Further directions also include the use of immunomodulatory agents (e.g., granulocyte colony stimulating factor), which would improve neutrophil number and function to alieviate immunological stress over the calving period and improve cow health and well-being. Further research could investigate these immunomodulatory mechanisms combined with optimised feeding and management strategies, which would result in improved health during the period of greatest risk of disease.

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Appendix A

A1.1 Neutrophils

The investigation into neutrophil function over the transition period is a key part of this thesis. Therefore, this appendix is included to supplement the review of the innate immune system. The majority of research into neutrophil function has been conducted on mouse and human cells. Therefore, much of the currently-available molecular research on neutrophil function needs to be extrapolated for use in bovine haematology research. Several good reviews discussing this research are available (e.g., Nathan, 2006; Kumar and Sharma, 2010; Mayadas et al., 2014). Paape et al. (2003) summarised the structure and function of the bovine neutrophil and provided a good overview, with particular focus on neutrophil structure and response to mastitis infection.

Neutrophils are the first leukocyte to be recruited from the blood to infected tissue. They first attach to adhesion molecules expressed by endothelial cells (Ley et al., 2007) and extravasate through the endothelial wall to the target pathogen in a directed manner via chemotaxis (Muller, 2013). Neutrophils must then be able to identify the invading pathogen (opsonisation) and engulf (phagocytose) the pathogen, where it is destroyed within the neutrophil by respiratory burst, enzymes, or antimicrobial peptides. They do not undertake repeated phagocytosis and their life span is relatively short. The half-life of a circulating neutrophil in mice is generally accepted as approximately 11 hours and in a healthy human, 6-8 hours. Pillay et al. (2010a) challenged this duration of neutrophil half-life through an in vivo study using deuterium-labelled water (^2H_2O) which was incorporated as ^{2}H-labelled adenosine into the DNA of cells in the bone marrow. The authors of this study concluded that the neutrophil half-life of mice is 12.5 hours and therefore, similar to previous data from mice (Basu et al., 2002). However, the circulating neutrophil half-life in humans was estimated as 3.8 days or an expected lifespan of 5.4 days, more than 10-fold longer than generally accepted. Unfortunately, the authors failed to adequately discuss this considerable discrepancy in contrast to *ex vivo* studies. For example, they did not acknowledge the possibility of deuterium-labelled adenosine being re-incorporated into newly synthesised neutrophils after the destruction of previously labelled neutrophils in the bone marrow (another site of neutrophil destruction), which would give the appearance of a longer life-span in urine or serum where neutrophil destruction was measured by ^{2}H-adenosine release by degraded cells. They also failed to adequately discuss neutrophil differentiation and maturation in the bone marrow, where the ^{2}H-adenosine is incorporated, and that bone marrow neutrophils were undoubtedly labelled also, thereby overestimating the lifespan in blood. Despite being highly cited, this paper is not without controversy (Tofts et al., 2011; Turner et al., 2011; Vrisekoop et al., 2011).

Neutrophils have traditionally been labelled as the 'foot-soldiers' of the innate immune system, their role being to seek out and destroy invading pathogens without any interaction with other cells of the adaptive immune system. It has been established that this simple explanation to describe the role of neutrophils is not accurate and these cells are indeed mediators of inflammation with the ability to signal and communicate with neighbouring cells and influence the adaptive immune system, reviewed in Kumar & Sharma (2010).

A1.1.1 The life and death of a neutrophil

Neutrophils are produced in the bone marrow from myeloid precursors. Up to 2×10^{11} cells per day are produced in a healthy human (Borregaard, 2010) and cow (Paape et al., 1979). Under normal circumstances approximately 1-2% of mature neutrophils are in circulation, therefore, there is a large mature pool of neutrophils stored in the bone marrow, primed for release after induction of an inflammatory event (Athens et al., 1961; Semerad et al., 2002). Neutrophils mature through the myeloid lineage as follows; myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and finally mature segmented polymorphonuclear cell.

A1.1.2 Neutrophil maturation

Within the bone marrow, there are three pools of neutrophils at different stages of maturation (da Silva et al., 1994). These are known as 1) the mitotic pool (myeloblasts, promyelocytes, and myelocytes), 2) the post-mitotic or maturation pool (metamyelocytes and band cells) and 3) the storage pool (segmented neutrophils and some band cells). Each of these cell types differ morphologically, for example, in size, nucleus morphology, and function. During the process of maturation, there is sequential acquisition of differing functional events indicative of differing stages of development. Van Merris et al. (2002) investigated functional events that occur during neutrophil maturation in bovine bone marrow. To do this, the authors investigated surface protein expression of CD11b and the Fc-IgG_2 receptor by flow cytometry, as well as phagocytic capacity, respiratory burst activity, and myeloperoxidase (MPO) activity. This study identified functional properties in cells at different stages of granulopoiesis: myeloperoxidase activity → Fc-IgG_2 receptor expression → CD11b expression → complement mediated phagocytosis → respiratory burst activity (Van Merris et al., 2002). Immature bovine neutrophils, i.e., prior to the band stage, do not exhibit phagocytic capability, whereas, this property is exhibited in immature human neutrophils as early as the promyelocyte stage (Silvaet al., 1989). This discrepancy highlights the functional differences between bovine neutrophils and human neutrophils at different stages of maturation, which is an important point of difference for this thesis.

A1.1.3 Neutrophil granules

Further markers of cells at different stages of neutrophil differentiation are the sequential appearance of granules, granule proteins, and transcription factors (Faurschou and

Borregaard, 2003). Granules are formed by the budding of small vesicles of the Golgi and can be identified from the promyelocyte stage. Granules at this stage were originally known as 'peroxidase-positive' granules due to the presence of MPO, they are also known as azurophil granules due to their affinity for azure A or simply "primary" granules (Bainton and Farquhar, 1968). At the transition from promyelocyte to myelocyte, the production of MPO ceases and the granules are known as 'peroxidase-negative' granules. Peroxidase-negative granules are further subdivided into specific or "secondary" granules which are observed in myelocytes and metamyelocytes, and gelatinase or "tertiary" granules in band cells. Peroxidase-negative granules contain three physiologically significant metalloproteinase (MMP) proteins; MMP-8, MMP-9, and MMP-23. Mature, segmented neutrophils have secretory vesicles packed with membrane proteins that are required in an acute inflammatory event. Such proteins include Mac-1 (CD11b/CD18), complement receptor 1, lipopolysaccharide/lipoteichoic acid-receptor (CD14), and FcγIII receptor (CD16).

To meet increased demand for neutrophils during an infection, a cytokine-controlled feedback mechanism is in place to regulate granulopoiesis. Granulopoiesis is controlled by granulocyte colony stimulating factor (G-CSF) which is under the control of IL-17A released by T cells (γδ and natural killer) (Forlow et al., 2001). IL-17A is in turn regulated by IL-23 released from tissue-resident macrophages and DCs at the infection site (Stark et al., 2005). During inflammation, neutrophils are recruited in response to cytokine and chemokine release, e.g., IL-1 and TNFα, and will undergo apoptosis after ingestion of bacteria. Stark et al. (2005) provided evidence in mice that phagocytic cells release cytokines after ingestion of apoptotic neutrophils to regulate neutrophil homeostasis. Apoptotic neutrophils are removed by macrophages and DCs, which leads to down-regulation of IL-23 production and therefore, curbs production of IL-17A and G-CSF, leading to reduced granulopoiesis to close the 'feedback loop' (Stark et al., 2005). Despite that much of the research is conducted in mice, the fundamental mechanisms are likely to be the same in cows. Administration of bovine G-CSF in calves stimulates neutrophil maturation in bone marrow with mature neutrophilia 24 hours after injection, followed by a 'left shift' with an increased number of band neutrophils present in the blood 2 to 3 days after treatment (Cullor et al., 1990). In this study by Cullor et al. (1990), 11 late lactation cows housed in concrete stalls offered alfalfa hay and grain were used, whereas in the current thesis, 45 (Holstein-Friesian and Jersey) periparturient/early lactation grazing cows offered fresh pasture or pasture silage and grain were used. The difference in housing and feed in the study by Cullor et al. (1990) might have influenced changes in the immune system due to stress or nutrition, respectively, as compared with the current thesis (Chapter 4).

A1.1.4 Neutrophil death

During an inflammatory event, neutrophils will undergo apoptosis in tissue and be removed by tissue-resident macrophages and DCs. In the absence of inflammation, neutrophils are resorbed from the blood stream by the spleen or liver by macrophages of the reticuloendothelial system or bone marrow (Furze and Rankin, 2008). A marker of neutrophil aging is the up-regulation of CXCR4 expression. The CXCR4 receptor is involved in neutrophil retention, demonstrated by the observation that administration of a CXCR4 antagonist (AMD3100) results in an increase in circulating neutrophils (Hendrix et al., 2004) and hematopoietic progenitor cells (Broxmeyer et al., 2005) in mice and humans. Furthermore, homozygous knockout of *CXCR4* in mice is embryonic lethal and the embryos exhibit hematopoietic defects (Ma et al., 1998; Zou, Kottmann, & Kuroda, 1998). Perhaps more applicable to the current study, was the investigation by Eash et al. (2009) who selectively deleted *CXCR4* in myeloid cells. Results indicated neutrophilia in peripheral blood of knockout mice and reduced homing of neutrophils to bone marrow, which further supports CXCR4 signalling as an important mediator of neutrophil retention in the bone marrow and as an indicator of neutrophil aging (Eash et al., 2009).

Once in the circulation, neutrophil apoptosis is controlled by the expression of several anti-apoptotic proteins and their pro-apoptotic counterparts. Human neutrophils express several cell cycle regulatory proteins such as cyclin dependent kinases (CDKs) (Rossi et al., 2006), survivin (Altznauer et al., 2004), pro- and anti-apoptotic proteins from the B cell lymphoma (Bcl-2) family, inhibitors of apoptosis (IAPs), and proliferating cell nuclear antigen, which is crucial for DNA synthesis and repair (Witko-Sarsat et al., 2010). Members of the Bcl-2 family that inhibit apoptosis include Bcl-2, Bcl-X_L, and Mcl-1 and members that are pro-apoptotic include Bad, Bax, Bcl-X_S, Bak, and Bik. Expression of Bad, Bax, Bak, and Bik is largely stable, whereas expression of Mcl-1 mRNA and protein is short-lived (~3 h) and is regulated by exogenous cytokines (Moulding et al., 2001). Therefore, prevention of apoptosis in neutrophils is largely reliant on Mcl-1. Mcl-1 expression is essential for the survival of myeloid-derived hematopoietic stem cells, evidence of which came from mice deficient in Mcl-1 (Zhou et al., 1998). Expression of Mcl-1 mRNA is greater in haematopoietic stem cells, which decreases with differentiation e.g., to common myeloid progenitor cells (Opferman et al., 2005). Collectively, research into Mcl-1 expression in neutrophils indicates it is critical for neutrophil survival, as reviewed by Murphy & Caraher (2015). The role of Mcl-1 in bovine neutrophils has not been investigated in depth. One study indicated that inhibition of NF-κB induces apoptosis in bovine neutrophils (Notebaert et al., 2005), which has also been demonstrated with human neutrophils (Ward et al., 1999). Therefore, we assume the onset of apoptosis in bovines is similar to that in mice and humans.

A1.1.5 Neutrophil extravasation

Inflammatory signals (such as pro-inflammatory cytokines and leukotrienes) are released in response to injured or infected tissue by sentinel leukocytes at the infection site (Day and Schultz, 2014). These signals induce changes in molecules on endothelial cells that enable neutrophil adhesion; reviewed in Springer (1994). Springer (1994) discusses the exit of neutrophils from the blood stream and binding of cell surface adhesion molecules to adhesion molecules on the apical surface of endothelial cells. That review identified neutrophil extravasation as a multistep process, however this was not always the case and has been built on from a two-step model (von Andrian et al., 1991). The process of neutrophil localisation is now widely regarded as a multistep process involving initial attachment or tethering, rolling, adhesion (arrest and firm adhesion), and transmigration (Ley et al., 2007; Kolaczkowska and Kubes, 2013). These steps are mediated by selectin and integrin molecules on the outer membrane of the neutrophil and apical membrane of the endothelial cell.

The selectin molecules, P-selectin and E-selectin, are present on activated endothelial cells for the arrest of neutrophils, and maximise neutrophil recruitment (Petri et al., 2008). They possess lectin domains that protrude far from the cell wall to form carbohydrate-protein bonds to neutrophils during the process of rolling (Kansas, 1996). L-selectin (CD62L) is an important selectin expressed on neutrophils and contributes to slow down and initiate neutrophil cell rolling on the endothelium. L-selectin also contributes to rolling by the ability to bind to previously arrested neutrophils on the surface of the endothelium, thereby facilitating further recruitment of neutrophils throughout inflammation (Bargatze et al., 1994). It is clear that L-selctin is important for neutrophil trafficking through experiments that block or alter L-selectin expression on neutrophils. For example, neutrophil infiltration into inflamed tissue is greatly reduced in L-selectin-deficient mice compared with wild-type mice (Tedder et al., 1995). Furthermore, blocking of L-selectin using monoclonal antibodies reduces neutrophil migration and has negative implications on clinical outcome (Ma et al., 1993; Bosse and Vestweber, 1994). These studies highlight the importance of L-selectin for homing to non-lymphoid tissues during inflammation.

After leukocyte rolling along the endothelium, inflammatory signals induce firm adhesion, which is mediated by integrin molecules like the B2 integrins CD11a (*ITGAL*), CD11b

(*ITGAM*), CD11c (*ITGAX*), and CD18 (*ITGB2*). These integrin molecules form heterodimeric membrane proteins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and inactivated-C3b receptor 4 or P150, 95 (CD11c/CD18) (Hynes, 1987, 1992). The importance of Mac-1, LFA-1 and P150, 95 β_2 integrin complexes is evident by the clinical manifestations of leukocyte adhesion deficiency (LAD). LAD is a rare genetic disorder presenting as recurrent bacterial infections, impaired pus formation, and impaired wound healing (Anderson and Springer, 1987). Early reports were recognised in the 1970's and noted defective neutrophil movement, chemotaxis, and phagocytosis in patients with recurring bacterial infections (Niethammer et al., 1976; Issekutz et al., 1979). Now, three LAD conditions have been identified: LAD I, II, and III, which are the result of defects in a variety of leukocyte adhesion molecules and therefore affect different steps of the adhesion cascade (Hanna and Etzioni, 2012). LAD II is a defect in selectins and, therefore, affects neutrophil rolling; LAD III is due to abnormal integrin activation; and LAD I, the most common, results from a β_2 integrin deficiency (CD18) and, therefore, affects firm adhesion as a result of defective integrin complexes Mac-1, LFA-1, and P150, 95 (Schmidt et al., 2013). LAD has not only been described in humans, but also animals (Kehrli et al., 1992; Zimmerman et al., 2013). Cows with LAD exhibit recurrent pneumonia, persistent neutrophilia, enteritis with bacterial overgrowth, and death at an early age (Nagahata et al., 1987; Takahashi et al., 1987). The molecular deficiencies underlying these disorders reveal the importance of a functional adhesion cascade for the efficient resolution of infection.

Ding et al. (1999) discussed the relative contribution of LFA-1 and Mac-1 in human neutrophil adhesion and emigration. These complexes have overlapping functions and their individual roles were investigated using mice with deficient LFA-1 (CD11a -/-), Mac-1 (CD11b -/-), and both LFA-1 and Mac-1 (CD18 -/-) (Ding et al., 1999). Results indicated that LFA-1 is likely to have a greater role in adhesion, whereas Mac-1 is likely to be more involved in steps after transendothelial migration. Integrin activation occurs through conformational change by activation of P-selectin glycoprotein ligand 1 (PSGL-1) or

CXCR2 upon binding of their respective ligands E/P-selectin or CXCL1 (Lefort and Ley, 2012). Activation of LFA-1 through CXCR2 signalling requires co-activators talin-1 and kindling-3 (Lefort et al., 2012).

Neutrophil adhesion and subsequent extravasation using the described molecules above is considered the classical neutrophil recruitment cascade. This has largely been elucidated using intravital microscopy, which allows in vivo visualisation of cells. Advancements in technology have indicated that there are exceptions to the classical recruitment (Megens et al., 2011). In several tissues (e.g., liver, lung, and brain), neutrophils can be recruited without selectins and/or integrins. For example, neutrophils recruited to the sinusoidal capillaries of the liver directly adhere and do not roll across the hepatic endothelium via CD44-hyaluronan interactions (McDonald et al., 2008). Whatever the mechanism, adhesion and extravasation is required for neutrophils to reach the site of infection and continue the inflammatory cascade.

A1.1.6 Summary

The literature reviewed in this appendix aims to justify the choice of gene targets for the neutrophil gene expression analysis experiments in chapters 4 and 5. The scope of the literature described here includes information pertinent to the capacity of circulating neutrophils, as the cells used in this thesis were isolated from whole blood. Circulating neutrophils have the capacity for destruction of bacteria, which becomes evident only when they migrate from circulation into tissue. By investigating gene expression in these cells, we can gather an understanding of their functional capabilities.

Appendix B

B1.1 Supplementary Tables

Supplementary Tables are to follow with table legends as below.

Supplementary Table 1. Primer information for genes investigated in neutrophils.
Gene symbol; presented in functional groups, gene name, mRNA accession number, gene ID, universal probe number and sequence (Roche, Germany), forward and reverse primers, the intron/exon boundary (if no boundary, only a single exon location is presented in brackets), amplicon size (number of nucleotides; nt), primer length, and the RT-qPCR efficiency using the Fluidigm Real-Time PCR Analysis software.

Supplementary Table 2. Effect of calving body condition and pre-calving feeding level on gene expression in circulating neutrophils.
Body condition score (BCS) comparison consists of cows calving at BCS 4.0 vs 5.0; 10-point scale (approximately 2.75 and 3.25 respectively, on a 5-point scale). Feeding level (FL) comparison consists of cows offered 75% vs 125% of maintenance requirements. Least squares means of overall treatment effect across all weeks are presented. Delta Ct was calculated relative to the geometric mean of two endogenous control genes (*RPL19* and *YWHAZ*). Statistical significance was calculated using repeated measures ANOVA for the effect of treatment (BCS or FL) and the interaction between treatment and time (Wk).

Supplementary Table 3. Raw normalised RNA sequencing read count data for differential gene expression analysis between risk groups. N.B., Table not included
Microsoft Excel file with normalised RNAseq count data (using EdgeR) for differential gene expression analysis between risk groups using repeated measures ANOVA. File also

demonstrates hierarchical grouping of cow groups; time point post-calving (week 0, 1, 4), risk group; ‘High Risk’ = hi, ‘Low Risk’ = lo, and individual cow ID number.

Supplementary Table 4. List of all proteins identified from LC-MS/MS analysis of plasma exosomes from ‘High Risk’ and ‘Low Risk’ cows.

Each tab of the Excel file contains a list of proteins identified in plasma exosomes of ‘High Risk’ (HR) and ‘Low Risk’(LR) cows at each time point; day of calving (d 0), week 1 post-calving (wk 1) and week 4 post-calving (wk 4). Lists consist of protein name, UniProt accession number, and associated gene ID (http://www.uniprot.org/).

Supplementary Table 5. List of all proteins identified from LC-MS/MS analysis with representative peptide accession number.

Control samples are MDBK cells cultured in media supplemented with 5% exosome-depleted foetal bovine serum. ‘Low Risk’ samples are MDBK cells cultured with exosomes isolated from plasma of ‘Low Risk’ cows and ‘High Risk’ samples are MDBK co-cultured with exosomes isolated from the plasma of ‘High Risk’ cows. Relative abundance (%) was calculated using the cumulative peptide signals for a protein relative to the total signal of that sample. Where no peptide was identified for a given protein, a numerical placeholder of 0.01% was used (grey values). Significance of Risk was calculated using two-sample t-test (proc mixed, SAS 9.3) if ≥ 2 cows expressed the protein with abundance $>0.01\%$ in either the ‘Low Risk’ or ‘High Risk’ group.

Supplementary Table 1. Primer information for genes investigated in neutrophils.

Function and gene	Gene name	Accession number	GeneID	Universal Probe #	Probe Sequence	Forward	Reverse	Exon Boundary	Amplicon Size (nt)	Fluidigm Efficiency
Antimicrobial										
BNBD4	Defensin, beta 4A	NM_174775.1	286836	112	CUGUCUGC	CTCCTCGCAGTCCTCTTCC	CGGCAGCTTTGAGGATTTC	Exon 1/2	77	2.09
DEFB1	Defensin, beta 1	NM_175703.3	281743	3	CUGCUGGG	TCCTCACACTTCTCTTCCTGGT	GACGGCAGCTTAGAGGATTACT	Exon 2/3	78	2.42
DEFB10	Beta-defensin 10	NM_001115084.1	100141457	115	UCUGGGUC	GCTCCTCTTGGTGGTCCTG	TTCCCCCAGCAGCTTAGATA	Exon 1/2	75	1.72
LAP	LAP lingual antimicrobial peptide	NM_203435	403090	3	CUGCUGGG	CTTGCGCTCCTCTTCCTG	GCAGCTTTGAGAATTTCTTACTCC	Exon 1/2	72	2.00
MMP8	MMP8 matrix metallopeptidase 8 (neutrophil collagenase)	XM_003585096	100336575	80	CCUGGAGA	CTGAACGTGACAGGGAAGC	CTCCACAGCGAGGCTTTCT	None (2)	64	2.04
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type	NM_174744	282871	61	CUGGGCAA	TCGACGATGAAGAGTTGTGG	CGCGTTTCCGAAGTAGGTC	Exon 4/5	65	1.97
MPO	Myeloperoxidase	NM_001113298	511206	25	CTCCTCCA	AAGTGGATACCTCGGTGGTG	GGCCAAGCCACTGTGAAG	Exon 3/4	113	1.87
PRKCB1	Protein kinase C beta type	NM_174587	282325	110	AGCCUCUG	ACCAGGGGATGAAATGTGAC	TGTGGGCTTGGATGTAGATG	Exon 4/5	123	1.85
SOD1	Superoxide dismutase,	NM_174615.2	281495	61	CTGGGCAA	TGGGCCAAAAGATGAAGAGA	ACGATGGCAACACCGTTT	Exon 3/4	75	1.85
TAP	Tracheal antimicrobial peptide	NM_174776.1	286837	144	CCTCTTCC	GAGGCTCCATCACCTGCTC	GCTTACAGGATTTCCTACTCCTTG	Exon 1/2	88	2.00
XBP1	X-box binding protein 1	NM_001034727	541236	78	CTCCAGCT	CCGGAAGAAAGCTCGAATG	TCTCGTAAAACGTGATTTTCTAACAA	Exon 1/2	96	1.75
Inflammation										
CCL2	C-C motif chemokine 2 precursor	NM_174006	281043	61	CUGGCAA	CCAGCAGCAAGTGTCCTAAA	GCTTGGGGTCTGCACATAAC	Exon 1/2	75	1.49
CCL3	C-C motif chemokine 3 precursor	NM_174511.2	282170	82	CAGAGGAG	ACTCCGTCTCGCAGCATC	CGGGCGACATAGGAGAAG	Exon 1/2	140	1.90
IL1R2	Interleukin-1 receptor type 2 precursor	NM_001046210	515700	111	CUCAGCCU	CATCTCTCCCCACCAGACC	TCCCAGAAACACCTTACATGG	Exon 6/7	73	1.89
IFNG	Interferon, gamma precursor	NM_174086	281237	81	CCAGGGCC	CTTTTGGGTTTTTCTGGTTCTTAT	GCTACATCTGGGCTACTTGCAT	Exon 1/2	95	1.86
IL10	Interleukin-10 precursor	NM_174088	281246	133	GGAGAAGG	TGCGAAAACAAGAGCAAGG	GGCTTTGTAGACACCCCTCTC	Exon 4/5	78	2.22
IL12A	Interleukin-12 subunit alpha precursor	NM_174355	281856	98	CTGTGCCT	CTGGCTTCTGGAAAGACCTC	CACGTGGTACATCTTCAAGTCC	Exon 5/6	78	2.52
IL1RN	Interleukin-1 receptor antagonist protein precursor	NM_174357	281860	15	GAGCAGGA	GATGAGATCAAGCTCAAGTTAGAGG	GTTGTCGAAGCGGATGAAG	Exon 3/4	96	1.94
IL23A	Interleukin-23 subunit alpha precursor	NM_001205688.1	511022	134	GAAGGAGG	CTGGAGTGCACACCTACCAA	CGGGGGACATCATCTGTAGT	Exon 1/2	81	1.88
IL6	Interleukin-6 precursor	NM_173923.2	280826	68	AGGAGCAG	CCCTCCAGGAACAGCTATGA	GGTAGGGAAAGCAGAAGTCATC	Exon 1/2	100	2.00
IL8	Interleukin 8 precursor	NM_173925.2	280828	15	TCCTGCTC	CTGGCTGTTGCTCTCTTGG	CTGTACTCATTCTTGACAGAACTGC	Exon 1/2	79	1.90
TNF	Tumor necrosis factor	NM_173966.3	280943	35	AGAAGAGGA	GCCCTCTGGTTCAAACACTC	GGGCTACCGGCTTGTTACTT	Exon 2/3	60	1.76
TRAF6	TNF receptor-associated factor 6	NM_001034661	539124	128	CAGGAGCT	CCAGGCTGTTCAGACTTTAAGC	CAGAGGCATGGTACATTGAGG	None (7)	61	1.93
Immune mediator										
CD14	Monocyte differentiation antigen CD14 precursor	NM_174008	281048	74	CUGCUGCC	ATGGTGTGCGTGCCCTAC	GGACACAACAGAACCCTGC	Exon 1/2	78	1.85
CXCR1	C-X-C chemokine receptor type 1	NM_174360	281863	157	ATGGTGGT	TCATCTTCCGTGAGGCCTAT	GGGTAGGACACGCATTATCATC	None (1)	104	1.90
CXCR1 & 2	C-X-C chemokine receptor type 1 and C-X-C chemokine receptor type :	2 genes		159	GATGCTGG	TAAGCCTCCTGGGAAACTCC	AGTGACAGAGCGACCAATCC			1.95
CXCR2	C-X-C chemokine receptor type 2	NM_001101285	782719	48	ACUGGGAA	GAATCATGGCTGAAACAAAATTTAC	GCCAAAATCTTCATCGCTGT	Exon1/2	77	1.90
CXCR4	C-X-C chemokine receptor type 4	NM_174301.3	281736	55	GGAGAGGA	CTGTGAGCAGAGGGTCCAG	AAGAATGTCCACCTCGCTTG	None (2)	60	1.93
FCER1G	High affinity immunoglobulin epsilon receptor subunit gamma precursc	NM_174537.3	282226	81	GGCCCTGG	TGCTCTTACTCCTTTTGGTTGA	GATGGCATCCAGGATATAGCA	Exon 1/2	74	1.97
FCGRIA	High affinity immunoglobulin gamma Fc receptor I precursor	NM_174538	282227	85	TCCAGGTC	GAAATCCACAGTGATTGGCTACT	GGGTCCCCTTCTGTGAAGAC	Exon 3/4	62	2.42
BOLADRA	Major histocompatibility complex, class II, DR alpha precursor	NM_001012677	506214	14	TCTCCCAG	AACACCCCAAACACCAATG	GTCAATGAAGCAGATGAGTGTGT	Exon 2/3	96	1.91
MYD88	Myeloid differentiation primary response 88	NM_001014382.2	444881	101	GAGGAGGA	GACGTGCTGATGGAACTGG	CCTGCTGCTGCTTCAGAATA	Exon 1/2	70	1.93
PTPRC	Receptor-type tyrosine-protein phosphatase C isoform 4 precursor	NM_001206523	407152	155	UUGCCUUC	TGGCTTAAACTTCTGGCATTC	TACCCAACCTTCTACTCAAGATGA	Exon 2/3	89	1.91
SYK	Tyrosine-protein kinase SYK	NM_001037465	515515	129	TTCTGGCT	TGGAACCTACAGGGTCAAGC	TGTGGTGGCGATCAGTTTC	Exon 2/3	78	1.89
TLR2	Toll-like receptor 2 precursor	NM_174197	281534	17	AGGAGCUG	GGGATTCCTACTGGGTGGAG	TAAAGGGAGGGTTGAAGTGC	None (2)	63	1.89
TLR4	Toll-like receptor 4 precursor	NM_174198.6	281536	39	AGGTGGAG	CAGAACTGCAAGTGCTGGATT	GGATAGGGTTTCCCGTCAGT	Exon 2/3	111	1.92
Adhesion										
CD44	CD44 antigen precursor	NM_174013	281057	60	CUUCCCCA	ACGTACTGCTTCAATGCCTCA	CAGGTCCGTGACCGATGTA	Exon 3/4	60	1.90
ITGA4	Integrin alpha-4 precursor	NM_174748.1	282882	20	CTGGCTGG	ACAGATGCAAGGTTGGGAAG	CAAGGATCTCCAAAAGGGCTA	Exon 2/3	76	1.86
CPNE3	Copine-3	NM_001099142.1	615304	8	CUGCCUUC	TCCATCCAATCCCTACTGTAATG	GTTGGTCGGTCCATAGAGTTTT	Exon 11/12	91	1.93
CD47	Leukocyte surface antigen CD47 precursor	NM_174708	282661	132	UGCUGCUC	GTGGCCCCTGGTAGTGGT	TGATTGCATTAAATATTAGTTGAGCTG	Exon 1/2	74	1.74
ITGAL	Integrin alpha-L precursor	NM_198221.2	281874	26	CUGGGCUG	CCCCACAAGTGACAACCTG	AGATGTTCTGGTCACATGTTCG	Exon 4/5	65	1.97
ITGAM	Integrin alpha-M precursor	NM_001039957	407124	66	GGCTGCTG	GGGCTTGTCTCTTGCATTTG	ACAGTGCACCAAATTTGCAA	Exon 4/5	75	1.97
ITGAX	Integrin alpha-X precursor	XM_00522497	100298252	147	UUGAUGGC	AGGACCCGGGTTTTTCTCT	GCTCTGTGTCCAAGTTGAAGC	Exon 1/2	70	1.95
ITGB2	Integrin beta-2 precursor	NM_175781	281877	42	GCUGGAUG	GCACACAAACTGGCAGAAAG	TGCAGACTTGGGGATGATCT	Exon 8/9	102	2.03
LGALS8	Galectin-8	NM_001045954	506977	83	GGUGGCUG	TCGTGATCATGGTCCTGAAA	GCAGGGTATGCCTTCCATT	Exon 4/5	60	1.96
SELL	L-selectin precursor	NM_174182	281485	37	CCAGGGCA	TGGGTCTGGATTATGCTCTGT	CGTTCTGCAGGGAAAATTACA	Exon 2/3	124	1.98
TLN2	Talin-2	XM_010811790.1	528252	139	TTGGCTTC	GGAGCTGAAAAGAGAATATTTCAGG	CGCGCCAATTTGACATACT	Exon 9/10	89	2.05
Maturation										
ANXA3	Annexin A3	NM_001035325.1	518050	66	GGCTGCTG	ACTGAGAGGACGAATGCACA	GAGAGATCACCCTTCAAGTCATC	Exon 4/5	95	1.88
BCL2	Apoptosis regulator Bcl-2	NM_001166486.1	281020	75	GGAGGCTG	GCACCTGCACACCTGGAT	CTAGGGCCATACAGCTCCAC	Exon 1/2	66	2.02
CASP8	Caspase-8	NM_001045970	507481	63	AGGAGGAG	TGGATCTGCTGCTTAACTACTTGTAT	CTGAAATCTGGAAAAGCAGAATC	Exon 4/5	114	2.13
CDC42	Cell division control protein 42 homolog precursor	NM_001046332.1	532712	58	CTCCATCC	TGTATTTCTAGTCTGCTTTTCAGTGGT	GTCTTTGGACAGTGGTGAGTTATC	Exon 3/4	96	1.86
DAXX	Death domain-associated protein 6	NM_001075155	504336	162	TCCTGGCC	TGATCCCGCACTGTCAGAT	GCCAAGCTTCGGTTCTCC	None (4)	60	1.84
FADD	FAS-associated death domain protein	NM_001007816.1	493720	135	ATGGCTTC	GGCACCTCGGAGTATCTGAC	GGTTCCGGGGGTACTTCTC	None (2)	60	2.05
FASLG	Tumor necrosis factor ligand superfamily member 6	NM_001098859.2	407111	49	GGCCACCA	GCCTGTGTCTCCTTGTGATG	AAATATCCCCAGCCCCAAT	None (1)	68	1.85
FCGR3A	Low affinity immunoglobulin gamma Fc region receptor III isoform 2 p	NM_001077402	281766	50	GCUCCAGA	CAGAGTCTGTGCAGATCACTGTT	CCAAGGTAGAAAGAATGACGAGA	Exon 4/5	71	1.93
KIT	Mast/stem cell growth factor receptor Kit precursor	NM_001166484	280832	153	GACGAGGA	GGATTTCCTCTTCGTTCTGCT	CTGGACTCACAGATGGCTGA	Exon 1/2	71	2.02
MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1 homolog	NM_001099206	788087	124	CTTCAGGC	CCCACCAGACTGCTGTTCTT	GGAGATCGGGGATTCCAT	None (1)	72	1.91
RIPK1	Receptor-interacting serine/threonine-protein kinase 1	NM_001035012	504727	100	CTGTGAGC	CCAACAAGGAGCCATACGAG	TTCCCAGACTTGATGCACAG	Exon 5/6	67	1.81
S100A9	Protein S100-A9	NM_001046328	532569	143	AGAAGAAGA	GCAAAAAGAGCTGCCAAACT	TGTGTCCAGATCCTCCATGA	Exon 2/3	82	1.93
THY1	Thy-1 membrane glycoprotein precursor	NM_001034765.1	614712	119	ACCACCAA	TCAAGGTCCTCTACCTGTCCA	CCAGAGAGGCGGAGTTCAC	None (3)	73	1.89

Transcription factor										
NCOR1	Nuclear receptor corepressor 1	NM_001193059	507663	18	CAGCAGGA	TCAGTACACCTTTCCCAGCA	AAGATGAGAAGAACGGTAATCAGG	Exon 2/3	70	1.88
NCR3	Natural cytotoxicity triggering receptor 3 precursor	NM_001040524	513769	69	GGAGGAAG	GAATGGGACCCTGCTGGT	ATAGAATCCAGCCCGAAGGA	Exon 4/5	82	1.98
NFKB1	Nuclear factor NF-kappa-B p105 subunit	NM_001076409	616115	103	CTCTTCCC	AGGACAGCTGGATGTGTAACTG	CCTCTTCATAAAATCGAATCTGG	Exon 9/10	94	1.92
PPARA	Peroxisome proliferator-activated receptor alpha	NM_001034036.1	281992	110	AGCCTCTG	CCCTGAACATCGAGTGTAGGAT	CAGCCGAATCGTTCTCCTAA	Exon 4/5	107	1.74
PPARD	Peroxisome proliferator-activated receptor delta	NM_001083636.1	353106	108	GAGAGCAG	TTCCAGCAGCTACACAGACC	CAGTTGGTCCAGCAGTGAGA	Exon 3/4	61	2.00
PPARG	Peroxisome proliferator-activated receptor gamma	NM_181024.2	281993	165	GAAGCCCA	GCGACTTAGCAATATTTATAGCTGTC	AGGCTTGCAGCAGATTGTCT	Exon 6/7	105	2.04
RXRA	Retinoic acid receptor RXR-alpha	XM_881943.5	507554	163	TGGACACC	TGCACGTACATCGCAACAG	TCTTGTCCATCTGCATGTCC	Exon 6/7	105	2.26
STAT3	Signal transducer and activator of transcription 3	NM_001012671.2	508541	130	CTGGACAC	ACCGGCCATTAGTCATCAAG	CTCAGGGAATTTAACCAGCAA	Exon 10/11	71	1.89
Leukotriene pathway										
ALOX5	Arachidonate 5-lipoxygenase	NM_001192792	404074	1	GCUCCAGG	GGAGTGGACTTTGTTCTGAACTACT	TTTTCTCAAAGTCGGCGAAG	Exon 4/5	109	1.82
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	NM_001076293	613869	26	CAGCCCAG	CACCTTCCTCGTCATGCTCT	CTTCTGCCTCACGAACAGGT	Exon 3/4	94	1.93
PLA2G4A	Cytosolic phospholipase A2	NM_001075864	525072	21	CAGAGCCA	TCTGGAAATGTCTCTTGAAGTTTG	TCCTTTCTCTGTTGTCTGAAAGC	Exon 6/7	96	2.09
Cellular metabolism										
ABCA1	ATP-binding cassette sub-family A member 1	NM_001024693.1	535379	103	CTCTTCCC	GGATATCCTGAAGCCAGTCCT	AAAGCTTTTGTGGCTTCACC	Exon 7/8	84	1.85
SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	NM_174602.2	282356	62	CAGCAGGT	GGCCTTTTTGTTAACCGTTTC	CATGAGCACAGCGGACAC	Exon 3/4	81	1.95
LDHA	L-lactate dehydrogenase A chain	NM_174099	281274	140	GGCAAAGA	CATGGCAGCCTTTTCCTTAG	AGCCTGGAGTTTGCTGTCAC	Exon 4/5	74	1.96
B cells										
CD19	B-lymphocyte antigen CD19 precursor	NM_001245998.1	517359	52	CTCCTCCC	CCTGGTGCTAATCATGAGGAA	AGTGCTCCACCCTATTCGAG	Exon 12/13	105	1.93
MS4A1	B-lymphocyte antigen CD20	NM_001077854	505653	100	CTGTGAGC	CAGGTGCCGTATATTAACATCAAC	GATCATCATCACTGCGAAATTG	Exon 5/6	126	1.88
T cells										
CD3E	CD3e molecule, epsilon (CD3-TCR complex) precursor	NM_174011.3	281054	10	GGAGGTGG	CACGCTGTACCTGAAAGCAA	ACACAGATGTCAACCACGATG	Exon 5/6	90	1.89
Did not work in neutrophils										
ARG1	Arginase-1	NM_001046154.1	513608	48	TTCCCAGT	ATGTGGACCCTGGGGAAC	ATGTTTCTTCCATCACCTTGC	Exon 5/6	105	-
Endogenous control genes										
ACTB	Actin, cytoplasmic 1	NM_173979.3	280979	122	TGCCCTGA	CATCACCATCGGCAATGA	TGATGGAATTGAAGGTAGTTTCG	Exon 3/4	107	1.88
B2M	Beta-2-microglobulin precursor	NM_173893	280729	7	CTTCTCCC	CCACCCCAGATTGAAATTGAT	CAGGTCTGACTGCTCCGATT	None (2)	66	1.89
G6PD	Glucose-6-phosphate 1-dehydrogenase	NM_001076124.2	538710	96	ACAGGCAG	AAAAGCCAACCTACAGATTTCG	TGAGCAGCAAGGTAGATTCG	Exon 3/4	103	1.91
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034.2	281181	9	CATCACCA	GAAGCTCGTCATCAATGGAAA	CCACTTGATGTTGGCAGGAT	Exon 4/5	67	2.00
GCH1	GTP cyclohydrolase 1	XM_010800681.1	286815	67	UGCUGGAG	AGCAATCATAGAGGCCTTGC	TTCTGCACTCCTCGCATTAC	Exon 3/4	87	1.87
GOLGA5	Golgin subfamily A member 5	NM_001098007	510412	157	ACCACCAU	CCAGCGTCAATTCTGTGAAC	TTTCATGGCCAGAATCTGAA	Exon 2/3	111	1.97
OSBPL2	Oxysterol-binding protein-related protein 2	NM_001035020	505328	125	CUUGGAGC	GCGTGTGGAGCATATTGAAG	GCTATGGGCATCGTGATCTT	Exon 4/5	64	1.90
PGK1	Phosphoglycerate kinase 1	NM_001034299.1	507476	71	GCAGCCAG	TGTCCCCATGCCTGATAAGT	CCACACAGTCCTTCAAGAACAA	Exon 3/4	95	1.98
RPL13A	60S ribosomal protein L13a isoform 1	NM_001076998.2	767925	157	ATGGTGGT	TCCCACCACCCTATGACAAG	CTTCAGACGCACAACCTTGA	Exon 5/6	68	1.89
RPL19	60S ribosomal protein L19	NM_001040516	510615	8	GAAGGCAG	GCGGATCCTCATGGAACAT	CGGGCTTCCTTGGTCTTAG	None (1)	105	1.89
RPL23	60S ribosomal protein L23	NM_001035014.2	504876	9	UGGUGAUG	CTCTGTGAAGGGGATCAAGG	GAATTACCACCGCTGGATGT	Exon 3/4	128	1.88
RPS9	40S ribosomal protein S9	NM_001101152.2	533892	138	ATCCACCA	TGAGGATTTCTTGGAGAGACG	ATGTTCACCACCTGCTTGC	Exon 4/5	126	1.85
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondı	NM_174178.2	281480	60	CTTCCCCA	CTGAAGCAGGTTTCAACACG	GTTGTCCTCCTCCATGTTCC	Exon 3/4	113	1.90
SMUG1	Single-strand selective monofunctional uracil DNA glycosylase	NM_001014958.1	539771	105	TGGGCATC	AATGATGAGCTGAGGCAACTG	GCGTATTCCACAGGGTTGTAG	None (2)	68	1.99
TBP	TATA-box-binding protein	NM_001075742.1	516578	106	AGCCAGAG	TTGTGCTTACCCACCAACAG	GGAGAACAATTCTCGGTTTGA	Exon 7/8	90	1.84
SNRPA	U1 small nuclear ribonucleoprotein A	NM_001046039.2	509802	24	GGGAGCTG	TTTTGTGGTCGGCACTCA	GCCATTGACAAAGTTGCTGA	Exon 1/2	87	2.08
UXT	Protein UXT	NM_001037471.2	525680	29	GGCAGAAG	GGCCCTTGGATATGGTTTTT	AGTCCTTGGTGAGGTTGTCG	Exon 5/6	110	1.90
YWHAZ	14-3-3 protein zeta/delta	NM_174814.2	287022	30	GGCTGAGG	TGAAAATGAAAGGAGACTACTACCG	GCTGTGACTGGTCCACAATC	Exon 3/4	84	1.90

Supplementary Table 2. Effect of calving body condition and pre-calving feeding level on gene expression in circulating neutrophils.

Function and gene	BCS 4.0	BCS 5.0	SED	*P* value	BCS*Wk	FL 75%	FL 125%	SED	*P* value	FL*Wk
Antimicrobial										
BNBD4	11.37	10.53	0.34	0.02	0.47	10.49	10.79	0.43	0.50	0.08
DEFB1	6.19	5.26	0.54	0.09	0.71	5.20	6.07	0.45	0.07	0.38
DEFB10	7.57	5.83	0.76	0.03	0.70	5.78	6.44	0.55	0.24	0.23
LAP	16.17	16.15	0.38	0.95	0.94	16.14	16.04	0.35	0.79	0.27
MMP8	9.37	8.45	0.45	0.05	0.12	8.50	8.56	0.54	0.91	0.42
MMP9	4.02	3.52	0.22	0.03	0.25	3.57	3.78	0.30	0.49	0.06
MPO	7.60	8.40	0.49	0.11	0.71	8.34	8.25	0.40	0.82	0.06
PRKCB1	2.34	2.35	0.11	0.87	0.94	2.37	2.47	0.10	0.37	0.73
SOD1	6.58	6.30	0.17	0.11	0.38	6.32	6.36	0.16	0.81	0.30
TAP	7.45	7.65	0.73	0.78	0.34	7.67	7.26	0.72	0.57	0.03
XBP1	17.33	17.59	0.26	0.31	0.08	17.53	16.99	0.39	0.17	0.06
Inflammation										
CCL2	12.80	12.83	0.66	0.96	0.78	12.71	12.38	0.70	0.64	0.81
CCL3	10.59	11.15	2.07	0.79	0.65	11.24	11.76	1.91	0.79	0.60
IL1R2	5.56	5.37	0.27	0.49	0.26	5.35	5.35	0.30	0.99	0.00
IFNG	21.29	19.74	1.74	0.38	0.21	20.07	20.08	2.08	1.00	0.64
IL10	4.14	3.65	0.22	0.03	0.86	3.66	3.78	0.23	0.63	0.02
IL12A	16.83	17.38	0.46	0.24	0.47	17.39	17.24	0.30	0.64	0.95
IL1RN	2.51	2.56	0.13	0.71	0.21	2.56	2.50	0.12	0.60	0.50
IL23A	3.43	3.92	0.17	0.01	0.92	3.91	3.74	0.16	0.29	0.11
IL6	7.77	7.63	0.30	0.64	0.15	7.69	7.92	0.31	0.47	0.59
IL8	2.25	2.35	0.10	0.34	0.67	2.36	2.26	0.10	0.35	0.79
TNF	5.56	5.81	0.24	0.32	0.58	5.82	5.90	0.19	0.66	0.98
TRAF6	2.65	2.75	0.13	0.41	0.57	2.74	2.72	0.12	0.86	0.28
Immune mediator										
CD14	13.66	13.67	1.18	1.00	0.04	13.39	12.66	1.32	0.58	0.96
CXCR1	2.92	3.02	0.17	0.55	0.80	3.06	3.12	0.18	0.71	0.38
CXCR1 & 2	30.12	30.71	0.45	0.20	0.37	30.70	30.25	0.41	0.29	0.25
CXCR2	3.83	3.80	0.19	0.88	0.87	3.81	3.83	0.19	0.93	0.62
CXCR4	4.48	4.49	0.17	0.96	0.40	4.51	4.39	0.15	0.44	0.64
FCER1G	6.39	6.36	0.44	0.96	0.44	6.38	6.57	0.40	0.63	0.14
FCGRIA	4.83	4.86	0.26	0.90	0.84	4.85	4.83	0.29	0.93	0.49
BOLADRA	2.90	3.04	0.12	0.26	0.96	3.05	2.96	0.11	0.46	0.24
MYD88	3.27	3.24	0.14	0.85	0.48	3.23	3.29	0.15	0.74	0.45
PTPRC	2.39	2.42	0.11	0.75	0.65	2.43	2.41	0.11	0.89	0.47
SYK	2.14	2.20	0.08	0.44	0.89	2.19	2.16	0.08	0.69	0.54
TLR2	2.54	2.53	0.16	0.96	0.96	2.53	2.59	0.13	0.66	0.45
TLR4	4.27	4.18	0.19	0.64	0.75	4.18	4.32	0.19	0.48	0.15
Adhesion										
CD44	2.29	2.39	0.11	0.37	0.75	2.37	2.38	0.12	0.98	0.01
ITGA4	4.38	4.51	0.13	0.34	0.48	4.51	4.55	0.13	0.73	0.67
CPNE3	5.88	5.75	0.25	0.60	0.06	5.79	5.72	0.21	0.72	0.96
CD47	5.15	5.28	0.20	0.49	0.16	5.31	5.33	0.18	0.91	0.18
ITGAL	2.58	2.51	0.11	0.57	0.51	2.52	2.58	0.11	0.55	0.62
ITGAM	2.50	2.64	0.14	0.36	0.98	2.61	2.51	0.13	0.44	0.55
ITGAX	2.64	2.62	0.11	0.86	0.13	2.64	2.76	0.13	0.35	0.41
ITGB2	2.02	2.03	0.09	0.87	0.36	2.05	2.10	0.07	0.47	0.76
LGALS8	3.20	3.18	0.12	0.84	0.83	3.17	3.21	0.12	0.77	0.99
SELL	3.01	3.04	0.16	0.83	0.61	3.04	3.03	0.16	0.97	0.35
TLN2	14.96	15.29	0.41	0.43	0.65	15.36	15.35	0.41	0.98	0.42
Maturation										
ANXA3	2.25	2.08	0.10	0.10	0.80	2.07	2.12	0.09	0.56	0.38
BCL2	3.22	3.36	0.15	0.38	0.83	3.35	3.42	0.14	0.58	0.61
CASP8	2.23	2.13	0.06	0.09	0.80	2.12	2.15	0.07	0.70	0.12
CDC42	1.94	1.91	0.09	0.74	0.45	1.89	1.99	0.09	0.30	0.11
DAXX	1.90	2.00	0.05	0.11	0.72	1.99	1.97	0.06	0.75	0.45
FADD	6.68	5.75	0.93	0.33	0.17	6.00	5.74	0.97	0.79	0.53
FASLG	3.88	4.10	0.15	0.16	0.84	4.10	4.08	0.17	0.87	0.46
FCGR3A	5.44	5.47	0.26	0.90	0.22	5.45	5.44	0.20	0.95	0.17
KIT	4.48	4.93	0.18	0.02	0.81	4.91	5.20	0.16	0.09	0.41
MCL1	3.13	3.13	0.15	0.97	0.36	3.12	3.12	0.15	0.99	0.40
RIPK1	2.62	2.63	0.13	0.91	0.35	2.63	2.69	0.11	0.60	0.29
S100A9	3.35	3.02	0.15	0.04	0.68	3.03	3.15	0.15	0.44	0.02
THY1	5.06	4.48	0.33	0.09	0.37	4.53	4.65	0.34	0.75	0.23
Transcription factor										
NCOR1	1.83	1.89	0.07	0.36	0.24	1.90	1.86	0.06	0.49	0.35
NCR3	2.87	2.98	0.16	0.51	0.46	2.98	2.96	0.13	0.84	0.38
NFKB1	1.94	1.97	0.08	0.79	1.00	1.97	1.95	0.08	0.75	0.52
PPARA	3.85	3.84	0.11	0.94	0.52	3.85	3.92	0.12	0.58	0.50
PPARD	3.41	3.22	0.17	0.27	0.51	3.22	3.35	0.17	0.46	0.32
PPARG	5.74	5.87	0.15	0.41	0.21	5.88	5.69	0.20	0.35	0.87
RXRA	4.95	5.17	0.20	0.29	0.38	5.22	5.14	0.20	0.68	0.61
STAT3	8.46	8.37	0.44	0.85	0.74	8.39	8.32	0.44	0.89	0.01
Leukotriene pathway										
ALOX5	4.73	5.00	0.37	0.46	0.83	4.91	5.05	0.33	0.68	0.33
ALOX5AP	2.97	2.65	0.14	0.03	0.41	2.66	2.82	0.17	0.35	0.03
PLA2G4A	3.02	2.93	0.14	0.51	0.40	2.93	2.95	0.16	0.89	0.04
Cellular metabolism										

ABCA1	2.93	3.15	0.18	0.21	0.01	3.16	3.01	0.19	0.41	0.90
SLC2A1	4.75	5.05	0.18	0.11	0.72	5.01	5.02	0.17	0.96	0.77
LDHA	3.39	3.31	0.14	0.59	0.61	3.32	3.34	0.14	0.87	0.20
B cells										
CD19	4.20	4.57	0.23	0.13	0.82	4.56	4.53	0.23	0.91	0.13
MS4A1	4.79	5.17	0.26	0.16	0.68	5.16	5.19	0.25	0.89	0.19
T cells										
CD3E	5.49	5.84	0.24	0.15	0.68	5.80	5.87	0.28	0.79	0.80
Endogenous control genes										
ACTB	2.62	2.58	0.10	0.72	0.68	2.59	2.66	0.10	0.48	0.80
B2M	4.63	4.95	0.20	0.14	0.25	5.01	4.77	0.18	0.18	1.00
G6PD	2.61	2.56	0.12	0.69	0.59	2.56	2.64	0.12	0.53	0.42
GCH1	3.15	3.12	0.12	0.79	0.50	3.11	3.27	0.11	0.18	0.06
GOLGA5	11.86	12.20	0.39	0.39	0.98	12.17	12.51	0.37	0.36	0.52
OSBPL2	2.93	2.93	0.11	0.98	0.55	2.94	2.93	0.11	0.88	0.22
RPL13A	2.80	2.89	0.10	0.37	0.65	2.89	2.90	0.11	0.93	0.34
RPL23	3.21	3.41	0.09	0.04	0.66	3.42	3.24	0.10	0.08	0.01
RPS9	2.84	2.76	0.11	0.48	0.94	2.74	2.75	0.11	0.93	0.39
SDHA	2.71	2.81	0.09	0.26	0.29	2.80	2.83	0.08	0.75	0.82
SMUG1	2.61	2.49	0.13	0.34	0.67	2.48	2.58	0.14	0.50	0.35
TBP	3.27	3.48	0.13	0.10	0.68	3.46	3.49	0.12	0.84	0.38
SNRPA	2.68	2.96	0.10	0.01	0.94	2.96	2.93	0.10	0.75	0.17
Did not work										
ARG1										
GAPDH										
PGK1										
UXT										

Supplementary Table 4. List of all proteins identified from LC-MS/MS analysis of plasma exosomes from High Risk (HR) and Low Risk (LR) cows.

Accession	Peptide Identification: HR Day 0	Gene
R9QSM8	Alpha-2-macroglobulin	A2M
B8Y9T0	Cumulus cell-specific fibronectin 1 transcript variant	FN1
B8Y9S9	Embryo-specific fibronectin 1 transcript variant	FN1
Q3SZZ9	FGG protein	FGG
A5PJE3	Fibrinogen alpha chain	FGA
F1MAV0	Fibrinogen beta chain	FGB
F1MGU7	Fibrinogen gamma-B chain	FGG
G5E5A9	Fibronectin	FN1
Q32S29	Histone H2B	H2B
A5D7N2	Histone H2B	HIST2H2BE
E1B8G9	Histone H2B	HIST3H2BB
E1BGW2	Histone H2B	HIST1H2BJ
F1MUD2	Histone H2B	HIST2H2BF
F1N453	Histone H2B	HIST2H2BE
F2Z4E8	Histone H2B	HIST1H2BB
F2Z4F9	Histone H2B	HIST1H2BN
G3MXP6	Histone H2B	LOC100336115
G8JL06	Histone H2B	HIST1H2BB
Q2KII5	Histone H2B	HIST1H2BI
F1MIF8	Histone H2B (Fragment)	
A4IFI0	IGLL1 protein	IGLL1
Q3SYR8	Immunoglobulin J chain	IGJ
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	IGL@
G3X690	Kinesin-like protein	KIF16B
G3N2Z6	Oncoprotein-induced transcript 3 protein	OIT3
F1MH22	Oncoprotein-induced transcript 3 protein	OIT3
G3MWJ7	Oncoprotein-induced transcript 3 protein	OIT3
A5PK72	Putative Uncharacterised protein	
F1MLW7	Uncharacterised protein	IGLL1
G3N0F3	Uncharacterised protein	
E1BNF9	Uncharacterised protein	SLAMF9
G3MZE0	Uncharacterised protein	
G5E5T5	Uncharacterised protein (Fragment)	IGHM
G3N0V0	Uncharacterised protein (Fragment)	
G5E513	Uncharacterised protein (Fragment)	IGHM
G3N028	Uncharacterised protein (Fragment)	
G3X7I5	Uncharacterised protein (Fragment)	

Accession	Peptide identification: HR Week 1	Gene name
G3MXP6	Histone H2B	LOC100336115
A5D7N2	Histone H2B	HIST2H2BE
E1B8G9	Histone H2B	HIST3H2BB
E1BGW2	Histone H2B	HIST1H2BJ
F1MUD2	Histone H2B	HIST2H2BF
F1N453	Histone H2B	HIST2H2BE
F2Z4E8	Histone H2B	HIST1H2BB
F2Z4F9	Histone H2B	HIST1H2BN
G8JL06	Histone H2B	HIST1H2BB
Q2KII5	Histone H2B	HIST1H2BI
Q32S29	Histone H2B	H2B
F1MIF8	Histone H2B (Fragment)	
Q3T101	IGL@ protein	IGL@
A4IFI0	IGLL1 protein	IGLL1
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	IGL@
Q2KHX5	Nuclear receptor coactivator 5	NCOA5
E1BMR0	Uncharacterised protein	NACC2
F1MM36	Uncharacterised protein	NCOA5
G3N0F3	Uncharacterised protein	
F6PMQ1	Uncharacterised protein	CCDC84
F1MLW7	Uncharacterised protein	IGLL1
G5E5H2	Uncharacterised protein	
G5E5V1	Uncharacterised protein	LOC100297594
E1B9N6	Uncharacterised protein	EVPL
F1MB40	Uncharacterised protein	LOC788599
G5E5T5	Uncharacterised protein (Fragment)	IGHM
G5E604	Uncharacterised protein (Fragment)	

Accession	Peptide identification: HR Week 4	Gene name
A5D7N2	Histone H2B	HIST2H2BE
E1B8G9	Histone H2B	HIST3H2BB
E1BGW2	Histone H2B	HIST1H2BJ
F1MUD2	Histone H2B	HIST2H2BF
F1N453	Histone H2B	HIST2H2BE
F2Z4E8	Histone H2B	HIST1H2BB
F2Z4F9	Histone H2B	HIST1H2BN
G3MXP6	Histone H2B	LOC100336115
G8JL06	Histone H2B	HIST1H2BB
Q2KII5	Histone H2B	HIST1H2BI
Q32S29	Histone H2B	H2B
F1MIF8	Histone H2B (Fragment)	
Q3T101	IGL@ protein	IGL@
A4IFI0	IGLL1 protein	IGLL1
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	IGL@
G3N0V2	Uncharacterised protein	KRT1
E1B991	Uncharacterised protein	KRT2
E1BMR0	Uncharacterised protein	NACC2
F1MLW7	Uncharacterised protein	IGLL1
G3MYU2	Uncharacterised protein	KRT77
G3MZ71	Uncharacterised protein	KRT2
G3N0F3	Uncharacterised protein	
G5E5H2	Uncharacterised protein	
G5E5V1	Uncharacterised protein	LOC100297594
G5E513	Uncharacterised protein (Fragment)	IGHM
G5E5T5	Uncharacterised protein (Fragment)	IGHM
F1N2J5	Uncharacterised protein (Fragment)	HAPLN4
G5E604	Uncharacterised protein (Fragment)	

Accession	Peptide identification: LR Day 0	Gene
F1MRD0	Actin, cytoplasmic 1	ACTB
B0JYQ0	ALB protein	ALB
B0JYN6	Alpha-2-HS-glycoprotein	AHSG
F1MIF8	Histone H2B (Fragment)	
Q32S29	Histone H2B	H2B
F2Z4E8	Histone H2B	HIST1H2BB
G8JL06	Histone H2B	HIST1H2BB
Q2KII5	Histone H2B	HIST1H2BI
E1BGW2	Histone H2B	HIST1H2BJ
F2Z4F9	Histone H2B	HIST1H2BN
A5D7N2	Histone H2B	HIST2H2BE
F1N453	Histone H2B	HIST2H2BE
F1MUD2	Histone H2B	HIST2H2BF
E1B8G9	Histone H2B	HIST3H2BB
G3MXP6	Histone H2B	LOC100336115
G3MYD7	Histone H3	LOC10029772
G3X807	Histone H4 (Fragment)	
A5D792	Histone H4	DCK
E1B7N2	Histone H4	HIST1H4I
G3N081	Histone H4	LOC100337473
E1B9M9	Histone H4	LOC525863
G3N2B8	Histone H4	LOC526226
E1BLC2	Histone H4	LOC781223
E1BBP7	Histone H4	
Q3T101	IGL@ protein	IGL@
A4IFI0	IGLL1 protein	IGLL1
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	IGL@
A6QNZ7	Keratin 10	KRT10
M0QVZ6	Keratin, type II cytoskeletal 5	KRT5
A4IFP2	KRT4 protein	KRT4
A5D7M6	KRT5 protein	KRT5
A4FV94	KRT6A protein	KRT6A
Q862P9	Similar to beta actin (Fragment)	
F1N6F5	Uncharacterised protein (Fragment)	CYP27C1
G5E513	Uncharacterised protein (Fragment)	IGHM
G5E5T5	Uncharacterised protein (Fragment)	IGHM
G3MXL3	Uncharacterised protein (Fragment)	KRT3
E1BIL2	Uncharacterised protein (Fragment)	KRT76
G3N0A8	Uncharacterised protein (Fragment)	UBR4
G5E604	Uncharacterised protein (Fragment)	
F1MLW7	Uncharacterised protein	IGLL1
G3N0V2	Uncharacterised protein	KRT1
G3MZ71	Uncharacterised protein	KRT2
E1B991	Uncharacterised protein	KRT2
M0QVY0	Uncharacterised protein	KRT6A
F1MUY2	Uncharacterised protein	KRT6C
F1N362	Uncharacterised protein	KRT84
G5E5V1	Uncharacterised protein	LOC100297594
E1BMR0	Uncharacterised protein	NACC2
G3MXX8	Uncharacterised protein	SCRT1
E1BNF9	Uncharacterised protein	SLAMF9
E1BHT5	Uncharacterised protein	UBR4
G3N0F3	Uncharacterised protein	
G5E5H2	Uncharacterised protein	

Accession	Peptide identification: LR Week 1	Gene
F1MK30	60S ribosomal protein L13	RPL13
Q3SZG7	60S ribosomal protein L13	RPL13
B0JYQ0	ALB protein	ALB
F1N4V6	ELAV-like protein (Fragment)	ELAVL3
F1MIF8	Histone H2B (Fragment)	
Q32S29	Histone H2B	H2B
F2Z4E8	Histone H2B	HIST1H2BB
G8JL06	Histone H2B	HIST1H2BB
Q2KII5	Histone H2B	HIST1H2BI
E1BGW2	Histone H2B	HIST1H2BJ
F2Z4F9	Histone H2B	HIST1H2BN
A5D7N2	Histone H2B	HIST2H2BE
F1N453	Histone H2B	HIST2H2BE
F1MUD2	Histone H2B	HIST2H2BF
E1B8G9	Histone H2B	HIST3H2BB
G3MXP6	Histone H2B	LOC100336115
G3MYD7	Histone H3	LOC100297725
Q3T101	IGL@ protein	IGL@
A4IFI0	IGLL1 protein	IGLL1
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	IGL@
Q05FF0	Interferon gamma receptor 2 (Fragment)	IFNGR2
A6QNR4	MTMR4 protein	MTMR4
G5E560	PH and SEC7 domain-containing protein 1	PSD
G3MZX1	Uncharacterised protein (Fragment)	C6orf132
G3N0M1	Uncharacterised protein (Fragment)	C6orf132
F1N6F5	Uncharacterised protein (Fragment)	CYP27C1
F1MIY4	Uncharacterised protein (Fragment)	GAS2L3
G5E513	Uncharacterised protein (Fragment)	IGHM
G5E5T5	Uncharacterised protein (Fragment)	IGHM
G3MZN6	Uncharacterised protein (Fragment)	PSD2
G5E604	Uncharacterised protein (Fragment)	
E1BLD1	Uncharacterised protein	HECTD1
F1MLW7	Uncharacterised protein	IGLL1
F1MZT1	Uncharacterised protein	LGI2
G5E5V1	Uncharacterised protein	LOC100297594
E1BNF9	Uncharacterised protein	SLAMF9
E1B793	Uncharacterised protein	STK36
E1B7F5	Uncharacterised protein	WDHD1
G5E5H2	Uncharacterised protein	
G3N0F3	Uncharacterised protein	

Accession	Peptide identification: LR Week 4	Gene
B0JYQ0	ALB protein	ALB
Q5GN72	Alpha-1-acid glycoprotein	AGP
R9QSM8	Alpha-2-macroglobulin	A2M
K4JBR5	Alpha-2-macroglobulin variant 1	A2M
K4JDT2	Alpha-2-macroglobulin variant 20	A2M
K4JR88	Alpha-2-macroglobulin variant 22	A2M
K4JF16	Alpha-2-macroglobulin variant 23	A2M
A5PJE3	Fibrinogen alpha chain	FGA
F1MAV0	Fibrinogen beta chain	FGB
F1MIF8	Histone H2B (Fragment)	
Q32S29	Histone H2B	H2B
F2Z4E8	Histone H2B	HIST1H2BB
G8JL06	Histone H2B	HIST1H2BB
Q2KII5	Histone H2B	HIST1H2BI
E1BGW2	Histone H2B	HIST1H2BJ
F2Z4F9	Histone H2B	HIST1H2BN
A5D7N2	Histone H2B	HIST2H2BE
F1N453	Histone H2B	HIST2H2BE
F1MUD2	Histone H2B	HIST2H2BF
E1B8G9	Histone H2B	HIST3H2BB
G3MXP6	Histone H2B	LOC100336115
G3MYD7	Histone H3	LOC100297725
Q3T101	IGL@ protein	IGL@
A4IFI0	IGLL1 protein	IGLL1
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	IGL@
A6QNR4	MTMR4 protein	MTMR4
F1MS24	Uncharacterised protein (Fragment)	CHM
F1N6F5	Uncharacterised protein (Fragment)	CYP27C1
G5E5T5	Uncharacterised protein (Fragment)	IGHM
G5E513	Uncharacterised protein (Fragment)	IGHM
F1N2I0	Uncharacterised protein	CHML
F1MLW7	Uncharacterised protein	IGLL1
E1B7S1	Uncharacterised protein	PARP12
E1BNF9	Uncharacterised protein	SLAMF9
G3N0F3	Uncharacterised protein	

Supplementary Table 5. List of all proteins identified from LC-MS/MS analysis with representative peptide accession number.

		Control				Low Risk							High Risk						Risk
Accession	**Protein Name**	1	2	**Mean**	**SD**	200	620	8639	9277	9307	**Mean**	**SD**	7671	8610	8616	9263	**Mean**	**SD**	*P*
P61603	10 kDa heat shock protein, mitochondrial	0.01	0.01	0.01	0.00	0.01	0.56	0.01	0.20	0.69	0.29	0.32	0.82	0.43	0.01	0.82	0.52	0.39	0.36
F1N6D5	116 kDa U5 small nuclear ribonucleoprotein component	0.01	0.01	0.01	0.00	0.01	0.01	0.05	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	
P63103	14-3-3 protein	0.51	0.01	0.26	0.35	0.41	0.80	0.76	0.44	0.47	0.58	0.19	0.01	0.01	0.01	1.11	0.29	0.55	0.30
E1BHA5	40S ribosomal protein	0.47	0.79	0.63	0.23	0.01	2.26	2.11	0.01	1.30	1.14	1.09	0.92	0.50	0.01	0.63	0.51	0.38	0.49
F1MUZ9	60 kDa heat shock protein, mitochondrial	0.45	0.71	0.58	0.18	0.55	0.44	0.09	0.01	0.42	0.30	0.24	0.01	0.49	0.01	0.71	0.30	0.35	0.99
E1BCL5	60S acidic ribosomal protein	0.01	0.01	0.01	0.00	0.01	0.58	0.09	0.01	0.07	0.15	0.24	0.01	0.11	0.01	0.68	0.20	0.32	0.80
Q3T087	60S ribosomal protein	0.72	0.55	0.63	0.12	0.20	0.85	0.48	0.01	0.33	0.37	0.32	0.01	0.38	0.01	1.00	0.35	0.47	0.93
Q0VCX2	78 kDa glucose-regulated protein	0.35	0.46	0.40	0.08	0.42	0.56	0.01	0.01	0.31	0.26	0.25	0.01	0.47	0.01	0.33	0.20	0.23	0.73
P60712	Actin, cytoplasmic	17.66	11.18	14.42	4.58	18.19	12.48	5.28	8.62	12.52	11.42	4.84	9.23	7.50	29.65	12.15	14.63	10.20	0.55
Q8SQH5	ADP/ATP translocase	0.01	0.01	0.01	0.00	0.09	0.01	0.01	0.01	0.21	0.07	0.09	0.01	0.01	0.01	0.01	0.01	0.00	0.25
B0JYQ0	ALB protein	5.71	2.28	4.00	2.43	6.73	1.45	1.74	2.75	1.66	2.87	2.22	2.56	0.97	13.12	0.45	4.27	5.96	0.64
Q5E962	Aldo-keto reductase family 1, member B1	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.06	0.02	0.03	
P16116	Aldose reductase	0.01	0.01	0.01	0.00	0.01	0.24	0.01	0.01	0.01	0.06	0.10	0.01	0.01	0.01	0.01	0.01	0.00	
Q5GN72	Alpha-1-acid glycoprotein	0.62	0.78	0.70	0.12	0.51	0.89	1.17	17.96	1.85	4.48	7.55	2.13	1.87	0.30	0.52	1.21	0.93	0.42
P34955	Alpha-1-antiproteinase	0.01	0.01	0.01	0.00	6.22	3.49	2.31	9.28	4.55	5.17	2.71	3.18	8.59	3.20	4.78	4.94	2.55	0.90
Q2KJF1	Alpha-1B-glycoprotein	0.45	0.43	0.44	0.02	0.34	0.36	0.01	0.82	0.01	0.31	0.33	0.01	0.63	0.01	0.40	0.26	0.31	0.84
B0JYN6	Alpha-2-HS-glycoprotein	11.82	11.51	11.66	0.22	15.52	6.89	7.82	21.42	16.62	13.65	6.17	26.87	29.76	3.73	12.61	18.24	12.24	0.48
Q7SIH1	Alpha-2-macroglobulin	0.01	0.01	0.01	0.00	0.01	0.01	0.01	31.29	0.44	6.35	13.94	0.01	0.18	0.01	0.01	0.05	0.09	0.40
A4ZZF8	Alpha-actinin	0.82	0.47	0.65	0.25	0.47	0.62	0.25	0.40	0.69	0.49	0.18	0.01	0.25	1.28	0.01	0.39	0.61	0.74
V6F832	Alpha-crystallin B chain	0.01	0.01	0.01	0.00	0.01	0.30	0.01	0.01	0.12	0.09	0.13	0.01	0.01	0.01	0.01	0.01	0.00	0.25
F1MB08	Alpha-enolase	1.15	0.72	0.94	0.31	1.37	1.06	0.01	0.01	0.70	0.63	0.61	0.01	0.73	0.69	0.91	0.59	0.40	0.90
Q3SZ57	Alpha-fetoprotein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	2.49	0.53	0.61	1.07	0.01	0.70	0.50	0.01	0.30	0.35	0.61

I6YIV1	Annexin	4.65	5.31	4.98	0.47	3.75	3.59	2.96	0.63	3.27	2.84	1.27	0.98	3.56	1.32	4.11	2.49	1.57	0.72
V6F9A2	Apolipoprotein A-I preproprotein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.16	0.01	0.04	0.07	0.01	0.05	0.01	0.01	0.02	0.02	
P81644	Apolipoprotein A-II	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.07	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.00	
E1B925	ARID1A	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	1.19	0.25	0.53	0.01	0.01	0.01	0.01	0.01	0.00	
F1MLB8	ATP synthase subunit alpha	0.65	0.68	0.67	0.02	0.68	0.73	0.45	0.01	0.51	0.48	0.29	0.01	0.23	0.59	0.57	0.35	0.28	0.53
Q0QEM9	ATP synthase subunit beta	0.29	0.44	0.36	0.11	0.33	0.86	0.15	0.01	0.56	0.38	0.34	0.01	0.14	0.46	0.01	0.16	0.21	0.28
P61283	Barrier-to-autointegration factor	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.07	0.02	0.03	
F1N6C0	Calmodulin	2.24	1.26	1.75	0.69	0.86	1.37	1.09	3.19	2.69	1.84	1.04	3.82	2.24	0.01	3.19	2.31	1.67	0.61
P00432	Catalase	0.01	0.01	0.01	0.00	0.18	0.01	0.80	0.01	0.01	0.20	0.34	0.01	0.01	0.01	0.01	0.01	0.00	0.31
G3MXG3	CCDC88A	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.13	0.01	0.04	0.06	
Q3T0Q6	Cellular nucleic acid-binding protein	0.01	0.01	0.01	0.00	0.01	0.13	0.01	0.01	0.19	0.07	0.08	0.34	0.01	0.01	0.08	0.11	0.16	0.64
Q5E9B7	Chloride intracellular channel protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.18	0.04	0.07	0.01	0.01	0.01	0.01	0.01	0.00	
F1MPU0	Clathrin heavy chain	0.08	0.01	0.04	0.05	0.01	0.01	0.01	0.01	0.09	0.03	0.03	0.01	0.04	0.01	0.01	0.02	0.02	
B0JYL8	Cofilin	0.67	1.05	0.86	0.27	0.85	0.01	0.82	0.60	0.67	0.59	0.34	0.01	0.96	0.01	1.08	0.51	0.59	0.82
G3X7A5	Complement C3	0.01	0.01	0.01	0.00	0.01	0.01	0.01	5.07	0.01	1.02	2.26	0.01	0.01	0.01	0.01	0.01	0.00	
Q3T0B6	Complement component 1 Q subcomponent-binding protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.07	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.00	
Q3T0E0	Copper transport protein ATOX1	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.04	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	
Q3MHY1	Cysteine and glycine-rich protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.26	0.06	0.11	0.01	0.01	0.01	0.01	0.01	0.00	
Q5EAD2	D-3-phosphoglycerate dehydrogenase	0.01	0.01	0.01	0.00	0.01	0.10	0.01	0.01	0.01	0.03	0.04	0.01	0.01	0.01	0.15	0.05	0.07	
A8E641	DPYSL5 protein	0.01	0.01	0.01	0.00	0.01	0.01	0.15	0.01	0.01	0.04	0.06	0.01	0.01	0.01	0.01	0.01	0.00	
O18787	Elongation factor	1.76	1.91	1.83	0.11	1.57	2.79	0.53	0.84	1.45	1.44	0.87	0.81	0.52	1.31	2.36	1.25	0.81	0.75
Q95M18	Endoplasmin	0.13	0.21	0.17	0.05	0.01	0.26	0.16	0.01	0.29	0.15	0.13	0.01	0.01	0.01	0.01	0.01	0.00	0.08

Q3SZ65	Eukaryotic initiation factor	0.01	0.01	0.01	0.00	0.15	0.12	0.01	0.01	0.15	0.09	0.07	0.01	0.01	0.01	0.17	0.05	0.08	0.48
Q2VYC3	Eukaryotic translation elongation factor	0.11	0.11	0.11	0.00	0.01	0.67	0.01	0.01	0.15	0.17	0.29	0.01	0.01	0.01	0.34	0.09	0.17	0.65
F1N647	Fatty acid synthase	0.01	0.01	0.01	0.00	0.01	0.17	0.01	0.01	0.01	0.04	0.07	0.01	0.01	0.01	0.05	0.02	0.02	
Q58D62	Fetuin-B	0.55	0.52	0.53	0.02	0.90	0.26	0.01	1.91	0.53	0.72	0.74	0.62	0.66	1.17	0.01	0.62	0.48	0.81
Q3SZZ9	FGG protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	5.50	0.01	1.11	2.45	0.01	0.01	0.01	0.01	0.01	0.00	
A5PJE3	Fibrinogen alpha chain	0.01	0.01	0.01	0.00	0.01	0.01	0.01	3.92	0.01	0.79	1.75	0.01	0.01	0.01	0.01	0.01	0.00	
F1MAV0	Fibrinogen beta chain	0.01	0.01	0.01	0.00	0.01	0.01	0.01	9.26	0.01	1.86	4.14	0.01	0.01	0.01	0.01	0.01	0.00	
P07589	Fibronectin	0.01	0.01	0.01	0.00	0.01	0.14	0.01	0.01	0.01	0.04	0.06	0.01	0.01	0.01	0.01	0.01	0.00	
F1N169	FLNA	0.01	0.01	0.01	0.00	0.01	0.44	0.01	0.01	0.01	0.10	0.19	0.01	0.01	0.01	0.38	0.10	0.18	
A6QLL8	Fructose-bisphosphate aldolase	0.01	0.01	0.01	0.00	0.30	0.01	0.01	0.01	0.15	0.10	0.13	0.01	0.01	0.01	0.01	0.01	0.00	0.23
P28801	Glutathione S-transferase P	0.01	0.01	0.01	0.00	0.01	0.83	0.01	0.01	0.14	0.20	0.36	0.01	0.01	0.01	0.01	0.01	0.00	0.33
P10096	Glyceraldehyde-3-phosphate dehydrogenase	2.74	3.16	2.95	0.30	2.53	1.60	1.37	1.97	2.33	1.96	0.48	1.64	0.93	3.86	2.59	2.26	1.27	0.64
P63243	Guanine nucleotide-binding protein subunit beta-2-like	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.24	0.01	0.07	0.11	
E9RHW1	Heat shock 27kDa protein	2.08	2.77	2.43	0.49	1.01	4.62	0.75	2.22	1.50	2.02	1.56	0.68	0.92	0.15	3.38	1.28	1.43	0.49
P19120	Heat shock cognate 71 kDa protein	2.89	2.83	2.86	0.05	3.03	3.38	1.40	0.83	2.24	2.18	1.07	0.94	1.67	3.30	2.17	2.02	0.99	0.83
G3MZ21	Hemoglobin fetal subunit beta	0.39	0.59	0.49	0.14	0.39	0.67	0.01	0.01	0.70	0.36	0.34	0.18	0.53	0.01	0.53	0.31	0.26	0.84
P01966	Hemoglobin subunit alpha	0.73	0.01	0.37	0.51	0.01	0.31	0.04	0.01	0.01	0.08	0.13	0.11	0.60	0.01	0.31	0.26	0.26	0.21
Q3ZC44	Heterogeneous nuclear ribonucleoprotein	0.24	0.47	0.36	0.16	0.84	0.47	0.13	0.71	0.89	0.61	0.31	0.43	0.84	0.15	0.69	0.53	0.31	0.71
P02253	Histone H1	0.30	0.01	0.16	0.21	0.26	0.01	2.61	0.01	0.17	0.61	1.12	0.01	0.44	0.01	0.15	0.15	0.20	0.45
E1BH22	Histone H2	2.13	4.87	3.50	1.94	1.26	3.00	5.12	1.62	1.05	2.41	1.70	3.13	4.89	0.40	4.34	3.19	2.00	0.55
G3N2P2	Histone H3/H3.3C	1.22	2.13	1.67	0.64	0.66	2.10	1.70	0.01	1.30	1.15	0.83	0.38	1.45	0.01	2.07	0.98	0.95	0.77
G3X807	Histone H4	2.07	1.58	1.82	0.35	1.00	2.34	2.62	0.93	1.37	1.65	0.78	1.80	1.70	0.34	3.85	1.92	1.45	0.73

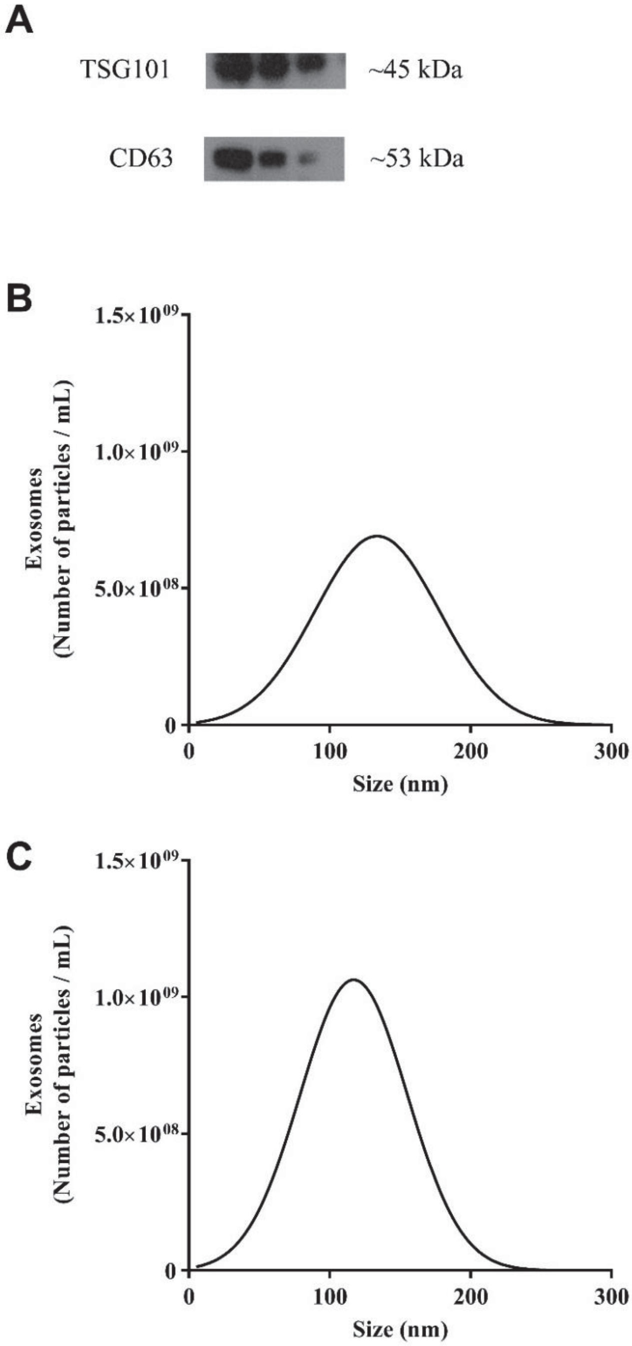
Q861P7	HLA-B-associated transcript	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.05	0.02	0.02	
A6H6Y0	HNRNPD protein	0.15	0.01	0.08	0.10	0.12	0.01	0.35	0.01	0.28	0.15	0.15	0.31	0.29	0.01	0.01	0.15	0.17	0.99
E1BF20	HNRNPH1	0.01	0.01	0.01	0.00	0.01	0.18	0.01	0.01	0.18	0.08	0.09	0.01	0.01	0.28	0.11	0.10	0.13	0.75
F1MY44	HNRNPM	0.01	0.01	0.01	0.00	0.01	0.13	0.01	0.01	0.22	0.08	0.10	0.01	0.01	0.01	0.01	0.01	0.00	0.22
Q2HJH4	HNRPDL protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.34	0.01	0.08	0.15	0.01	0.01	0.01	0.01	0.01	0.00	
B0JYP6	IGK protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	1.34	0.01	0.28	0.60	0.01	0.01	0.01	0.01	0.01	0.00	
Q3T101	IGL@ protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	1.38	0.01	0.28	0.61	0.01	0.01	0.01	0.01	0.01	0.00	
A4IFI0	IGLL1 protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	5.90	0.01	1.19	2.63	0.01	0.01	0.01	0.01	0.01	0.00	
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.76	0.01	0.16	0.34	0.01	0.01	0.01	0.01	0.01	0.00	
G3X8D6	Iodothyronine deiodinase	0.01	0.01	0.01	0.00	0.01	0.01	0.20	0.01	0.01	0.05	0.08	0.01	0.01	0.01	0.01	0.01	0.00	
B9VPZ5	Lactoferrin	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.07	0.01	0.03	0.03	
Q3B7M5	LIM and SH3 domain protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.34	0.08	0.15	0.74	0.01	0.01	0.01	0.19	0.36	
B0JYN3	L-lactate dehydrogenase	0.64	0.71	0.67	0.05	0.65	0.59	0.47	0.28	0.56	0.51	0.14	0.22	0.38	0.01	0.01	0.16	0.18	0.01
A0JNP8	LOC516032 protein	0.01	0.01	0.01	0.00	0.01	0.01	0.08	0.01	0.01	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.00	
Q6PVY3	Macrophage migration inhibitory factor	0.32	0.01	0.16	0.22	0.11	0.69	0.84	0.01	0.65	0.46	0.37	0.01	0.50	0.01	0.59	0.28	0.31	0.46
Q865V6	Macrophage-capping protein	0.01	0.01	0.01	0.00	0.06	0.12	0.06	0.01	0.10	0.07	0.04	0.01	0.09	0.01	0.09	0.05	0.04	0.52
G5E5F7	MAGEB4	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.04	0.02	0.02	
Q0QF29	Malate dehydrogenase	0.01	0.01	0.01	0.00	0.01	0.49	0.01	0.01	0.01	0.11	0.21	0.01	0.01	0.01	0.01	0.01	0.00	
Q8MII4	Metallothionein	0.16	0.01	0.09	0.11	0.08	0.01	0.10	0.01	0.03	0.05	0.04	0.23	0.19	0.01	0.01	0.11	0.12	0.29
P60661	Myosin light polypeptide	0.01	0.01	0.01	0.00	0.01	1.17	0.44	0.01	0.63	0.45	0.48	0.94	0.54	0.01	1.25	0.69	0.54	0.52
Q5E9E2	Myosin regulatory light polypeptide	0.01	0.01	0.01	0.00	0.01	0.01	0.32	0.01	0.41	0.15	0.20	0.41	0.37	0.01	0.40	0.30	0.19	0.30
F1MTV9	Nucleophosmin	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.19	0.06	0.09	
Q3T0Q4	Nucleoside diphosphate kinase B	0.01	0.01	0.01	0.00	0.14	0.20	0.01	0.01	0.01	0.07	0.09	0.01	0.65	0.01	0.01	0.17	0.32	0.54
A6H767	Nucleosome assembly protein 1-like	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.14	0.04	0.06	

A6QNL5	PDIA6 protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.05	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	
P62935	Peptidyl-prolyl cis-trans isomerase	0.95	2.05	1.50	0.78	0.77	1.67	1.52	0.01	1.76	1.15	0.75	0.87	1.89	0.01	2.00	1.19	0.94	0.94
Q9BGI4	Peroxiredoxin	0.07	0.01	0.04	0.04	0.01	0.78	0.01	0.01	0.39	0.24	0.34	0.01	0.50	0.01	0.01	0.13	0.25	0.62
P12234	Phosphate carrier protein, mitochondrial	0.01	0.01	0.01	0.00	0.01	0.09	0.01	0.01	0.01	0.03	0.04	0.01	0.01	0.01	0.01	0.01	0.00	
P13696	Phosphatidylethanolamine-binding protein	0.01	0.01	0.01	0.00	0.01	0.01	0.26	0.01	0.19	0.10	0.12	0.01	0.45	0.01	0.01	0.12	0.22	0.84
Q3T0P6	Phosphoglycerate kinase	0.24	0.01	0.13	0.17	0.42	0.41	0.01	0.01	0.26	0.22	0.20	0.01	0.13	0.01	0.32	0.12	0.15	0.42
Q3SZ62	Phosphoglycerate mutase	0.01	0.01	0.01	0.00	0.01	0.47	0.01	0.01	0.01	0.10	0.20	0.01	0.01	0.01	0.01	0.01	0.00	
Q3SYT9	Poly(RC) binding protein	0.15	0.01	0.08	0.10	0.01	0.35	0.01	0.01	0.01	0.08	0.15	0.01	0.01	0.01	0.08	0.03	0.03	
E1B9K1	Polyubiquitin-C	0.01	0.01	0.01	0.00	0.01	0.65	0.01	0.01	0.50	0.24	0.31	0.01	0.01	0.01	0.78	0.20	0.39	0.89
E1BHJ0	Profilin	0.18	0.30	0.24	0.08	0.26	0.61	0.29	1.61	0.42	0.64	0.56	0.01	0.46	0.01	0.49	0.24	0.27	0.24
Q2HJH9	Programmed cell death protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.08	0.02	0.03	0.35	0.01	0.01	0.10	0.12	0.16	0.24
Q3T165	Prohibitin	0.01	0.01	0.01	0.00	0.01	0.12	0.01	0.01	0.01	0.03	0.05	0.01	0.21	0.01	0.08	0.08	0.09	0.38
F1MXP8	Prosaposin	0.01	0.01	0.01	0.00	0.01	0.28	0.32	0.01	0.51	0.23	0.22	1.39	0.26	0.01	0.64	0.58	0.60	0.26
Q3ZBF7	Prostaglandin E synthase	0.01	0.01	0.01	0.00	0.01	0.01	0.12	0.01	0.01	0.03	0.05	0.01	0.01	0.01	0.01	0.01	0.00	
F1MMK9	Protein AMBP	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.22	0.04	0.06	0.09	0.01	0.03	0.01	0.06	0.03	0.02	0.54
A5D7E8	Protein disulfide-isomerase	0.07	0.01	0.04	0.04	0.01	0.01	0.06	0.01	0.25	0.07	0.10	0.01	0.01	0.01	0.01	0.01	0.00	0.31
Q862H7	Protein S100	0.16	0.01	0.08	0.10	0.10	0.36	0.56	0.66	1.52	0.64	0.54	0.21	0.21	0.01	0.46	0.22	0.18	0.18
A6QPM9	PTMS protein	0.01	0.01	0.01	0.00	0.01	0.01	0.18	0.01	0.01	0.04	0.08	0.34	0.01	0.01	0.01	0.09	0.16	
Q3ZC87	Pyruvate kinase	2.62	2.93	2.77	0.22	2.50	2.28	1.13	1.69	1.93	1.91	0.53	1.79	1.30	5.09	2.12	2.58	1.71	0.43
Q0IIL9	Receptor protein-tyrosine kinase	0.01	0.01	0.01	0.00	0.01	0.01	0.07	0.01	0.01	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.00	
Q3SZN8	Ribonuclease/angiogenin inhibitor	0.13	0.24	0.18	0.08	0.12	0.14	0.01	0.01	0.20	0.10	0.08	0.01	0.06	0.01	0.01	0.02	0.02	0.14
Q0QEV5	Ribosomal protein L18	0.19	0.31	0.25	0.08	0.32	0.28	0.07	0.01	0.27	0.19	0.14	0.01	0.24	0.01	0.36	0.15	0.17	0.75
S5G966	Ribosomal protein L23a Y-linked protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.28	0.01	0.01	0.21	0.13	0.14	0.10
Q58DM9	Ribosomal protein L27a	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.00	

Q3ZBA4	RNA binding motif (RNP1, RRM) protein	0.01	0.01	0.01	0.00	0.01	0.01	0.16	0.01	0.01	0.04	0.07	0.53	0.01	0.01	0.01	0.14	0.26	
Q3SZ20	Serine hydroxymethyltransferase	0.01	0.01	0.01	0.00	0.01	0.12	0.01	0.01	0.01	0.03	0.05	0.01	0.01	0.01	0.01	0.01	0.00	
G3X6N3	Serotransferrin	10.40	3.40	6.90	4.95	3.77	1.69	0.77	9.53	2.46	3.65	3.47	3.77	4.00	8.69	1.75	4.55	2.94	0.69
Q2KJH6	Serpin H1	0.01	0.01	0.01	0.00	0.01	0.11	0.01	0.01	0.01	0.03	0.04	0.01	0.01	0.01	0.01	0.01	0.00	
A1L5B7	SERPINE1 mRNA binding protein	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.25	0.01	0.29	0.14	0.15	0.09
Q2TBR3	SET nuclear oncogene	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.10	0.01	0.01	0.03	0.04	
E1BQ37	SFPQ	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.05	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	
G3X6S5	SH3 domain-binding glutamic acid-rich-like protein	0.01	0.01	0.01	0.00	0.09	0.01	0.11	0.01	0.08	0.06	0.05	0.18	0.17	0.01	0.01	0.09	0.09	0.52
Q862P4	Similar to elongation factor-1-gamma	0.01	0.01	0.01	0.00	0.23	0.01	0.01	0.01	0.01	0.05	0.10	0.01	0.01	0.31	0.01	0.09	0.15	
E1BNF9	SLAMF9	0.01	0.01	0.01	0.00	0.01	1.49	0.01	0.01	0.01	0.31	0.66	0.01	0.01	0.01	0.01	0.01	0.00	
Q3ZCH0	Stress-70 protein, mitochondrial	0.40	0.38	0.39	0.01	0.30	0.55	0.01	0.01	0.19	0.21	0.23	0.01	0.01	0.01	0.45	0.12	0.22	0.56
Q3ZBZ8	Stress-induced-phosphoprotein	0.01	0.01	0.01	0.00	0.01	0.01	0.10	0.01	0.01	0.03	0.04	0.24	0.01	0.01	0.01	0.07	0.12	
P00442	Superoxide dismutase [Cu-Zn]	0.01	0.01	0.01	0.00	0.10	0.01	0.13	0.01	0.16	0.08	0.07	0.17	0.15	0.01	0.15	0.12	0.07	0.45
F1MCT8	SYNCRIP	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.08	0.02	0.03	0.01	0.01	0.01	0.07	0.02	0.03	
Q3ZBH0	T-complex protein 1 subunit	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.14	0.04	0.06	0.01	0.01	0.01	0.32	0.09	0.15	
G8JKZ8	Thioredoxin	0.20	0.01	0.11	0.13	0.40	0.45	1.02	0.30	0.67	0.57	0.29	1.18	0.69	0.01	0.83	0.68	0.49	0.69
P35705	Thioredoxin-dependent peroxide reductase, mitochondrial	0.01	0.01	0.01	0.00	0.01	0.37	0.01	0.01	0.01	0.08	0.16	0.01	0.01	0.01	0.01	0.01	0.00	
F1N3A1	Thrombospondin-1	0.01	0.01	0.01	0.00	0.01	0.11	0.01	0.01	0.15	0.06	0.07	0.01	0.01	0.01	0.21	0.06	0.10	0.97
G8JL04	Thymosin beta 4 X-linked	0.45	0.01	0.23	0.31	0.01	0.01	0.01	0.01	0.28	0.06	0.12	0.01	0.01	0.01	0.01	0.01	0.00	
P21752	Thymosin beta-10	2.73	0.01	1.37	1.92	1.31	0.01	3.61	0.01	0.01	0.99	1.57	13.94	1.15	0.01	0.01	3.78	6.79	0.40
F1MDH3	Talin-1	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.16	0.04	0.07	0.01	0.01	0.01	0.01	0.01	0.00	

Q5E9F5	Transgelin-2	0.09	1.06	0.58	0.69	0.37	0.34	0.27	0.01	0.55	0.31	0.20	0.01	0.86	0.01	0.62	0.38	0.43	0.76
Q3ZBT1	Transitional endoplasmic reticulum ATPase	0.01	0.01	0.01	0.00	0.01	0.14	0.01	0.01	0.27	0.09	0.12	0.01	0.01	0.73	0.29	0.26	0.34	0.32
A5PJ79	Transketolase	0.61	0.47	0.54	0.10	0.01	0.28	0.01	0.01	0.01	0.06	0.12	0.01	0.24	0.01	0.19	0.11	0.12	0.57
O46375	Transthyretin	0.01	0.01	0.01	0.00	0.01	0.20	0.30	0.89	0.22	0.32	0.33	0.01	0.13	0.01	0.43	0.14	0.20	0.38
Q5E956	Triosephosphate isomerase	0.58	0.85	0.71	0.19	0.64	0.81	0.48	0.01	0.70	0.53	0.31	0.01	0.54	0.01	0.83	0.35	0.41	0.48
P81947	Tubulin	4.14	2.10	3.12	1.44	3.07	5.82	1.67	4.89	4.46	3.98	1.63	1.92	1.72	6.94	4.12	3.67	2.43	0.83
P48427	Tubulin-specific chaperone A	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.24	0.01	0.01	0.01	0.07	0.12	
P62992	Ubiquitin-40S ribosomal protein S27a	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.35	0.59	0.01	0.24	0.28	0.11
P63048	Ubiquitin-60S ribosomal protein L40	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.00	1.73	0.01	0.01	0.01	0.44	0.86	
P62866	Ubiquitin-like/S30 ribosomal fusion protein	0.01	0.01	0.01	0.00	0.01	0.24	0.29	0.35	0.21	0.22	0.13	0.53	0.17	0.01	0.01	0.18	0.25	0.76
P48616	Vimentin	1.62	3.64	2.63	1.43	3.42	3.98	2.14	0.01	4.28	2.76	1.74	1.51	0.80	1.39	2.75	1.61	0.82	0.27
I7CT57	Vitamin D binding protein	0.20	0.17	0.19	0.02	0.23	0.26	0.10	0.94	0.23	0.35	0.34	0.87	0.29	0.22	0.01	0.35	0.37	0.99
Q3ZBS7	Vitronectin	0.01	0.01	0.01	0.00	0.22	0.16	0.01	0.01	0.01	0.08	0.10	0.01	0.10	0.01	0.17	0.07	0.08	0.88
P68002	Voltage-dependent anion-selective channel protein	0.01	0.01	0.01	0.00	0.01	0.12	0.01	0.01	0.01	0.03	0.05	0.01	0.09	0.01	0.01	0.03	0.04	

B.1.2 Supplementary Figures



Supplementary Figure 1. Validation of exosomes isolated from plasma of 'High Risk' and 'Low Risk' cows. Figure legend over page.

Supplementary Figure 1. Validation of exosomes isolated from plasma of 'High Risk' and 'Low Risk cows' (previous page). Exosomes were characterised after enrichment from the 100,000 × *g* pellet by buoyant density centrifugation. (**A**) Representative Western blot for exosome markers: TSG101 and CD63. (**B**) Representative Nanosight NS500 measurement of exosomal particle-size distribution from plasma of 'Low Risk' dairy cows. (**C**) Representative Nanosight NS500 measurement of exosomal particle-size distribution from plasma of 'High Risk' dairy cows.

Appendix C

DRC 16 forms

Included in this appendix are the statements of contribution to Doctoral thesis containing publications. Forms are included for Chapters 2-7.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Mallory Ann Crookenden

Name/Title of Principal Supervisor: Alan Murray

Name of Published Research Output and full reference:
Evaluation of endogenous control gene expression in bovine neutrophils by reverse transcriptase-quantitative PCR using microfluidics gene expression arrays. Journal of Dairy Science. Accepted. Volume/Issue TBC.

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  All experimental design, cellular and molecular work, results interpretation, and manuscript write-up was completed by M. A. Crookenden.

Candidate's Signature

11/05/17
Date

Principal Supervisor's signature

11/5/17
Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Mallory Ann Crookenden

Name/Title of Principal Supervisor: Alan Murray

Name of Published Research Output and full reference:
M.A. Crookenden, A. Heiser, A. Murray, V.S.R. Dukkipati, J.K. Kay, J.J. Loor, S. Meirer, M.D. Mitchell, K.M. Moyes, C.G. Walker and J.R. Roche. 2016. Parturition in dairy cows temporarily alters the expression of genes in circulating neutrophils. Journal of Dairy Science. 99(8):6470-6483. doi: 10.3168/jds.2015-10877

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  M. A. Crookenden contributed to experimental design. All cellular and molecular work, manuscript write-up and results interpretation was completed by M. A. Crookenden.

Candidate's Signature

11/05/17
Date

Ala Murry
Principal Supervisor's signature

11/5/17
Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Mallory Ann Crookenden

Name/Title of Principal Supervisor: Alan Murray

Name of Published Research Output and full reference:
M. A. Crookenden, C. G. Walker, A. Heiser, A. Murray, V. S. R. Dukkipati, J. K. Kay, S. Meier, K. M. Moyes, M. D. Mitchell, J. J. Loor, and J. R. Roche. 2016. Effects of precalving body condition and prepartum feeding level on gene expression in circulating neutrophils. J. Dairy Sci. 100(3):2310-2322. doi: 10.3168/jds.2016-12105

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  M.A. Crookenden contributed to the study design. Cellular and molecular work, manuscript write-up, and results interpretation was completed by M. A. Crookenden.

Molly Crookenden

Candidate's Signature

11/05/17

Date

Ala Murray

Principal Supervisor's signature

11/5/17

Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Mallory Ann Crookenden

Name/Title of Principal Supervisor: Alan Murray

Name of Published Research Output and full reference:
Effects of metabolic health on the transcriptome of circulating neutrophils during the transition period.
M.A. Crookenden, C.G. Walker, K.M. Moyes, B. Kuhn-Sherlock, K. Lehnert, A. Heiser, A. Murray, V.S.R. Dukkipati, J.J. Loor, and J.R. Roche.
Submitted to BMC Genomics May, 2017.

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  M.A. Crookenden had input into the study design and sample selection. Cellular and molecular work, manuscript write-up, and results interpretation was completed by M. A. Crookenden.

Candidate's Signature

11/05/17
Date

Ala Murray
Principal Supervisor's signature

11/5/17
Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Mallory Ann Crookenden

Name/Title of Principal Supervisor: Alan Murray

Name of Published Research Output and full reference:
M.A. Crookenden, C.G. Walker, H. Peiris, Y. Koh, A. Heiser, J.J. Loor, K.M. Moyes, A. Murray, V.S.R. Dukkipati, J.K. Kay, S. Meier, J.R. Roche and M.D. Mitchell. 2016. SHORT COMMUNICATION: Evidence that circulating exosomal proteins represent metabolic state in transition dairy cows. J. Dairy Sci. 99(9): 7661-7668. doi: 10.3168/jds.2015-10786.

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  M.A. Crookenden contributed to study design. Results interpretation and manuscript write-up was completed by M. A. Crookenden.

Candidate's Signature

11/05/17
Date

Principal Supervisor's signature

11/5/17
Date

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Name of Candidate: Mallory Ann Crookenden

Name/Title of Principal Supervisor: Alan Murray

Name of Published Research Output and full reference:
M. A. Crookenden, C. G. Walker, H. Peiris, Y. Koh, F. Almughlliq, K. Vaswani, S. Reed, A. Heiser, J. J. Loor, J. K. Kay, S. Meier, S.S. Donkin, A. Murray, V. S. R. Dukkipati, J. R. Roche, and M. D. Mitchell. 2016. Effect of circulating exosomes from transition cows on Madin-Darby bovine kidney cell function. J. Dairy Sci. In Press. doi: 10.3168/jds. 2016-12152.

In which Chapter is the Published Work: Chapter 7

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:
  Study design and cellular and molecular work was completed by M. A. Crookenden with technical assistance. Manuscript write-up, and results interpretation was completed by M. A. Crookenden.

Candidate's Signature

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Date

Principal Supervisor's signature

11/5/17
Date