SINGLE NUCLEOTIDE POLYMORPHISMS IN BOVINE CHEMOKINE AND TOLL-LIKE RECEPTORS; IMPACTS ON DISEASE SUSCEPTIBILITY AND PRODUCTIVITY IN DAIRY CATTLE

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2012

Abstract

Bovine mastitis is recognised worldwide as the most important and costly disease affecting dairy cattle. The reduction of herd mastitis rates is crucially needed to improve animal welfare and profitability, and lessen the reliance on antibiotics.

Single nucleotide polymorphisms (SNPs) within genes that have a critical role in the innate immune response, such as Toll-like receptors (TLRs) and the chemokine receptors CXCR1 and CXCR2, could impact on establishment and progression of intramammary infection, and therefore influence an animal's susceptibility to disease. The genetic selection of animals with favourable TLR and CXCR1/2 mutations, with no impact on production traits, could be incorporated into dairy breeding programmes.

In order to investigate any associations with clinical mastitis (CM) incidence and milk quality and quantity, this study identified and analysed SNPs alongside actual CM and production data from a Holstein-Friesian herd. This revealed 46 SNPs, 9 of which are novel, within bo*TLR1*/*4*/*5*, bo*CD14*, bo*CXCR1* and bo*CXCR2*; selected SNPs were then tested for association with CM. This is the first report of bo*TLR1* SNPs and a non-coding bo*CXCR1* SNP that associate significantly with susceptibility to CM. Favourable linkage of reduced CM with increased milk fat and protein was observed, indicating selection for these markers would not be detrimental to milk quality.

Furthermore, this study provides evidence that some of these SNPs underpin functional variation in bovine TLR1 and CXCR1, and possibly underlie an immunological mechanism for disease susceptibility. SNPs in bo*TLR1* and bo*CXCR1* were significantly associated with impaired transcript levels in milk somatic cells. In addition bo*TLR1* SNPs associated with impaired cytokine responses from cell populations when exposed to ligand or heat-killed mastitis-causing bacteria. The potential impact of bo*TLR1* variation on the immune response to *Staphylococcus aureus* is demonstrated, and this has implications for boTLR1-mediated immune responses to other pathogens.

Publications from this thesis

Russell, C. D., S. Widdison, et al. (2012). "Identification of single nucleotide polymorphisms in the bovine Toll-like receptor 1 gene and association with health traits in cattle." Vet Res **43**(1): 17.

Acknowledgements

Firstly this doctoral thesis would not have been possible without the guidance, support, encouragement and patience of my principal supervisor, Dr. Tracey Coffey. I am indebted to her for overseeing my scientific development, helping me pursue this wonderful window of opportunity and providing a platform to establish a career in molecular research. In addition the guidance and thought provoking advice of my 'second' principal supervisor, Prof. Jamie Leigh, was highly valued, not to mention his unsurpassed enthusiasm for sharing his knowledge (whenever he could) on *Streptococcal* mastitis. The good advice, support and friendship of my second supervisor, Dr. Stephanie Widdison, has been invaluable on an academic and a personal level, particularly her multilingual skills at conferences and teaching of scientific writing, for which I am extremely grateful.

A special mention to the many people who have shared their expertise and offered kind support and friendship during my time at IAH Compton and University of Nottingham, especially current and past members of the bovine genomics group and the farm staff at the Mayfield dairy, in particular Jo Semmens for her technical assistance. This work would not have been possible without the financial and academic support from a BBSRC-funded Doctoral Training Partnership through the IAH and University of Nottingham. I would also like to thank my music library for the lab trances that helped complete the many late night DNA/RNA extractions.

Nobody has been more important to me in the pursuit of this project than my family and friends. Their collective support outside of the thesis, taking an interest in my work and financially aiding me, was invaluable to my happiness outside of science allowing me to finish this marathon journey. Thank you to my Dad who continues to be a role model and now shoulders this mantle with Berni, my father-in-law. Above all, I would like to thank my wife Kari-ann. This adventure began with our engagement, involved our marriage, and earlier this year our family expanded to include our beloved daughter Phoebe; the next adventure has only begun. I am blessed to be able to share it with you.

Phoebe May, my beautiful daughter, thank you for inspiring and reorienting my life.

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List of abbreviations

AP- 1 – Activator protein-1

CDS – Coding sequence

CM – Clinical mastitis

cDNA – Complementary DNA

CXCR – Chemokine (CXC motif) receptor

EBV – Estimated breeding value

ELISA – Enzyme-linked immunosorbent assay

FACS – Fluorescence-activated cell sorting

GATA-1 – GATA binding factor-1

HK – Heat-killed

IMI – Intramammary infection

LD – Linkage disequilibrium

LPS – Lipopolysaccharide

LRR – Leucine-rich repeat

MEC – Mammary epithelial cell

MØ - Macrophage

MyD88 – Myeloid differentiation primary response gene (88)

NMR – National milk records

NS – Non-synonymous

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

Oct-1 – Octamer-1 binding protein

PAM_3 - Pam_3CSK_4 (Cys-Ser-$(Lys)_4$) x 3TFA

PAMP – Pathogen associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PMN – Polymorphonuclear cell

PRR – Pathogen recognition receptor

Q-PCR – Quantitative PCR

QTL – Quantitative trait locus

RFLP – Restriction fragment length polymorphism

RT – Room temperature

SCC – Somatic cell count

SNP – Single nucleotide polymorphism

tSNP – Tagging SNP

Sp1 – Specificity Protein 1

TBS – Transcription binding site

TIR – Toll/Interleukin-1 receptor

TLR – Toll-Like receptor

T_M – Melting temperature

UTR – Untranslated region

Chapter 1.0 - Introduction

1.1 Bovine mastitis

1.1.1 Bovine mastitis is an economically important disease

Bovine mastitis is a disease in cattle resulting from inflammation of the mammary gland which can lead to swelling and hardening of the udder, changes in the composition and quality of the milk and permanent damage to the mammary tissue (Zhao and Lacasse 2008). Mastitis is generally the result of teat duct invasion and intramammary establishment of bacteria, whereupon host detection initiates inflammatory responses in an attempt to eliminate infection. The ubiquitous presence of mastitis-causing bacteria in or on the cow and around the farm environment means the most effective disease control measures often rely on farm management practices to limit the duration of bacterial infection and to restrict contagious spread of pathogens through the herd. Commonly this involves isolation of affected cows from the milking herd to either allow to self-cure or to treat with an appropriate antibiotic regimen. The severity of a seemingly untreatable long-term infection or the damage and permanent loss of productivity from repeated infection can result in premature culling of the cow. Losses due to reduction in milk yield, discarding of 'unhealthy' milk, increased mortality, culling and treatment costs make mastitis the most economically important disease of dairy cattle worldwide, with annual losses to industry in the United Kingdom (UK) alone estimated at £300 million (Viguier, Arora et al. 2009). The disease further raises major issues for animal welfare and food product safety (Oliver, Jayarao et al. 2005), as well as concerns over the widespread and sometimes inappropriate use of antibiotics that may promote the development of resistance in mastitis-causing bacteria (Goni, Vergara et al. 2004; Vanderhaeghen, Cerpentier et al. 2010). Bovine mastitis continues to be a significant problem and the development of additional long term and complementary control measures to further reduce incidence will be of great benefit to the industry, the welfare of the animal and the biosecurity of the consumer.

1.1.2 Nature and current trends of disease

Bovine mastitis is a common but complex and multifactoral disease that varies considerably in its occurrence and severity, and can have an infectious or non-infectious aetiology. This can be attributed to a variety of factors best viewed as an interrelationship or imbalance between the cow, pathogen and the environment. Udder inflammation is predominantly a pathophysiological response to a diverse range of causative agents, with up to 137 different organisms identified as causes of mastitis (Watts 1988). Bacteria account for the vast majority of mastitis cases and in most countries, including the UK, leading bacterial causes are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* (Bradley, Leach et al. 2007). Pathogen nature and duration of infection determine, to some extent, the degree of mastitis severity. Contributory host factors include cow productivity, nutrition, age, lactation stage, immune function and status, while environmental or management factors including season, region, housing, hygiene and herd size can all further influence both the presence of pathogen and the pathogen/host response (Sordillo, Shafer-Weaver et al. 1997; Waage, Sviland et al. 1998; Barkema, Schukken et al. 1999; Ericsson Unnerstad, Lindberg et al. 2009; Tenhagen, Hansen et al. 2009).

Mastitis can be subdivided into categories based on parameters of disease presentation; clinical or subclinical mastitis, and the type or origin of the causative pathogen; environmental or contagious. Defining cases by these broad criteria can aid interpretation and management of disease. Clinical mastitis (CM) typically manifests with an acute onset of mild to severe inflammation, visible as swelling, pain and redness of the affected udder quarter/s with adverse changes to the milk such as clotting. As severity increases, milk production continues to decrease, tissue damage due to bacterial toxins can ensue and the cow can become systemically ill with potential for progression to septicaemia and death.

Subclinical mastitis shows no outward signs of disease, however infection can become chronic and progress with clinical episodes that can cause permanent hardening and damage to the udder. Subclinical cases are confirmed by bacteriology and culture of a mastitic pathogen, along with an indirect approach via the detection of a raised milk somatic cell count (SCC), from the affected quarter/s.

Mastitis can also be classed as contagious or environmental, depending on the origin of infection. Environmental mastitis is caused by opportunistic pathogens not adapted to survival within the host, which spread from the environment to the cow and include *E. coli*, *Klebsiella* spp., and *S. uberis*. Opportunistic pathogens typically invade and multiply to elicit a host immune response resulting in successful elimination. Contagious mastitis is caused by pathogens that survive on or in the udder, with infection spread by direct quarter to quarter or cow to cow contact, or via fomites in the milking parlour. Subclinical mastitis is often associated with contagious pathogens, including *S. agalactiae* and *S. aureus*. Whilst defining mastitis in this way can be convenient at a management level, at a pathogen level this distinction is less practical. In theory all 'environmental' pathogens could exhibit contagious spread within and between cows. Infection with the opportunist *S. uberis*, for example, can be difficult to manage and control if subclinical infection remains undetected for a prolonged period, allowing a greater opportunity for contagious spread. In herds where contagious transmission is being controlled, infection by *S. uberis* is likely to result from environmental sources, including faecal matter originating from the cows (Lopez-Benavides, Williamson et al. 2007). An overview of the general disease presentation for pathogens commonly isolated from bacterial mastitis is presented in Table 1.1.

Table 1.1 - Common causes of bacterial mastitis and associated patterns of disease

Pathogen	Gram Stain	Origins	Presentation and nature of disease
Escherichia coli	-ve	Environmental	Almost always clinical, infection can vary from mild and self-curing, to severe and acute toxic, requiring treatment and neutralisation of endotoxins. High SCC.
Streptococcus uberis	+ve	Environmental*	Cause of both clinical and subclinical infections that may become persistent. Moderate to high SCC rise.
Staphylococcus aureus	+ve	Contagious	Chronic subclinical infection with sporadic clinical episodes. Can become persistent and difficult to treat, causing long-term udder damage and culling of the animal. Variable SCC rises.
Streptococcus dysgalactiae	+ve	Environmental*	Often associated with teat damage, infection mostly clinical and easy to treat.
Streptococcus agalactiae	+ve	Contagious	Cause of both clinical and subclinical infections, can be mildly chronic. High SCC rise. Easy to treat and control.
Coagulase-negative *Staphylococci* (CNS)†	+ve	Contagious	Considered a commensal, rarely causes clinical infection, commonly subclinical. Moderate increase in SCC. Often isolated from well managed herds.
Klebsiella spp.	-ve	Environmental*	Commonly associated with dirty conditions. Coliform mastitis.
Enterobacter spp.	-ve	Environmental	Can be difficult to treat, coliform mastitis.
Pseudomonas spp.	-ve	Environmental	Can cause severe and chronic mastitis. Sourced from contaminated water.
Corynebacterium bovis†	+ve	Contagious	Considered a common commensal, rarely causes clinical infection. Slight SCC elevation. Presence may be protective to infection from other pathogens
Mycoplasma bovis	N/A	Contagious	Usually introduced into a herd, can cause severe clinical infection.

*Can display contagious spread. †Considered a minor pathogen and may not be actual cause of CM episodes.

Overt CM can be the result of a new infection or the progression of an existing subclinical one. It is recorded and analysed within a herd by prevalence, the proportion of a population having CM at a given time or by incidence with the number of cases over a period of time (rate) often presented as CM cases /100 cows /year. Regular recording of incidence is essential when comparing national and historical CM data trends, which appear to be evolving due to the emerging importance of different pathogens (Bradley 2002). A 'Five Point Plan' control strategy initiated in the 1960s to improve animal hygiene and husbandry led to a thirty year progressive decline in UK dairy herd mastitis from around 150 cases /100 cows /year, to 40 cases /year (Kingwill, Neave et al. 1970; Kossaibati, Hovi et al. 1998). A 2007 survey of dairy herds across England and Wales suggested that the incidence has since increased to between 47-65 cases /100 cows /year (Bradley, Leach et al. 2007). The survey further highlights the limited success of the 'Five Point Plan' in controlling only contagious pathogen spread, since opportunistic pathogens such as *E. coli* and *S. uberis* predominate and are now the leading cause of CM in the UK (Figure 1.1) and common causes of mastitis worldwide (Bradley, Leach et al. 2007). Although *S. aureus* continues to be a major cause of subclinical mastitis, a larger proportion of cases resulted in the isolation of the opportunist *S. uberis* (Bradley, Leach et al. 2007), highlighting the shift in environmental pathogen importance. Vaccination programmes may be required to further control mastitis (Leigh 1999), however an effective vaccine has yet to be established (Prenafeta, March et al. 2010). The use of teat sealants and improvement of bedding systems and herd energy balance are potential solutions to lowering disease (Huxley, Green et al. 2002; Ericsson Unnerstad, Lindberg et al. 2009; Moyes, Drackley et al. 2010). However an additional long term and complementary strategy to reduce CM incidence is the genetic selection of cattle that are less susceptible to mastitis.

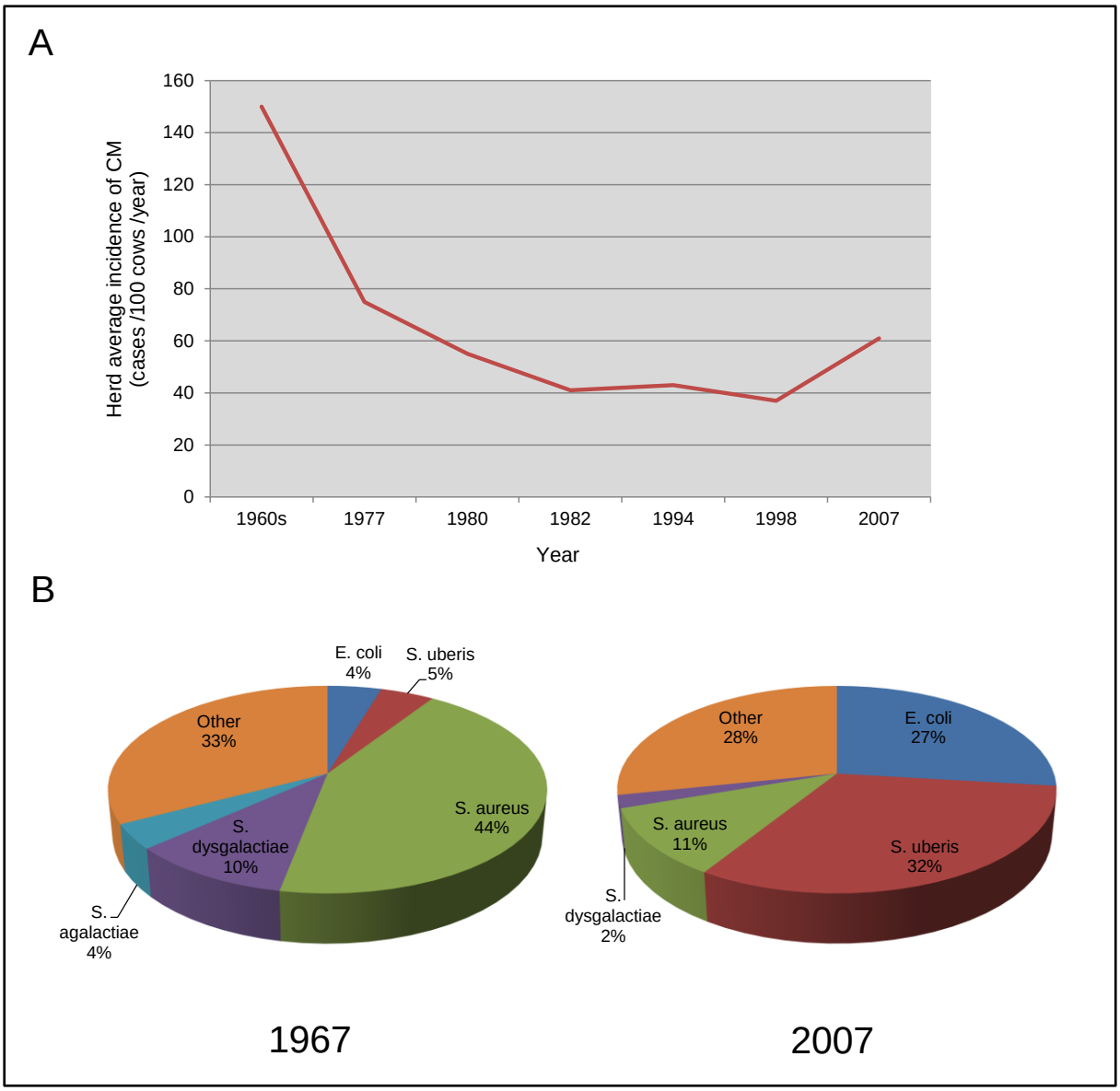


Figure 1.1 - The changing trends for UK clinical mastitis and importance of key pathogens

(A) Effectiveness of the 'Five Point Plan' at reducing UK herd averages for CM incidence since implementation in the 1960s. (B) The shift in pathogen importance in the past 40 years: comparison of common causes of CM (%) from UK dairy herds in 1967 and 2007 (Bradley 2002; Bradley, Leach et al. 2007).

1.1.3 Current breeding strategies require accurate CM markers

Dairy products are a major part of the food chain for the majority of the world's population and growing demand and reduced profit margins continue to influence breeding for favourable production traits in dairy herds. High yields, good milk fat and protein content and a reduced SCC are financially rewarded qualities that have all been optimised largely because of genetic selection (Shook 2006). The heritability of these and other traits, including growth, fertility, calving ease and weight, are all correlated and indexed into estimated breeding values (EBVs). These are used to evaluate and market the performance of sires to yield progeny of commercial value. However selective breeding on the basis of optimal production traits could be detrimental to the health and well-being of the cow (Simianer, Solbu et al. 1991). Holstein-Friesians are the favoured commercial dairy breed and herds in many countries are significantly inbred due to the lack of an expansive Holstein semen gene pool (Kantanen, Olsaker et al. 2000; Zenger, Khatkar et al. 2007). Any heritable genetic dysfunctions and associated traits passed on consecutively through a dairy herd could have a negative impact on disease such as mastitis. The ability to select cattle less susceptible to mastitis, with little or no impact on milk production, will considerably benefit both the industry and the animal.

A lack of comprehensive phenotypic data on CM currently limits many breeding programmes which select on the basis of a lowered milk SCC, the number of somatic cells per millilitre (mL) of milk, as a surrogate of CM. As well as accounting for subclinical cases, SCC is more routinely recorded and is estimated to have low to moderate heritability as a breeding trait (Rupp and Boichard 1999). However there are limitations when relying on SCC data, as arguably the use of current recordings as a proxy for intramammary infection (IMI) status is not absolutely correlated with bacterial presence (Schepers, Lam et al. 1997; Heringstad, Gianola et al. 2006; Koeck, Miglior et al. 2012). Furthermore, selective breeding

for a reduced SCC is selecting against the immune cell which, as the first line of immune defence, can eliminate IMI in the absence of overt clinical signs. Dairy herds selected for a reduced SCC are consequently becoming more susceptible to opportunistic infections (Barkema, Schukken et al. 1998; Suriyasathaporn, Schukken et al. 2000).

The ability to select animals that are likely to exhibit a reduced level of actual CM incidence, whilst maintaining production traits such as milk yield, would therefore be more beneficial than simply the selection of animals by SCC. The identification of single nucleotide polymorphisms (SNPs) in genes involved with the mammary innate immune response are receiving particular interest as DNA markers for CM susceptibility and associated production traits (Sharma, Leyva et al. 2006; Pant, Schenkel et al. 2008; Verschoor, Pant et al. 2009; Beecher, Daly et al. 2010; Chen, Yang et al. 2010; Skelding, Schenkel et al. 2010; He, Chu et al. 2011). SNPs are an abundant form of heritable DNA point base mutations (Brookes 1999) often linked with gene and protein dysfunction that may underlie disease. For example a coding non-synonymous (NS) SNP, that translates into an amino acid substitution, may impact the protein's tertiary structure; whereas a non-coding SNP that lies within DNA regions containing promoter and regulatory sequences can affect gene splicing, transcription, and translation. Large-scale genome SNP analyses in cattle have demonstrated substantial genetic variance within and between breeds (Gibbs, Taylor et al. 2009; Stothard, Choi et al. 2011), and extensive reviews highlighting the links between SNPs, both coding and non-coding, and livestock diseases have been published (Ibeagha-Awemu, Kgwatalala et al. 2008). Many SNPs may remain undetected with those already identified requiring verification, particularly in dairy breeds. Several CM association studies have analysed the association of SNPs within key genes with reduced SCC as a surrogate for CM in dairy cows (Sharma, Leyva et al. 2006; Pant, Schenkel et al. 2008; Verschoor, Pant et al. 2009; Beecher, Daly et al. 2010; Chen, Yang et al. 2010; Skelding, Schenkel et al. 2010; He,

Chu et al. 2011). Using actual CM incidence, the identification of SNPs associated with reduced susceptibility may improve accuracy for genomic selection of these phenotypes.

1.2 Innate immune responses of the mammary gland

1.2.1 Overview of mammary defence

The mammary gland possesses a variety of anatomical, cellular and humoral defence mechanisms to help prevent the invasion and establishment of pathogens (Sordillo, Shafer-Weaver et al. 1997; Rainard and Riollet 2006). Pathogens usually gain entry to the quarter via the teat canal by breach of the teat orifice. This varies in penetrability before, during and after lactation periods, and is further influenced by teat morphology, lactation and mastitis history (Seykora and McDaniel 1985). A keratin waxy plug, formed from the stratified squamous epithelial lining within the duct, seals the teat canal during dry periods and between milkings (Craven and Williams 1985; Paulrud 2005). This forms an effective physical and microbicidal barrier to invasion. However, damage to the plug, often from excessive milk flux from multiple lactations, can temporarily or permanently increase the penetrability of the teat canal (Schultze and Bright 1983). Pathogens which colonise the canal can then invade and multiply in the mammary gland cistern, where they encounter the second line of mammary gland defence that enlists numerous soluble and cellular components. Although the added presence of non-specific soluble factors in milk, including lactoferrin, complement and lysozyme, can target pathogens and supplement mammary defence (Rainard and Riollet 2006), it is the cells of the innate immune system that have a significant role in the detection of pathogens and initiation of early immune response mechanisms (Sordillo, Shafer-Weaver et al. 1997; Oviedo-Boyso, Valdez-Alarcon et al. 2007).

Immune cells found in the milk of uninfected udders are predominantly macrophages (MØ), with polymorphonuclear neutrophils (PMNs) present in much smaller numbers (Lee,

Wooding et al. 1980). Together these leukocytes account for the majority of somatic cells in 'normal' milk and collectively function to detect and phagocytise pathogens and subsequently initiate an appropriate immune response. Following establishment of IMI, continued interaction with pathogens induces resident leukocytes and mammary epithelial cells (MEC) to synthesise and release soluble factors including the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin (IL) -6 and the neutrophil chemoattractant, CXCL8 (formerly IL-8). Expression of cytokines and other immune markers function to recruit circulating leukocytes to the foci of infection and enhance their bactericidal activity. Production of CXCL8, for example, induces chemotaxis of neutrophils from surrounding blood vessels into the mammary gland. During mastitis the mass migration of neutrophils is vital to contain infection. Increasing neutrophil numbers makes them the dominant cell type in infected quarters, resulting in the observed increase in milk SCC. The migration and continued presence of MØ is also important to activate adaptive immunity, if needed, and limit tissue damage by ingesting apoptotic neutrophils that would otherwise release harmful reactive oxygen species. If the IMI is resolved efficiently, migration of neutrophils ceases with potentially only a mild inflammatory episode and minimal or no clinical signs.

Persistence of an infection can occur if the neutrophil influx is ineffective, either because of a poor migratory response, or because a pathogen is highly resistant to PMN phagocytosis, for example strains of *S. uberis* (Field, Ward et al. 2003). Continued pathogen growth within the mammary gland can lead to sustained leukocyte migration and further complications such as the development of chronic inflammation or the potentially fatal build-up of bacterial toxins. The ability of a pathogen to evade certain components of the innate response may therefore require opsonins, MØ or an influx of lymphocytes for specific cell-mediated and/or humoral responses to control infection; activated T cells have been observed

during *S. aureus* and *S. uberis* mastitis (Soltys and Quinn 1999; Rivas, Schwager et al. 2007) although their exact immunological role is unknown. Early and effective pathogen detection however is likely to have an influential role in the development of an appropriate mammary immune response.

1.2.2 Innate immune components of mammary immunity

Our knowledge of the innate immunity of the bovine mammary gland has recently grown in complexity (Rainard and Riollet 2006). Resident leukocytes and MEC differentially express an array of effector molecules for the initiation and progression of an immune response following infection. Analysis of cytokine profiles in infected animals has highlighted that the pathogen specificity of the immune response and the location within the gland of that response potentially correlate to the outcome of infection. (Swanson, Stelwagen et al. 2009; Rinaldi, Li et al. 2010; Rinaldi, Li et al. 2010a). These immune responses, which appear to be conserved between cattle breeds challenged with the same pathogen (Bannerman et al. 2009), involve cell synthesis of cytokines and other immune effector molecules that are induced by, and targeted at, innate receptor proteins that reside on mammary, neutrophil, MØ and lymphocyte cell surfaces. GermLine-encoded pattern recognition receptors (PRR) are responsible for the innate recognition of pathogen-associated molecular patterns (PAMPs), and include proteins from the Toll-like receptor (TLR) family. The detection of PAMPs by TLRs, and the subsequent initiation of signalling pathways to induce cytokine production, can promote chemotactic migration of cells, including neutrophils and MØ, from surrounding blood vessels. This is facilitated by the chemokine receptor protein family, responsible for activating and directing cell movement into the mammary gland. Impairment in TLR and chemokine receptor function or expression will have a negative impact on the outcome of infection by mastitis-causing pathogens.

1.3 Toll-like Receptors

1.3.1 Background, structure and function

Mammalian Toll-like receptors are a key family of innate immune proteins and a major class of PRR, serving principal recognition and signalling functions critical to host defence mechanisms (Medzhitov 2001). The Toll protein originally demonstrated immune importance in *Drosophila* (Lemaitre, Kromer-Metzger et al. 1995; Rosetto, Engstrom et al. 1995) promoting the discovery and study of a human 'Toll-like' homologue (now designated as TLR4), establishing mammalian Toll as a crucial mediator of inflammatory and adaptive responses (Medzhitov, Preston-Hurlburt et al. 1997). Ten human (hu) TLRs have since been identified, each possessing their own ligand recognition repertoire, allowing the receptor to differentiate between an array of PAMPs from bacterial, viral, fungal and protozoan sources (Werling and Jungi 2003; Kawai and Akira 2005).

TLRs are a structurally conserved family of type I transmembrane glycoproteins, characterised by an extracellular leucine-rich repeat (LRR) domain, a short transmembrane region and an intracellular Toll/interleukin-1 receptor (TIR) domain (Figure 1.2). The LRRs are short amino acid sequence motifs with hydrophobic intervals, and are responsible for determining the TLR's extracellular horseshoe-shaped structure that is required for the interaction and binding of ligands (Kobe and Deisenhofer 1995; Kobe and Kajava 2001; Matsushima, Tanaka et al. 2007; Botos, Segal et al. 2011). Recognition of a TLR ligand elicits a conformational change, which leads the intracellular TIR domain to recruit and activate internal cytoplasmic adaptor proteins. This initiates downstream signalling events and gene transcription, directing production of cytokines, chemokines and other effector molecules for a regulated immune response (Werling and Jungi 2003; Pasare and Medzhitov 2005).

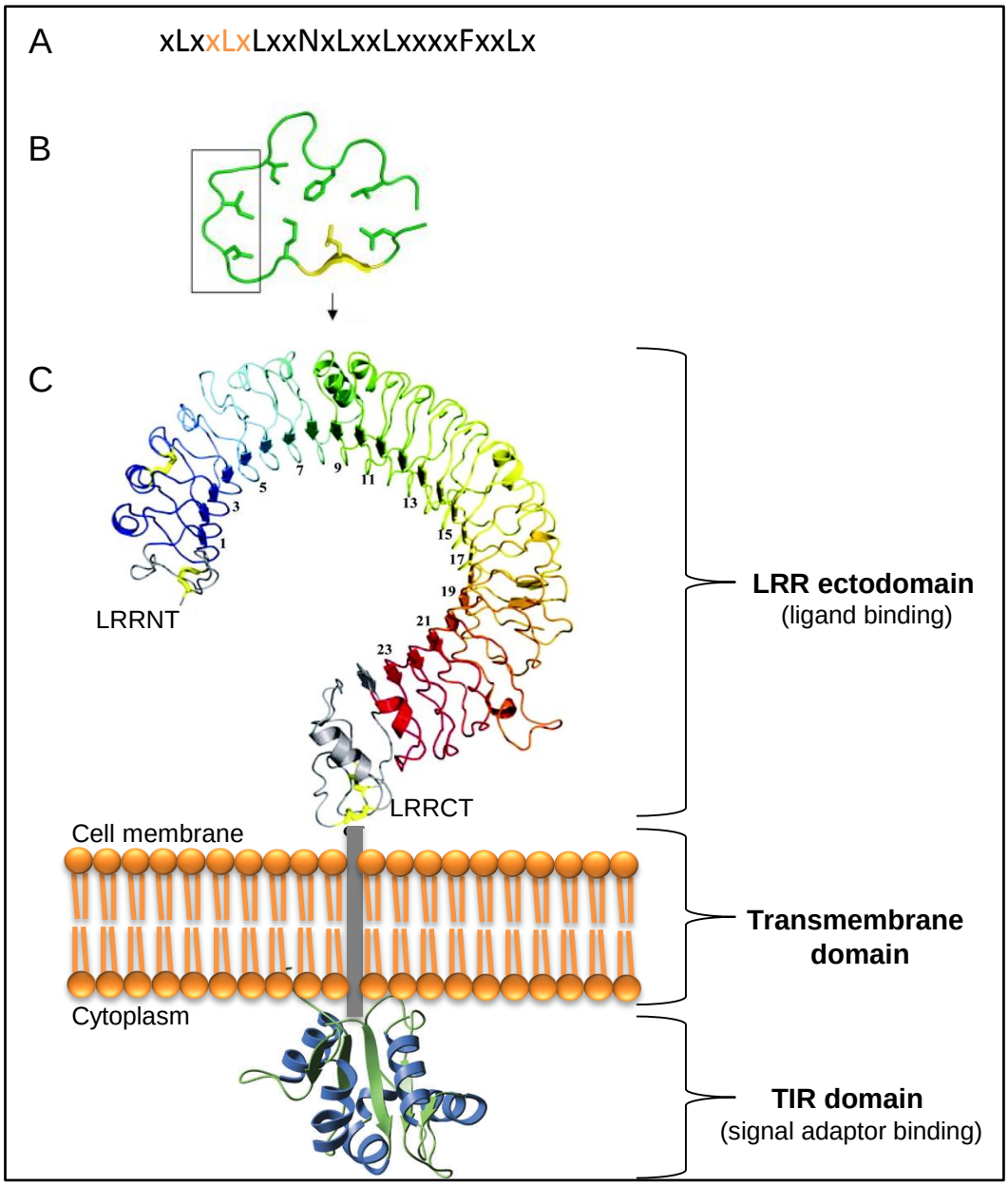


Figure 1.2 - Overview of a typical TLR structure

(A) LRR consensus sequence for TLR3 with highlighted residues forming the β strand. L denotes leucine residues, N and F an asparagine and phenylalanine residue respectively (Botos, Segal et al. 2011). (B) A LRR loop from human TLR3 with conserved residues forming a hydrophobic core. The boxed region forms the surface that interacts with the ligand (Botos, Segal et al. 2011). (C) Ribbon diagrams of the human TLR3 ectodomain, showing 23 LRRs coloured blue to red (numbered within convex) capped by LRR N-terminal (LRRNT) and LRR C-terminal (LRRCT) motifs (grey) (Bell, Botos et al. 2005), and the TIR intracellular domain.

Ten functional bovine (bo) TLRs have been characterised (Coffey et al. unpublished) (McGuire, Jones et al. 2006), revealing close (83-90%) nucleotide identity to their human orthologues (Menzies and Ingham 2006). In general, the mammalian TLR family can be divided into two subgroups depending on their extracellular or intracellular localisation. TLRs 3, 7, 8 and 9 are primarily located on intracellular endosomal membranes, where they mostly detect internalised pathogen nucleic acids, such as single-stranded (TLRs 7 and 8) and double-stranded (TLR3) RNA, and unmethylated CpG DNA (TLR9). TLRs 1, 2, 4, 5, 6 and 10 are sited on the cell membrane where they predominantly interact with microbial patterns, namely bacterial cell wall components, including lipoproteins (TLR2) and lipopolysaccharide (LPS) (TLR4), and proteins within the bacterial flagellum (TLR5). TLRs require dimerisation to themselves or other cell surface receptors/molecules to extend ligand recognition and/or enhance their signalling potential. Heterodimerization of TLR2 with TLR1 or TLR6 expands its spectrum of ligand recognition to include triacylated and diacylated lipoproteins respectively. A TLR2 and TLR10 heterodimer has also been postulated (Hasan, Chaffois et al. 2005), since TLR10 shows close identity and ancestry to TLR1 and TLR6, with all three encoding genes sharing a common locus in humans and cattle (Opsal, Vage et al. 2006). The absence of a functional TLR10 mouse equivalent (Hasan, Chaffois et al. 2005) has precluded the use of knockout methodologies to determine a ligand(s) for TLR10/TLR2. Evidence that TLR10 may share TLR1 ligands has been reported, however a signalling mechanism has yet to be determined (Guan, Ranoa et al. 2010). TLR2 further collaborates with the lectin family receptor Dectin-1 to enhance inflammatory responses towards β-glucan-containing particles (Gantner, Simmons et al. 2003). TLR4 is involved in LPS signalling with the co-receptor CD14 (Chow, Young et al. 1999) and requires the accessory molecules LPS-binding protein (LBP) and MD-2 to enhance LPS interactions (Shimazu, Akashi et al. 1999). TLRs 1-10 and their principal ligands are summarised in Table 1.2.

Table 1.2 - Mammalian Toll-like receptors and their principal ligand targets

TLRs	LRRs*	Localisation	Ligand/s	Target Microbes	SNP Links to disease
TLR1 (+*TLR2*)	19	CM	Triacylated Lipopeptides	Bacteria (Gram +ve and Gram–ve)	Leprosy, TB
TLR2	19	CM	Lipoprotein/Lipopeptides, Mycoplasma, Lipoteichoic acid, Peptidoglycan, Glycolipids, Porins	Bacteria (+ve)	Leprosy, TB, Sepsis, Rheumatic fever
TLR2 (+*Dectin-1*)	-	CM	Zymosan	Fungi	Fungal infections (Plantinga, van der Velden et al. 2009)
TLR3	23	Endo	dsRNA, ssRNA	Viruses	COPD, asthma (Ritter, Mennerich et al. 2005)
TLR4 (+*CD14*, *MD-2*)	21	CM/Endo	LPS, Lipid A, Fusion protein (RSV)	Bacteria (-ve) and viruses	RSV, Sepsis, Malaria (Mockenhaupt, Cramer et al. 2006)
TLR5	20	CM	Flagellin	Bacteria (+ve and –ve)	Legionnaire's disease
TLR6 (+*TLR2*)	19	CM	Diacylated Lipopeptides, Lipoteichoic acid, Soluble Tuberculosis Factor (STF), Zymosan	Bacteria (+ve) and Fungi	Prostate cancer (Sun, Wiklund et al. 2005), TB
TLR7	25	Endo	ssRNA	Viruses	HIV progression
TLR8	25	Endo	ssRNA	Viruses	HIV progression
TLR9	25	Endo	Unmethylated CpG motifs	Bacteria and Viruses	HIV progression
TLR10 (+*TLR2?*)	19	CM/extracellular	Unknown, (Triacylated Lipopeptides?)		Asthma (Lazarus, Raby et al. 2004)

TLR ligand identification is expanding as new affinities continue to be discovered for the receptors. CM = cellular membrane, Endo = endosomal membrane, ssRNA = single-stranded RNA, dsRNA = double-stranded RNA, CpG = Cytosine phosphate-guanosine, TB = tuberculosis, COPD = chronic obstructive pulmonary disease, HIV = human immunodeficiency virus, RSV = respiratory syncytial virus. *Number of LRR from huTLR orthologues, boTLR LRRs yet to be fully characterised. LRR and ligand information (Manavalan, Basith et al. 2011). Unless citation given, TLR disease information (Misch and Hawn 2008).

Five TLRs and their ligand/dimer complexes have been modelled; TLR1/TLR2, TLR2/TLR6, TLR4/MD-2, and TLR3 (Jin, Kim et al. 2007; Liu, Botos et al. 2008; Kang, Nan et al. 2009; Park, Song et al. 2009), and these serve as templates for the prediction of structures and complexes of other TLRs. In addition, they reveal the importance of agonist binding to induce TLR dimers or conformational changes in existing dimers, furthering our understanding of the TLR signalling mechanism. For example, binding of double-stranded RNA and LPS initiates the formation of TLR3 and TLR4 homodimers respectively, allowing the cytoplasmic TIR domains to dimerise and recruit specific adaptor proteins, initiating downstream signalling (Jin and Lee 2008; Botos, Segal et al. 2011). Specific adaptor proteins include Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL or TIRAP), TIR-domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Carpenter and O'Neill 2009). Briefly, TLR TIR domains initiate one of two signal-transduction pathways according to their use of MyD88 or TRIF to mobilise nuclear factors such as NF-κB for pro-inflammatory cytokine production (Figure 1.3). The MyD88-dependent pathways are used by all TLRs (except TLR3), whereby TIR domains associate directly with MyD88 or indirectly via another adapter protein, TIRAP (MAL). The MyD88-independent pathways are utilised by TLR3 and endosome-bound TLR4, since their TIR domains associate with the TRIF adaptor protein, and activate additional nuclear factors, such as IRF3 for interferon production. Only TLR4 can utilise both pathways by associating with MyD88 at the cell surface, and then following LPS interaction, internalise on endosomes to permit indirect TIR association with TRIF via TRAM (Tanimura, Saitoh et al. 2008). The above outlines the general TLR signalling pathway. However, this pathway involves many more components as part of a highly regulated and complex network (Kawai and Akira 2007) with potential crosstalk to other innate receptors (Kawai and Akira 2011) and the complement system (Hajishengallis and Lambris 2010).

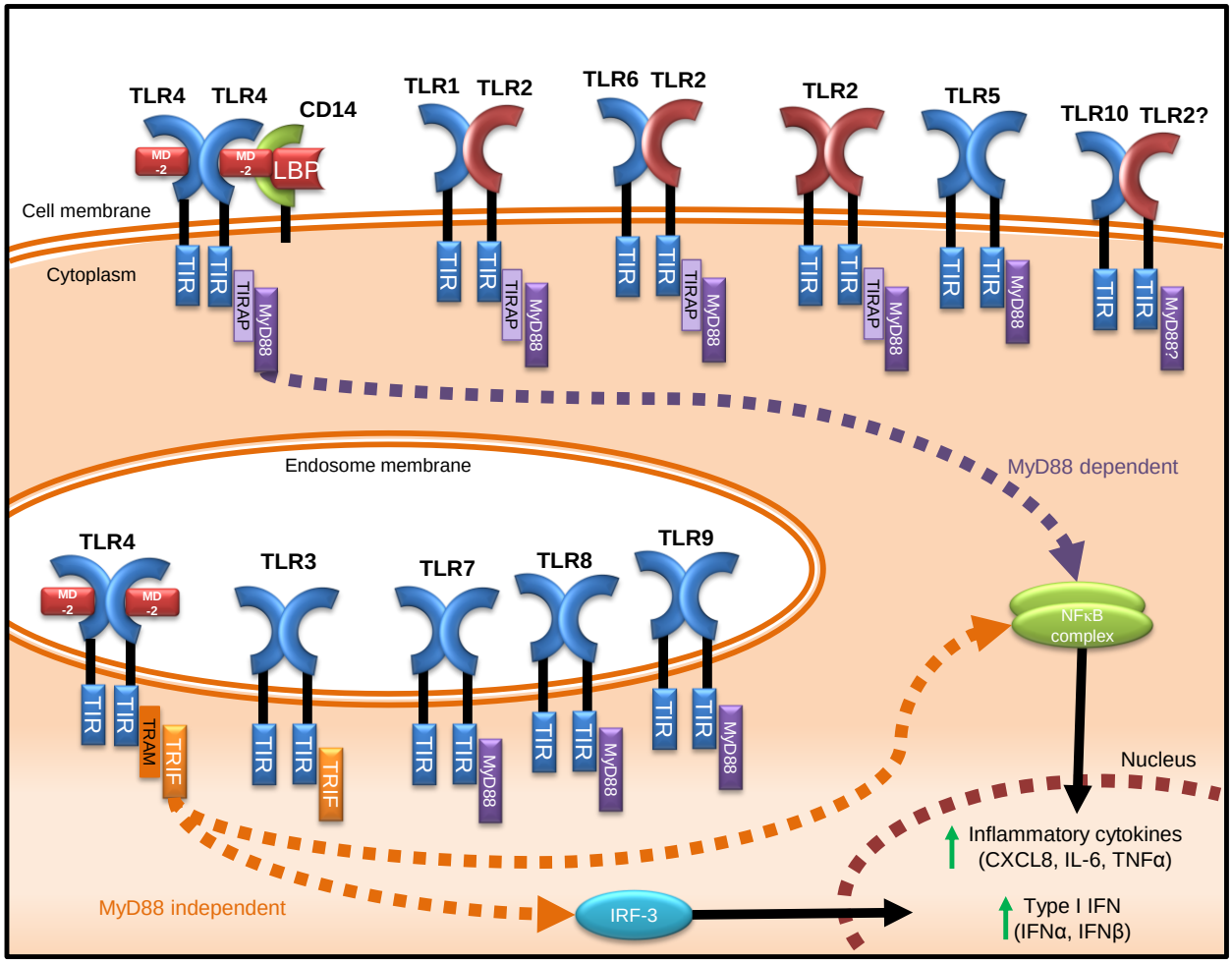


Figure 1.3 - Overview of mammalian TLR dimers and their signalling mechanisms

TLR homodimers and heterodimers allow TIR domains to initiate one of two signal transduction pathways according to their direct or indirect association with MyD88 or TRIF adapter proteins. TLR4 utilises both MyD88-dependent and MyD88-independent signal pathways, which subsequently mobilise nuclear factors such as NF-κB or IRF3 for pro-inflammatory cytokine or interferon (IFN) production.

1.3.2 SNPs in TLRs can predispose to disease

The effects of SNPs within mammalian *TLR*s are dependent on their location. Within the extracellular ligand-binding or intracellular TIR domains they can cause weakening of the interaction of PAMP ligands and signalling leading to an unregulated or insufficient immune response and a higher risk of infection (Schroder and Schumann 2005). Extensive reviews have been published to highlight hu*TLR* SNP markers that predispose severity and susceptibility to a spectrum of bacterial diseases (Misch and Hawn 2008), including leprosy, TB, and Legionnaire's disease (Table 1.2).

Bovine *TLRs* are highly polymorphic between cattle breeds (Cargill and Womack 2007; Seabury, Cargill et al. 2007; Seabury and Womack 2008). In addition, bo*TLR* SNPs are linked with cattle disease, including infectious bovine keratoconjunctivitis (Kataria, Tait et al. 2011) and *Mycobacterium avium paratuberculosis* (MAP) infection. The single or cumulative presence of NS SNPs within and around ectodomain LRR motifs in boTLRs 1, 2 and 4, and a TIR missense SNP in boTLR2, may impact PAMP recognition of, and increased susceptibility to, MAP infection (Mucha, Bhide et al. 2009; Ruiz-Larranaga, Manzano et al. 2011). There is potential to expand these studies to identify non-coding SNPs that may interrupt gene promoter sites or microRNA sites, splice sites or other regulatory motifs, potentially interfering with messenger RNA (mRNA) regulation and protein expression. Polymorphic variants of bo*TLR*s may impact on receptor expression, function and response and, therefore, the innate immune response to mastitis-causing pathogens and contribute towards differences in the pathology of disease.

1.4 Chemokines and Chemokine Receptors

The chemokine system functions by selectively activating and directing cell migration, serving vital roles in both homeostasis and host inflammatory responses. This is accomplished by the chemokines, a subfamily of cytokines that can induce and direct chemotaxis in a cell(s)-specific manner, through the interaction and activation of the appropriate chemokine G-protein linked 7-transmembrane serpentine receptor/s (Allen, Crown et al. 2007). The chemokines are grouped into one of four families, CC, CXC, XC and CX_3C, depending on the positioning of the first two highly conserved and structurally determining cysteine amino acid pairs, as illustrated in Figure 1.4. Based on the chemokine family they bind, receptors are subsequently classified CCR, CXCR, XCR or CX_3CR. In humans 20 chemokine receptors, CCR1-10 (including two variants of CCR3), CXCR1-7, XCR1 and CX_3CR1, have so far been identified, recognising the 50 chemokines (ligands) currently known (Table 1.3). The chemokine system is highly promiscuous, as multiple chemokines can bind the same/multiple receptors within their structure-based subfamily, to add robustness to the system (Mantovani 1999). In addition, evidence of receptor/ligand dimerisation (Allen, Crown et al. 2007) and non-signalling 'decoy' receptors (e.g. DARC and D6), now classified as atypical chemokine receptors (Graham, Locati et al. 2012), provide further complexity. This allows attenuation or fine-tuning of a specific chemokine gradient for cell-dependent activation and migration. All bovine homologues for the chemokine receptor repertoire have now been fully characterised, along with the identification of an additional bovine chemokine receptor closely related to human CCR1; named CCR like-1 or CCRL1 (Widdison, Siddiqui et al. 2010). The bovine chemokine system therefore, whilst retaining high similarity to that in human, exhibits species diversity with potential for novel functions (Widdison and Coffey 2011) (Table 1.3).

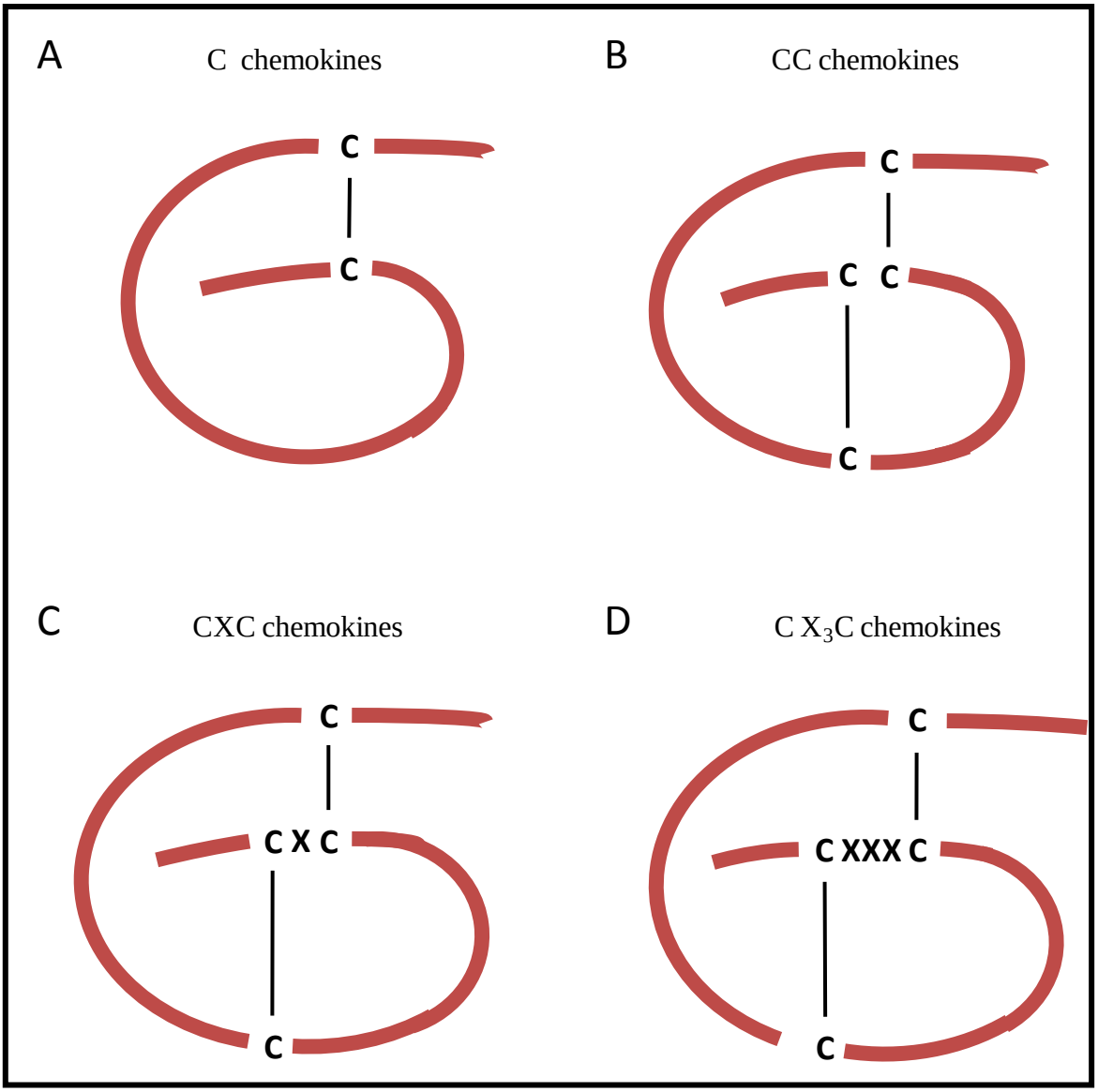


Figure 1.4 - Chemokine classes

The four classes of chemokines are illustrated (A-D) with red lines representing a basic tertiary structure, and black lines representing the disulphide bonds that bridge adjacent cysteine (C) residues - X represents any non-cysteine amino acids, with the exception of proline.

Table 1.3 - Chemokine receptors and their known ligands

Receptor	Chemokine Ligand/s	Total
CCR1	**CCL3**, **CCL3LI**, **CCL5**, **CCL7**, **CCL13**, *CCL14*, *CCL15*, *CCL16*, **CCL23**	9
CCRL1†	Unknown	-
CCR2	**CCL2**, **CCL7**, **CCL8**, **CCL13**	4
CCR3	**CCL5**, **CCL7**, **CCL11**, *CCL15*, *CCL16*, **CCL24**, **CCL26**	7
CCR4	CCL17, CCL22	2
CCR5	**CCL3**, **CCL3LI**, **CCL4**, **CCL5**, **CCL8**	5
CCR6	CCL20	1
CCR7	*CCL19*, CCL21	2
CCR8	**CCL1**	1
CCR9	*CCL25*	1
CCR10	*CCL27*, **CCL28**	2
CXCR1	**CXCL6**, **CXCL7**, **CXCL8**	3
CXCR2	**CXCL1**, **CXCL2**, **CXCL3**, **CXCL5**, **CXCL6**, **CXCL7**, **CXCL8**	7
CXCR3	*CXCL4*, **CXCL9**, **CXCL10**, **CXCL11**, *CXCL12*	5
CXCR4	*CXCL12*	1
CXCR5	*CXCL13*	1
CXCR6	**CXCL16**	1
CXCR7*	**CXCL11**, *CXCL12*	1
XCR1	XCL1, XCL2	2
CX_3CR1	**CX_3CL1**	1
DARC*	**CCL2**, **CCL7**, **CCL8**, **CCL11**, **CCL13**, *CCL14*, *CCL16*, CCL17, **CXCL1**, **CXCL5**, **CXCL6**, **CXCL7**, **CXCL8**, **CXCL9**, **CXCL11**, *CXCL13*	16
D6*	**CCL2**, **CCL3LI**, **CCL4**, **CCL5**, **CCL7**, **CCL8**, **CCL11**, **CCL13**, *CCL14*, CCL17, CCL22	11
CCX-CKR*	*CCL19*, CCL21, *CCL25*	3

†Novel receptor identified in cattle (Widdison, Siddiqui et al. 2010). *Atypical chemokine receptors. Chemokines in bold are inflammatory chemokines, in italics are homeostatic chemokines, underlined are both inflammatory and homeostatic. Content sourced from Allen, Crown *et al*. (2007) and Mantovani, Savino *et al*. (2010).

Chemokines have homeostatic and/or inflammatory roles. Pro-inflammatory chemokines are up-regulated in response to antigen and direct the migration of leukocytes from the blood, across the endothelium and into the affected tissue. Homeostatic chemokines are constitutively expressed to coordinate the continuous basal level of cell migration required. Roles range from the development of B and T lymphocytes by trafficking their migration to secondary lymphoid organs, to the constant cell monitoring of at risk areas, such as skin and lungs, to maintain immune surveillance (Moser and Ebert 2003; Moser and Willimann 2004). Because of their key role in maintaining health and modulating disease, extensive reviews have been published to highlight the links between chemokine receptors in inflammatory disorders (e.g. atherosclerosis and allergy), infectious diseases (e.g. malaria and HIV), and progression of tumour malignancy (e.g. breast cancer) (Murdoch and Finn 2000; Mantovani, Savino et al. 2010; Bonecchi, Locati et al. 2011). Chemokine receptor genetic variability can also influence disease severity and susceptibility. The presence of SNPs within CCR5 has been associated with more severe bronchiolitis due to the receptor's influence on inflammation (Hull, Rowlands et al. 2003). SNPs in CCR2 have been linked to sarcoidosis (Spagnolo, Renzoni et al. 2003) and poor myocardial health, including heart attack risk (Petrkova, Cermakova et al. 2003).

This project primarily focuses on the two chemokine receptors CXCR1 and CXCR2, which have affinity for the chemokine CXCL8.

1.5 Summary

The early detection of mastitis-causing pathogens and the subsequent initiation of appropriate immune responses help prevent onset of disease, and require innate immune receptors which reside on immune cell surfaces to discriminate microbial patterns and facilitate cell migration. Functional expression of TLRs on resident mammary leukocytes and epithelia is important in the initiation and control of innate immune responses towards mastitis-causing bacteria (Goldammer, Zerbe et al. 2004; De Schepper, De Ketelaere et al. 2008; Ibeagha-Awemu, Lee et al. 2008; Yang, Zerbe et al. 2008). TLR interaction with pathogens leads to the secretion of immune effector molecules, including NF-κB-mediated production of the pro-inflammatory chemokine CXCL8 (Farhat, Sauter et al. 2008). During mastitis, synthesis of CXCL8 increases rapidly (Lee, Bannerman et al. 2006), providing a potent chemotactic gradient to facilitate movement of neutrophils. Efficient recruitment of neutrophils into the mammary gland is vital if IMI is to be controlled, and therefore relies on the functioning of the CXCL8 chemokine receptors, CXCR1 and CXCR2, that are expressed on neutrophil cell surfaces (Figure 1.5). Variations in TLRs or these chemokine receptors in the form of SNP mutations may affect the immune response and contribute towards observed differences in disease pathogenesis. Investigation of potential variation may highlight a role in mastitis disease and therefore their identification may provide suitable genetic markers for selection. Our approach to the control of bovine mastitis, which has long been considered a management problem, has now evolved into the search for such genetic risk factors (Su, Guldbrandtsen et al. 2010). To complement current and novel control strategies, selecting animals with favourable TLR and CXCR mutations could further reduce herd mastitis rates, crucially needed for improving animal welfare, dairy herd sustainability and profitability, and for the decreased farm use of antibiotics.

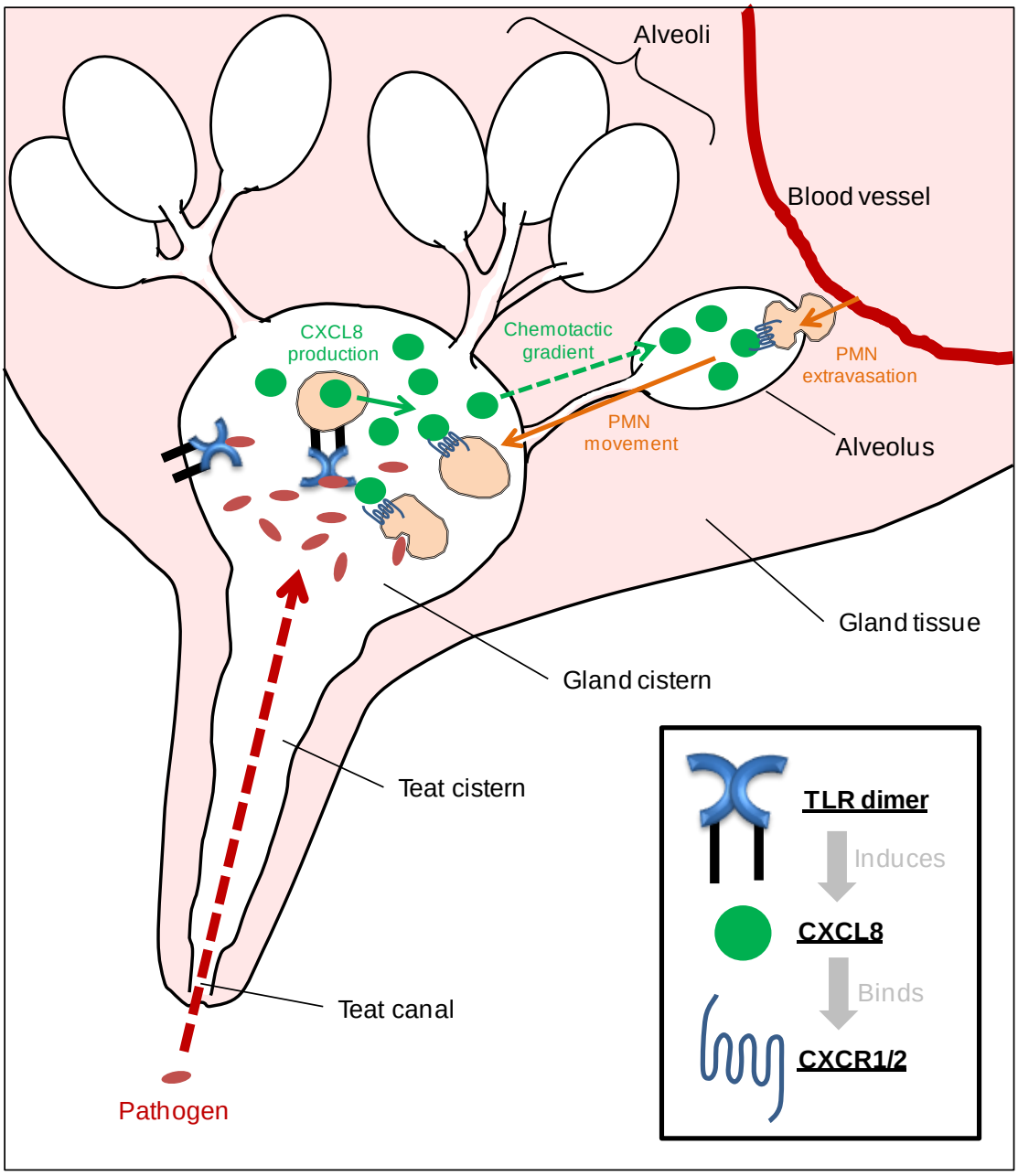


Figure 1.5 - Schematic summarising the importance of mammary TLR and neutrophil CXCR during mastitis in an udder quarter

Bacterial invasion via breach of the teat canal may enable establishment of infection in the gland cistern. Efficient recruitment of PMN is vital for containing infection; their chemotaxis relies on PAMP recognition by TLR residing on MEC and resident milk leukocytes to initiate production of CXCL8 which acts as the chemoattractant. This generates a potent chemotactic gradient to attract PMNs from surrounding blood vessels (extravasation) and direct them into the gland (chemotactic movement), and is facilitated by chemokine receptors CXCR1 and CXCR2 which bind CXCL8 to activate and direct cell migration.

1.5.1 Bos taurus genome assembly

The most complete sequencing of the *Bos taurus* genome, annotation build Btau_4.0, was published in 2009 and is available in GenBank under accession number AAFC03. This assembly version, built from two Hereford cows using a mixture of hierarchical shotgun (bacterial artificial chromosome (BAC) clones) and whole-genome shotgun (WGS) sequencing methods, totals 2.98 Gb in length with >90% of the assembly placed on 30 cow chromosomes (Liu, Qin et al. 2009). Refinements of the Btau_4.0 version assembly, including Btau_4.5 assembly (NCBI) and the Ensembl prediction build Bos_taurus_UMD_3.1 (Partipilo, D'Addabbo et al. 2011), have been released incorporating further WGS reads to improve on chromosome scaffolding. Assembly UMD_3.1 has a slightly reduced genome length of 2.7 Gb largely due to removal of duplicate reads; however, unassigned sequences still amount to 4-6% of the assembly. Bovine post-genomic resources are available through NCBI and Ensembl, allowing accurate BLAST (Basic Local Alignment Search Tool) of DNA sequence reads to the latest cow genome assembly.

1.5.2 Alternate approaches to mapping mastitis resistance in cattle

Association of singular gene SNPs to a disease trait may lend greater accuracy when identifying specific genetic determinants; however, this can be a laborious and time-consuming method. For multifactoral disease traits such as CM susceptibility, multiple genes spread across the genome may directly and/or indirectly have an influence and therefore associate with disease. An alternative and cost effective approach is to perform genome-wide association studies (GWAS) using suitable SNP array technologies. GWAS allows examination of many (>60k) SNP markers, including some of known disease importance, in different individuals (often case-control comparisons) to identify a SNP or group of SNPs that associates frequently with disease (Matukumalli, Lawley et al. 2009). Wide genome coverage from SNP arrays provides a robust platform for mapping disease genes and

quantitative trait loci (QTLs) - stretches of genomic DNA containing or linked to the genes underlying a phenotypic trait such as disease susceptibility. QTL mapping involves observing the inheritance of a large set of SNP markers with a trait; however, marker spacing and inheritance patterning (e.g. haplotypes) often result in QTLs that span large genomic regions containing multiple genes. Mastitis and milk QTLs, for example, are spread over most chromosomes due to the number of genetic and environmental factors that contribute to the animal's phenotype (Ogorevc, Kunej et al. 2009). Genome-wide QTL studies have revealed the bo*TLR6-TLR1-TLR10* gene cluster to be situated within a dense QTL region for CM and a number of milk production traits (Klungland, Sabry et al. 2001; Ogorevc, Kunej et al. 2009), whilst bo*TLR4*, bo*CD14*, bo*CXCR1* and bo*CXCR2* have all similarly been highlighted as candidates for association with mastitis susceptibility (Ogorevc, Kunej et al. 2009). Despite QTLs narrowing genomic focus, analysis of individual genes within each QTL is still necessary.

1.6 Objectives of this project

The objectives of this study were to identify SNPs in bovine genes encoding the cellular membrane-localised PRRs, boTLRs 1, 2, 4, 5, and 6, and boCD14, all known to interact with bacterial PAMPs, and genes encoding boCXCR1 and boCXCR2 that are critical to the recruitment of neutrophils. These genes were investigated for SNPs within coding and non-coding regions including introns, 5'UTR and 3'UTR, and adjacent genomic upstream/downstream regions. In order to investigate any associations with mastitis incidence and milk quality and quantity, identified SNPs were analysed alongside health and production data from a Holstein-Friesian herd. This study aims to challenge the following hypothesis:

SNPs in receptors critical to the innate immune response of the mammary gland can influence an animal's susceptibility to disease as well as milk production traits.

Chapter 2.0 - Materials and Methods

2.1 Sample population

The herd of Holstein-Friesian cows used for this study (Mayfield Dairy, Institute for Animal Health, Compton, UK) contributes to the National Milk Records (NMR) (www.nmr.co.uk), which submits production records for each animal to a database accessed through the InterHerd software programme (University of Reading/PAN Livestock services team, UK). This, along with health data held locally, provided comprehensive information for each cow within the herd. All animal data were recorded within the years 2001-2010, during which the mean CM incidence for the milking herd was 64 cases/100 cows/year, above the historic national average for England and Wales, estimated at 47 cases (Bradley, Leach et al. 2007). The Mayfield herd trends for CM incidence and mean SCC between the years 2001 and 2010 are illustrated in Figure 2.1. CM incidence had declined to a low of 30 cases /100 cows /year in 2005, before increasing to 100 cases /100 cows /year in 2009, following the establishment of a larger milking herd. Mean milk SCC per cow showed a steady rise during the years 2001 – 2004 to peak above 200,000 cells /mL, and then declines, to remain at ~150,000 cells for five consecutive years before returning above 200,000 in 2010. This trend suggests a very weak positive correlation with CM incidence (Figure 2.1). The herd maintained a steady level in milk quality with mean milk fat and protein percentages showing little change during 2001-2010 (Figure 2.2). Production has decreased however, with reduced 305d milk yield averages in 2009 and 2010 (Figure 2.2), years coinciding with raised CM incidence (Figure 2.1).

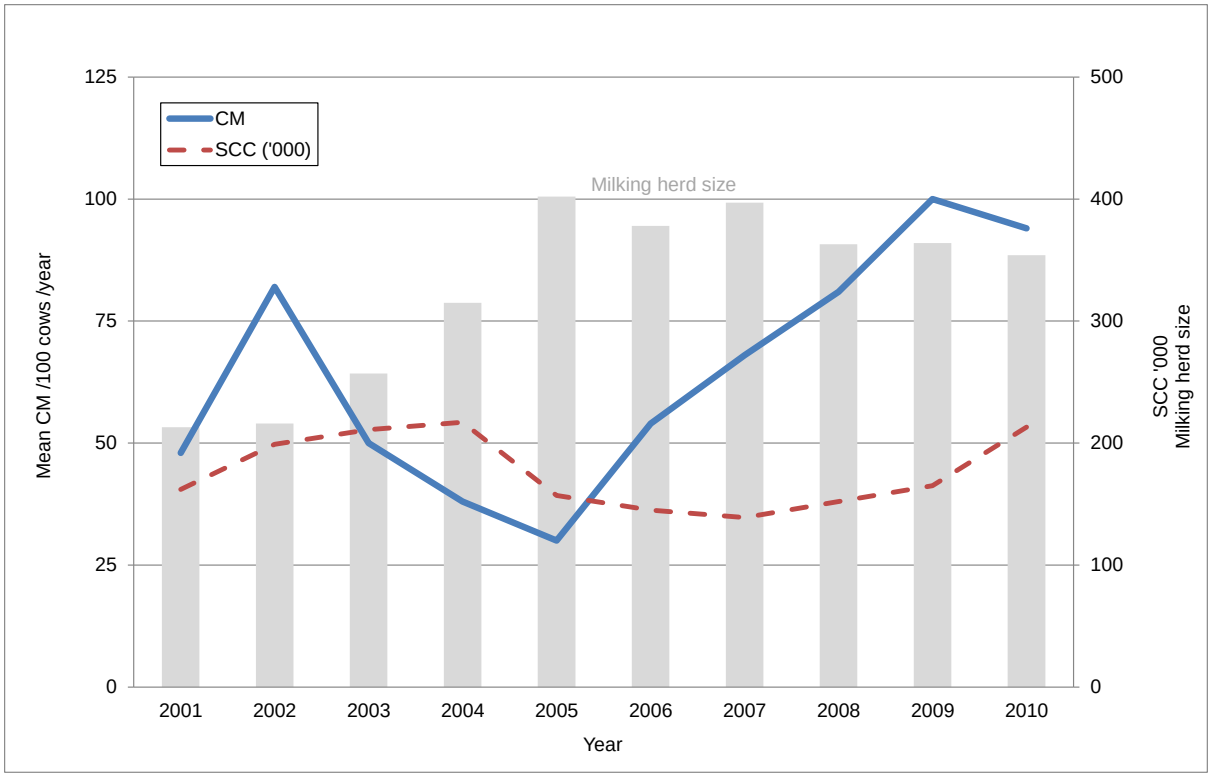


Figure 2.1 – Trends for mean herd CM incidence and SCC for cows within and up to their third parity at the Mayfield dairy between the years 2001 and 2010

Mean CM incidence (blue line) presented as cases per 100 cows/year (primary Y axis), and mean milk SCC (red dashed line) presented on the secondary Y axis (‘000s) are representative of all cows that were within and up to their third parity for that year. Milking herd size (grey bars using the secondary Y axis) is representative of cows of any parity. Weak positive correlations of 0.029 and 0.07 were detected between CM incidence and SCC, and CM and herd size respectively (Pearson correlation whereby +1.0 = strong positive correlation).

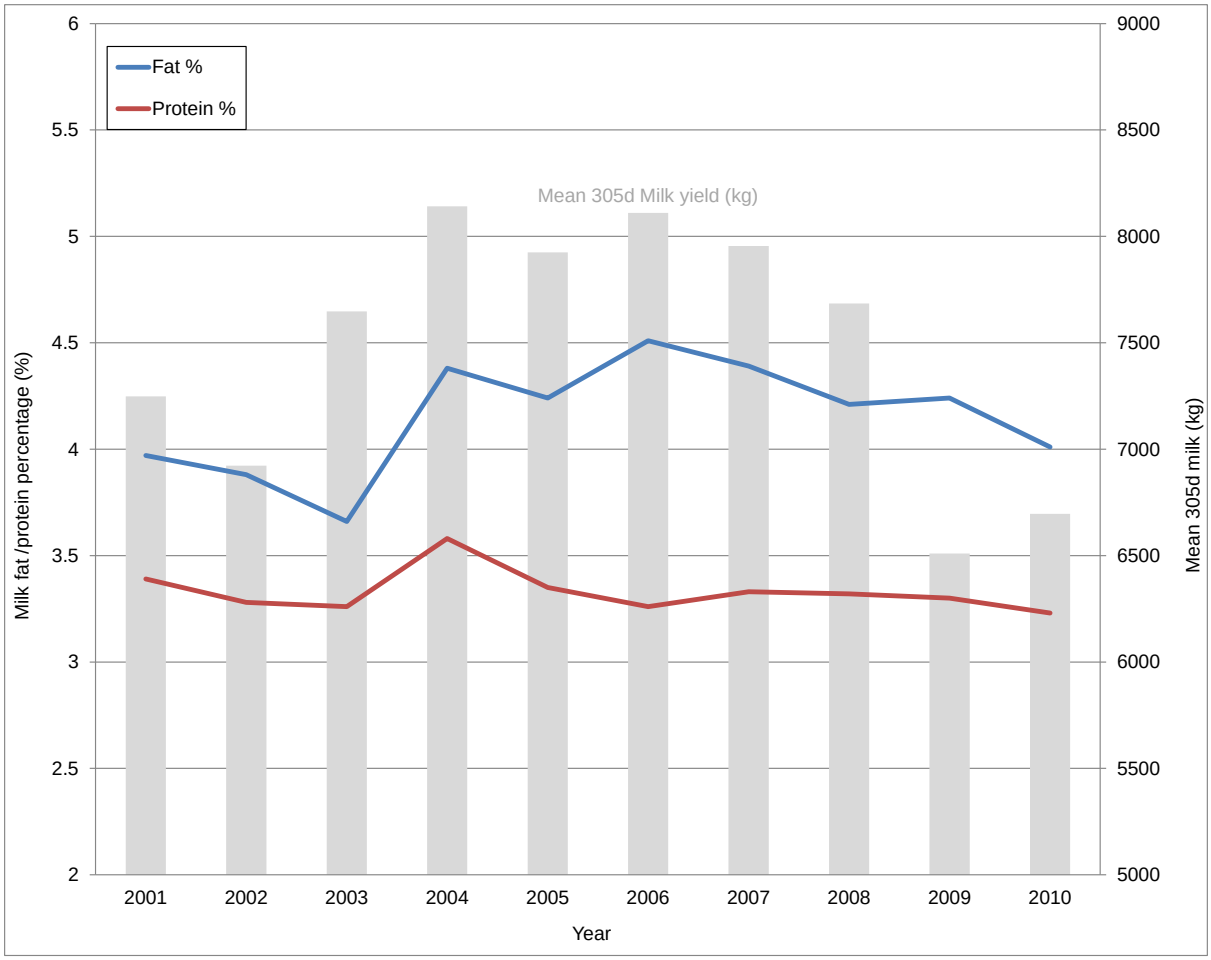


Figure 2.2 – Herd trends for milk production and quality at the Mayfield dairy between the years 2001 and 2010

Milk quality presented as mean milk fat (blue line) and protein (red line) percentages (primary Y axis). Milk production (grey bars) presented as mean 305d yields (secondary Y axis). Positive correlations of 0.436 and 0.502 were detected between milk yield and fat percentage and milk yield and protein percentage respectively, however these were not significant. A significant negative correlation (P>0.05) of -0.73 was detected between milk yield and CM incidence data from Figure 2.1.

2.2 Isolation and extraction of genomic DNA from milk somatic cells

Fresh milk samples (50 mL) were stored on ice prior to centrifugation (3000 *g* /5 min at 4°C). Supernatants were carefully removed along with any residual fat layer. Remaining casein micelles were removed using an adaptation of a procedure devised previously (Murphy, Shariflou et al. 2002). In brief, cell pellets were resuspended in 1.5 mL Phosphate Buffered Saline (PBS) GIBCO^{TM} (Life Technologies Ltd. Paisley, UK) / Ethylenediaminetetra-acetic acid (EDTA) (Sigma-Aldrich Chemical Co. Poole, Dorset, UK) solution (1 mL PBS, 300 µL 0.5 M EDTA and 200 µL Tris-EDTA (TE) buffer – 10 mM-Tris-HCl-1 EDTA pH 7.6), mixed well and allowed to stand at room temperature (RT) for 10 min. Cells were re-pelleted by centrifugation (7000 *g* /1 min /RT), the supernatant discarded and cells resuspended in 1.5 mL of TE buffer before a final centrifugation step (7000 *g* /1 min) and resuspension in 200 µL PBS.

Milk somatic cells were processed using the DNeasy Blood and Tissue Kit (Qiagen Ltd., Crawley, UK) for genomic DNA isolation. Briefly, 20 µL proteinase K solution (Qiagen) was added to the cells resuspended in PBS and mixed well before overnight incubation at 4°C. To ensure RNA-free genomic DNA, 4 µL RNase A (100 mg/mL, Qiagen) was added following overnight incubation and samples left for 2 min at RT. DNA was then extracted according to manufacturer's instructions (Qiagen). The quantity and quality of DNA was analysed using a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA).

2.3 Sequencing and identification of SNPs

Specific primers for polymerase chain reaction (PCR) amplification of coding and non-coding genomic regions were designed based on either the *Bos taurus* genome annotation build Btau_4.0 (NC_007304.4), an assembly built from two Hereford cows (Liu, Qin et al. 2009), or the Ensembl prediction build Bos_taurus_UMD_3.1, a refinement of the Btau_4.0

assembly (Partipilo, D'Addabbo et al. 2011). Sequencing of PCR amplicons from a DNA pool comprising animals with high (greater than six lifetime CM cases) or no mastitis case histories was initially chosen as a method of accelerating detection of disease-associated SNPs. Selected animals were unrelated and shared similar ages and lactation histories. Sequencing of purified PCR products (protocols detailed below) was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Samples were sequenced by capillary electrophoresis on an ABI PRISM 310 Genetic Analyser (Applied Biosystems), and analysed using the Sequencher^{TM} package version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). All primers (Table 2.1) were designed using Primer3 software (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/) and synthesised by Sigma-Genosys Ltd. (Haverhill, UK). All SNP offsets are given relative to their position (bp) from the 'A' nucleotide of the ATG codon of each gene, as based on the Human Genome Variation Society guidelines (http://www.hgvs.org/mutnomen/recs.htmL).

2.3.1 PCR

PCR amplification of DNA was performed using DreamTaq™ DNA polymerase (Fermentas UK, York, UK) according to the manufacturer's protocol. Typically, each 50 µL reaction contained 2 µL of each primer (10 pM), 5 µL of 10X DreamTaq™ Buffer (+ 20 mM $MgCl^{2+}$), 1 µL 10 mM dNTPs (Fermentas), 34.75 µL Ambion® DEPC-treated water (Life Technologies), 5 µL 50-100 ng template DNA and 0.25 µL (1.25 units - U) DreamTaq™ DNA polymerase. An initial DNA denaturation step, at 95°C for 3 min, was followed by 30-34 cycles of the following; denaturation at 95°C for 30 s, annealing at appropriate temperature (see below) for 30 s and extension at 72°C for 1 min/kilo base (kb) of expected product size. This was followed by a final extension at 72°C for 10 min. Oligonucleotide primer annealing temperatures were estimated by subtracting 2-3°C from their melting

temperature (T_m); calculated using the Wallace rule: T_m (°C) = 4°C x (number of G's and C's in the primer) + 2°C x (number of A's and T's in the primer). Annealing temperatures were optimised by performing PCRs over a range of temperatures. All PCRs were run with a negative template control (addition of H_2O in place of DNA).

2.3.2 DNA electrophoresis

DNA was visualised by electrophoresis in agarose (electrophoresis grade, Sigma-Aldrich) gels of 0.75-2% (weight/volume) made with 1X Tris-borate-EDTA (TBE): 1 M Tris, 0.90 M Boric acid, 100 mM EDTA, and stained with GelRed™ (Biotium Inc., Hayward, CA, USA) stock solution diluted at 1:10,000. DNA was loaded into lanes; diluted 5:1 with loading dye: Blue/Orange 6X loading dye – 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 44, 10 mM tris-HCl (pH 7.5, 50 mM EDTA (pH 8.0) (Promega UK, Southampton, UK). A 1 kb DNA Step ladder (Promega) was used for qualitative measurement of DNA product size. Gels were run in electrophoresis tanks containing 1X TBE and GelRed™ at 1:10,000, and results visualised on a 300 nm transilluminator.

2.3.3 PCR purification

PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen) according to the manufacturer's protocol.

2.3.4 DNA gel extractions

Gel extraction and purification of DNA was carried out using the QiaQuick Gel Extraction Kit (Qiagen) according to manufacturer's protocol.

Table 2.1 - Primers used for PCR amplification of genomic regions and RFLP PCR digests

Gene	Application	Genomic Region	Annealing Temp (°C)	Forward primer (5'- 3')	Reverse primer (5'- 3')
Bo*TLR1*	Genotyping	5' upstream region to 5' UTR1	59	CCTCTGTCCTGTTGGCAGTC	GCCTTGTTCCCTGTGGTAGA
	Genotyping	5'UTR1 to 5'UTR intron 1	60	CAGACTGCCAACAGGACAGA	CCTTCTCCAGGGGATCTTTC
	Genotyping	5' UTR3 to 5'UTR intron 3	60	AAGGAGGACCCCTGTCTAGG	CAATGTTGGCATGGAGTGAG
	Genotyping	5' UTR intron 3 to CDS +218bp	59	GCCTCCTTACTGTGCCTGAG	TGGTTGTTTCCAGGGATAAGTT
	Genotyping	5' UTR intron 4 to CDS +820bp	59	GGACATGGTTAGGAGGTGGA	GTACGCCAAACCAACTGGAG
	Genotyping	CDS +650 to +1760bp	59	GCACCACAGTGAGTCTGGAA	TTCTTCACCCAGGCAGAATC
	Genotyping	CDS +1671to 3' UTR region	60	AGGGCTGGCCTGAGTCTTAT	CTGGGGCCTGAAAAGACATA
	Genotyping	CDS +2160 to 3' UTR region	58	ACTTTGCCCACCACAATCTC	CTGGGGCCTGAAAAGACATA
	RFLP	SNP C+798T *Mbo* II digest	59	GCACCACAGTGAGTCTGGAA	GTACGCCAAACCAACTGGAG
	RFLP	SNP A+1762G *Bcl* I digest	60	AGGGCTGGCCTGAGTCTTAT	TTCTTCACCCAGGCAGAATC
Bo*TLR2*	Genotyping	5' UTR to 5'UTR intronic region	59	CCAGATTCCACCCTCTCAAG	AAACCAACGCAGCAGAATTT
	Genotyping	CDS +167 to + 1156bp	59	GCCTTGACCTGTCCAACAAT	AGGCATCCTTACAGGCTGAG
	Genotyping	CDS +2137 to 3' UTR region	60	TTTGTGAAGAGCGAGTGGTG	CAATTTGGTCGCAGTTTTGA
Bo*TLR4*	Genotyping	5' UTR to intron 1	61	GCCACAGTTCCTGGGTAGTC	TTTGATTCCTGCGGTTTCTT
	Genotyping	Intron 1 to intron 2	61	TTGGATGGATGGATGTTTGA	CAGCACCATGCTCAGGTTTA
	Genotyping	Intron 2 to CDS +720bp	61	TTGCCGGACTTCTTTGTTTC	AAGAAGGGCTTGTAGACTTCCT
	Genotyping	CDS +2110bp to 3' UTR region	61	AGCAGGAACACCTACCTGGA	GAGGGGAATCTGAATCAGGAG
Bo*TLR5*	Genotyping	5' upstream region to CDS +242bp	59	GCTCACATGGTCACCACAAG	AAACTGAGTTCCCAGCTCCA
	Genotyping	CDS +2302bp to 3' downstream region	58	TTCCTTAGAGACGGGTGGTG	GTCGATGCAATGGGAGAGAT
Bo*TLR6*	Genotyping	5' upstream region to intron 1	59	GGCAGAGTGAACACAGGTGAA	CATCAGCAGAGGCATTCTCA
Bo*CD14*	Genotyping	5' UTR region to CDS+154bp	59	ATGGAAAGTACCCAGGCTGA	GACGGCAACCATACACTGAA
	Genotyping	CDS +986bp to 3' UTR region	58	GACCTGACTCTGGACGGAAAT	TCCTTCCAGGCATGTTTACC
Bo*CXCR1*	Genotyping	5' intron to CDS +249	59	ATCTGAATGCCCAGACATGG	AGTGACAGAGCGACCAATCC
	Genotyping	CDS +553 to 3' UTR	59	GAGGCCTATCAACCACCGTA	TTCCTGGCTGACAAGGAAAG
Bo*CXCR2*	Genotyping	5' upstream to intron1	60	AGGAGCATGGTCAGAGAGGA	GAGGTGTGGGGATAGATGGA
	Genotyping	CDS +935 to 3' downstream	59	TCAACCCCCTCATCTACGTC	AATGCCATCACCCTTGAAAC

CDS = Coding sequence.

2.4 Restriction fragment length polymorphism (RFLP) - PCR analysis

In addition to sequencing of PCR amplicons, the bo*TLR1* SNPs +798C>T and +1762A>G were genotyped using RFLP analysis of PCR products using restriction enzymes. Restriction enzyme *Mbo*II, which recognises 5'- GAAGA$(N)_8$▼- 3', digests DNA in the presence of the C allele for SNP +798C>T. Restriction enzyme *Bcl*I, which recognises 5'- T▼GATCA- 3', digests DNA in the presence of the A allele (Isoleucine) for the NS SNP +1762A>G (Li, Shi et al. 2009). Restriction digests were performed with 2 µL 10X buffer, 10 µL PCR product, 2 U of appropriate restriction enzyme (New England Biolabs UK, Hitchin, UK) and X amount of Ambion® DEPC-treated water (Life Technologies) to a final volume of 20 µL. Digests were incubated at the recommended temperature for 2 h, with subsequent heat inactivation of the enzyme at 65°C for 20 min. Homozygous and heterozygous samples, confirmed by sequencing, were included as controls.

2.5 Identifying associations between SNPs and CM and milk quality

Identified SNPs were correlated to health or milk productivity data available through the InterHerd software program and NMR data. These included CM case histories, milk SCC, mean 305d milk yields and mean milk protein/fat concentrations. All animal data were recorded within and up to the first three lactations only. This recording parameter represents the average number of parities per animal and further limits the influence of age and any farmer bias of culling on CM within each genotyped population. Mean SCC were transformed logarithmically as NEWSCC = LOG2 (SCC/100 000) +3. This model accounts for the skewness of data distribution (Rupp and Boichard 1999). Mastitis was investigated in three ways, percentage of population affected, mastitis rate and recurrence rate, parameters recommended for effective farm monitoring of mastitis (Edmondson and Blowey 1998). Percentage of population affected was calculated by the presence or absence of CM cases per animal for their first three lactations. Mastitis rate, or mean incidence of CM, is presented as

cases/cow/year ([total number of cases/total number of milking days]*365) during the first three lactations. CM was defined as an individual clinically apparent event, coupled with a rise in milk SCC and/or isolation of pathogen, recorded on a single day within any/all quarters during a lactation period. Repeated cases within the same quarter were noted (recurrence rate = number of repeat cases/total number of mastitis cases) but only regarded as a new case after seven CM-free days return to milking. In the event of an animal having CM history, the total number of milking days was adjusted to take into account the 14 day animal recovery period per CM case. Other factors, such as the number of cases within the first 14 days of each new lactation (often the result of infection during dry-off period), were analysed for significant differences between genotypes.

2.6 Selection of genotyped cows for functional assays

Samples collected from genotyped cows selected for functional assays were from healthy, mid-lactating, age-matched cattle that had recorded no IMI in the two weeks prior to sample collection. Health of each cow two weeks post-sample collection was also recorded, and in the event of an animal having IMI within this time-frame, data was not used.

2.7 TLR ligands

All TLR ligands, Pam_3CSK_4 (PAM_3), lipoteichoic acid from *S. aureus* (LTA) and lipopolysaccharide (LPS) EB Ultrapure (InvivoGen, San Diego, CA, USA), were reconstituted in sterile endotoxin-free water, according to the manufacturer's instructions, to a concentration of 1 mg/mL, and stored at -20°C. PAM_3 is a synthetic triacylated lipoprotein and potent stimulus of TLR1/2 complexes. LTA is a TLR2 stimulus and LPS stimulates TLR4.

2.8 Stimulation of whole blood with TLR ligands

Whole blood (WB) was collected into heparin (final conc. 10 U/mL) and stimulated using an adaptation of a procedure devised previously (Kovach, Yee et al. 1990). Briefly, 400 µL of heparinised blood were pipetted into 1.5 mL tubes. 100 µL of TLR ligand, further diluted in filtered PBS to appropriate concentrations (PAM_3 - 250 ng/mL, LPS - 100 ng/mL, LTA - 1 µg/mL), were added to each blood aliquot and vortexed. The tubes were then incubated at 37°C for 24 h with gentle agitation. Following incubation, a further 800 µL of filtered PBS was added to each tube. Cellular components were pelleted by centrifugation at 800 *g* for 15 min, allowing removal of residual diluted plasma.

2.9 Isolation of PMN leukocytes from bovine blood

PMN leukocytes were isolated from 50 mL blood samples. Briefly, blood was collected into EDTA (final concentration of ~1.5% weight/volume), centrifuged at RT (1000 *g* /15 min) and the plasma and buffy coat phases carefully removed. For every 2.5 g of lower blood phase, 10 mL of H_2O were added. The mixture was agitated and left for 40 s before adding half the water volume of NaCl/PO_4 reagent (2.7% NaCl/PO_4 reagent: 2.7 g NaCl into 10 mL 0.132 M phosphate buffer (18.74 g Na_2HPO_4 and 17.96 g KH_2PO_4 into 1 L H_2O, pH 6.8) and made up to 100 mL with H_2O) to restore isotonic strength. PMNs were pelleted by centrifugation (125 *g* /10 min /RT), the supernatant discarded and the cells resuspended in 1.5 mL of PBS. This was repeated twice to wash the cells. Typically 1×10^8 PMN cells were obtained per 50 mL sample.

2.10 Stimulation of PMNs with TLR ligands

Isolated PMNs were seeded to 1×10^6 /1 mL into culture media: RPMI 1640 + GlutaMAX™ with 10% heat-inactivated foetal calf serum (FCS) (both Invitrogen, Life Technologies, UK) and 100 U mL^{-1} /100 μg mL^{-1} Penicillin/Streptomycin (Sigma, St Louis, MO, USA). Prior to stimulation, seeded cells were incubated at 37°C, in 5% CO_2 for approximately 18 h in 24-well tissue culture plates. PAM_3 was added to the cells in 100 μL of culture media, at final concentrations of 100 ng/mL, 250 ng/mL and 500 ng/mL. Negative controls included a media control (no ligand) and LPS (100 ng/mL), as a non-TLR1 stimulant. Stimulated cells (4 h and 8 h post-stimulation) were lysed and total RNA extracted as detailed in section 2.15.1. Supernatants were taken 24 h post-stimulation for detection of cytokine production by ELISA as detailed in section 2.17.

2.11 Isolation of CD14+ leukocytes from bovine blood

CD14+ leukocytes were isolated from 50 mL blood samples. Briefly, heparinised blood (final concentration 10 U/mL) was collected and separated over a density gradient (Histopaque 1083; Sigma-Aldrich) by centrifugation (1000 *g* /30 min /RT) to enrich the peripheral blood mononuclear cell (PBMC) percentage. Following careful removal of the buffy coat phase (PBMCs) into sterile PBS (50 mL), cells were pelleted by centrifugation (125 *g* /10 min /4°C), the supernatant discarded and the cells resuspended in 50 mL of PBS. This was repeated twice to wash the cells. Cells were aliquoted 1×10^7/mL in FCS containing 10% Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich) and stored in liquid nitrogen (-80°C).

Monocytes (CD14+ cells) were enriched from PBMCs following incubation with monoclonal anti-human (mouse IgG2a) CD14 Microbeads (Miltenyi-Biotech Ltd. Surrey, UK) and the labelled cells isolated using a MiniMACS® column (Miltenyi-Biotech)

according to the manufacturer's instructions. Typically 1×10^5 CD14+ cells were obtained from 1×10^6 PBMCs.

2.12 Flow cytometric analysis of isolated CD14+ cells

The purity of each isolated CD14+ cell population was evaluated by flow cytometric analysis following incubation with an anti-human (mouse IgG2a) CD14+ antibody, conjugated with the fluorochrome fluorescein isothiocyanate (FITC) (Miltenyi-Biotech). An appropriate FITC-conjugated mouse IgG2a isotype control antibody (Miltenyi-Biotech) was used to distinguish specific from non-specific fluorescence. In addition, cells from each CD14+ sort were incubated with propidium iodide (PI) solution (Sigma-Aldrich) for assessment of cell viability. Briefly, samples from each cell sort were incubated with the appropriate amount of primary antibody for 10 min at +4°C; or PI solution (25 µg/mL) for 5 min at +4°C. Stained cells were washed in refrigerated (2-8°C) cell sorting buffer solution: PBS/0.5% Bovine Serum Albumin (BSA) (Sigma-Aldrich)/ 2mM EDTA pH 7.2, and pelleted (300 *g*/10 min /RT) prior to the removal of supernatant. This step was repeated twice before final suspension of cells in an appropriate volume of FACSflow fluid (BD Biosciences, San Jose, CA, USA). Cytometric analysis was performed on a BD FACSCanto™ II flow cytometer (BD Biosciences). Ten thousand events were collected for each sample, and the data analysed using BD FACSDiva™ software (BD Biosciences).

2.13 Bacterial strains and preparation of heat-killed bacterial reagents

The four strains of bacteria used for this study represent three highly important pathogens and causative agents of bacterial mastitis: *S. uberis, E. coli* (strain P4) and *S. aureus* (strain M60). Two strains of *S. uberis* were used, strain 0140J (strain ATCC BAA-854/0140J, originally

isolated from a clinical case of bovine mastitis in the UK and known to be of high virulence), and strain EF20 (a strain isolated from a healthy udder and infrequently associated with pathogenesis). Both *S. uberis* isolates have been used in previous studies (Hill 1988; Egan, Kurian et al. 2010). *E. coli* and *S. aureus* strains were both virulent isolates from cases of bovine mastitis (BramLey 1976; Anderson 1977).

Bacteria were grown overnight in Todd Hewitt broth (Oxoid Ltd., Cambridge, U.K.) at 37°C to an exponential phase of growth. All cultures were of similar optical densities of between 0.9-1.0 OD_{550nm}. Bacterial numbers were calculated for each aliquot prior to heat treatment via plate spreading of serial dilutions and colony counting (typically 1×10^9 colony forming units (CFU) /mL). Aliquots containing 1 mL of overnight culture were incubated in a water bath for 1 h at 60°C for *E. coli* P4, or 70°C for *S. uberis* 0140J, *S. uberis* EF20 and *S. aureus* M60, to heat-inactivate/kill the bacterium. Swabs from each heat-killed (HK) aliquot were plated and incubated overnight at 37°C to confirm zero growth of colonies and effectiveness of treatment. HK bacteria from each aliquot were pelleted (125 *g* /5 min) and washed twice in sterile PBS before final suspension in 1 mL of PBS.

2.14 Stimulation of CD14+ cells with TLR ligands and pathogens

Isolated CD14+ cells were seeded to 1×10^5 /1 mL in culture media: RPMI 1640 + GlutaMAXTM with 10% FCS and 100 U mL-1 /100 µg mL-1 Gentamycin (Sigma). Prior to stimulation, seeded cells were incubated at 37°C, in 5% CO_2 for approximately 2 h in 24-well tissue culture plates. PAM_3 was added to the cells in 100 µL of culture media, at a final concentration of 250 ng/mL; shown to be effective in inducing a TLR1-mediated response (see section 2.9). Negative controls included a media control (no ligand) and LPS (100 ng/mL), as a non-TLR1 stimulant. The equivalent of ~10^6 of HK bacterium in 100 µL of

culture media was added to CD14+ cells, giving an equivalent of 10:1 multiplicity of infection (MOI). Supernatants were taken 24 h post-stimulation for detection of cytokine production by ELISA.

2.15 RNA isolation and cDNA synthesis

2.15.1 RNA isolation

Pelleted cell samples were lysed in RLT buffer for total RNA extraction using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Samples were DNase-treated (DNAfree, Ambion®, Life Technologies) and 1 µL of the sample was quantified on the NanoDrop 8000 spectrophotometer. Integrity of RNA (and mRNA) was additionally visualised on agarose gels by assessing the clarity of 28S and 18S rRNA bands.

2.15.2 First strand complementary DNA (cDNA) synthesis

Total RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen Ltd. UK) using the recommended protocol. Typically, each 20 µL reaction contained ~1 µg total RNA, 1 µL of oligo dT (500 µg/mL) (Promega UK), 1 µL 10 mM dNTPs (Fermentas), and X amount of Ambion® DEPC-treated water to an initial volume of 12 µL. The mixture was incubated at 65°C for 5 min and stored on ice for 1 min, before the addition of 4 µL of 5X First-Strand buffer, 2 µL of 0.1 M dithiothreitol (DTT) and 1 µL (20 U) Ribonuclease inhibitor (RNAsin) (Promega UK). Following incubation at 42°C for 2 min, 1 µL (200 U) of SuperScript II was carefully added, followed by continued incubation at 42°C for 50 min before inactivating the reaction by incubation at 70°C for 15 min. Complementary RNA was removed from cDNA by the addition of 2 U Rnase H (Invitrogen) and incubated at 37°C for 20 min. Quantification of cDNA was carried out using the NanoDrop 8000 spectrophotometer.

2.16 Q-PCR analysis

Quantitative PCR (Q-PCR) was performed with the ABI Prism 7500 FAST Sequence Detection System (Applied Biosystems) using FAST Universal Mastermix (Applied Biosystems) according to the manufacturer's protocol. Taqman® probe and primers for bovine *GAPDH*, *CXCL8*, *CXCR1*, *CXCR2*, *IL-6*, *IL-10*, *TNF-α* and *Bcl2A1* have been described previously (Collins, Howard et al. 1999; Werling, Collins et al. 2002; Widdison, Watson et al. 2008; Widdison, Watson et al. 2009; Widdison, Watson et al. 2011). Taqman® probe and primers for bovine *TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, *TLR10* and *RPLP2* were designed from sequenced templates, avoiding any SNPs, and wherever possible, spanning intron/exon boundaries, using the Primer3 software (Rozen and Skaletsky 2000). All primers and probes (Table 2.2) were synthesised by Sigma-Genosys Ltd. and Eurogentec Ltd. (Romsey, UK) respectively. Probes were labelled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). 100 ng of cDNA template from each animal were tested in triplicate and quantified by comparison with a standard curve from plasmid DNA of known copy number. Relative target gene expression was then calculated by normalising to the combined mean expression levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein, large, P2 (*RPLP2*). Both genes are commonly used as reference genes for Q-PCR, and *GAPDH* expression stability has been shown in bovine cells responding to inflammatory stimuli (Bougarn, Cunha et al. 2011). Expression stability of bo*RPLP2*/bo*GAPDH* ratios were observed for Q-PCR assays involving PAM_3 stimulated PMNs in this study (Appendix I). Analysis was performed using Microsoft® Excel 2008 (Microsoft Co., Redmond, WA, USA) and Prism® 5.04 software (Graph Pad Software, Inc, CA, USA).

Table 2.2 - Primers and probes used for quantitative PCR (QPCR) assays

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Probe (5'- 3')
Bo*TLR1*	GCACCACAGTGAGTCTGGAA	GTACGCCAAACCAACTGGAG	TGTGTGCTTGATGATAATGGGTGTCCT
Bo*TLR2*	ACGACGCCTTCGTGTCCTAC	GCTCCTGGACCATGAGGTTC	CGAGCGGGATTCCTACTGGGTGG
Bo*TLR4*	TGGAGGACATGCCAGTGCT	CACCGACACACTGATGATCGT	AGTTTCAGGAACGCCACTTGTCAGCTG
Bo*TLR5*	CTAGACCTGGGTGGAAGTCAG	AGGGATGAAGGTAAAGACTCTGAA	TTCCTGTGGTCTCTCCGATGCTG
Bo*TLR6*	CCTGCCCATCTGTAAGGAAT	TAGGTGCAAGTGAGCAATGG	TTGGCAACTTGACCCAACTGAATTTC
Bo*TLR10*	TGGTTGGATGGTCAGATTCA	CAGGGCAAATCAAAGTGGA	CCATTGTTGTCATGCTCGTTCT
Bo*CXCR1*	AGGGGTTTGAGGATGAGTTTG	CAGTGCTTATCTCACAGGGACT	TCTTCTGTGGGTGGCGTGCC
Bo*CXCR2*	ACCTGCCCTCTATTCTAACAGA	GCATGCTTATTGATATCCAGAATTT	CTGCCCCATGTCGGCCAG
Bo*GAPDH*	CATGTTCCAGTATGATGATTC	GAGCTTCCCGTTCTCTGC	CGGCAAGTTCAACGGCAC
Bo*RPLP2*	TCAACAAGGTCATCAGTGAGC	CCGATACCCTGAGCAATGA	CGTCCTCGATGTTCTTTCCGTGG
CXCL8	TGAAGCTGCAGTTCTGTCAA	TGGAAAGGTGTGGAATGTGT	TGAGTACAGAACTTCGATGCCAATGCA
IL-6	CAGCTATGAACTCCCGCTTCA	AGGAGCAGCCCCAGGG	AAGCGCCTTCACTCCATTCGCTGTC
IL-10	GGTGATGCCACAGGCTGAG	AGCTTCTCCCCCAGTGAGTTC	CACGGGCCTGACATCAAGGAGCA
TNF-α	CGGTGGTGGGACTCGTATG	GCTGGTTGTCTTCAAGCTTCA	CAATGCCCTCATGGCCAACGG
Bcl2A1	TTTCTTTGTGGCGGAGTTC	AAAAGTCAGCCAGCCAGATT	ACAAACCCATTTTCCCAGCCTCC

2.16.1 Plasmid DNA purifications

For preparation of quantitative PCR gene standards, a single colony of the appropriate transformed isolate expressing the target gene was grown in 10 mL of Luria-Bertani (LB) media with 100 U mL^{-1} of ampicillin. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's protocol.

2.17.1 Detection of CXCL8

Levels of bovine CXCL8 were determined by luminescent ELISA, using a pair of commercial human IL-8-specific antibodies (R&D Systems, Oxon, UK) according to a modified luminescence-based ELISA described previously (Kwong, Hope et al. 2002). In brief, black Nunc MaxiSorp flat-bottom 96-well microplates were coated with 100 µL/well of capture antibody at 2 µg/mL diluted in coating buffer (0.05 M carbonate/bicarbonate buffer pH 9.6) (Sigma-Aldrich), and incubated overnight at RT. Wells were washed five times with 300 µL of PBS + 0.05% Polyoxyethylensorbitan monolaurate (Tween-20) (Sigma-Aldrich), and then blocked with 300 µL PBS + 1% sodium casein (Sigma-Aldrich) solution, made fresh per assay, for 2 h at RT. Wells were washed five times before adding 100 µL of sample to be assayed, diluted in reagent diluent solution (PBS + 1% sodium casein + 0.05% Tween-20) and incubated for 1 h at RT. Samples were tested in triplicate, with recombinant bovine CXCL8 (Kingfisher Biotech, Inc., MN, USA) as the standard. Wells were washed five times before adding 100 µL biotinylated secondary antibody, at 0.1 µg/mL in blocking buffer, and then incubated for 1 h at RT. Wells were washed five times before adding 1/500 streptavidin-horseradish peroxidase (SA-HRP) (Amersham, GE Healthcare, Buckinghamshire, UK) in reagent diluent, and incubated for 45 min at RT. Wells were washed five times before the addition of 100 µL luminol - SuperSignal Femto Maximum Sensitivity Substrate (Pierce,

Thermo Fisher Scientific, Northumberland, UK). The relative light unit value was read on an Anthos LUCY 1.0 luminometer (Anthos Labtec, Salzburg, Austria).

2.17 ELISA

2.17.2 Detection of IL-6

Levels of bovine IL-6 were determined using a commercial tetramethylbenzidine (TMB) ELISA kit according to the manufacturer's protocol (Thermo Fisher Scientific), using clear Nunc MaxiSorp flat-bottom 96-well microplates, and reagents and incubation times as detailed in section 2.16.1. Samples were tested in triplicate, with the provided recombinant bovine IL-6 as the standard. After washing from SA-HRP incubation, TMB liquid substrate (Sigma-Aldrich) was added, and stopped with the addition of 2N H_2SO_4 once colourisation of the lowest standard was observed. The OD_{450nm} was read using an Anthos LUCY 1.0 luminometer (Anthos Labtec).

2.17.3 Quantifying protein levels

The amount of protein present in the samples was calculated using a standard curve generated from six standards of known concentrations. Standard curve analysis was carried out using Prism® 5.04 software (Graph Pad Software) using the average OD reading of the triplicates. A standard curve was generated by log transforming (X=Log(X)) the concentrations of the standards used and plotting these against the OD readings by nonlinear regression using a sigmoidal dose-response (variable slope) curve. The unknown concentrations of the samples were interpolated from their OD readings using the standard curve, and anti-log transformed (X=10^X) into concentration.

2.18 Statistical analyses

2.18.1 Genotypic frequencies and LD between alleles

Genotypic frequencies were tested for deviation from Hardy-Weinberg equilibrium according to Chi^2 test and level of significance at 1 degree of freedom. Haplotypes and linkage disequilibrium (LD) scores (based on the r^2 statistic), were analysed using the Haploview programme (Barrett, Fry et al. 2005). r^2, the square of the correlation coefficient, measures the specific relationship between the alleles of two loci. An r^2 value ranges between 0, no LD, and 1, absolute LD. In the event of alleles sharing r^2 scores of 0.9–1.0 (high to complete LD that allows for a small degree of spurious genotyping), a tagging SNP (tSNP) is selected as a proxy for the allele linkage group.

2.18.2 Association analysis

Owing to substantial positive skewness (due to zero values) of data distribution, the mean CM rate was transformed: NEWCM = LOG10 ([CM+1/milking days]*365)

This is based upon the model NEWX=LOG10(X+1) as suggested for data transformation (Tabachnick and Fidell 2007). A genotypic model was used for separate genotype pair-wise comparisons. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons for CM rate, milking traits and functional data means (Q-PCR and ELISA) were performed using Prism® 5.04 software (Graph Pad Software). CM events across all lactations (CM recorded = 1, no recorded CM = 0) between genotypes were pair-wise tested for significance by a Chi^2 odds ratio test using Minitab® 16 software (Minitab Statistical Software, Inc, PA, USA). Calculation of Pearson correlation coefficients between factors and analysis of CM response in a General linear model (GLM) was performed using Minitab® 16 software.

Chapter 3.0 - Identification of bovine *TLR1* SNPs – association with mastitis and production traits in cattle

This chapter expands published data (Russell, Widdison et al. 2012).

3.1 Introduction

3.1.1 Bovine Toll-like receptor 1

BoTLR1 is a member of a unique TLR sub-family that includes boTLR6 and boTLR10. Phylogenetic analysis of bo*TLR1*, *6* and *10* reveals a common ancestry with all three genes located in tandem forming a ~69kb bo*TLR6-TLR1-TLR10* gene cluster on *B. taurus* chromosome 6 (bta6) (Opsal, Vage et al. 2006). All encoded proteins have the ability to form heterodimers with boTLR2 to expand the repertoire of recognised bacterial lipoproteins. TLR1/TLR2 complexes mediate cellular responses to natural triacylated lipoprotein structures (Takeuchi, Sato et al. 2002; Omueti, Beyer et al. 2005), which are cell wall constituents of Gram-positive and Gram-negative bacteria (Babu, Priya et al. 2006). TLR6/TLR2 complexes expand ligand recognition to diacylated lipoproteins, although in cattle the boTLR1/boTLR2 heterodimers may be further activated by diacylated lipoproteins (Werling, Jann et al. 2009). Whilst additional investigation is required to uncover their function, mammalian TLR10/TLR2 complexes may extend recognition of TLR1 agonists via an unknown signalling mechanism (Guan, Ranoa et al. 2010). Current evidence demonstrates that bovine MEC are able to sense triacylated lipoproteins through the boTLR1/boTLR2 heterodimer (Porcherie, Cunha et al. 2012). Triacylated lipoproteins are present on cell membranes of important mastitis-causing pathogens including *E. coli* and *S. aureus* (Kurokawa, Lee et al. 2009; Kovacs-Simon, Titball et al. 2011), suggesting that boTLR1 plays a significant role in mammary immune defence (Figure 3.1).

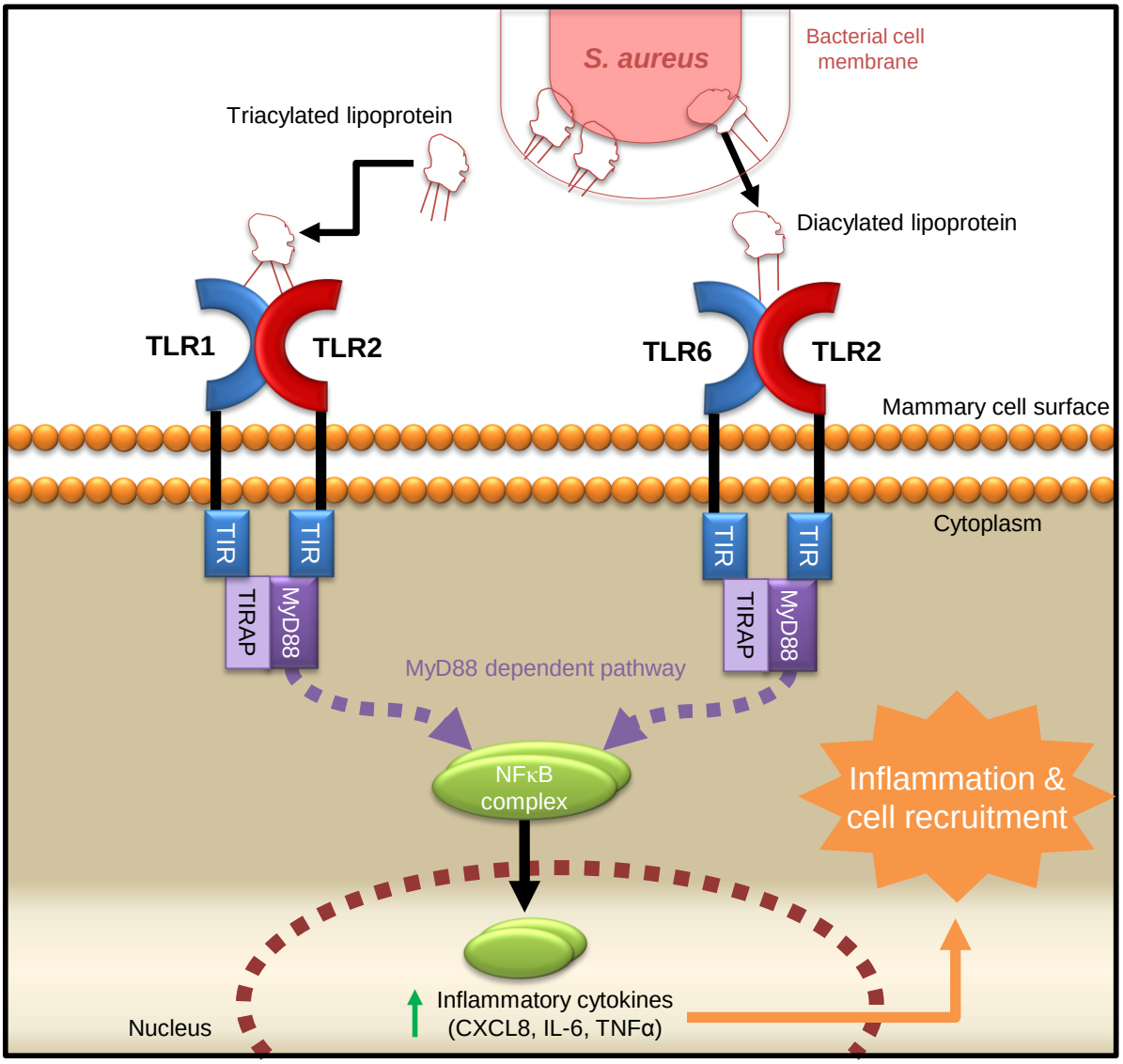


Figure 3.1 - Overview of TLR1/TLR2 heterodimer signalling

Lipoproteins embedded within the cell membrane of bacterial pathogens constitute a major exogenous ligand group for mammalian TLR2, whereby association with TLRs 1 and 6 expands ligand recognition to triacylated and diacylated lipoproteins respectively. TLR2 heterodimerisation is important for specific ligand interactions with both TLR ectodomains and subsequent association of cytoplasmic TIR domains with adapter proteins MyD88 and TIRAP, initiating MyD88-dependent signalling cascades (generalised as dashed arrows) leading to the translocation of transcription factor NF-κB (others include AP-1 and IRF-5) into the nucleus. This initiates synthesis of cytokines leading to a mammary immune response that includes recruitment of leukocytes and lymphocytes into the gland to eliminate the pathogen.

TLR1 variants have been postulated to underlie altered immune responses to a wide spectrum of bacterial pathogens. In humans, a NS *TLR1* SNP located within the transmembrane domain, which had an impact on TLR1 trafficking, NF-κB activity and cytokine output, was found to have strong associations with susceptibility to and progression of infection by Gram-positive bacteria and *Mycobacterium leprae* (Hawn, Misch et al. 2007; Johnson, Lyle et al. 2007; Misch, Macdonald et al. 2008; Wurfel, Gordon et al. 2008). Polymorphisms affecting bo*TLR1* may therefore predispose excessive or weakened immune responses to pathogens and hence could confer an increased risk of mammary infection.

3.1.2 BoTLR1 and health and disease in cattle

Genome-wide quantitative trait locus (QTL) studies revealed the bo*TLR6-TLR1-TLR10* gene cluster to be situated within a dense QTL region for CM and a number of milk production traits (Klungland, Sabry et al. 2001; Ogorevc, Kunej et al. 2009). Furthermore, bo*TLR1* has been highlighted as a strong candidate for underlying QTL regions for resistance to disease, such as mastitis (Jann, King et al. 2009). Despite strong QTL evidence, associative studies between bo*TLR1* and disease are limited. The bo*TLR1* coding sequence (CDS) is highly polymorphic, containing many SNPs across several cattle breeds (Seabury, Cargill et al. 2007). Four SNPs within the CDS of bo*TLR1* were identified in Holstein cows (Li, Shi et al. 2009), but only the previously identified (Seabury, Cargill et al. 2007) NS SNP +1762A>G (reported as +1596G>A) demonstrated highly significant differences in SCC between all genotype populations. The +1762A>G mutation, encoding an isoleucine to valine change within the receptor's transmembrane domain, has also been associated with bovine TB in a Chinese Holstein herd (Sun, Song et al. 2012). Further investigation is required to verify such studies, and to establish associations with novel bo*TLR1* SNPs/haplotypes. More importantly, the ability to use data describing actual CM incidence, rather than a surrogate such as SCC, in

associations with specific genetic markers, has the potential to yield more accurate information.

3.1.3 Rationale for research

The existing limited study of bo*TLR1* SNPs provides scope for further investigation. Existing NS bo*TLR1* SNP +1762A>G associations can be expanded upon and extended into the identification of novel SNPs in the coding or non-coding regions, including introns, 5'UTR and 3'UTR, and adjacent genomic upstream/downstream regions. SNPs identified may interrupt gene promoters or microRNAs, splice sites or other regulatory motifs, potentially interfering with mRNA regulation and protein expression (Song, Chen et al. 2006). The influence of the +1762A>G SNP may be polygenic, linked with additional SNPs and inherited as a haplotype.

The aims for this part of the research were to analyse the impact of existing and novel bo*TLR1* SNPs on CM incidence and milking traits in a Holstein-Friesian herd. In addition, potential differences in relative mRNA levels between observed genotypes were quantified. These differences may influence boTLR1 activity and support existing genotype associations.

3.2 Results

3.2.1 Characterisation of boTLR1 SNPs

The genomic organisation of bo*TLR1* was confirmed using cDNA sequencing and analysis of NCBI EST data, mapped to the Btau_4.0 sequence (NC_007304.4). As described previously (Opsal, Vage et al. 2006), the full length bo*TLR1* mRNA transcript comprises five exons and four introns. The CDS for full length bo*TLR1*; a 2379bp transcript corresponding to a 793 amino acid (aa) chain (Farhat, Riekenberg et al. 2010), is located within exon 5. Sequencing of bo*TLR1* within a Holstein-Friesian herd (*n* = 246) revealed eleven SNPs; five of which are novel (Table 3.1). Eight of the eleven SNPs were exonic, six were located in the CDS, of which only one was a NS change (+1762A>G, Iso > Val) positioned within the transmembrane domain, while the remaining two were located in the non-coding 3'UTR. The three non-exonic SNPs were identified within the introns of the 5' UTR (Figure 3.2).

3.2.2 RFLP-PCR analysis

The bo*TLR1* SNPs +798C>T and +1762A>G were genotyped using RFLP analysis of PCR products, using the restriction enzymes *Mbo*II and *Bcl*I respectively. These proved to be an effective method for genotyping animal samples, with representative results of a typical *Bcl*I digest illustrated in Figure 3.3.

3.2.3 Analysis of LD patterns in boTLR1 for tagging SNP selection

Seven bo*TLR1* SNPs: -79T>G, +798C>T, +1762A>G, +2097T>C, +2100C>T, +2463C>T, and +2731A>G (Figure 3.2) were detected within the herd (*n* = 156, 246, 202, 100, 100, 225 and 232 cows respectively) and included for LD analysis and tagging SNP selection. A graphical representation of the LD between these SNPs is shown in Figure 3.4. All SNPs were defined as one haploblock, with one linkage group identified.

Table 3.1 - Identified SNPs within bo*TLR1* from Holstein-Friesians

SNP	Location	Genomic (Bta6) position	Amino acid position	Amino acid	Restriction site presence	Previously reported	dbSNP ID
-1205A>G	Intron3	60440366	-	-	-	No	-
-1151T>A	Intron3	60440312	-	-	-	No	-
-79T>G	Intron4	60439240	-	-	-	No	-
+798C>T	Exon5	60438363	266	Phe-Phe	*Mbo*II*	Yes [1]	ss73689409
+1641A>C	Exon5	60437520	547	Ser-Ser	*Bst*X-I	Yes [2]	ss73689413
+1716G>A	Exon5	60437445	572	Lys-Lys	*Bsi*Y	Yes [2]	ss73689414
+1762A>G	Exon5	60437324	587	Ile-Val	*Bcl*I*	Yes [2]	ss73689415
+2100C>T	Exon5	60436989	700	Phe-Phe	-	Yes [2]	ss73689417
+2103T>C	Exon5	60436986	701	Val-Val	*Bsm*AI	Yes [2]	ss73689418
+2463C>T	3'UTR	60436698	-	-	*Ms*II	No	-
+2731A>G	3'UTR	60436430	-	-	-	No	-

Abbreviations: Bta6 = *B. taurus* chromosome 6, dbSNP = Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/projects/SNP). All bo*TLR1* sequences were aligned with the published Btau_4.0 sequence NC_007304.4 (underlined allele denotes the published sequence). Light shading - synonymous coding SNPs, dark shading - non-synonymous coding SNP. All bo*TLR1* SNP offsets are given relative to their position (bp) from the A nucleotide of the first ATG codon. *Restriction sites used for RFLP-PCR genotyping.

[1]Opsal, Vage et al. 2006, [2]Seabury, Cargill et al. 2007.

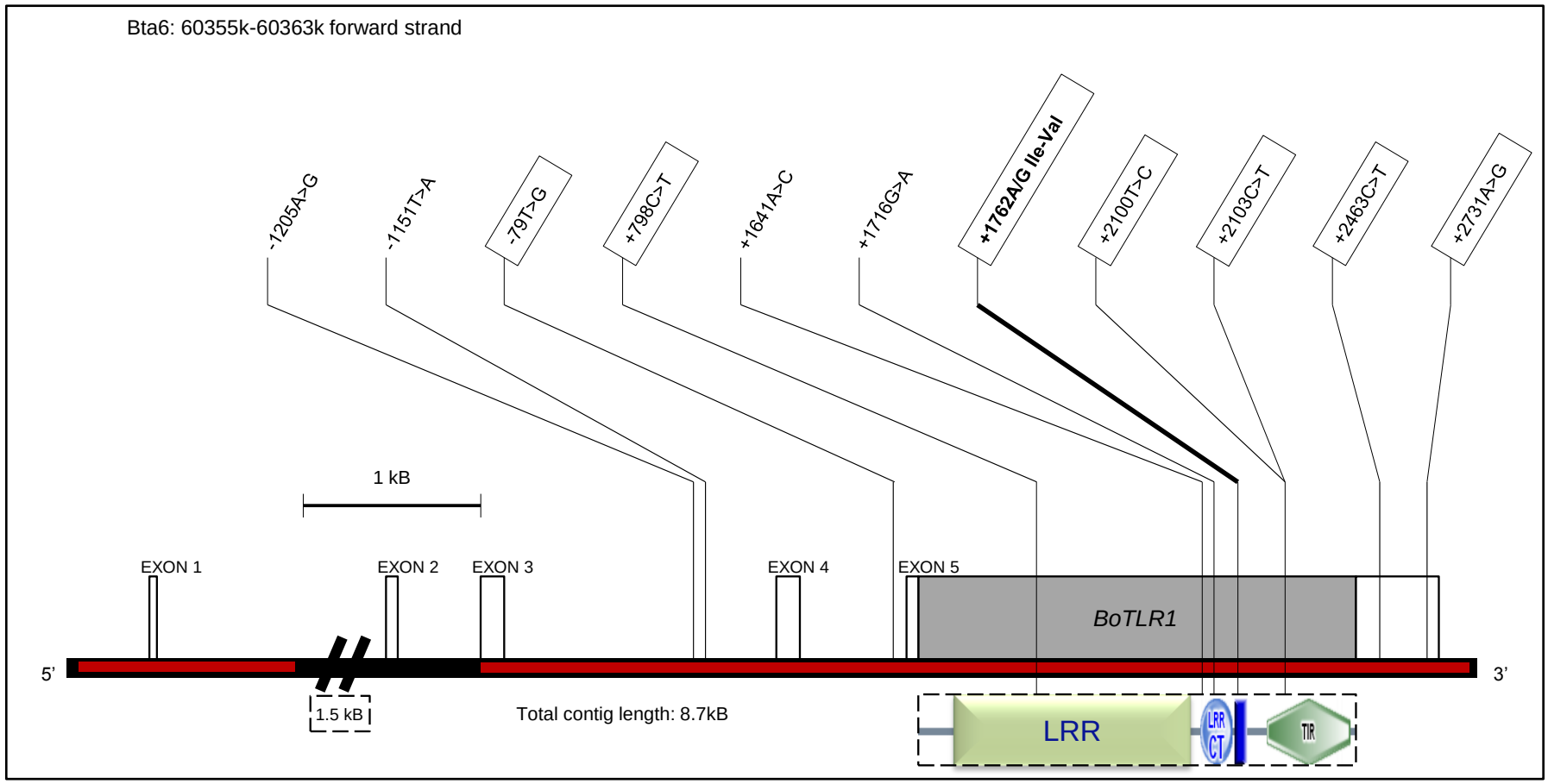


Figure 3.2 - Schematic illustrating the positions of identified SNPs within the bovine *TLR1* gene

Grey box represents coding exon (CDS = 2379bp), open boxes represent non-coding untranslated exonic regions (UTR). A TLR1 protein domain architecture diagram, designed using web-based programmes http://smart.embl-heidelberg.de/ and http://www.lrrfinder.com/, is illustrated below the CDS. The non-synonymous +1762A>G SNP (in bold), is located within the transmembrane domain (vertical black bar). LRR - leucine-rich repeat domains (19 in total), LRR C - leucine-rich repeat C terminal domain, TIR - Toll-interleukin 1-resistance domain. Sequence shown from Btau_4.0 sequence (NC_007304.4), Bta6: 60355k-60363k. Red line represents total sequencing coverage (~6.8kb). Boxed SNPs have been genotyped extensively.

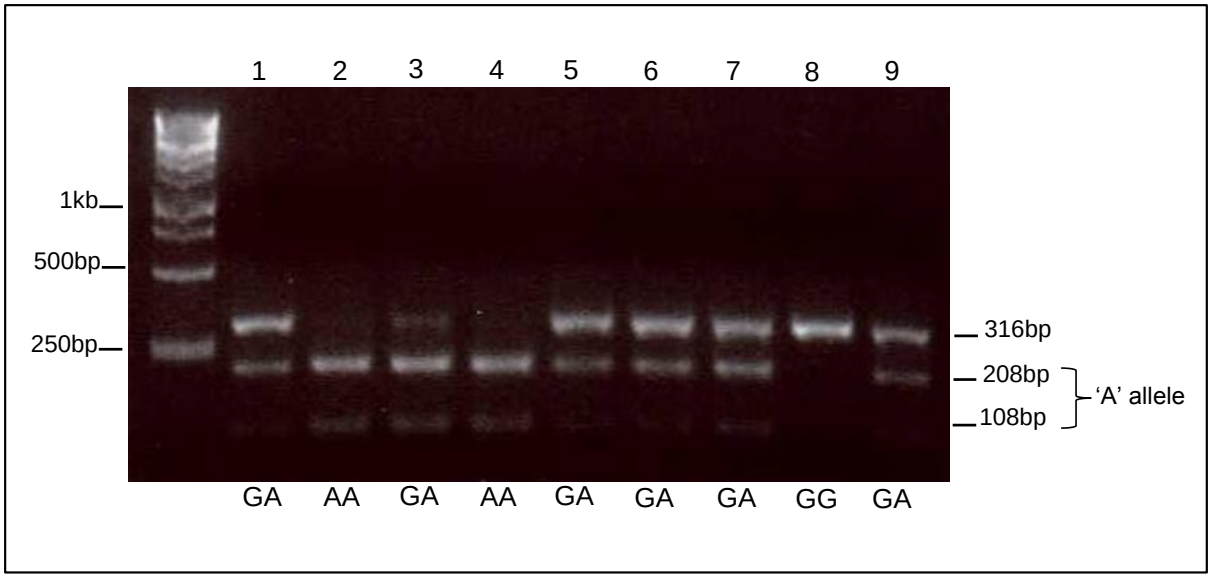


Figure 3.3 - RFLP-PCR product patterns following *Bcl*I digest of the +1762A>G site

Agarose (2%) gel separation of DNA digest products from nine animal samples (1-9 above gel) to determine the genotype (below gel) for the NS bo*TLR1* SNP +1762A>G. The restriction enzyme *Bcl*I cuts only in the presence of the A allele (isoleucine), whereby digest of a 316bp PCR transcript yielded the following product sizes (shown on the right) for each genotype: GG = 316bp (no cut), GA = 316bp, 206bp and 108bp, AA = 208bp and 108bp.

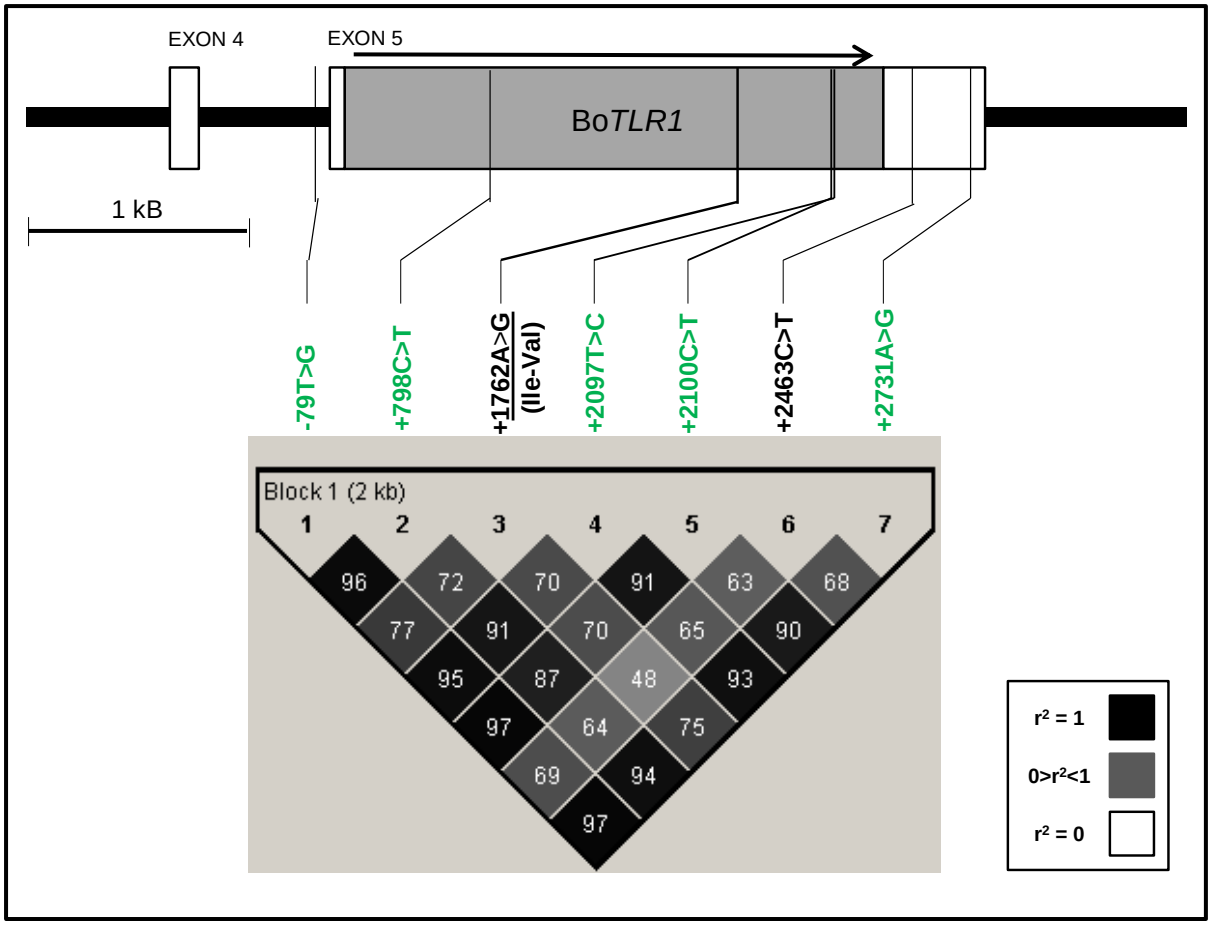


Figure 3.4 - Linkage disequilibrium (LD) plot for seven extensively genotyped SNPs in relation to the bo*TLR1* gene model

Grey box represents bo*TLR1* coding exon, open boxes represent non-coding UTR. The NS SNP is underlined. The graphical representation, generated by Haploview, of the r^2 LD relationship between each SNP is expressed on a grey-scale gradient; where black = perfect LD (r^2 = 1.0) and white = very low LD (<0.1). The r^2 value for each pair-wise comparison is shown. SNP positions highlighted green represent a linkage group whereby each marker is in strong to near complete linkage disequilibrium (LD >0.9) with one another. All SNPs were defined as one haploblock.

SNPs 1, 2, 4, 5, and 7 are in strong LD (>0.9) with each other, and include the intronic SNP -79T>G, synonymous coding SNPs +798C>T, +2097T>C and +2100C>T, and the 3'UTR SNP +2731A>G. As a result, the -79T>G marker was selected as the tSNP for these five SNPs (Table 3.2). Two singleton SNPs were also identified, the NS +1762G>A, and the 3'UTR +2463C>T. LD between the tSNP -79T>G and the NS +1762A>G was moderate (~0.8). LD between SNPs -79T>G and +2463C>T was lower. The lowest LD was observed between SNPs +1762A>G and +2463C>T (Figure 3.4). Consequently, analysis of association with traits for tSNP -79T>G, and SNPs +1762A>G and +2463C>T was performed individually.

Table 3.2 – Summary of selected tSNP marker for bo*TLR1* linkage group

Linkage group	tSNP marker	Tagged SNPS
1	t-79T>G	+798C>T +2097T>C +2100C>T +2731A>G

3.2.4 Analysis of genotypes and allelic frequencies of boTLR1 markers selected for association analysis

The three bo*TLR1* SNPs, -79T>G, +1762A>G and +2463C>T, were detected within the herd (*n* = 246, 202 and 225 respectively). The genotypic and allelic frequencies for each SNP are similar, with the heterozygote consistently the most abundant genotype (Table 3.3). All SNPs were found to be in agreement with Hardy-Weinberg equilibrium. Preliminary haplotype construction for SNPs -79T>G, +1762A>G and +2463C>T yielded two major haplotypes of $G_{79}\underline{A}_{1762}\underline{C}_{2463}$ (frequency 0.42) and $\underline{T}_{79}G_{1762}T_{2463}$ (frequency 0.41) (underlined allele denotes the published Btau_4.0 sequence NC_007304.4). Owing to the low numbers of typed individuals in each haplotype, association analysis cannot be performed.

Table 3.3 - Genotype and allele frequencies including significance from Hardy-Weinberg equilibrium for bo*TLR1* SNPs selected for trait association

SNP	Genotype	*n* =	Genotype Frequency	HWE	Allele	Allele Frequency
-79T>G[1]	TT	78	0.32	0.885	T	0.543
	TG	122	0.49		G	0.456
	GG	46	0.19			
	Total	246				
+1762A>G[2]	AA	54	0.27	0.49	A	0.505
	GA	96	0.47		G	0.495
	GG	52	0.26			
	Total	202				
+2463C>T	CC	64	0.28	0.163	C	0.507
	CT	102	0.46		T	0.493
	TT	59	0.26			
	Total	225				

[1]SNP genotyped by RFLP-PCR using +798C>T proxy. [2]SNP genotyped by RFLP-PCR. Hardy-Weinberg equilibrium (HWE) not significant at 1 degree of freedom.

3.2.5 Analysis of boTLR1 SNPs with clinical mastitis and milk production traits

The three boTLR1 SNPs -79T>G, +1762A>G and +2463C>T were analysed for association with CM and milk production traits during their first three lactations, as illustrated in Table 3.4. Each SNP was further tested for association with CM per individual parity (P1, P2 and P3), as illustrated in Table 3.5.

3.2.5.1 Association analysis of boTLR1 tSNP -79T>G

Analysis of the tSNP -79T>G revealed the TG and GG genotypes associated with an increased rate of CM (0.73 and 0.75 cases/cow/year, respectively) compared with the TT genotype (0.40 cases/cow/year) (Table 3.4). The association with CM rate was found to be significantly (P<0.05) higher for the heterozygote (TG) population, the most abundant genotype, when compared with homozygote (TT) animals. The proportion of animals within each genotyped population having any episodes of CM during their first three lactations was highest in the GG genotype (70%) compared with TT and TG genotypes (58% and 60%, respectively), although this was not statistically significant.

Analysis per individual parity revealed the higher CM rate genotype, GG, to significantly associate with increased CM incidence during the second (P2) parity (P<0.05) when compared to the common homozygous TT genotype, with rates of 0.63 compared to 0.24 respectively (Table 3.5). Although when compared to the lowered CM rate of TT cows at P2, TG animals also displayed raised incidence of 0.59, only near significance (P<0.1) was detected. However significance was detected between these genotypes for the proportion of animals within each population having any CM during P2; with the highest in the TG heterozygotes (35%) compared with TT (16%). TT and TG cows exhibited a highly significant increase in CM incidence by P3 when compared to P1 and P2 data (Figure 3.5). Over half of the genotyped TG population (56%) had at least one CM event during P3,

however only near significance was detected ($P<0.1$) when compared to the TT and GG genotypes (37% and 29% respectively).

Analysis for association with production traits revealed that the TG and GG genotypes of the tSNP -79T>G associated significantly with reduced milk fat (TG = $P<0.05$, GG = $P<0.01$) and protein (TG = $P<0.01$, GG = $P<0.001$) concentrations when compared to those observed in the TT genotype (Table 3.4). No differences in either milk yield or mean SCC were detected between any of the genotyped populations described.

Table 3.4 – Association of bo*TLR1* SNP genotypes with CM and milk production traits over the first three lactations

SNP	Gen.	*n* =	P % with CM[1]	Mean CM rate[2]	*P* =[3]	Mean SCC[4]	*P* =[3]	Mean 305d milk yield[4]	*P* =[3]	Mean milk fat (%)[4]	*P* =[3]	Mean milk protein (%)[4]	*P* =[3]
-79T>G	TT	78	58%	0.40 (± 0.11)		2.88 (± 0.84)		7868 (± 1181)		4.15 (± 0.41)		3.28 (± 0.19)	
	TG	122	60%	0.73 (± 0.18)*	< 0.05	2.76 (± 0.74)	-	8058 (± 1139)	-	4.02 (± 0.38)*	< 0.05	3.2 (± 0.17)*	< 0.01
	GG	46	70%	0.75 (± 0.24)	< 0.1	2.91 (± 0.78)	-	7983 (± 1273)	-	3.89 (± 0.41)*	< 0.01	3.16 (± 0.18)*	< 0.001
+1762A>G	AA	54	62%	0.74 (± 0.27)	< 0.1	2.87 (± 0.69)	-	7836 (± 1104)	-	3.95 (± 0.42)	< 0.1	3.17 (± 0.18)	-
	GA	96	61%	0.70 (± 0.18)	< 0.1	2.84 (± 0.76)	-	8104 (± 1282)	-	4.04 (± 0.41)	-	3.21 (± 0.19)	-
	GG	52	59%	0.38 (± 0.16)		2.84 (± 0.85)		8113 (± 1132)		4.13 (± 0.41)		3.25 (± 0.18)	
+2463C>T	CC	64	67%	0.73 (± 0.22)	-	2.90 (± 0.75)	-	7841 (± 1067)	-	4.02 (± 0.45)	-	3.19 (± 0.18)*	< 0.05
	CT	102	61%	0.74 (± 0.17)*	< 0.05	2.84 (± 0.81)	-	8137 (± 1206)	-	3.98 (± 0.45)	-	3.18 (± 0.2)*	< 0.05
	TT	59	59%	0.36 (± 0.11)		2.82 (± 0.83)		7940 (± 1262)		4.13 (± 0.38)		3.27 (± 0.18)	

Abbreviations: Gen. = genotype, P % = population percentage, CM = clinical mastitis, SCC = somatic cell count. *Column presence indicates significant association ($P<0.05$). [1]No significance was detected between genotypes by Chi^2 odds ratio test. [2]Mean ± 95% confidence interval given in column parentheses. [3]*P* values analysed by ANOVA and Tukey's multiple comparisons, showing only results tested against the underlined (lower CM rate) genotype; – (dash) denotes no significance. [4]Standard deviation from the mean given in column parentheses.

Table 3.5 - Association of bo*TLR1* SNP genotypes with CM at first (P1) second (P2) and third (P3) parity

SNP	Gen.	*n*=	P1 P % with CM[1]	P1 Mean CM rate[2]	*P* =[3]	P2 P % with CM[1]	P2 Mean CM rate[2]	*P* =[3]	P3 P % with CM[1]	P3 Mean CM rate[2]	*P* =[3]
-79T>G	TT	78	20%	0.26 (±0.18)		16%	0.24 (±0.12)		37%	0.87 (±0.33)	
	TG	122	19%	0.54 (±0.20)	-	35%*	0.59 (±0.22)	< 0.1	56%†	1.15 (±0.51)	-
	GG	46	13%	0.53 (±0.42)	-	26%	0.63 (±0.37)*	<0.05	29%	0.88 (±0.61)	-
+1762A>G	AA	54	22%	0.44 (±0.26)	-	26%	0.60 (±0.35)	-	47%	1.24 (±0.52)	-
	GA	96	27%	0.57 (±0.25)	-	34%	0.62 (±0.23)	< 0.1	44%	1.14 (±0.40)	-
	GG	52	18%	0.27 (±0.16)		22%	0.28 (±0.16)		41%	0.74 (±0.31)	
+2463C>T	CC	64	32%	0.58 (±0.26)	-	35%	0.80 (±0.42)	< 0.1	47%	1.64 (±1.24)	-
	CT	102	25%	0.66 (±0.35)	-	30%	0.69 (±0.31)	< 0.1	48%	1.35 (±0.41)	-
	TT	59	22%	0.28 (±0.16)		19%	0.21 (±0.10)		42%	0.96 (±0.54)	

Abbreviations: Gen. = genotype, P % = population percentage, CM = clinical mastitis, P1 = parity 1, P2 = parity 2, P3 = parity 3. [1]Column presence of * represents detection of significance between genotypes by Pearson Chi^2 odds ratio test ($P<0.05$), with positioning of * alongside the genotype contributing most to the Chi^2. Column presence of † represents detection of near significance between genotypes ($P<0.1$), with positioning of † alongside the genotype contributing most to the Chi^2. [2]Mean±95% confidence interval given in column parentheses. [3]*P* values analysed by ANOVA and Tukey's multiple comparisons, showing only results tested against the underlined (most abundant) homozygous genotype; – (dash) denotes no significance.

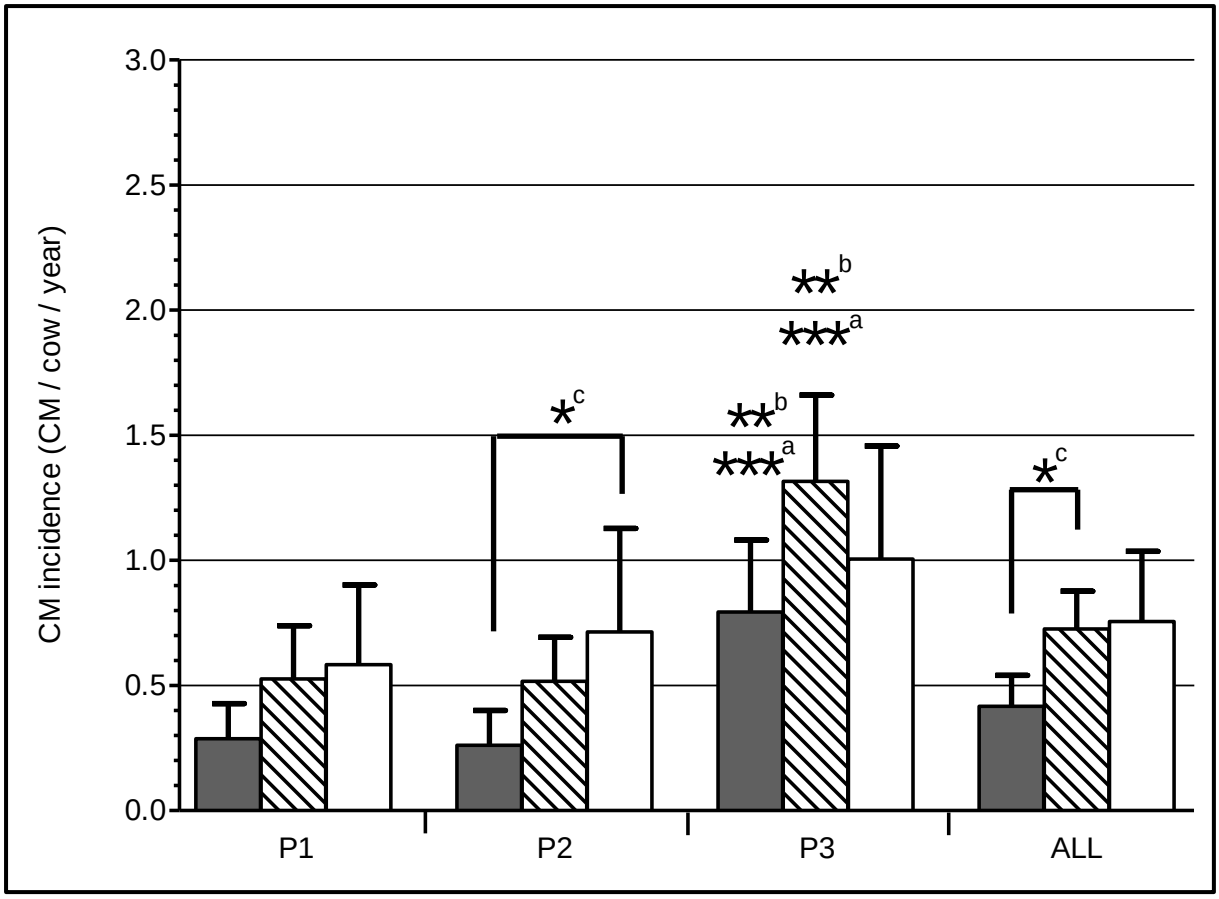


Figure 3.5 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*TLR1* -79T>G tSNP variant populations: TT, TG and GG

Data from each variant population are presented as a bar, with positive whiskers representing 95% confidence interval of the mean. Grey bars – TT animals (*n* =78), hatched bars – TG animals (*n* =122) and open bars – GG animals (*n* =46). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. [c] = tested between the indicated genotypes. Genotypic means differ significantly at the following levels: **P*<0.05, ***P*<0.01 ****P*<0.001, by ANOVA and Tukey's multiple comparisons.

3.2.5.2 Association analysis of boTLR1 SNP +1762A>G

Analysis of the NS transmembrane domain SNP +1762A>G, demonstrated AA and AG animals had higher rates of CM (0.74 and 0.70 cases/cow/year, respectively) compared with GG animals (0.38 cases/cow/year), although these differences were not significantly different. The proportion of animals within each genotyped population having any CM during their first three lactations was similar across all three genotypes (Table 3.4).

Analysis of CM incidence per individual parity revealed the higher disease rate genotypes AA and AG demonstrated increased CM incidence at P1, P2 and P3 when compared to the homozygous GG genotype; however no significance was detected (Table 3.5). The lower CM rate genotype GG exhibited a significant increase in CM incidence by P3 when compared to their P1 and P2 data; AA and AG animals exhibited a significant P3 increase when compared to their P1 data only (Figure 3.6).

Similar to trends with the tSNP -79T>G genotypes, the genotypes of the NS SNP +1762A>G with higher CM rate, AA and AG, demonstrated with lower milk fat and protein concentrations; however these differences were not statistically significant (Table 3.4). No differences in either milk yield or mean SCC were detected between any of the genotyped populations described.

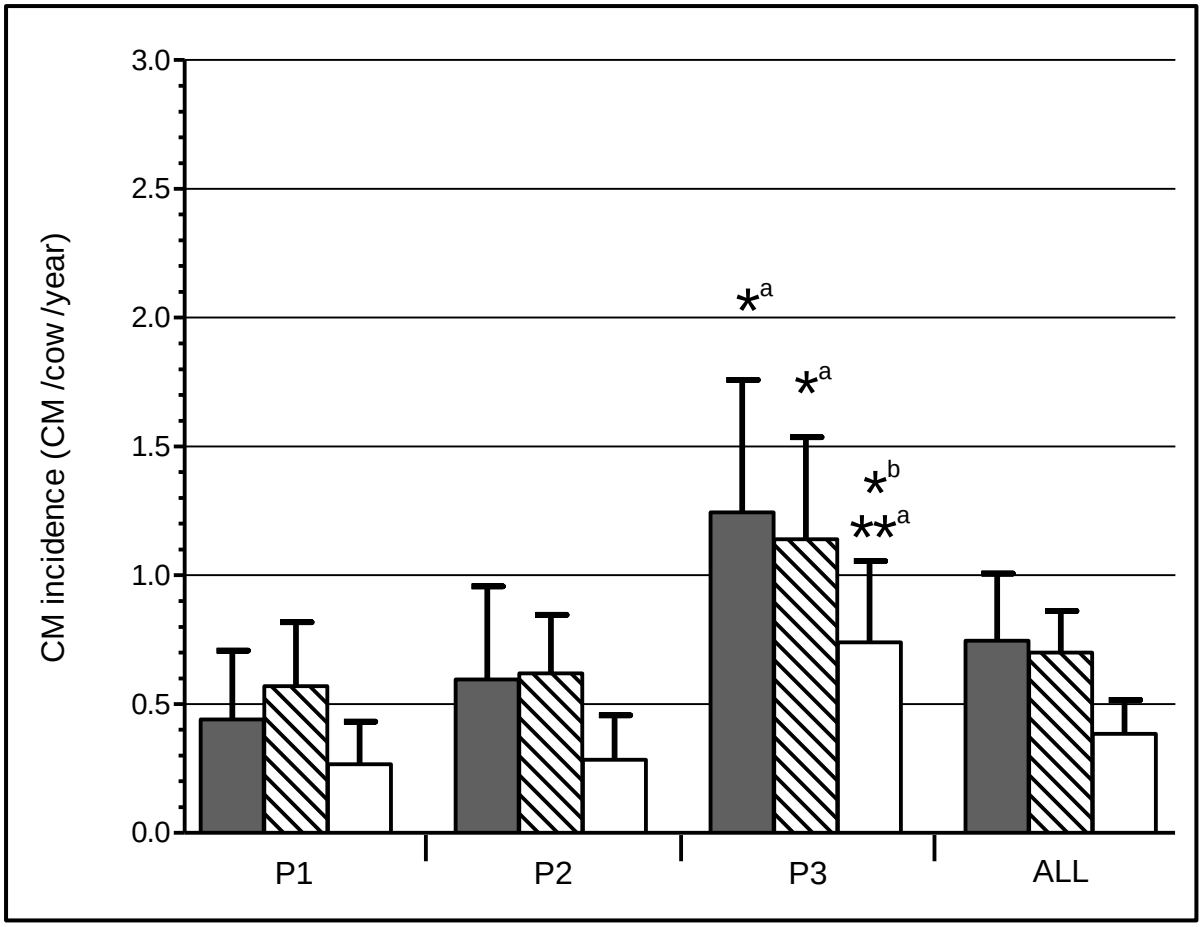


Figure 3.6 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*TLR1* +1762A>G SNP variant populations: AA, AG and GG

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – AA animals (*n* =54), hatched bars – AG animals (*n* =96) and open bars – GG animals (*n* =52). P1 = first parity, P2 = second parity, P3 = third parity, and ALL = all parities. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Genotypic means differ significantly at the following levels: **P*<0.05, ***P*<0.01 by ANOVA and Tukey's multiple comparisons.

3.2.5.3 Association analysis of boTLR1 SNP +2463C>T

Analysis of the SNP +2463C>T located within the 3'UTR revealed that CC and CT animals had higher rates of CM (0.73 and 0.74 cases/cow/year) compared to TT animals (0.36 cases/cow/year) (Table 3.4). However, only the increase seen in the most abundant genotype, heterozygous CT cows, was shown to be statistically significant ($P<0.05$). The proportion of animals within each genotyped population having any CM during their first three lactations was highest in the CC genotype (67%) compared with CT and TT genotypes (61% and 59%, respectively), although this was found not to be statistically significant.

Analysis of CM incidence per individual parity revealed the higher CM rate genotypes, CC and CT, tended to associate with increased CM incidence during P2 ($P<0.1$) when compared to the homozygous TT genotype, with rates of 0.80 and 0.69 cases/cow/year compared to 0.24 respectively (Table 3.5). Owing to lowered CM at P1 and P2, only TT cows exhibited a significant increase in incidence by P3 when compared to their P1 and P2 data (Figure 3.7).

Analysis of production traits within the genotyped animals revealed that higher CM genotypes CC and CT produced milk with significantly lower fat and protein concentration ($P<0.05$) when compared with the TT population (Table 3.4). No differences in either milk yield or mean SCC were detected between any of the genotyped populations described.

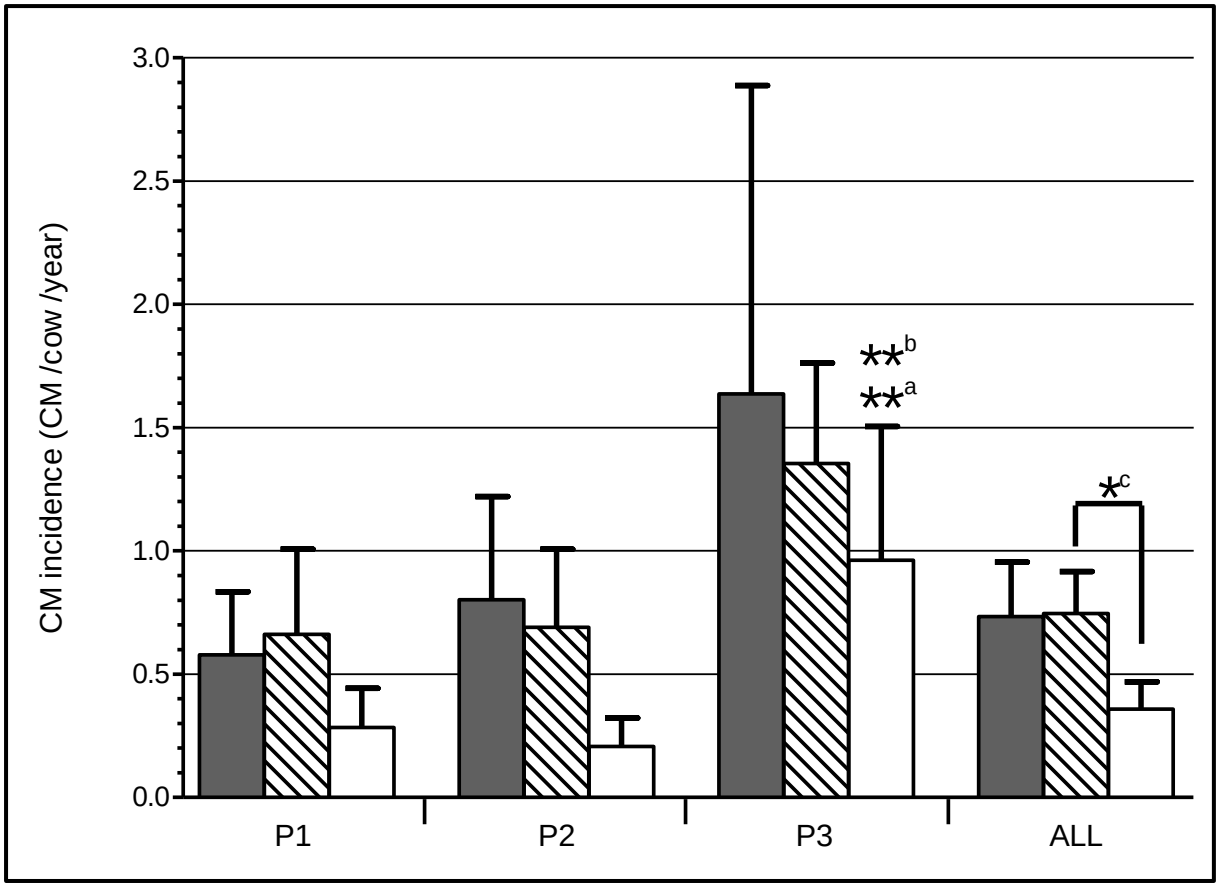


Figure 3.7 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*TLR1* +2463C>T SNP variant populations: CC, CT and TT

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – CC animals (*n* =64), hatched bars – CT animals (*n* =102) and open bars – TT animals (*n* =59). P1 = first parity, P2 = second parity, P3 = third parity, and ALL = all parities. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. [c] = tested between the indicated genotypes. Genotypic means differ significantly at the following levels: **P*<0.05, ***P*<0.01, by ANOVA and Tukey's multiple comparisons.

3.2.6 Multiple comparisons

Correlation coefficient analysis was performed revealing a weak positive correlation between CM and SCC (Pearson coefficient = 0.198) and weak negative correlations between CM and milk fat and protein percentages (Pearson coefficient = -0.2 and -0.1 respectively). All significant associations between individual SNP genotypes and CM were found to survive adjustments for each factor using a general linear model (GLM).

3.2.7 Q-PCR analysis of basal boTLR1 mRNA abundance

Milk somatic cells from cows with representative bo*TLR1* -79T>G tSNP genotypes were assessed for differences in basal *boTLR1* expression. All animals were of similar age and stage of lactation and none had any known health issues. Q-PCR assays were performed using RNA from five representative animals of each genotype. Cows with the GG genotype demonstrated lower *boTLR1* mRNA abundance ($P<0.01$) compared with either the TT or TG genotypes (Figure 3.8). Due to close homology and potential for co-expression, the abundance of *boTLR6* and *boTLR10* mRNA was also measured and revealed no significant differences. In addition, to exclude the influence of unidentified bo*TLR2* variance on *boTLR1* expression the abundance of *boTLR2* mRNA was also measured and revealed no significant differences when segregated by bo*TLR1* -79T>G tSNP genotypes (Figure 3.9).

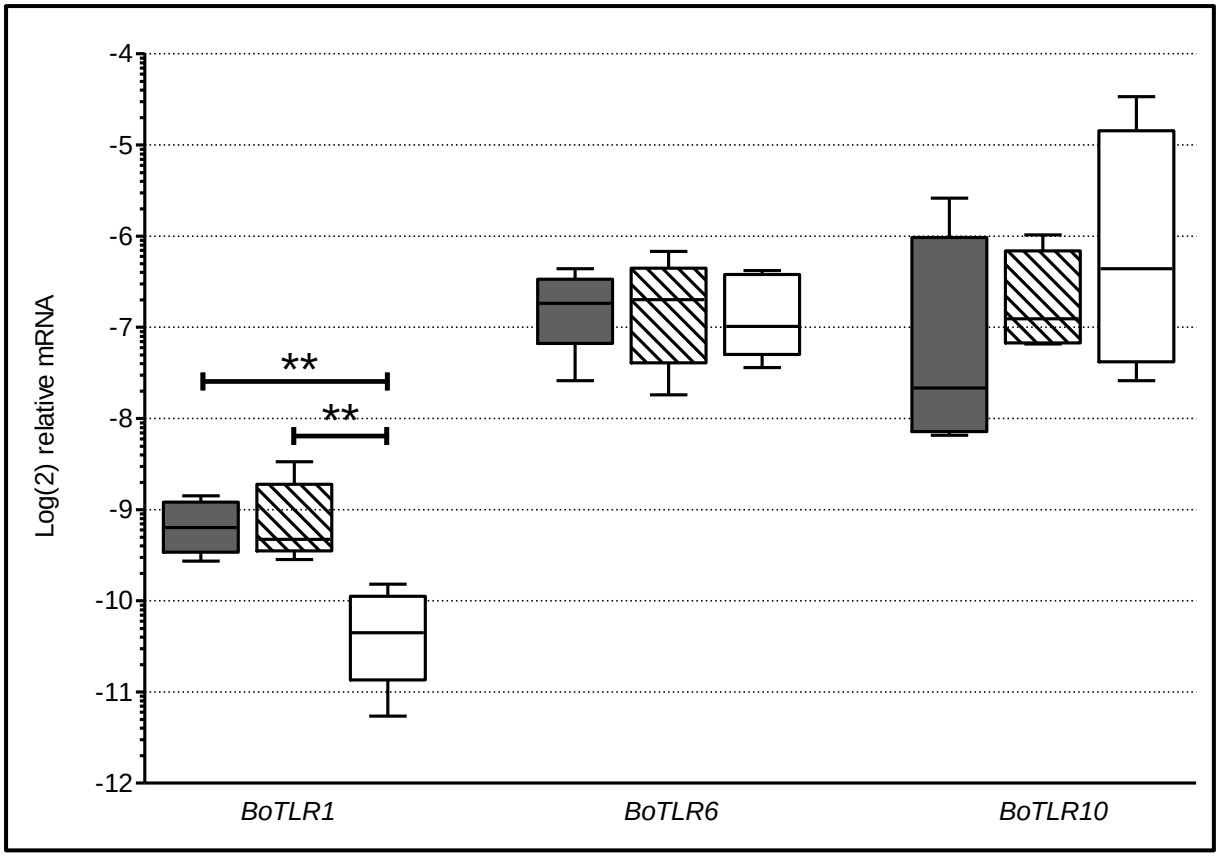


Figure 3.8 - Q-PCR data illustrating basal mRNA abundance levels of *boTLR1*, *boTLR6* and *boTLR10* in milk somatic cells grouped into the three bo*TLR1* -79T>G tSNP variant populations: TT, TG and GG

Grey boxes – TT animals, hatched boxes – TG animals, and open boxes – GG animals (n =5). **Genotypic means differ significantly (P<0.01 by ANOVA and Tukey's multiple comparisons).

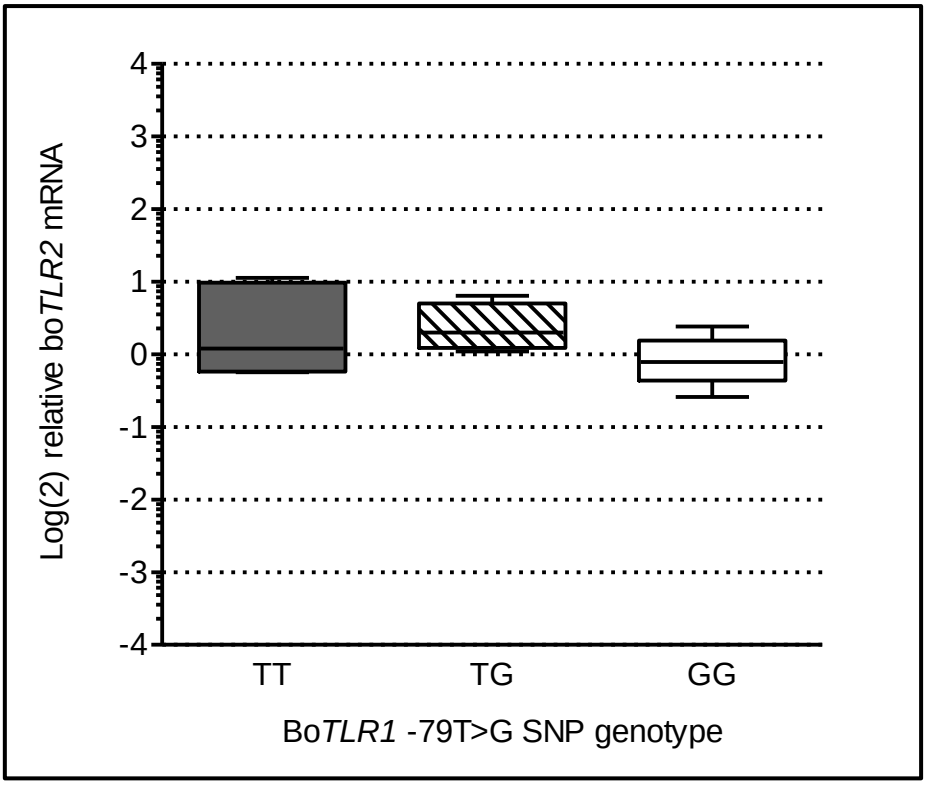


Figure 3.9 - Q-PCR data illustrating basal mRNA abundance levels of *boTLR2* in milk somatic cells grouped into the three bo*TLR1* -79T>G tSNP variant populations: TT, TG and GG

Grey boxes – TT animals, hatched boxes – TG animals, and open boxes – GG animals (*n* =5).

3.2.8 In-silico analysis for putative transcription binding sites

The AliBaba2.1 (TRANSFAC 4.0 sites) software programme was used to search for putative transcription binding sites (TBS) located immediately upstream of the ATG start codon for bo*TLR1*. The upstream bo*TLR1* SNPs identified in this study were analysed for any potential impact on predicted TBS. Figure 3.10 illustrates analysis of the 5' intronic SNP -79T>G to determine the presence (T allele) or absence (G allele) of several putative DNA binding sites for transcription factors and enhancer proteins, including matched sites for AP-1-related c-Jun, Octamer-1 (Oct-1), CCAAT/enhancer binding protein alpha (C/EBPalp) and TATA binding protein (TBP).

A

*Bos_taurus*_ Btau_4.0 Primary Assembly Sequence

```
-155 TCATTTAATGAGTCTCTAGTAAAAAAATGTTTAATGTCAGTCATGCTTAATTTAATATAG
                      =====Hb===
                                        ====AP-1===
                                           ==CRE-BP1=
                                             ===c-Jun==
                                             ===c-Fos==
                                                   ==Oct-2.1=
                                                        ====
                SNP-79T>G
 -95 TCATTTAATGCTGACTTTTTTACCCCTAGGATCTGTACTGTATCCAGATGCTCTGAAGAA
     GCN4==
            ===c-Jun==
            =C/EBPalp=
            ===Oct-1==
                   ====TBP===
                    =C/EBPalp=
                    =====Hb===
                                      =====GR===

 -35 TAGCAACTACCCTTAGGAATGTCTACTGTTACAAAatg...
```

B

Alternate Allele Sequence

```
-155 TCATTTAATGAGTCTCTAGTAAAAAAATGTTTAATGTCAGTCATGCTTAATTTAATATAG
                      =====Hb===
                                        ====AP-1===
                                           ==CRE-BP1=
                                             ===c-Jun==
                                             ===c-Fos==
                                                   ==Oct-2.1=
                                                        ====
                SNP-79T>G
 -95 TCATTTAATGCTGACTGTTTTACCCCTAGGATCTGTACTGTATCCAGATGCTCTGAAGAA
     GCN4==

                                      =====GR===

 -35 TAGCAACTACCCTTAGGAATGTCTACTGTTACAAAatg...
```

Figure 3.10 - Location and impact of upstream bo*TLR1* -79T>G SNP in relation to putative transcription binding sites for both published allele (A) and alternate allele (B) sequences

The AliBaba 2.1 software programme was used to predict (75% match threshold or 70% match threshold) transcription factor binding sites (TBS) in the bo*TLR1* upstream region. Upstream nucleotide sequence shown in upper case, purple text represents 5'UTR. ATG start codon shown by lower case. Numbering indicates nucleotide position from the start codon. SNP position highlighted in red. Positions of putative TBS are aligned below the nucleotide sequence. TBS abbreviations: Hb = hunchback, AP-1 = Activator Protein-1, CRE-BP1 = cyclic AMP response element – binding protein 1, c-jun and c-fos = JUN and FOS proteins which dimerise to form AP-1, Oct-2.1 and Oct-1 = Octamer transcription factor-1 and -2, GCN4 = General control protein GCN4, C/EBPalp = CCAAT/enhancer binding protein alpha, TBP = TATA-binding protein, GR = glucocorticoid receptor.

3.3 Discussion

Three SNPs within exonic and non-exonic regions of bo*TLR1* were analysed for association with susceptibility to CM, identifying significant associations for the tSNP -79T>G and the 3'UTR SNP +2463C>T. Rates of CM for genotypes -79 TT and +2463 TT were much lower in comparison to the homozygous genotypes (-79 GG and +2463 CC) and significantly lower than their respective heterozygous genotypes. Dairy cows have been subjected to intense selection pressures for higher milk yields and reduced SCC that may unintentionally have been detrimental to CM susceptibility (Suriyasathaporn, Schukken et al. 2000). In this study no significant differences were observed between identified bo*TLR1* SNPs, including the NS SNP +1762A>G, for mean 305d milk yields or SCC. This contradicts a study of 208 Chinese Holsteins, in which the NS SNP +1762A>G associated with highly significant differences in milk SCC. Animals with an AA (isoleucine) at this position had lower SCC, and were described as more resistant to mastitis as a result (Li, Shi et al. 2009). In contrast, our results suggest the GG (valine) at this location is the more favourable genotype in terms of having a lower CM incidence in comparison to AA. Although this discrepancy may reflect breed and herd variation, it may also demonstrate the dichotomy that the SCC, which is the often used surrogate measure of mastitis, is also the presence of the effector cell which can eliminate infection in the absence of overt clinical signs. In agreement with GG animals being a more disease-resistant genotype, analysis of a further Chinese Holstein herd associated AA and AG variants with bovine TB susceptibility (Sun, Song et al. 2012). The raised CM rates observed in this study for +1762A>G AA and AG cows, although not significant, will benefit from genotyping in a larger herd.

All bo*TLR1* SNP genotypes that were associated with a lowered rate of CM displayed significantly greater milk fat and protein concentrations when compared to the other two genotypes. It is unclear whether a functional association exists between milk fat and protein

concentrations and mastitis susceptibility. Interestingly the casein gene cluster is located ~28Mb from the bo*TLR6-TLR1-TLR10* gene cluster on Bta6 (Threadgill and Womack 1990). Protein and fat contents are financially rewarded milk qualities and breeding for these factors could influence bo*TLR1* genotypes.

In addition to being one of the first studies to examine association between bo*TLR1* SNPs and actual CM incidence, evidence of variable receptor expression between the defined genotypes is also demonstrated. In this study animals segregated by -79T>G tSNP genotypes exhibited significantly lower bo*TLR1* expression in GG animals when compared with TT or TG. This difference, which appeared to be specific for bo*TLR1* as it was not seen for paralogues bo*TLR6* or bo*TLR10*, or dimer partner bo*TLR2*, may indicate interference with transcription. Preliminary analysis for causal mutations, using the programme MOTIF (http://motif.genome.jp) and TRANSFAC 4.0 programme AliBaba2.1 (www.gene-regulation.com/pub/programs.htmL), highlighted the -79T>G SNP as part of several candidate DNA binding motifs, including putative matches for AP-1-related c-Jun, Octamer-1 (Oct-1) and C/EBPalp. A similar search for putative sites immediately 5' of the ATG start codon of *TLR1* in the human system revealed almost identical presence of Oct-1, Hb, AP-1, and C/EBPalp sites. Several putative Oct-1 sites were also detected immediately 5' of the ATG start codon in porcine *TLR1*, suggesting species conservation and importance for Octamer binding. Oct-1 is thought to work closely with AP-1 and GATA-1 as important regulatory elements for TLR expression (Roger, Miconnet et al. 2005). However this motif, along with others, was only identified with the T variant and was absent in those with the G substitution. Although the potential for -79T>G SNP determining Oct-1 regulation of bo*TLR1* expression is an attractive hypothesis, it is also possible that this tSNP may simply be a marker for other unidentified causal mutations. Furthermore, the animals used for the Q-PCR assays in this study all conform to observed haplotypes: TT = $T_{79}G_{1762}T_{2463}$ and GG =

$G_{79}A_{1762}C_{2463}$, with TG animals heterozygous for each SNP. In essence +1762 AA and +2463 CC variants, genotypes with increased CM, have reduced bo*TLR1* expression.

Reduced boTLR1 stimulation by pathogens in -79 GG cows may equate to a dampened inflammatory response thus enabling establishment and persistence of infection. In terms of influence upon mastitis susceptibility, GG cows have a higher CM rate than TT cows; however heterozygous TG cows have a similar rate to GG cows, despite expressing bo*TLR1* at a similar level to a TT cow. This suggests that differences in bo*TLR1* transcript levels alone may not account for the variation in CM incidence, and/or that post-transcriptional and post-translational regulation may also be affected by the cumulative presence of SNPs; a high level of linkage was observed within the herd between bo*TLR1* SNPs identified in this study. The NS SNP +1762 Iso>Val substitution within the transmembrane domain warrants targeted structure/function studies to verify its impact on TLR1 signalling. SNPs within the 3'UTR, including the +2463C>T SNP that significantly associated with CM in this study, could also interfere with microRNA (miRNA) post-transcriptional regulation. Although *TLR1* miRNAs have been predicted for human and mouse systems (www.microrna.org/) there is no suitable bovine miRNA database. LD between the tSNP -79T>G and the 3'UTR SNP +2463C>T was moderate to high (~0.7) therefore their individual associations with disease and reduced gene expression could be influenced by their cumulative presence. Furthermore, influence of unidentified polymorphisms within bo*TLR1* and additional related genes such as bo*TLR2* and bo*TIRAP* should be considered. Despite this study not showing any correlation between bo*TLR2* mRNA levels and observed bo*TLR1* variation, mutations within boTLR2 may still influence boTLR1 expression and function.

In conclusion the findings presented here demonstrate an association between SNPs common to bo*TLR1* and CM susceptibility. Furthermore, we present evidence that several of these SNPs underpin variation in bo*TLR1* expression. The complexity of mastitis infection

suggests a polygenic and multi-factorial immune response comprising many different proteins which will in turn interact with one another. The ability to detect significant associations with CM for a single gene indicates the importance of boTLR1-related responses in the control of bacterial disease in cattle. A bovine population with impaired bo*TLR1* expression may also be more susceptible to other diseases and health issues. Preliminary evidence for this comes from our study where it was observed that animals with the -79 GG variant seemed to be prematurely culled compared to other populations (data not shown). Furthermore the NS SNP +1762A>G, in moderate to high LD (>0.8) with the tSNP -79, has been associated with TB susceptibility (Sun, Song et al. 2012).

The potential of SNPs within bo*TLR1* to act as genetic markers for altered susceptibility to mastitis warrants further investigation. The favourable linkage of lowered CM with increased milk fat and protein concentrations in the bo*TLR1* variants demonstrated here indicates that selection for lowered CM using these markers would not be detrimental to milk quality, as has been previously suggested in studies linking SNPs with increased SCC as a marker for CM susceptibility.

Chapter 4.0 - SNPs in bo*TLR1* underlie the dampening of innate immune responses

This chapter expands published data (Russell, Widdison et al. 2012).

4.1 Introduction and Rationale

SNPs identified within bo*TLR1* are potential markers for impaired bo*TLR1* expression and CM susceptibility. Defined by their -79T>G tSNP genotype, the TT variant population was observed to have significantly lowered CM incidence when compared to heterozygotes, whilst a GG variant population, which tended to show raised incidence compared to TT cows, demonstrated lowered basal levels of bo*TLR1* transcripts in milk somatic cells. Currently there are no boTLR1 antibodies available to directly compare levels of protein expression. To ascertain the functional consequence of potentially reduced bo*TLR1* expression, production of cytokines known to be induced by TLR1 was determined from cells stimulated with the ligand PAM_3, a lipoprotein which has an affinity for TLR1/TLR2 heterodimers (Jin, Kim et al. 2007), as illustrated in Figure 4.1. A recent chimeric study demonstrated the importance of the TLR1 TIR domain for the signalling of immune responses to PAM_3 and heat-killed bacteria, since, although PAM_3 can bind TLR10/TLR2, signalled production of cytokines CXCL8 and IL-6 requires a TLR1 cytoplasmic domain (Guan, Ranoa et al. 2010).

In the human system, a number of functional studies facilitating the analysis of TLR1 variants exist. The well-documented NS hu*TLR1* SNP 1805T>G, encoding an isoleucine to serine substitution within the receptor's transmembrane domain, impacts signalling and function. It has been observed across several studies that cells from GG (serine) variants display impaired NF-κB activity, reduced receptor trafficking and surface expression and reduced cytokine responses (Hawn, Misch et al. 2007; Johnson, Lyle et al. 2007; Misch, Macdonald et al. 2008; Wurfel, Gordon et al. 2008; Randhawa, Shey et al. 2011). These investigations detected lowered cytokine production of IL-6, CXCL8 and TNF-α from whole blood and PBMCs provided by GG variants, following *in vitro* stimulation with PAM_3 (at varying concentrations) and extracts of *Mycobacterium tuberculosis* and *M. leprae*.

Functional studies analysing differences in the response of boTLR1 between polymorphic variant populations have yet to be documented in cattle. Evidence of increased production of the important chemokine CXCL8 has recently been reported in bovine MEC exposed to PAM_3 (Porcherie, Cunha et al. 2012). The objective of this part of the study was to ascertain any functional consequences of bo*TLR1* polymorphic variation on the resulting cytokine response, following ligand-receptor binding. In addition, variances in cytokine responses to HK pathogens of economic importance were analysed between the bo*TLR1* -79T>G tSNP variants.

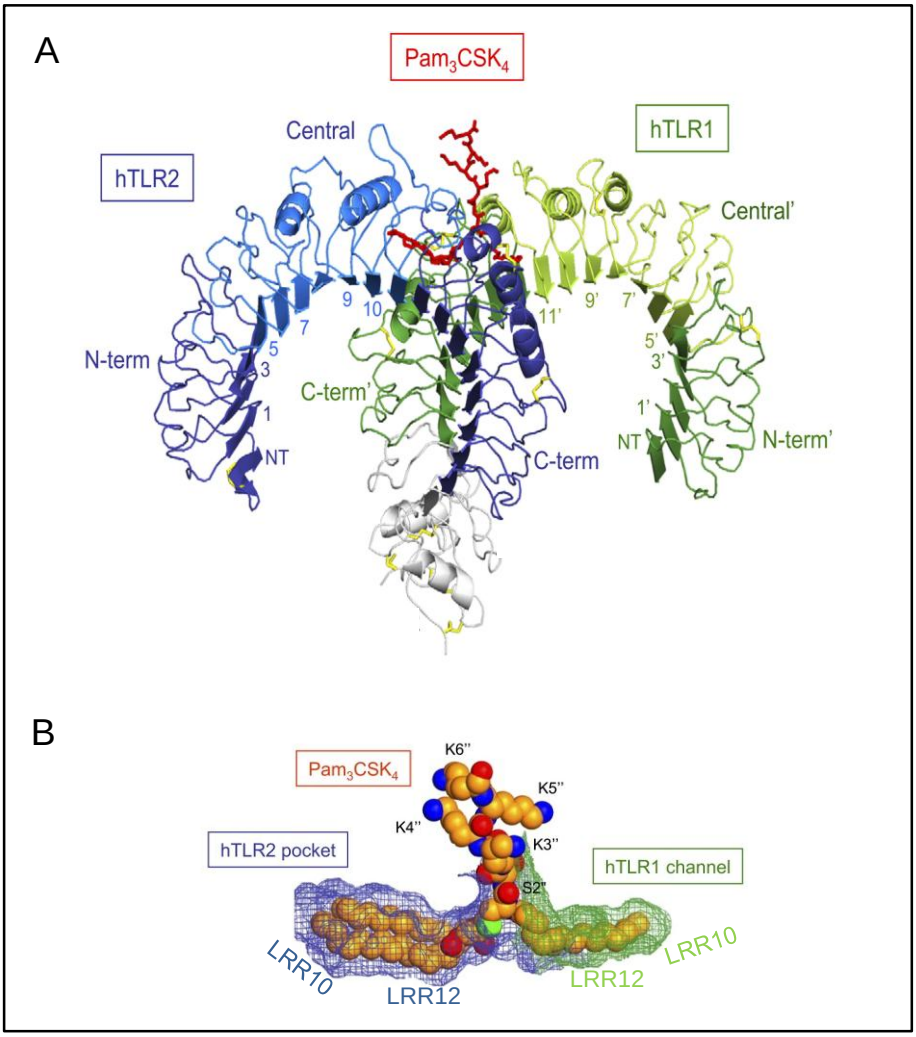


Figure 4.1 - Overall structure of the human (h) TLR1-TLR2-Pam_3CSK_4 complex

(A) Side view of the complex; the TLR1 COOH-terminal (C-term), central and NH_2-terminal (N-term) fragments are in green, the TLR2 fragments are in blue, and the triacylated Pam_3CSK_4 (PAM_3) lipopeptide is in red. LRR domains belonging to the TLR1 proteins are labelled with apostrophes. (B) The shape of the PAM_3-binding pocket is shown in mesh. Molecular surfaces that belong to TLR1 and TLR2, including LRR positions, are depicted in green and blue respectively. PAM_3 is shown as a space-filling model (orange), with two 'tails' specific for TLR2 and one 'tail' specific for TLR1. Images and information (Jin, Kim et al. 2007).

4.2 Results

4.2.1 Variation in cytokine production to a TLR1 ligand in whole blood

Whole blood samples from nine animals (three representatives from each bo*TLR1* -79T>G tSNP genotype) were stimulated for 24 h with the TLR1-specific ligand PAM_3 and non-specific ligands LPS (TLR4) and LTA (TLR2). Supernatants were assayed for CXCL8 production by ELISA as a marker for receptor stimulation. CXCL8 data demonstrated significantly ($P<0.05$) higher levels of CXCL8 produced by cells from the cows with the TT genotype than those with the TG and GG genotypes, following stimulation with PAM_3 (Figure 4.2). Levels of CXCL8 production following LPS and LTA exposure were similar across all three genotypes, with no significant differences.

4.2.2 Variation in cytokine production to a TLR1 ligand in PMNs

PMN-enriched cell populations from 12 animals (four representatives from each bo*TLR1* -79T>G tSNP genotype) were stimulated for up to 24 h with PAM_3, at 100 ng/mL, 250 ng/mL and 500 ng/mL, and LPS (100 ng/mL) as a non-TLR1 stimulant. FACS analysis of PMN samples suggested minimal difference in cell populations and purity obtained from each animal (Figure 4.3). CXCL8 and IL-6 mRNA abundance and protein production were measured as primary markers for TLR1 stimulation.

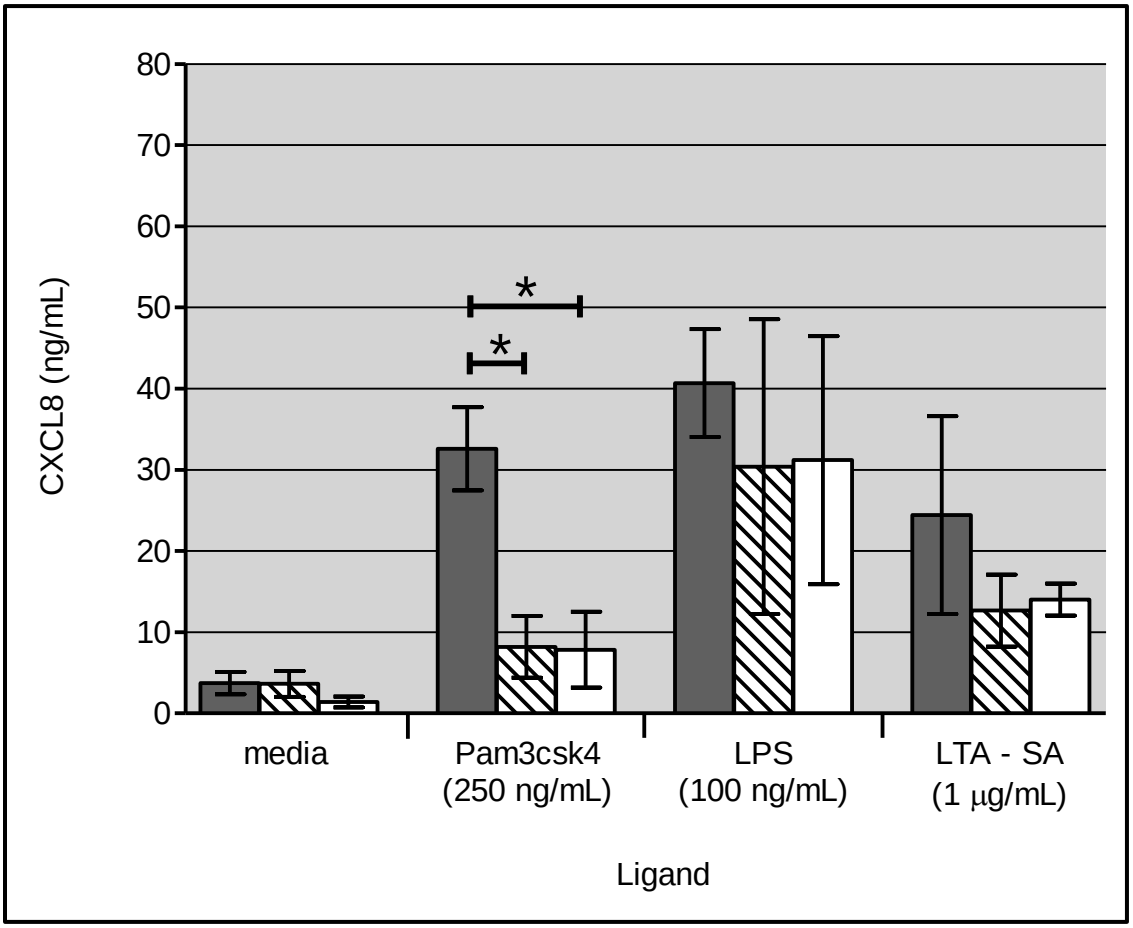


Figure 4.2 - ELISA data following stimulation with TLR ligands in bovine whole blood from animals representing the three bo*TLR1* -79T>G SNP variant populations: TT, TG and GG

ELISA data of CXCL8 production following +24 h stimulation. Data from each variant population (n = 3) are presented as a bar representing mean production, with error bars represent the SEM. Grey bars – TT animals, striped bars – TG animals, and open bars – GG animals. *Genotypic means differ significantly (P<0.05 by ANOVA and Tukey's multiple comparisons).

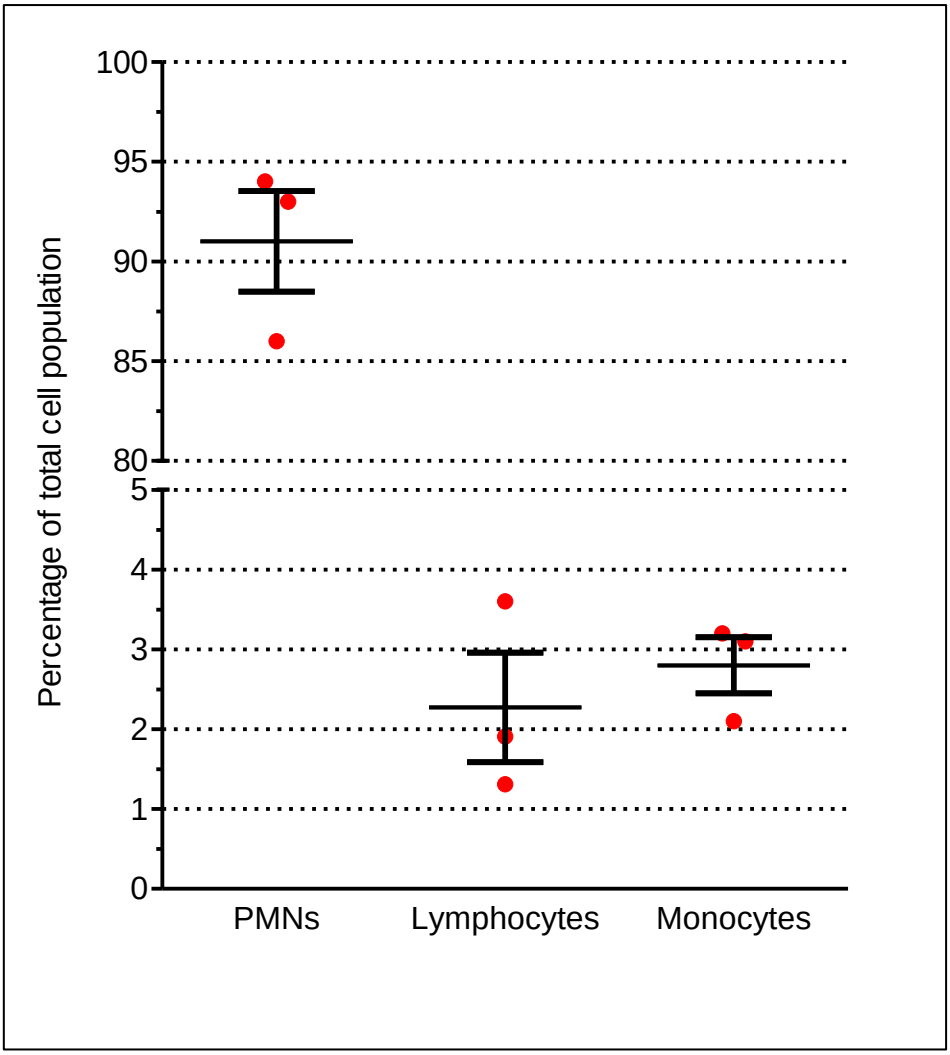


Figure 4.3 - Cell populations and purity of enriched PMN samples

Each red point represents FACS analysis data from one animal, with horizontal lines to indicate genotype mean and standard error.

Q-PCR analysis indicated that animals with the GG genotype had lower levels of *CXCL8* mRNA post-stimulation with PAM_3, at all concentrations, compared to equivalent samples from animals with either the TT or TG genotypes. Samples from animals that were homozygous TT showed significantly greater ($P<0.05$) *CXCL8* mRNA abundance 4 h post-stimulation with PAM_3, at concentrations of 250 and 500 ng/mL, when compared to GG homozygotes (Figure 4.4A). Homozygous GG animals generally demonstrate reduced *IL-6* abundance 4 h post-stimulation; however no significant differences were detected between genotypes across all ligand concentrations (Figure 4.4B). A similar trend in *CXCL8* and *IL-6* data was observed from cells stimulated for 8 h. Animals that were homozygous TT displayed significantly greater *CXCL8* ($P<0.05$) and *IL-6* ($P<0.01$) mRNA abundance 8 h post-stimulation with 500 ng/mL PAM_3 when compared to GG homozygotes (Figure 4.5A and B). Analysis of an additional pro-inflammatory cytokine, TNF-α, demonstrated exposure to PAM_3 at all ligand concentrations had little effect on *TNF-α* transcript levels at both 4 h and 8 h post-stimulation (Figure 4.4C and Figure 4.5C).

Supernatants (24 h post-stimulation) were assayed for CXCL8 and IL-6 production by ELISA. CXCL8 data showed a similar trend to gene expression data obtained by Q-PCR, with significantly ($P<0.05$) lower levels of CXCL8 produced by cells from the GG variants than those with the TT genotype, following stimulation with 500 ng/mL PAM_3 (Figure 4.6A). Individual variation was largest in samples from TG heterozygotes, with higher concentrations of PAM_3 resulting in increased variability in CXCL8 production. IL-6 data showed significantly ($P<0.05$) lower levels produced by cows with the GG genotype than the TT genotype following stimulation with 500 ng/mL PAM_3 (Figure 4.6B). Overall, stimulation with TLR1 ligand across all concentrations did not increase cytokine production in GG variants. The response to LPS, used to confirm the specificity of the observed response

to PAM_3, was variable across all genotypes but did not segregate by the defined genotypes (Table 4.1).

The mRNA abundance of the anti-apoptotic factor *Bcl2A1* was investigated as an additional marker to highlight differences in boTLR1 stimulation. Microarray studies have demonstrated importance for *Bcl2A1* during immune responses (Widdison, Watson et al. 2011) and transcriptional differences have been observed between *S. uberis*-infected and non-infected mammary glands (Coffey and Leigh unpublished). Following stimulation with PAM_3, Q-PCR analysis showed greater levels of *Bcl2A1* in animals with the TT genotype compared to those with either the TG or GG genotypes. Significantly ($P<0.05$) greater levels of mRNA were detected in samples from animals with the TT genotype compared to those with the TG genotype, following 4 h stimulation with PAM_3 at 250 ng/mL (Figure 4.4D). A statistically significant difference between *Bcl2A1* mRNA levels from TT animals compared to lowered levels from animals with either the TG or GG genotype was detected at 4 h and 8 h post-stimulation with PAM_3 at 500 ng/mL (Figure 4.4D and Figure 4.5D).

Q-PCR analysis indicated no significant differences between genotypes for transcript levels of the anti-inflammatory cytokine *IL-10*. Homozygous GG animals tended to demonstrate reduced *IL-10* levels at 4 h post-stimulation with PAM_3, across all concentrations (Figure 4.4E); however by 8 h post-stimulation, a small increase in transcript levels was detected showing little variability between genotypes (Figure 4.5E). Bo*TLR1* transcripts increase slightly at 4 h post-stimulation, with the data trend showing marginally greater expression from TT animals compared to TG and GG following stimulation with PAM_3 at 100 ng/mL (no statistical significance detected); although bo*TLR1* levels and genotypic variance decreases following exposure at the higher concentrations (Figure 4.4F). Bo*TLR1* transcript levels at 8 h post-stimulation are minimal with no significant variability between genotypes across all ligand concentrations (Figure 4.5F).

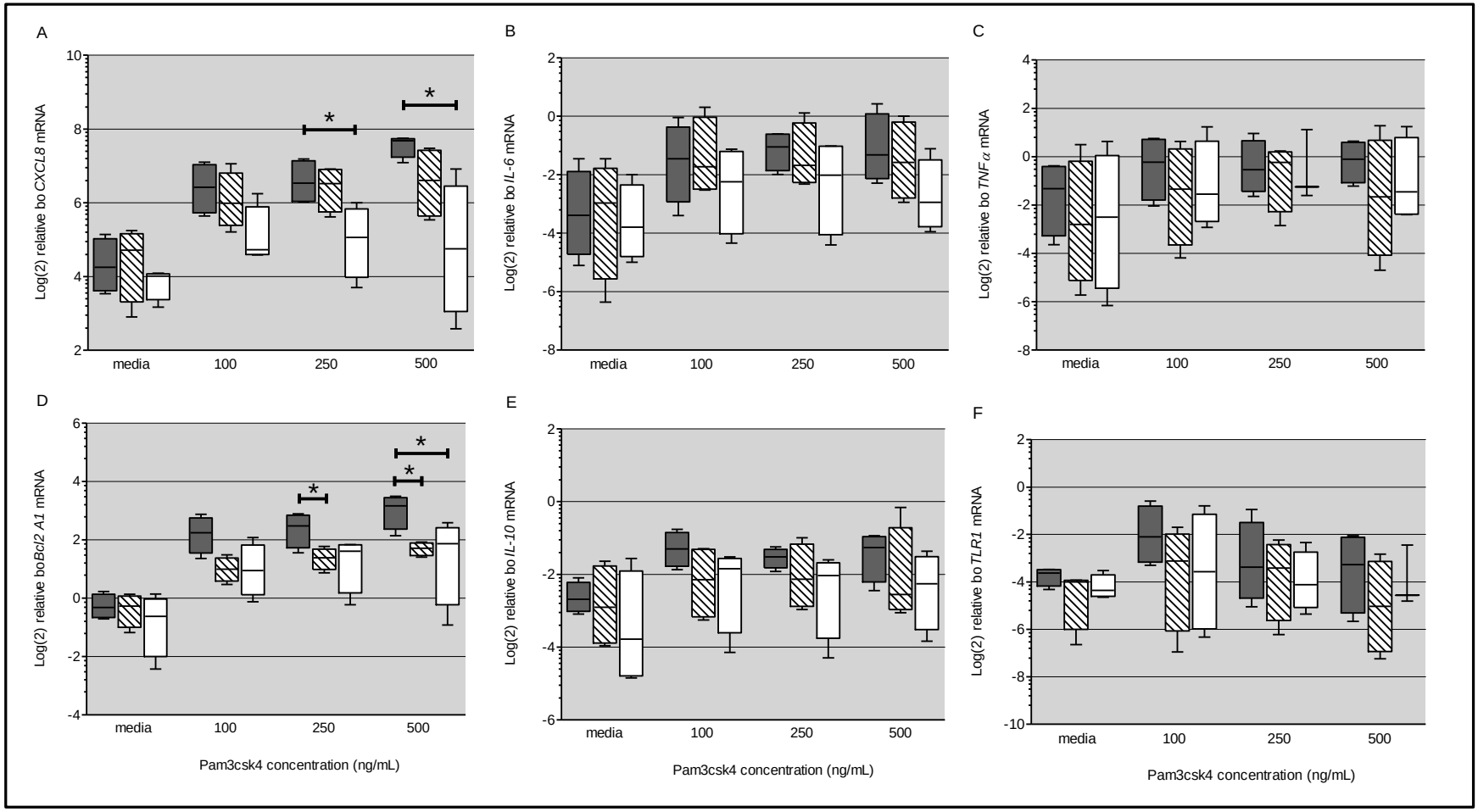


Figure 4.4 - Q-PCR data following +4 h stimulation with the TLR1 ligand PAM_3 in bovine PMNs grouped into the three bo*TLR1* -79T>G SNP variant populations: TT, TG and GG

Q-PCR data of *CXCL8* (A), *IL-6* (B), *TNF-α* (C), *Bcl2A1* (D), *IL-10* (E) and *TLR1* (F) mRNA abundance following +4 h stimulation. Data from each variant population (n = 4) is presented as a box and whisker plot, with the ends of the whiskers representing the minimum and maximum data values. Grey box plot – TT animals, hatched box plot – TG animals, and open box plot – GG animals. Lack of box plot indicates data missing from one animal. *Genotypic means differ significantly (P<0.05 by ANOVA and Tukey's multiple comparisons).

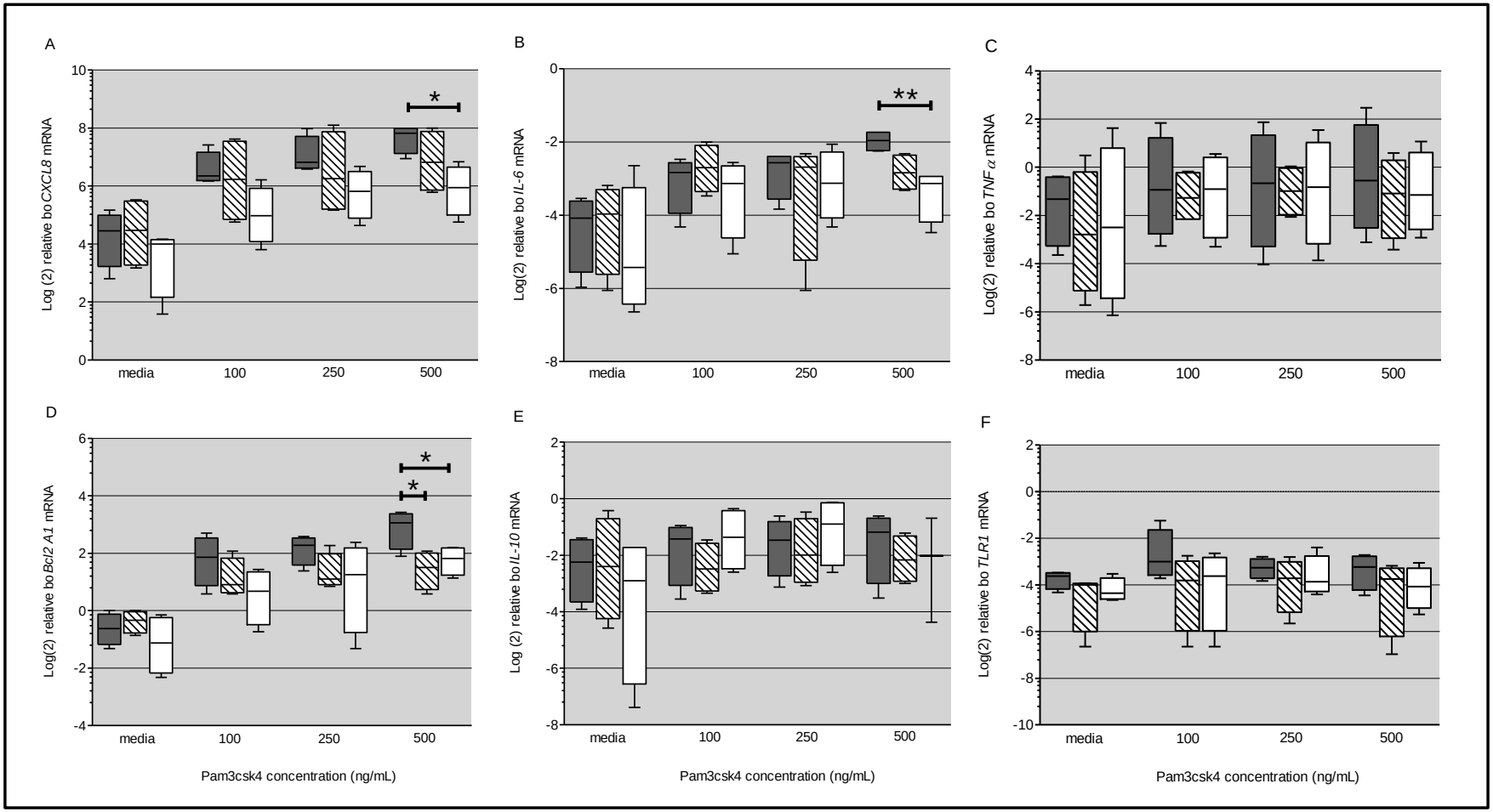


Figure 4.5 - Q-PCR data following +8 h stimulation with the TLR1 ligand PAM_3 in bovine PMNs grouped into the three bo*TLR1* -79T>G SNP variant populations: TT, TG and GG

Q-PCR data of *CXCL8* (A), *IL-6* (B), *TNF-α* (C), *Bcl2A1* (D), *IL-10* (E) and *TLR1* (F) mRNA abundance following +8 h stimulation. Data from each variant population (n = 4) is presented as a box and whisker plot, with the ends of the whiskers representing the minimum and maximum data values. Grey box plot – TT animals, hatched box plot – TG animals, and open box plot – GG animals. Lack of box plot indicates data missing from one animal. Genotypic means differ significantly at levels *P<0.05 and **P<0.01, by ANOVA and Tukey's multiple comparisons.

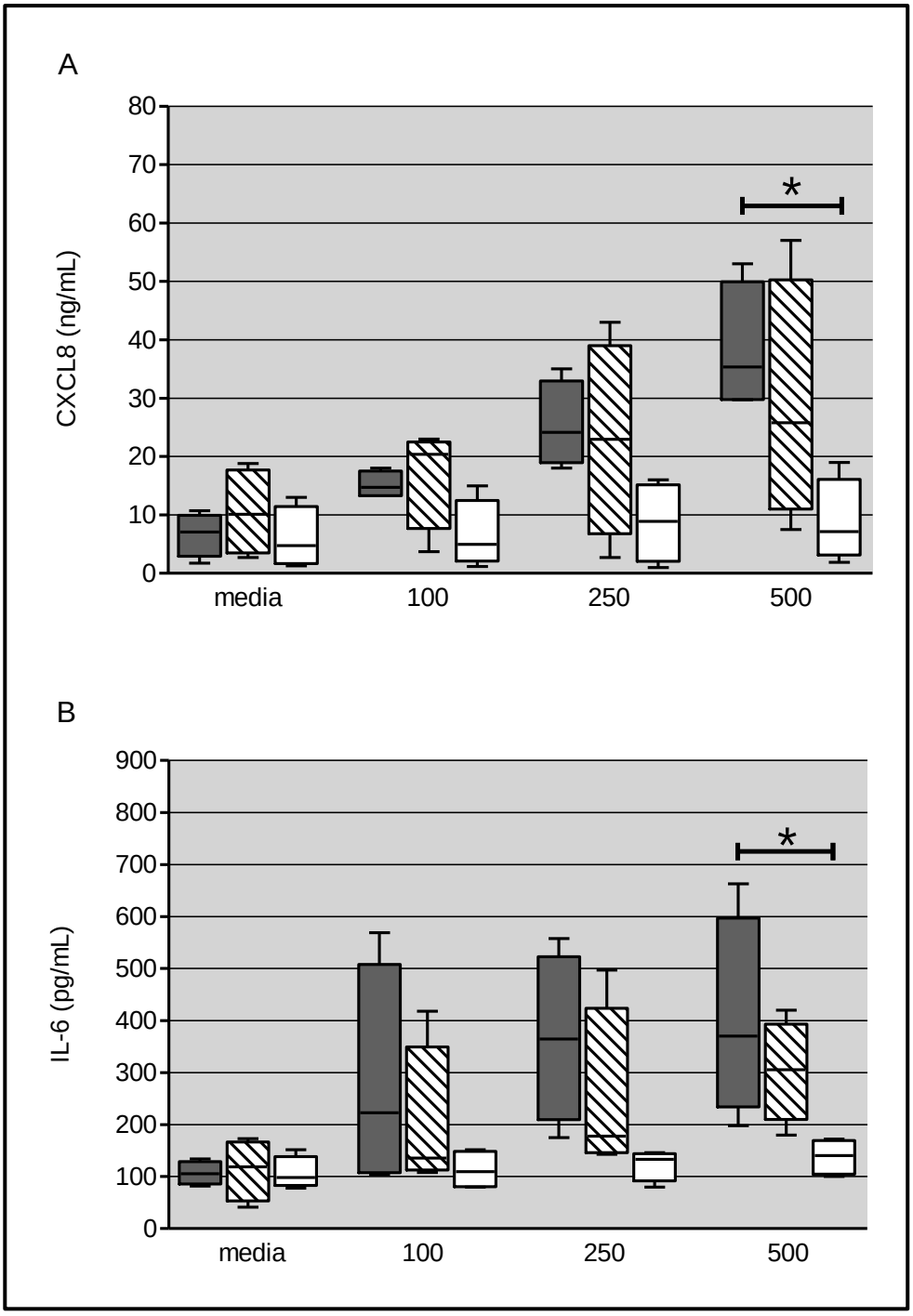


Figure 4.6 - ELISA data following stimulation with the TLR1 ligand PAM_3 in bovine PMNs grouped into the three bo*TLR1* -79T>G SNP variant populations: TT, TG and GG

ELISA data of CXCL8 (A) and IL-6 (B) production following + 24 h stimulation. Data from each variant population (n = 4) is presented as a box and whisker plot, with the ends of the whiskers representing the minimum and maximum data values. Grey box plot – TT animals, hatched box plot – TG animals, and open box plot – GG animals. *Genotypic means differ significantly (P<0.05 by ANOVA and Tukey's multiple comparisons).

Table 4.1 - Cytokine expression and production levels following PMN stimulation with LPS

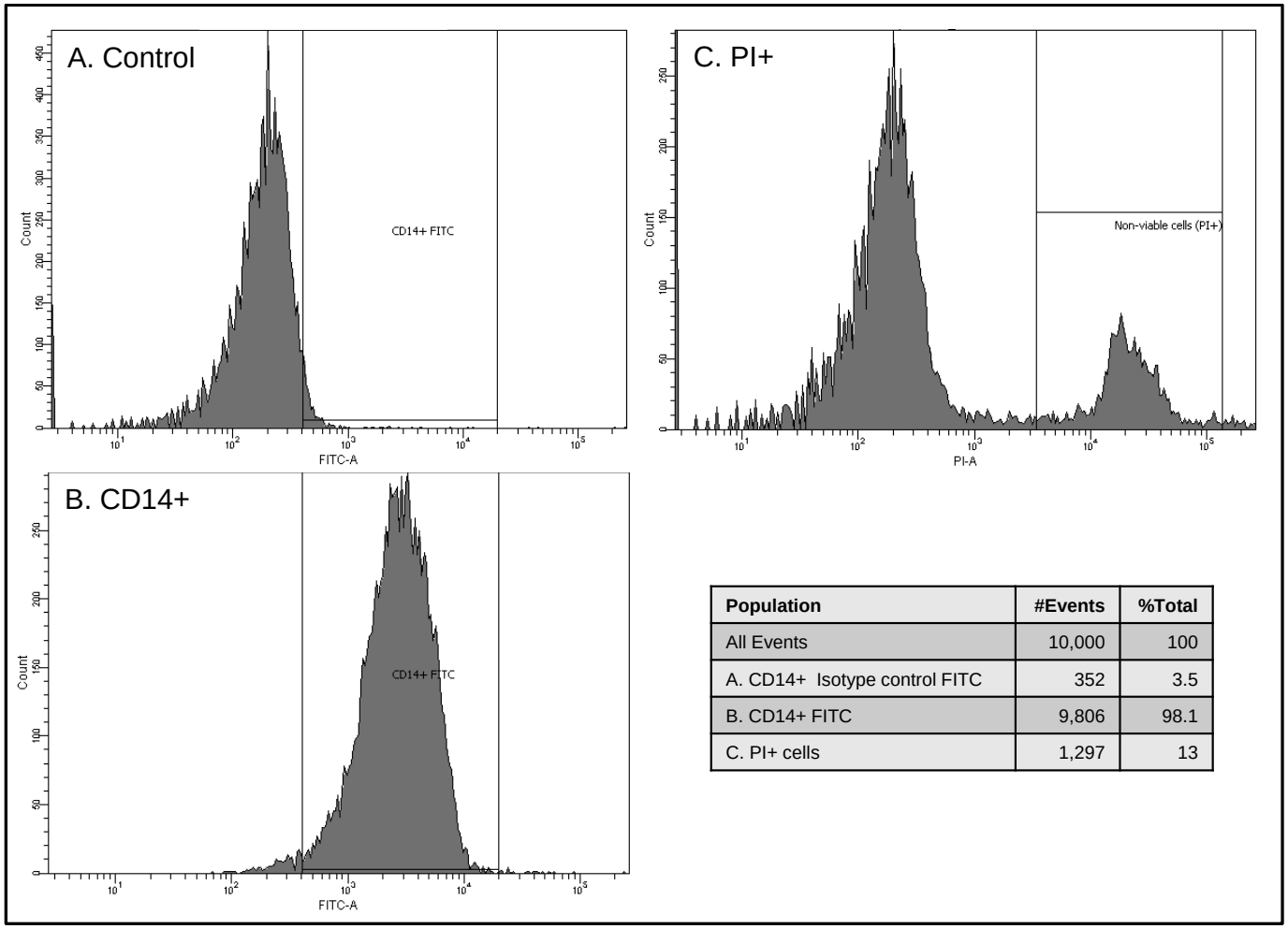
	bo*TLR1* -79T>G SNP genotype		
	TT	TG	GG
CXCL8 4 h	75 (48-126)	135 (47-186)	94 (34-160)
IL-6 4 h	0.3 (0.21-0.41)	0.2 (0.19-0.22)	0.2 (0.11 -0.28)
TLR1 4 h	0.08 (0.01-0.14)	0.06 (0.01-0.1)	0.018 (0.01-0.02)
TNF-α 4 h	-	-	-
IL-10 4 h	0.34 (0.17-0.64)	0.24 (0.14-0.43)	0.2 (0.17-0.24)
Bcl2 A1 4 h	4.4 (3.4-6.9)	4.4 (3.7-7.4)	5 (2.5-8.9)
CXCL8 (ng/µL)	24 (19-50)	50 (20-59)	38 (31-50)
IL-6 (pg/µL)	390 (220-656)	382 (167-407)	420 (346-462)

Mean relative expression levels of *CXCL8*, *IL-6* and *Bcl2 A1* transcripts and mean CXCL8 and IL-6 production levels (+24 h) following PMN stimulation with the TLR4 ligand LPS from animal samples segregated by tSNP bo*TLR1* -79T>G genotypes: TT, TG and GG. Minimum and maximum values are given in parenthesis (min-max). No significant differences ($P<0.05$) were observed between any of the genotype means using one-way ANOVA and Tukey's multiple comparison tests.

4.2.3 Variation in cytokine production following pathogen stimulation in blood-derived CD14+ cells

CD14+ cell populations isolated from nine animals, three representative animals for each bo*TLR1* -79T>G tSNP genotype, were stimulated for 24 h with the TLR1 ligand PAM_3, TLR4 ligand LPS, and four strains of HK bacteria; *E. coli* P4, *S. aureus* M40, and *S. uberis* 0140J and EF20, representing the leading causative agents of bacterial mastitis. Prior to stimulation, the purity of each isolated CD14+ cell population was evaluated by flow cytometric analysis following incubation with FITC-conjugated CD14+ antibodies (Figure 4.7). All isolated CD14+ cell populations display >97% purity, with marginal differences in viable cell counts (Figure 4.8).

Supernatants were assayed for CXCL8 production by ELISA. Data showed significantly (P<0.05) higher levels of CXCL8 production by cells from cows with the TT genotype than those with the TG and GG genotypes, following stimulation with PAM_3 (Figure 4.9). Levels of CXCL8 production following LPS exposure revealed no significant differences between the three genotypes. Significantly higher levels of CXCL8 were produced by cows with the TT (P<0.01) and TG (P<0.05) genotypes compared with GG animals, following stimulation with HK *S. aureus*. CXCL8 production in response to HK *E. coli* was high in all three genotypes, with greater levels observed from TT and TG cows compared to GG, however no significance was detected. CXCL8 responses from CD14+ cells to both strains of *S. uberis* were low, with no significance differences observed between the defined genotypes (Figure 4.9).



Population	#Events	%Total
All Events	10,000	100
A. CD14+ Isotype control FITC	352	3.5
B. CD14+ FITC	9,806	98.1
C. PI+ cells	1,297	13

Figure 4.7 - Analysis of isolated CD14+ cell populations following incubation with FITC-conjugated CD14+ antibodies
The purity of each isolated CD14+ cell population was evaluated by flow cytometric analysis. (A) An isolated CD14+ population following incubation with a FITC-conjugated mouse IgG2a isotype control antibody. Example displays lowered fluorescence from the gated CD14+ FITC antibody population. (B) An isolated CD14+ population following incubation with an anti-human (mouse IgG2a) CD14+ FITC antibody, showing a sample purity of >98%. (C) An isolated CD14+ population following incubation with propidium iodide (PI) for assessment of cell viability, with the example displaying an 87% viable population.

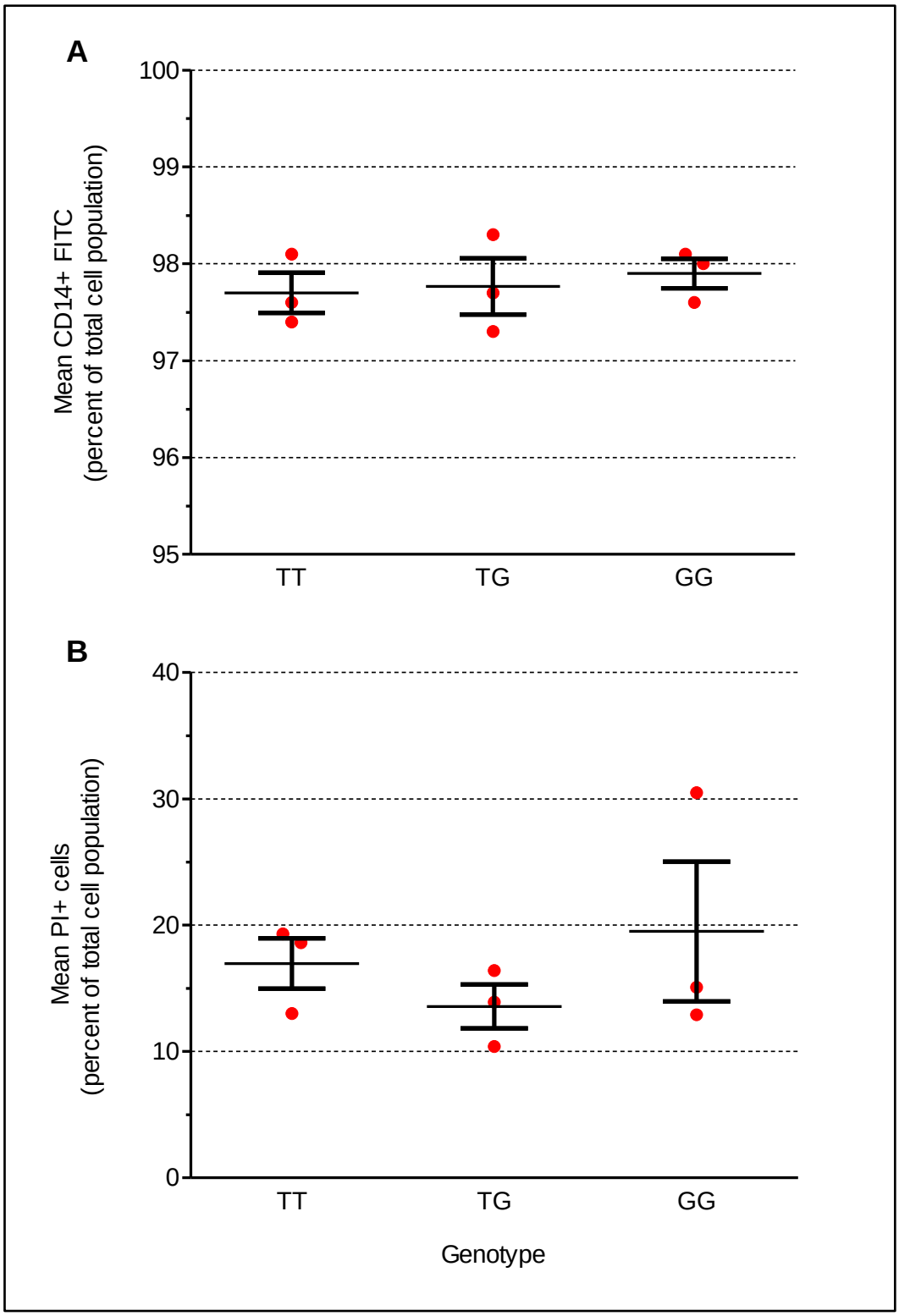


Figure 4.8 - Purity and viable cell percentages for isolated CD14+ cell populations grouped into the three bo*TLR1* -79T>G SNP variant populations: TT, TG and GG

(A) Percentage of isolated CD14+ cell populations positive for CD14+ FITC. (B) Percentage of isolated CD14+ cell populations positive for propidium iodide (PI+). Each red point represents data from one animal/sort, with horizontal lines to indicate genotype mean and standard error.

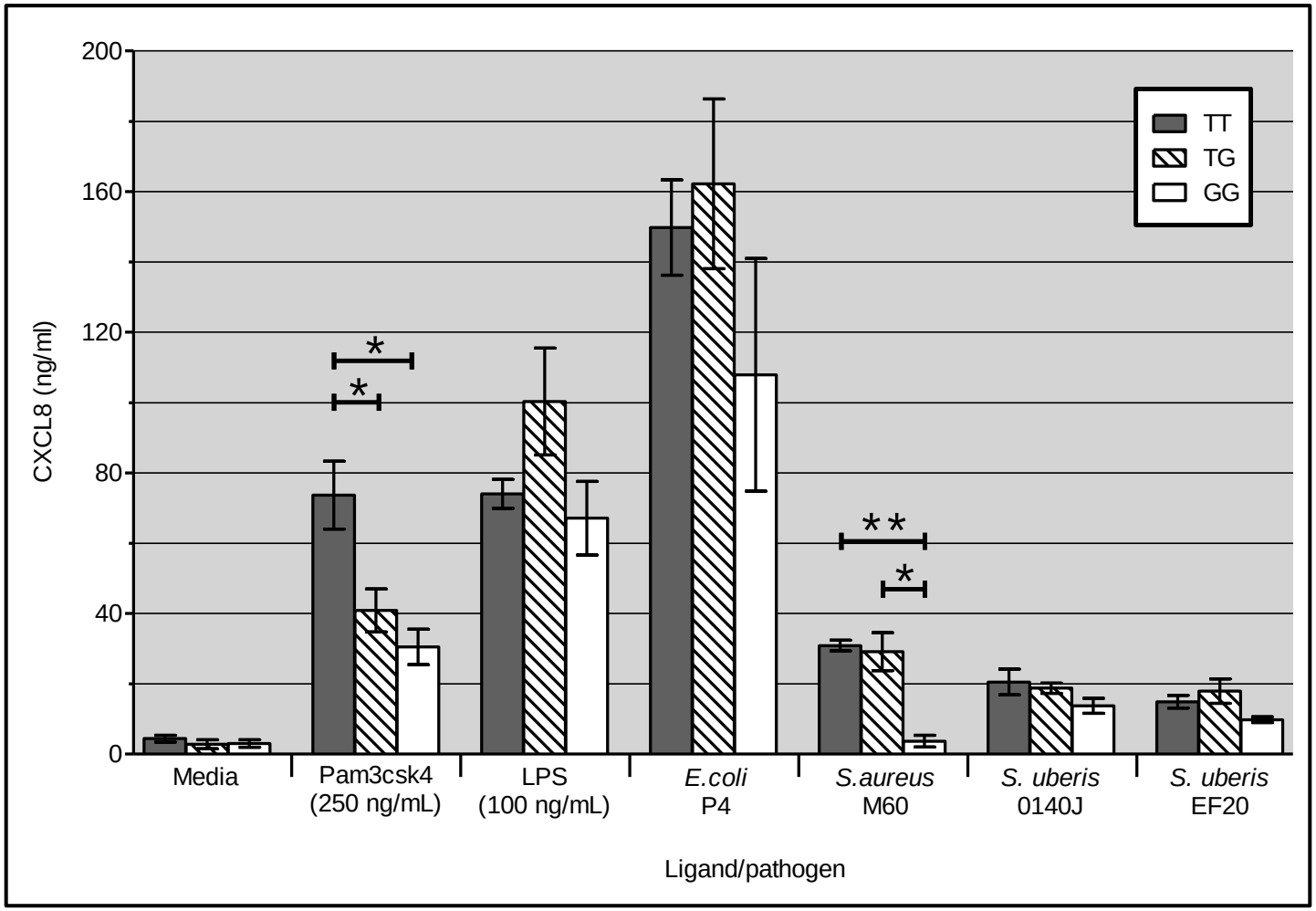


Figure 4.9 - ELISA data of CXCL8 production in CD14+ cell populations

Analysis of CXCL8 production following +24 h stimulation with TLR ligands and heat-killed bacteria of blood derived CD14+ cells grouped into the three bo*TLR1* -79T>G SNP variant populations: TT, TG and GG. Grey bars – TT animals, striped bars – TG animals, and open bars – GG animals (n = 3). Data bars represent the mean of three animals, with error bars representing the SEM. Contributing data from each animal are averaged responses from three independent stimulations. Genotypic means differ significantly at the levels *(P<0.05) and **(P<0.01) by ANOVA and Tukey's multiple comparisons.

4.3 Discussion

To ascertain the functional consequence of potentially reduced bo*TLR1* expression, production of cytokines known to be induced by boTLR1 was determined from cells stimulated with the TLR1 ligand PAM_3. An initial study using whole blood samples, three from each representative bo*TLR1* -79T>G tSNP genotype, revealed significantly greater CXCL8 production following stimulation with PAM_3 from TT variants when compared to TG and GG cows. These differences were specific to boTLR1 as they were not observed following stimulation with boTLR4 or boTLR2 ligands. Enriched bovine PMN populations were obtained to expand these primary findings. Across all time-points and ligand concentrations analysed, PAM_3 stimulation of PMNs from animals segregated by -79T>G tSNP genotypes revealed lower levels of CXCL8 and IL-6 mRNA and protein from animals of a GG genotype and consistently high CXCL8 and IL-6 response from those with TT genotypes. Cytokine production from cells from the TG animals was the most variable. Used as an additional marker of effective boTLR1 stimulation, significantly greater levels of *Bcl2A1* were detected in animals with the TT genotype compared to those with the TG or GG genotypes. A target of NF-κB activity (Liang, Zhou et al. 2004), *Bcl2A1* encodes a pro-survival protein and is up-regulated in neutrophils following stimulation with TLR ligands including PAM_3 (Francois, El Benna et al. 2005). All genotypes were additionally stimulated with LPS, and no differences were detected in levels of CXCL8, IL-6 or *Bcl2A1*. This further indicates that response differences observed following stimulation with PAM_3 were influenced by variation in boTLR1. In addition, although potential binding of PAM_3 with boTLR10 is unable to signal for CXCL8 or IL-6 synthesis (Guan, Ranoa et al. 2010), no genotypic variation was observed for bo*TLR10* transcript levels in previous data (Figure 3.8), further suggesting that boTLR10 expression and function will have limited influence on the production and measurement of cytokines in PAM_3 assays.

Small amounts of CXCL8 protein were detected in media controls. This may be the result of cell agitation during the sorting process, or simply represent a basal level of expression in resting PMN. A study analysing neutrophil responses to bacteria demonstrated small yet detectable amounts of CXCL8 in granules from resting human neutrophils (Faldt, Dahlgren et al. 2002).

Q-PCR data from the PMN studies suggests PAM_3 had little effect on *TNF-α* and *IL-10* transcript levels at both 4 h and 8 h post-stimulation, and failed to segregate by the defined genotypes. This may be due to the manner by which TLRs respond and signal for cytokine. Induction of *IL-10* is TLR ligand-specific and, in agreement with other findings (Samarasinghe, Tailor et al. 2006; Hawn, Misch et al. 2007; Wurfel, Gordon et al. 2008), this study demonstrates poor induction of *IL-10* following LPS and PAM_3 stimulation. Due to limited supernatant, quantification of TNF-α and IL-10 proteins were not performed. Results from these assays could have verified any genotypic variance between 24 h protein levels for these cytokines. A further interesting observation from the PMN assays was the detection of continually low levels of bo*TLR1* transcript following PAM_3 exposure. This may result from the neutrophil's inefficiency to induce bo*TLR1* expression and/or the result of bo*TLR1* being highly regulated following stimulation. A separate study demonstrated either unchanged or decreased bo*TLR* expression profiles in bovine immune cells following ligand exposure, despite effective cytokine responses (Guo, Zhao et al. 2009). Inducible levels of bo*TLR1* may therefore have little impact on TLR1-directed inflammatory responses, and vice versa.

PMNs contain a high proportion of neutrophils, short-lived cells whose primary role is the phagocytosis of microbes to contain and eliminate infection. The mass migration of neutrophils into the mammary gland, interpreted by an elevation in milk SCC, remains a critical immune event during mastitis. Pathogen interaction with neutrophil TLRs stimulates the cell to synthesise cytokines that influence their own activity and survival, recruit

additional immune cells and modulate the immune response (Hayashi, Means et al. 2003; Paape, Bannerman et al. 2003; Parker, Whyte et al. 2005; Kobayashi 2008). The greater expression of *Bcl2A1* in animals with the TT genotype may promote PMN survival to complement the increased cytokine output. Reduced boTLR1 stimulation by pathogens in GG cows may equate to a dampened inflammatory response enabling establishment and persistence of infection.

In terms of influence on mastitis susceptibility, data detailed in Chapter 3.0 revealed GG cows to have a higher CM rate than TT cows; however heterozygous TG cows have a similar rate to GG cows, despite expressing bo*TLR1* at a similar level to a TT cow. TG animals showed intermediary cytokine responses to TLR1 ligand and, like GG animals, lower expression of *Bcl2A1*. This may suggest that differences in bo*TLR1* transcript levels alone may not account for the variation in receptor response, and/or that post-transcriptional and post-translational regulation may also be affected by the cumulative presence of SNPs. As detailed in Chapter 3.0, a high degree of linkage was observed between bo*TLR1* SNPs, and included the identification of a linkage group represented by tSNP -79T>G. The animals used for the functional assays in this study all conform to observed haplotypes: TT = $T_{79}G_{1762}T_{2463}$and GG = $G_{79}A_{1762}C_{2463}$, with TG animals heterozygous for each SNP. In terms of being causative of a variable TLR1 response the NS SNP +1762 Iso>Val is an unlikely candidate; both isoleucine and valine are non-polar branched amino acids with hydrophobic properties. It is therefore unlikely that a substitution within the transmembrane domain would impact the tertiary structure; although further targeted structure/function studies are required to verify this. Furthermore, influence of the 3'UTR SNP +2463C>T, which was associated with CM (detailed in Chapter 3.0), unidentified polymorphisms within bo*TLR1* and additional related or target genes such as bo*TLR2*, *TIRAP*, *NF-κβ* and *CXCL8* should be considered. Mutations within boTLR2 may influence boTLR1 expression and function; a

recent study identified several NS bo*TLR2* SNPs within a Holstein-Friesian herd potentially impacting LRR and TIR protein domains important for interactions and responses to PAM_3 (Ruiz-Larranaga, Manzano et al. 2011).

To assess for genotypic variance in a cytokine response to pathogens associated with mastitis, enriched CD14+ cell populations were isolated from blood collected from three representative animals from each bo*TLR1* -79T>G tSNP genotype, and stimulated for 24 h with ligand (PAM_3 and LPS), HK *E. coli*, HK *S. aureus* and HK *S. uberis* (strains 0140J and EF20). *E. coli* and *S. aureus* strains were both virulent isolates from cases of bovine mastitis (BramLey 1976; Anderson 1977). *S. uberis* 0140J, a virulent strain, and *S. uberis* EF20, an isolate of low virulence, are frequently used in experimental models of infection in dairy cattle (Hill 1988; Leigh, Ward et al. 2004; Egan, Kurian et al. 2010; Moyes, Drackley et al. 2010). Control data confirmed similar purity and viable cell counts between each CD14+ cell population. Increased CXCL8 production levels in response to TLR ligands were demonstrated in all cell populations. Although LPS responses were similar between -79T>G tSNP genotypes, significantly higher levels of CXCL8 were produced by CD14+ cells from cows with the TT genotype than those with the TG and GG genotypes, following stimulation with PAM_3.

Analysis of results following stimulation with the HK bacteria demonstrated a genotype-specific response to *S. aureus* but not to *E. coli* or *S. uberis*. Significantly higher levels of CXCL8 were produced by CD14+ cells from TT ($P<0.01$) and TG ($P<0.05$) variants than those from GG cows, following stimulation with *S. aureus*. While levels of CXCL8 produced by cells from the TT and TG variants following stimulation with *S. aureus* were very similar, very low levels were detected in the cells from the GG animals. These findings may further support the importance of boTLR1 in the detection of *S. aureus* and the establishment of an immune response. The major structural form of lipoproteins in *S. aureus*

strains is triacylated (Asanuma, Kurokawa et al. 2011). These include triacyl forms of SitC that are likely targets for TLR1/TLR2; a separate study analysing cells co-transfected with TLR1/TLR2 demonstrated high CXCL8 activity following exposure to HK *S. aureus* (Guan, Ranoa et al. 2010). Blood-derived CD14+ cells consist mainly of monocytes, precursors of MØ (Ziegler-Heitbrock, Ancuta et al. 2010) that are the predominant cell type in 'healthy' milk and important in the detection of pathogens invading the mammary gland (Oviedo-Boyso, Valdez-Alarcon et al. 2007). The novel findings from this study suggest an impaired mammary boTLR1 response by somatic cells, including MØ, from a GG cow could have severe implications in the pathogenesis of *S. aureus* mastitis and potentially other bacterial diseases such as susceptibility to infection by *Mycobacterium bovis*; a recent study has linked bo*TLR1* variants to bovine TB (Sun, Song et al. 2012).

Data from *in vitro* CD14+ cell assays also highlighted a pathogen-specific cytokine response which appeared to correlate with observations of pathogen-dependent immune responses during intramammary infection (Bannerman 2009). CXCL8 production in response to *E. coli*, mainly induced through boTLR4 following interactions with *E. coli* LPS, was significantly elevated for all three genotypes in this study, with trends for a larger increase in TT and TG variants despite no statistical significance. CXCL8 production from CD14+ cells exposed to *S. aureus* and *S. uberis* strains was greatly reduced in comparison to *E. coli*, similar to published observations (Bannerman 2009; Swanson, Stelwagen et al. 2009; Rinaldi, Li et al. 2010). These results may reflect the differences that exist in the pathogenesis of disease. An acute and heightened inflammatory response is more often attributed to *E. coli* infection whereas reduced inflammatory responses reflect the persistency of infection with *S. aureus* mastitis (Riollet, Rainard et al. 2001; Bannerman 2009).

Responses to stimulation with each of the HK *S. uberis* strains were similar between the virulent 0140J and non-virulent EF20 strains, each resulting in low levels of CXCL8

production. Although potentially unexpected, work to characterise the cytokine response profiles of somatic cells following intramammary challenge with 0140J and EF20 is on-going; with preliminary data suggesting CXCL8 production is substantially increased in cows infected with 0140J between 24 h and 48 h time-points, compared to only minor increases in cows infected with EF20 (Coffey and Leigh unpublished). The addition of a 36 h post-stimulation time-point, therefore, further proven to be sufficient for raised production of CXCL8 during *S. uberis* infection (Bannerman, Paape et al. 2004; Bannerman 2009), may have identified differences in CXCL8 production between the different bo*TLR1* genotypes following stimulation with the two HK strains.

It is important to emphasise that results from the CD14+ cell assays are from analysis of a single cytokine measured at a single time-point. These assays would benefit from greater sample numbers, an additional time-point, variation of MOI, and the analysis of further pro-inflammatory and immunoregulatory cytokines, as well as cytokines such as the IL-1 family and IL-2, which are produced by activated MØ to stimulate T-cell lymphocytes. Influx of activated T lymphocytes has been observed during *S. aureus* and *S. uberis* mastitis (Soltys and Quinn 1999; Rivas, Schwager et al. 2007). In addition, it is important to note that HK pathogens will elicit different cytokine responses compared to the live pathogen, bacterial lysate preparations or even *in vivo* conditions.

In conclusion, these findings present evidence that SNPs common to bo*TLR1*, and associated with CM susceptibility, may underpin functional variation in boTLR1. To the author's knowledge this is the first study to demonstrate genotype-specific variation in cytokine responses from bovine whole blood and enriched cell populations when exposed to PAM_3. Furthermore, the potential impact of bo*TLR1* variation on the immune response to *S. aureus* is demonstrated here and has implications for other pathogens, warranting further study. A rapid immune response, conferred by the favourable bo*TLR1* SNP -79 TT variant

(Table 4.2), could reduce detrimental clinical manifestations of diseases via an efficient yet controlled influx of inflammatory cells to neutralise pathogens and control infection, with potential implications for *S. aureus* mastitis. An efficient boTLR1 response to *S. aureus* will be vital to limit its internalisation and survival in bovine mammary cells (Hebert, Sayasith et al. 2000). A bovine population with impaired boTLR1 expression and function may also be more susceptible to other diseases and health issues, as demonstrated in a recent study linking bo*TLR1* variants to bovine TB (Sun, Song et al. 2012).

Table 4.2 - Overview and comparison of results from boTLR1 functional studies

Subject	Measure	Time-points	Bo*TLR1* -79T>G tSNP genotype TT	TG	GG
Milk somatic cells	Bo*TLR1* (basal)		++	++	+/-*
PMN cells challenged with PAM_3	*CXCL8*	4 h, 8 h	+++	++	+*
	CXCL8	24 h	+++	++/+	+/-*
	IL-6	4 h, 8 h	++/+	++/+	+/-*
	IL-6 (pg/µL)	24 h	+++/+	+	-*
	Bo*TLR1* (induced)	4 h, 8 h	+	+/-	+/-
	Bcl2 A1	4 h, 8 h	++	-*	+*
CD14+ cells	CXCL8 *E. coli*	24 h	+++	+++	++
	CXCL8 *S. aureus*	24 h	++/+	++/+	-*
	CXCL8 *S. uberis*	24 h	+	+	+
Herd population	CM incidence (cases/100 cows/year)		40	73*	75

The tSNP -79 TT variants, with lowered CM incidence, demonstrated effective boTLR1 function and expression with favourable cytokine and survival factor responses to ligand and HK bacteria. Average relative expression levels of *CXCL8*, *IL-6*, *Bcl2 A1* and *boTLR1* transcripts and CXCL8 and IL-6 production levels (+24 h) following stimulation with the TLR1 ligand PAM_3 and HK bacteria from animal samples segregated into bo*TLR1* tSNP -79T>G genotypes: TT, TG and GG. Milk somatic cell assay and herd population CM data detailed in Chapter 3.0. Level of expression/immune responses are graded +++, ++ or + (high to low), - denotes negligible expression. Blue shading corresponds to grading system from dark (+++) to light (+) *Significance differences between genotype means detected against TT genotype.

Chapter 5.0 - Identification of bovine *TLR4*, *CD14* and *TLR5* SNPs – association with mastitis and production traits in cattle

5.1 Introduction

5.1.1 Toll-like receptor 4 and CD14

TLR4 was the first TLR to be described in mammals (Medzhitov, Preston-Hurlburt et al. 1997) and, together with its co-receptor CD14, is involved in the cell surface recognition of lipopolysaccharide (LPS), a cell wall constituent of Gram-negative bacteria (Chow, Young et al. 1999). TLR4/CD14 may further recognise mannan (Flo, Ryan et al. 2002) and fusion F protein from respiratory syncytial virus (RSV) (Kurt-Jones, Popova et al. 2000). Cell surface recognition of LPS by TLR4 requires additional accessory molecules; soluble LPS-binding protein (LBP) presents LPS to CD14, which in turn presents LBP/LPS to the myeloid differentiation factor 2 (MD-2) protein, which associates with TLR4 to optimise LPS interactions (Shimazu, Akashi et al. 1999; Park, Song et al. 2009). Complete presentation of LPS to TLR4 leads to receptor dimerisation and activation of MyD88-dependent signalling pathways, which mobilise nuclear factors such as NF-κB for inflammatory cytokine production. Unlike other TLR members, TLR4 then internalises in intracellular endosomes, and utilises MyD88-independent (TRIF adaptor protein) signalling pathways (Tanimura, Saitoh et al. 2008) to activate further nuclear factors, such as IRF3 for interferon production, inducing innate and adaptive immune responses to LPS (Figure 5.1).

Polymorphic variation of TLR4 can alter signalling and affect receptor function, as demonstrated by the human mutation D299G, a NS SNP encoding an aspartic acid to glycine substitution within the TLR4 extracellular domain. Despite having no impact on LPS binding or TLR4 cell surface expression levels, the mutation compromises MyD88 and TRIF recruitment to the TIR domain following LPS interaction, subsequently impacting TLR4 signalling (Figueroa, Xiong et al. 2012). These findings are important as they provide an underlying immunological mechanism for previous studies associating D299G with disease

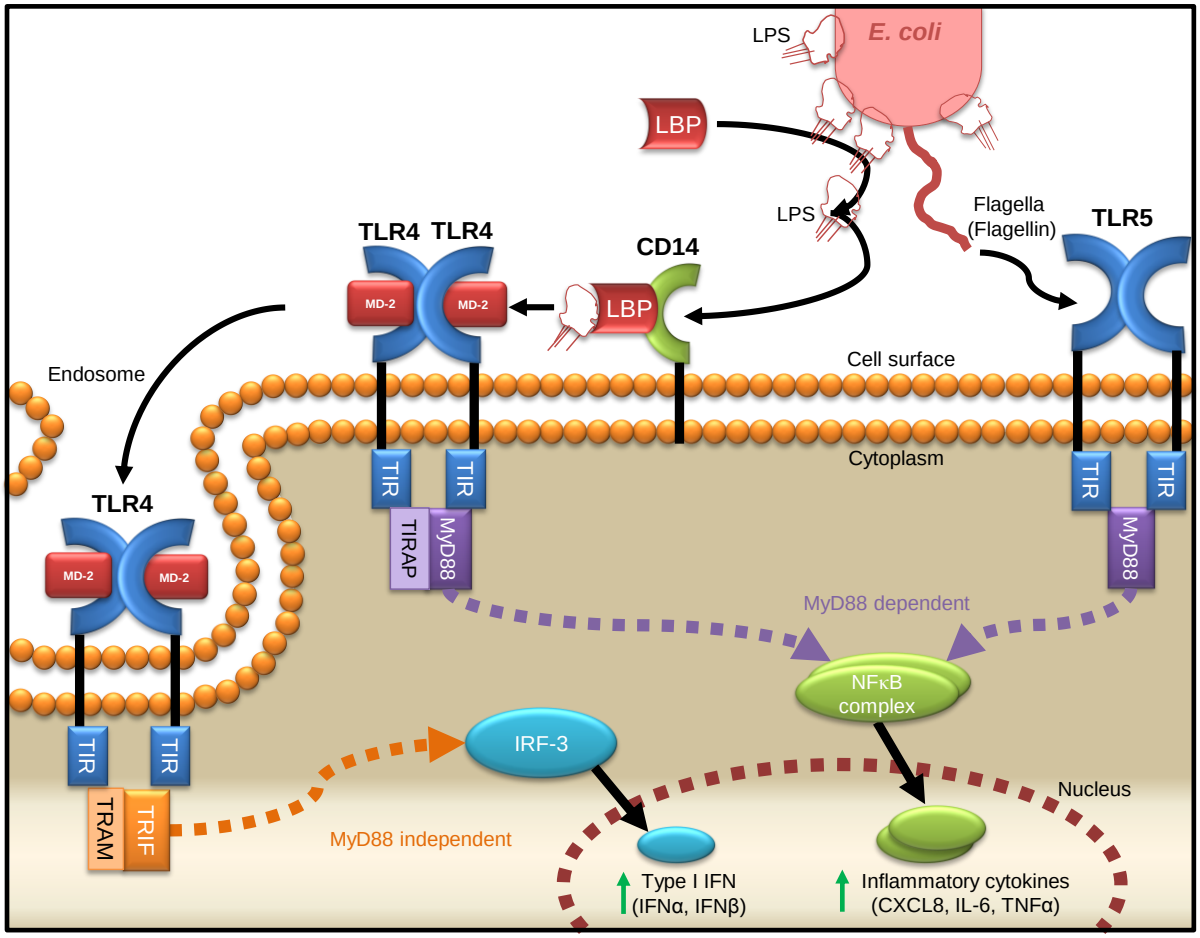


Figure 5.1 - Overview of the TLR4/CD14 and TLR5 signalling pathway

LBP, CD14 and MD-2 help TLR4 interact with LPS at the plasma membrane, activating intracellular adaptor molecule MyD88 via the TIR adaptor protein (TIRAP) which associates with the receptor's TIR domain (Fitzgerald, Rowe et al. 2003). Controlled by CD14 (Zanoni, Ostuni et al. 2011), TLR4 then internalises within an endosome and replaces MyD88 with TRIF, which is activated by the TIR domain via TRIF-related adaptor molecule (TRAM) (Fitzgerald, Rowe et al. 2003). MyD88-dependent and -independent (TRIF) pathways initiate specific signalling cascades (generalised as grey dashed arrows), leading to the translocation of transcription factors NF-κB and IRF-3 into the nucleus. This induces transcriptional activity and up-regulation of effector molecules such as inflammatory cytokines and interferons (IFNs). TLR5 recognises flagellin, a monomeric component of flagella, and signals via MyD88 only.

susceptibility including Gram-negative bacterial infection, sepsis, RSV and malaria (Misch and Hawn 2008). As well as coding SNPs, non-coding *TLR4* SNPs located within the 3'UTR impact on gene expression and function (Song, Chen et al. 2006). Polymorphisms within *CD14* have also been associated with diseases and inflammatory conditions such as asthma (Smit, Siroux et al. 2009) and heart disease (Shimada, Watanabe et al. 2000).

In cattle, there are numerous reports linking bo*TLR4* variation with disease, including infectious bovine keratoconjunctivitis (IBK) (Kataria, Tait et al. 2011) and MAP infection (Mucha, Bhide et al. 2009; Ruiz-Larranaga, Manzano et al. 2011). BoTLR4 has been implicated in the initiation of mammary immune responses towards Gram-negative infections such as *E. coli* mastitis (De Schepper, De Ketelaere et al. 2008; Buitenhuis, Rontved et al. 2011). *In vitro* studies, investigating bovine MEC responses, have demonstrated increased expression of bo*TLR4* following LPS exposure (Ibeagha-Awemu, Lee et al. 2008) and during *S. aureus* mastitis (Goldammer, Zerbe et al. 2004). Intriguingly, significant up-regulation of bo*TLR4* was similarly observed in neutrophils following intramammary challenge with *S. uberis* (Moyes, Drackley et al. 2010), implicating a potential role for boTLR4 in Gram-positive mammary infections.

5.1.2 Association of bovine TLR4 and CD14 SNPs with health and disease in cattle

Due to the importance of the TLR4 response in mammary defence, the presence of SNPs within bo*TLR4* and bo*CD14* has been implicated in dairy health and disease. Bo*TLR4*, a large gene (~12kb) located on chromosome 8, comprises three exons, with two introns dividing a CDS that is 2523bp in length corresponding to an 841aa chain, with 5' and 3'UTRs identified. This gene is highly polymorphic, revealing over 30 SNPs across several cattle breeds (White, Taylor et al. 2003). A more recent study genotyped bo*TLR4* SNPs in a Canadian Holstein bull population and analysed data for associations with dairy health (Sharma, Leyva et al. 2006). The NS +2021C>T SNP, causing a Thr to Ile substitution within

the transmembrane domain, was associated with SCC, with the authors observing an increase in SCC in heterozygous CT genotyped cows when compared to CC cows. Another study, genotyping six breeds that included a small Holstein-Friesian herd, failed to find associations for +2021C>T with SCC although the CC genotype was associated with decreased milk fat and protein percentages (Beecher, Daly et al. 2010).

Non-coding bo*TLR4* SNPs have been identified and tested as markers for dairy health. An intronic bo*TLR4* SNP common to three Chinese cattle breeds associate with SCC (Wang, Xu et al. 2007). A non-coding 5' SNP, encoding a transverse mutation (G<>C) at position -226bp relative to the bo*TLR4* start codon, lies within a potential promoter region and likely transcription factor binding site. Analysis of SNP -226G>C in Holstein bulls associated the rarer C allele with a 2% decline in lactation persistency when compared with the G allele (Sharma, Leyva et al. 2006). Persistency of lactation refers to the rate of decline in daily milk production following peak yield, with cows that are lactation persistent generally more disease resistant (Sharma, Leyva et al. 2006).

The bo*TLR4* -226G>C SNP may prove a potential target for future association studies as it has a negative functional impact on bo*TLR4* at the transcriptional level following LPS exposure (Sharma, Mount et al. 2008), and predisposes animals to MAP infection (Ruiz-Larranaga, Manzano et al. 2011). The -226G>C SNP, along with others, could be genotyped in the Mayfield herd to determine whether its frequency can be related to further physiological/health traits, milk standards and susceptibility and persistence of mastitis. Additionally, novel bo*TLR4* SNPs may also exist since SNP identification to date has only involved small DNA pools from differing breeds.

The TLR4 co-receptor CD14 will provide an additional potential target for disease-associated SNPs. Bo*CD14*, 1.4kb in length and located on chromosome 7, comprises two

exons with an intron dividing a CDS that is 1122bp in length corresponding to a 374aa chain, with short 5' and 3'UTRs identified. A previous study identified variation within bo*CD14* and impaired expression of its protein on the surface of neutrophils (Ibeagha-Awemu, Lee et al. 2008a). A NS SNP, causing an asparagine to aspartic acid substitution at +612 to the start codon (reported as 1908A>G), is located in an LRR domain that could potentially affect protein expression and pathogen recognition. SNP +612A>G was genotyped in another herd but failed to associate with SCC, although the G allele did associate with lowered milk fat and protein percentages (Beecher, Daly et al. 2010). Bo*CD14* has been highlighted as a 'mutational hotspot' in cross-breed cattle (Pal, Sharma et al. 2011), and may impact on TLR4 responses to IMI.

Despite a comparative marker map study finding no significant association between clinical mastitis and bo*TLR4* in a study of 768 Norwegian Red bulls (Opsal, Lien et al. 2008), the authors acknowledged the possibility of bo*TLR4* influencing mastitis in other populations and cattle breeds. Indeed, a cattle database of candidate genes for milk production and mastitis has recently been developed to support future genomic approaches to udder health studies. The review highlights bo*TLR4* and bo*CD14* as promising candidates for association with mastitis resistance/susceptibility (Ogorevc, Kunej et al. 2009).

5.1.3 Toll-like receptor 5

TLR5 is important for the recognition of, and inducing MyD88-signalling in response to, flagellin (Figure 5.1), the protein monomer that makes up the filament of bacterial flagella found on nearly all motile bacteria. TLR5 has demonstrated links to disease in humans; a common stop codon polymorphism, located within the ligand-binding domain of human TLR5, compromises the receptor's ability to signal flagellin. This SNP has been associated with susceptibility to pneumonia caused by *Legionella pneumophila* (Hawn, Verbon et al.

2003), and conversely with protection against systemic lupus erythematosus (Hawn, Wu et al. 2005).

Bo*TLR5* is located on chromosome 16, and comprises a single exon containing the CDS that is 2577bp in length corresponding to an 859aa chain. 5' and 3'UTRs have yet to be identified. Bo*TLR5* has been studied in several cattle breeds, with coding and non-coding regions shown to be highly polymorphic (Seabury, Cargill et al. 2007). However, little is known of the functional impact of these SNPs. Furthermore, evidence of a role for boTLR5 responses during mastitis have yet to be verified, especially as it is yet not known if mastitis-causing bacteria, such as *E. coli*, are flagellated within the mammary gland.

5.1.4 Rationale for research

Existing evidence of genetic variability within bo*TLR4* and bo*CD14* can be expanded on and tested for correlation with CM and production traits. The ability to use actual recorded CM data may lend more accuracy to existing associations between bo*TLR4*/bo*CD14* SNPs and SCC. Extended gene coverage and subsequent analysis of further SNPs yet to be identified in the coding or non-coding regions, that may be breed-specific, could reveal additional disease-associated markers. Functional impact from bo*TLR4* -226C>G and bo*CD14* +612A>G SNPs could be polygenic, linked with further SNPs and inherited as a haplotype. In addition, little work exists associating bo*TLR5* SNPs with disease and production traits in cattle, providing potential for further work.

The aims for this part of the research were to analyse the impact of existing and novel bo*TLR4*, bo*CD14* and bo*TLR5* SNPs on CM incidence and production traits in the Mayfield Holstein-Friesian herd. Candidate genes were investigated for SNPs within coding and non-coding regions including introns, 5'UTR and 3'UTR, and adjacent genomic upstream/downstream regions. SNPs identified may interrupt gene promoter sites or micro

RNA sites, splice sites or other regulatory motifs, potentially interfering with mRNA regulation and protein expression (Song, Chen et al. 2006). Any differences in relative mRNA levels between observed genotypes will be quantified, and may correlate with existing evidence of TLR functional variation, and contribute towards differences in mastitis disease pathology and milk quality and quantity.

5.2 Results

5.2.1 Characterisation of boTLR4, boTLR5 and boCD14 SNPs

The genomic organisation of the three bovine genes, bo*TLR4*, bo*TLR5* and bo*CD14*, were confirmed using internal cDNA sequencing and NCBI EST data, mapped to the Ensembl prediction build, Bos_taurus_UMD_3.1 assembly. Sequencing of 5' and 3' flanking gene regions (traversing both coding and non-coding parts) using a Holstein-Friesian DNA pool revealed a total of 13 SNPs, one of which is novel, and one insertion/deletion (in/del) (Table 5.1). Three SNPs were identified within bo*TLR4*, the well characterised 5'UTR SNP, -226G>C, the novel +96C>T SNP and +4620A>G SNP, which lie intronic between exons 1 and 2 (Figure 5.2). Three non-coding exonic SNPs were identified in bo*CD14*, one located within the 5' UTR and two within the 3'UTR, all previously reported (Figure 5.3). Bo*TLR5* was more polymorphic, with seven SNPs and one in/del identified, all previously reported (Seabury 2007). As illustrated in Figure 5.4, three of these SNPs and the in/del are located 5' upstream from the ATG start codon. A further three SNPs are 3' downstream of the stop codon. The remaining SNP, the coding synonymous bo*TLR5* +2459G>A, is located at amino acid position +820 (glutamine).

Table 5.1 - Identified SNPs within analysed bovine gene regions from Holstein-Friesians

Bo Gene	SNP	Type	Genomic position (Chr:bp)	Amino acid position	Amino acid	Previously reported	RefSNP ID
TLR4	-226G>C	5' UTR	8:108829143	-	-	Yes[1]	rs29017188
	+96C>T	Intronic	8:108829465	-	-	No	-
	+4620A>G	Intronic	8:108833989	-	-	Yes[2]	-
CD14	-5C>T	5'UTR	7:53448908	-	-	Yes[3]	rs41255516
	+1305A>G	3'UTR	7:53447598	-	-	Yes[3]	rs136324317
	+1325G>T	3'UTR	7:53447578	-	-	Yes[3]	rs109566936
TLR5	-311G>A	5'Upstream	16:27306627	-	-	Yes[4]	rs55617432
	-277T>C	5'Upstream	16:27306593	-	-	Yes[4]	rs55617326
	-235T>C	5'Upstream	16:27306551	-	-	Yes[4]	rs55617294
	-211-/A	5'Upstream	16:27306527	-	-	Yes[4]	rs55617154
	+2459G>A	Coding	16:27303858	820	Glu-Glu	Yes[4]	rs55617337
	+2620A>G	3' /Intronic	16:27303697	-	-	Yes[4]	rs55617176
	+2672C>T	3' /Intronic	16:27303645	-	-	Yes[4]	rs55617288
	+3019G>C	3'/ Intronic	16:27303298	-	-	Yes[4]	rs55617158

Abbreviations: Chr:bp = Bta chromosome: chromosomal positioning in base pairs, refSNP = Reference Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/projects/SNP). Genomic positioning according to the Bos_taurus_UMD_3.1 assembly (underlined allele denotes the published sequence). Light shading - synonymous coding SNPs. All SNP offsets are given relative to their position (bp) from the A nucleotide of the first ATG codon. [1]Sharma, Leyva et al. 2006, [2]White, Taylor et al. 2003, [3]Ibeagha-Awemu, Lee et al. 2008a, [4]Seabury, Cargill et al. 2007.

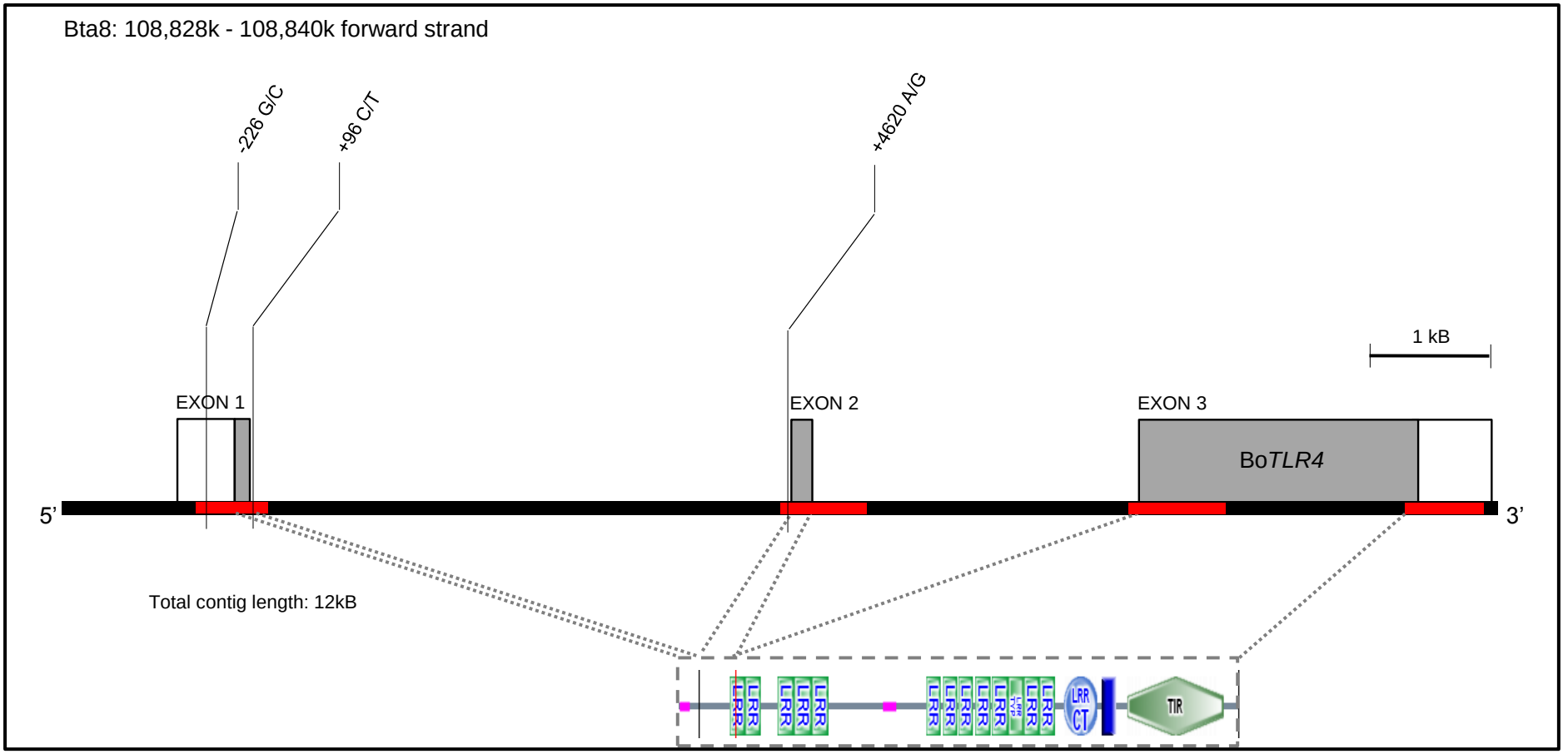


Figure 5.2 - Schematic showing the positions of identified SNPs within the bovine *TLR4* gene

Grey boxes represent coding exons (CDS = 2523bp), open boxes represent exonic non-coding untranslated regions (UTR). A TLR4 protein domain architecture diagram (designed using http://smart.embl-heidelberg.de/) is illustrated below the CDS, showing the transmembrane domain (vertical blue bar), LRR - leucine-rich repeat domains, LRR C - leucine-rich repeat C terminal domain, TIR - Toll-interleukin 1-resistance domain. Red lines represent sequencing coverage (total = 2904bp). Sequence and alignment from Bos_taurus_UMD_3.1 Primary Assembly (AC_000165.1).

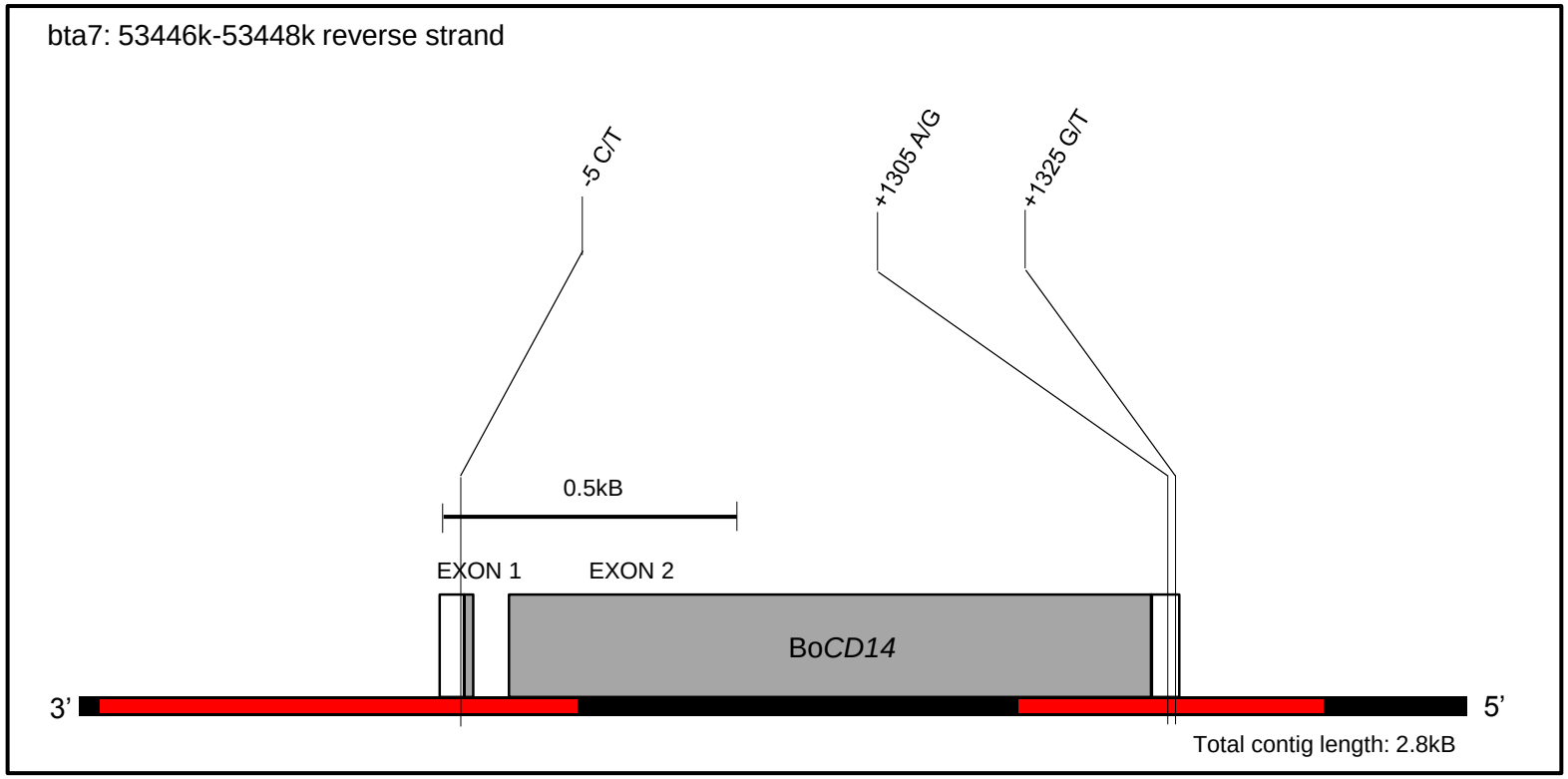


Figure 5.3 - Schematic showing the positions of identified SNPs within the bovine *CD14* gene

Grey boxes represent coding exons (CDS = 1122bp), open boxes represent exonic non-coding untranslated regions (UTR). Red lines represent sequencing coverage (total = 1259bp). Sequence and alignment from Bos_taurus_UMD_3.1 Primary Assembly (AC 000164.1_minus strand).

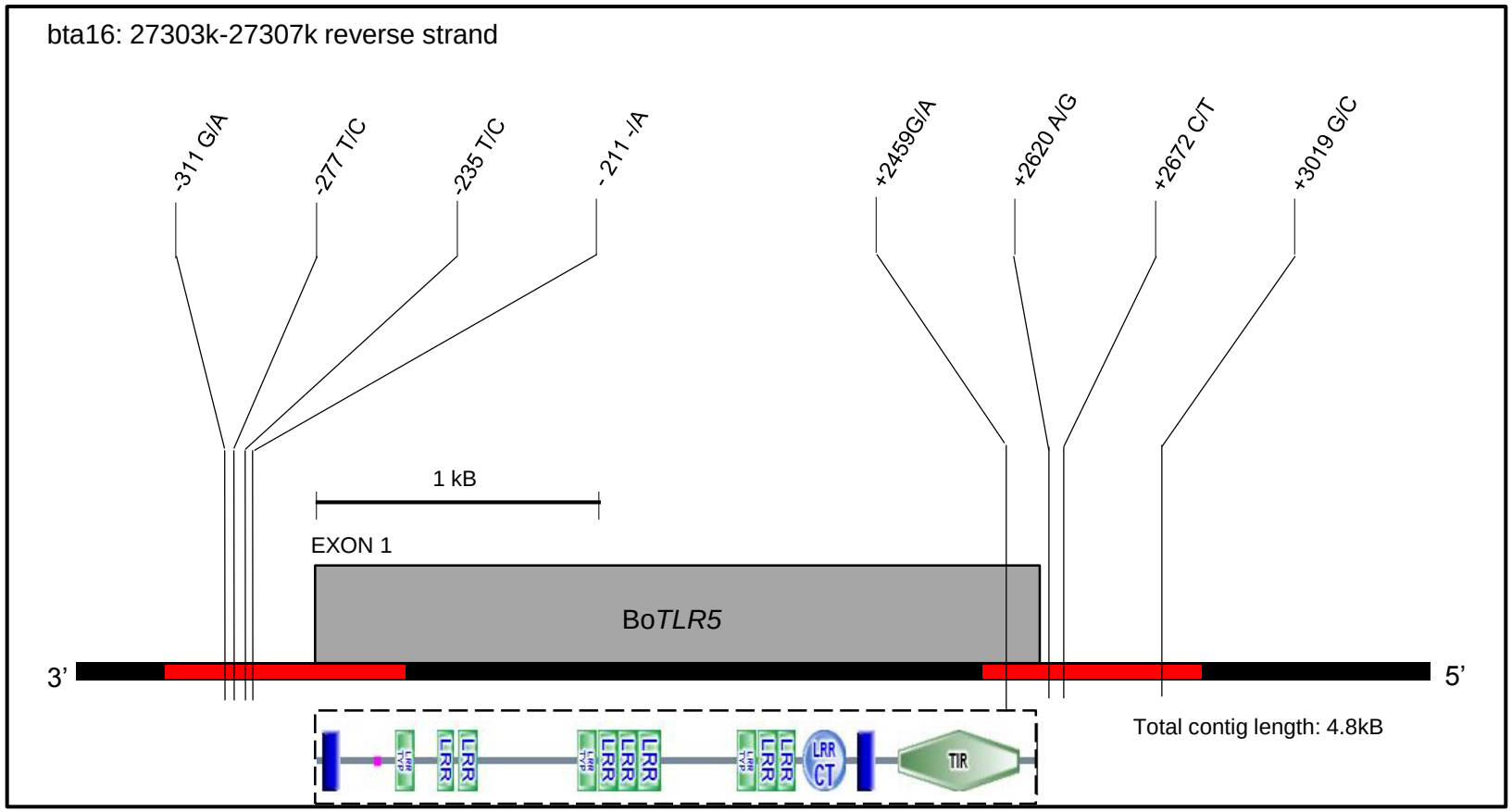


Figure 5.4 - Schematic showing the positions of identified SNPs within the bovine *TLR5* gene

Grey box represents the coding exon (CDS = 2577bp). A TLR5 protein domain architecture diagram (designed using http://smart.embl-heidelberg.de/) is illustrated below the CDS, showing the transmembrane domain (vertical blue bar), LRR - leucine-rich repeat domains, LRR C - leucine-rich repeat C terminal domain, TIR - Toll-interleukin 1-resistance domain. Red lines represent sequencing coverage (total = 1656bp). Sequence and alignment from Bos_taurus_UMD_3.1 Primary Assembly (AC_000173.1_minus strand).

5.2.2 Analysis of LD patterns in boTLR5 for tSNP selection

All seven SNPs within the bo*TLR5* candidate region were included for initial LD analysis and tSNP selection. A graphical representation of the LD between SNPs is shown in Figure 5.5. One haploblock was defined containing SNPs 1, 2 and 3, all in perfect LD (r^2=1) with each other. As a result, the -311G>A marker was selected as the tSNP for this linkage group, which also contains SNPs -277T>C and –235T>C. The A in/del at position -211 was in complete LD with the tSNP -311G>A. An A nucleotide insertion occurred only in the presence of the G allele. The four remaining bo*TLR5* SNPs (4-7) are in moderate to low LD with -311G>A tSNP (Figure 5.5).

5.2.3 Analysis of genotypic and allelic frequencies of markers selected for association analysis

Four markers were selected for further study, two candidate SNPs from bo*TLR4*, -226G>C and +96C>T, the bo*CD14* SNP -5C>T, and the bo*TLR5* tSNP -311G>A; all were extensively genotyped within the herd (*n* =103, 101, 131 and 112 respectively). The genotypic and allelic frequencies for all four selected markers are shown in Table 5.2. Two SNP markers, bo*TLR4* -226G>C and bo*CD14* -5C>T, display a common homozygous population whereby one allele is more abundant (bo*TLR4* - G and bo*CD14* - C allele frequencies 0.62 and 0.69 respectively) compared to their rarer respective allele. The other two SNPs, bo*TLR4* +96C>T and bo*TLR5* -311G>A, were not in agreement with the Hardy-Weinberg equilibrium, with the former having a very low T allele frequency (0.08) and the latter a low heterozygous population (0.20), when compared to their respective homozygous populations (Table 5.2). However, all four SNPs were taken forward for association analysis.

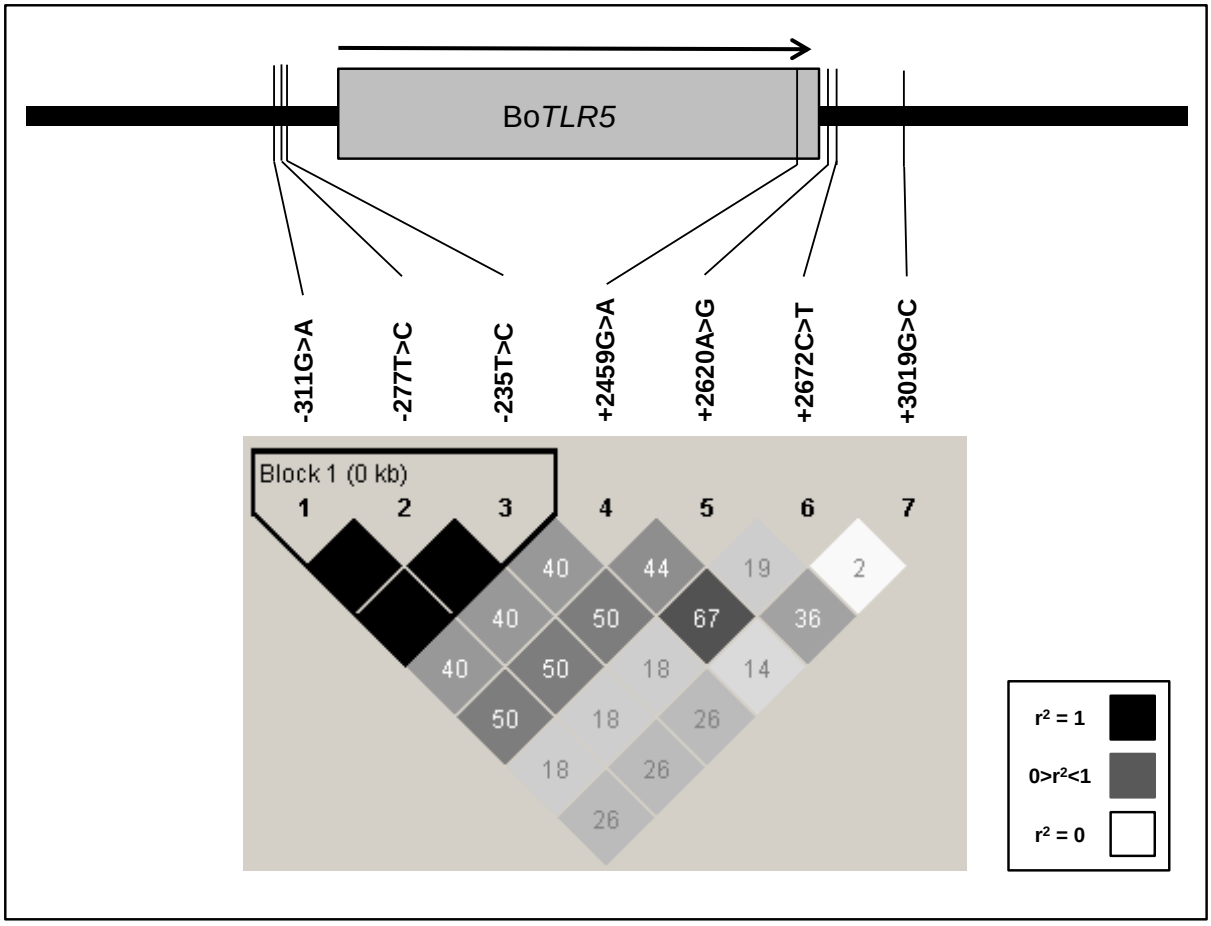


Figure 5.5 - Linkage disequilibrium (LD) plot for genotyped SNPs in relation to the bo*TLR5* gene model

Grey box represents bo*TLR5* coding exon. The graphical representation, generated by Haploview, of the r^2 LD relationship between each SNP is expressed on a grey-scale gradient; where black = perfect LD (r^2 = 1.0) and white = very low LD (<0.1). The r^2 value for each pair-wise comparison is shown, where no value indicates r^2 = 1. SNPs 1-3 were defined as a haploblock.

Table 5.2 - Genotype and allele frequencies including significance from Hardy-Weinberg equilibrium for candidate gene SNPs selected for trait association

Gene	SNP	Genotype	*n* =	Genotype Frequency	HWE	Allele	Allele Frequency
Bo*TLR4*	-226G>C	GG	45	0.44	2.46	G	0.62
		GC	40	0.39		C	0.38
		CC	18	0.17			
		Total	103				
	+96C>T	CC	88	0.87	10.42*	C	0.92
		CT	10	0.10		T	0.08
		TT	3	0.03			
		Total	101				
Bo*CD14*	-5C>T	CC	64	0.48	0.62	C	0.69
		CT	52	0.40		T	0.31
		TT	15	0.12			
		Total	131				
Bo*TLR5*	-311G>A	GG	43	0.38	38.25*	G	0.49
		GA	23	0.20		A	0.51
		AA	46	0.42			
		Total	112				

*Hardy-Weinberg equilibrium (HWE) significant at 1 degree of freedom ($P<0.001$).

5.2.4 Association of selected bovine SNPs with CM and milk production traits

The four SNPs, bo*TLR4* -226G>C and +96C>T, bo*CD14* -5C>T, and bo*TLR5* tSNP -311G>A, were analysed for association with CM and milk production traits during the first three lactations, as illustrated in Table 5.3. The four SNPs were further tested for association with CM per individual parity (P1, P2 and P3), as illustrated in Table 5.4.

5.2.4.1 Association analysis of boTLR4 SNP -226G>C

Association analysis of the bo*TLR4* SNP -226G>C with CM revealed no significant differences between genotypes over the first three lactations. The data trend showed the rarer CC genotype to have higher CM incidence (0.57 cases/cow/year) when compared with heterozygous GC and the abundant GG population (both 0.47 cases/cow/year) (Table 5.3). The proportion of animals within each genotyped population having any CM during their first three lactations was lowest for GC cows (47%) when compared with homozygous GG and CC genotypes (67% and 61% respectively).

Analysis for SNP association with CM per individual parity revealed no significant differences between genotypes (Table 5.4). In all three genotypes, CM incidence was significantly increased at P3 when compared to their P1 and P2 incidence (Figure 5.6). Analysis of production traits revealed no significant differences between genotypes for milk SCC, yield, fat and protein concentrations (Table 5.3).

Table 5.3 - Association of SNP genotypes with CM and milk production traits over the first three lactations

SNP	Gen.	*n* =	P % with CM[1]	Mean CM rate[2]	*P* =[3]	Mean SCC[4]	*P* =[3]	Mean 305d milk yield[4]	*P* =[3]	Mean milk fat (%)[4]	*P* =[3]	Mean milk protein (%)[4]	*P* =[3]
Bo*TLR4*	GG	45	67	0.47 (±0.16)		2.93 (±0.70)		8289 (±1239)		4.05 (±0.47)		3.22 (±0.18)	
-226G>C	GC	40	47	0.47 (±0.20)	-	2.88 (±0.72)	-	8380 (±1081)	-	4.05 (±0.38)	-	3.20 (±0.17)	-
	CC	18	61	0.57 (±0.31)	-	3.02 (±1.01)	-	7957 (±1315)	-	4.23 (±0.51)	-	3.29 (±0.20)	-
Bo*TLR4*	CC	88	56	0.47 (±0.12)		2.93 (±0.77)		8266 (±1176)		4.08 (±0.45)		3.23 (±0.18)	
+96C>T	CT	10	78	0.70 (±0.49)	-	2.94 (±0.84)	-	8363 (±1365)	-	4.13 (±0.56)	-	3.23 (±0.21)	-
	TT	3	33	0.37 (±1.53)	-	2.61 (±0.76)	-	7985 (±1657)	-	4.04 (±0.10)	-	3.06 (±0.11)	-
Bo*CD14*	CC	64	53	0.59 (±0.19)		2.89 (±0.73)		8297 (±1170)		3.99 (±0.43)		3.20 (±0.17)	
-5C>T	CT	52	52	0.47 (±0.20)	-	2.77 (±0.84)	-	8186 (±995)	-	4.09 (±0.41)	-	3.21 (±0.19)	-
	TT	15	67	0.47 (±0.27)	-	2.67 (±0.78)	-	8170 (±1462)	-	3.83 (±0.48)	-	3.09 (±0.16)	-
Bo*TLR5*	GG	43	60	0.57 (±0.20)	-	2.78 (±0.73)	-	7748 (±1081)	-	4.06 (±0.44)	-	3.23 (±0.19)	-
t-311G>A	GA	23	70	0.58 (±0.26)	-	3.03 (±0.71)	-	8158 (±1369)	-	4.01 (±0.52)	-	3.26 (±0.18)	-
	AA	46	61	0.57 (±0.20)		2.72 (±0.76)		7837 (±1367)		4.07 (±0.50)		3.23 (±0.21)	

Abbreviations: Gen. = genotype, P % = population percentage, CM = clinical mastitis, SCC = somatic cell count. [1]No significance was detected between genotypes by Chi^2 odds ratio test. [2]Mean±95% confidence interval given in column parentheses. [3]*P* values analysed by ANOVA and Tukey's multiple comparisons, showing only results tested against the underlined (most abundant) homozygous genotype; – (dash) denotes no significance. [4]Standard deviation from the mean given in column parentheses.

Table 5.4 - Association of SNP genotypes with CM at first (P1) second (P2) and third (P3) parity

SNP	Gen.	*n* =	P1 P % with CM[1]	P1 Mean CM rate[2]	*P* =[3]	P2 P % with CM[1]	P2 Mean CM rate[2]	*P* =[3]	P3 P % with CM[1]	P3 Mean CM rate[2]	*P* =[3]
TLR4	GG	45	13	0.26 (±0.21)		20	0.30 (±0.19)		47	1.02 (±0.44)	
-226G>C	GC	40	10	0.19 (±0.20)	-	25	0.32 (±0.21)	-	38	0.97 (±0.48)	-
	CC	18	17	0.20 (±0.23)	-	22	0.34 (±0.35)	-	59	1.11 (±0.63)	-
TLR4	CC	88	13	0.24 (±0.14)		21	0.29 (±0.13)		44	0.97 (±0.30)	
+96C>T	CT	10	10	0.14 (±0.33)	-	40	0.66 (±0.63)	-	60	1.34 (±1.26)	-
	TT	3	0	0.00 (±0.00)	-	0	0.00 (±0.00)	-	33	1.51 (±6.51)	-
CD14	CC	64	21	0.39 (±0.22)		28	0.46 (±0.24)		38	1.04 (±0.42)	
-5C>T	CT	52	17	0.25 (±0.18)	-	27	0.52 (±0.32)	-	37	0.71 (±0.32)	-
	TT	15	20	0.25 (±0.29)	-	21	0.31 (±0.31)	-	50	1.06 (±0.82)	-
TLR5	GG	43	27	0.30 (±0.17)	-	23	0.35 (±0.24)	-	48	1.04 (±0.42)	-
t-311G>A	GA	23	22	0.35 (±0.31)	-	43	0.67 (±0.42)	-	41	0.71 (±0.32)	-
	AA	46	15	0.34 (±0.26)		24	0.29 (±0.18)		51	1.06 (±0.82)	

Abbreviations: Gen. = genotype, P % = population percentage, CM = clinical mastitis. [1]No significance was detected between genotypes by Chi^2 odds ratio test. [2]Mean±95% confidence interval given in column parentheses. [3]*P* values analysed by ANOVA and Tukey's multiple comparisons, showing only results tested against the underlined (most abundant) homozygous genotype; – (dash) denotes no significance.

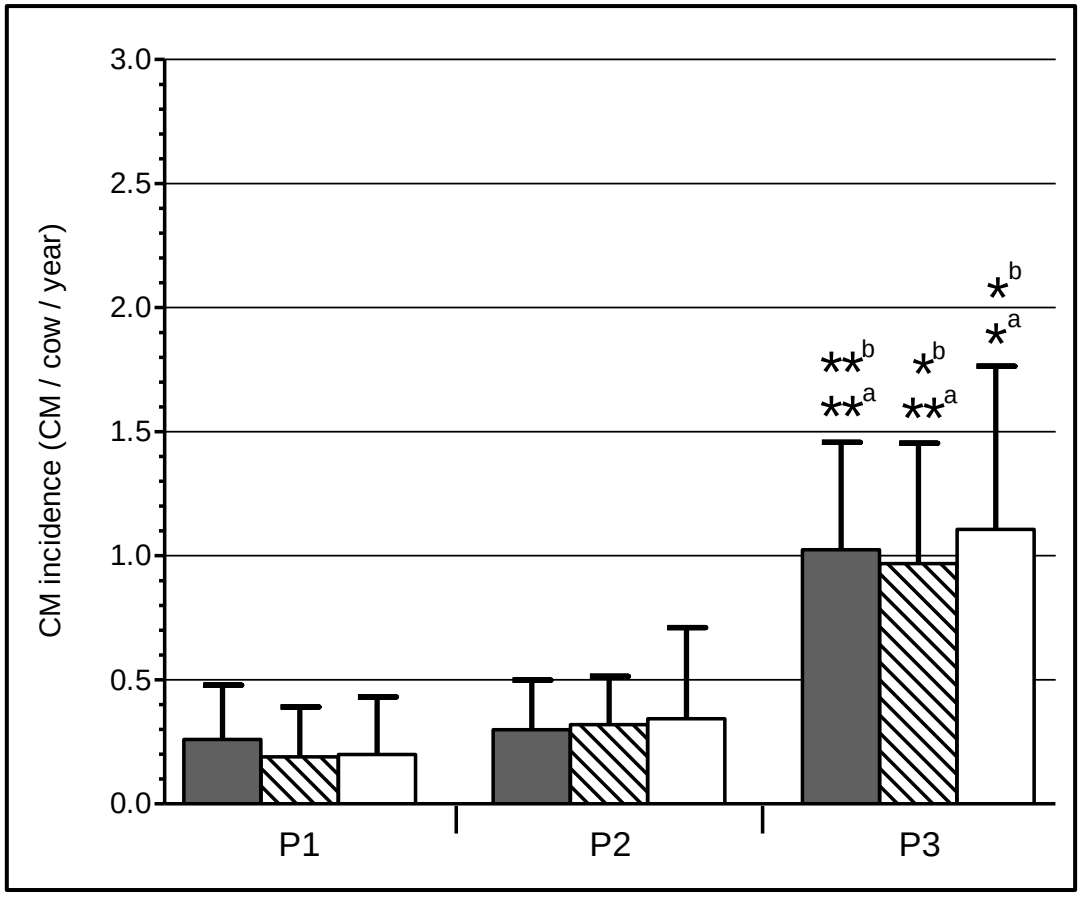


Figure 5.6 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*TLR4* -226G>C SNP variant populations: GG, GC and CC

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – GG animals (*n* =45), hatched bars – GC animals (*n* =40) and open bars – CC animals (*n* =18). P1 = first parity, P2 = second parity and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Genotypic means differ significantly at the following levels: **P*<0.05, ***P*<0.01, by ANOVA and Tukey's multiple comparisons.

5.2.4.2 Association analysis of boTLR4 SNP +96C>T

Association analysis of the bo*TLR4* SNP +96C>T with CM revealed no significant differences between genotypes over the first three lactations. The data trend showed the rare CT genotype to have higher CM incidence (0.70 cases/cow/year) when compared to the abundant CC population (0.47 cases/cow/year) (Table 5.3). The proportion of animals within each genotyped population having any CM during their first three lactations was again higher for CT cows (78%) when compared to homozygous CC genotypes (56%). Due to low population numbers for the even rarer TT genotype, no trend was observed.

Analysis of SNP association with CM per individual parity revealed no significant differences between genotypes (Table 5.4). Animals from the CC genotype displayed significantly increased CM incidence at P3 when compared to their P1 and P2 incidence (Figure 5.7). The rare TT population contributed data to P3 only, which displays an exaggerated CI whisker because of the low animal numbers (Figure 5.7). Analysis of production traits revealed no significant differences between genotypes for milk SCC, yield, fat and protein concentrations (Table 5.3).

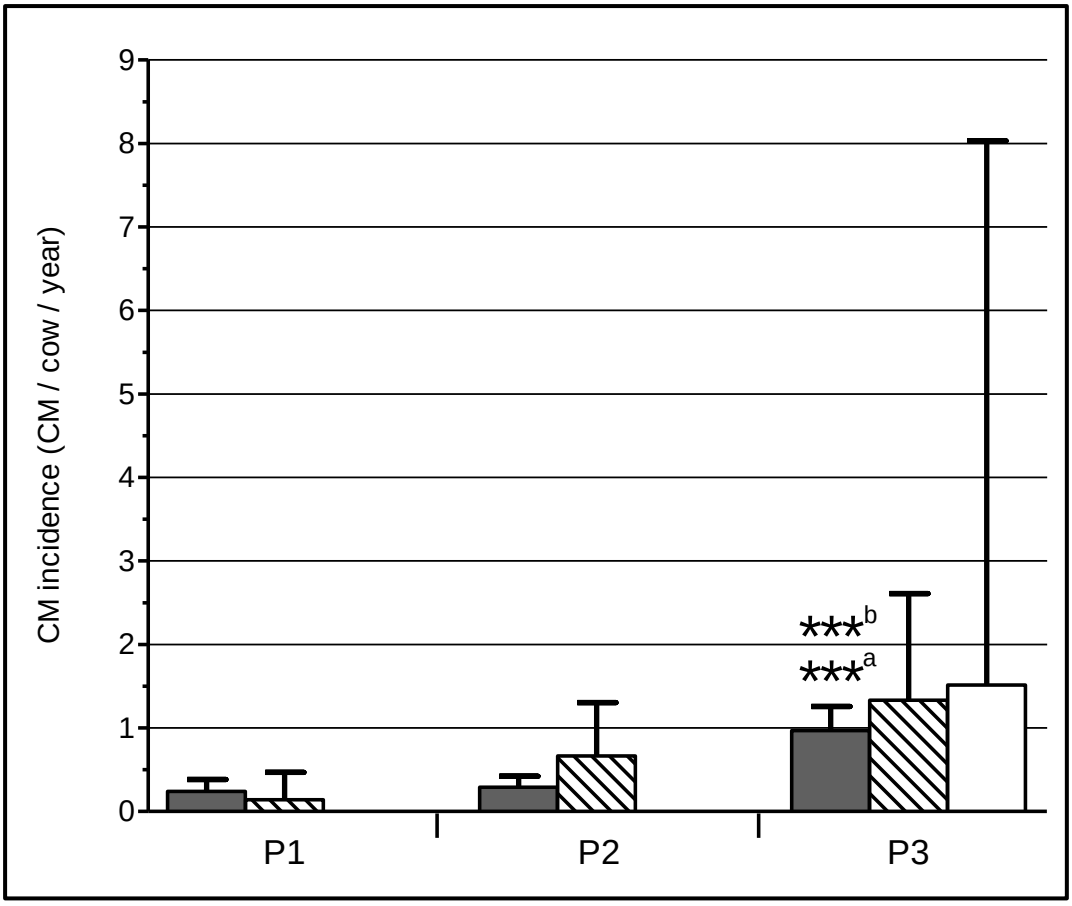


Figure 5.7 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*TLR4* +96C>T SNP variant populations: CC, CT and TT

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – CC animals (*n* =88), hatched bars – CT animals (*n* =10) and open bars – TT animals (*n* =3). P1 = first parity, P2 = second parity and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. ****P*<0.001 by ANOVA and Tukey's multiple comparisons.

5.2.4.3 Association analysis of boCD14 SNP -5C>T

Association analysis of the bo*CD14* -5C>T SNP with CM revealed no significant differences between genotypes over the first three lactations. The data trend showed the most abundant CC genotype to have higher CM incidence (0.59 cases/cow/year) when compared to heterozygous CT and the rarer TT population (both 0.47 cases/cow/year) (Table 5.3). The proportion of animals within each genotyped population having any CM during their first three lactations was higher for TT cows (67%) when compared to CT and CC genotypes (52% and 53% respectively).

Analysis for SNP association with CM per individual parity revealed no significant differences between genotypes (Table 5.4). The highest CM incidence for each genotype was observed at P3 (Figure 5.8). However, only the CC population showed a significantly increased P3 incidence of 1.04 cases/cow/year when compared with a P1 incidence of 0.39 cases ($P<0.01$), and a P2 incidence of 0.46 cases ($P<0.05$). Analysis of production traits revealed no significant differences between genotypes for milk SCC, yield, fat and protein concentrations (Table 5.3); however, the TT genotype tended to associate with reduced milk protein when compared to CC and CT cows ($P<0.1$).

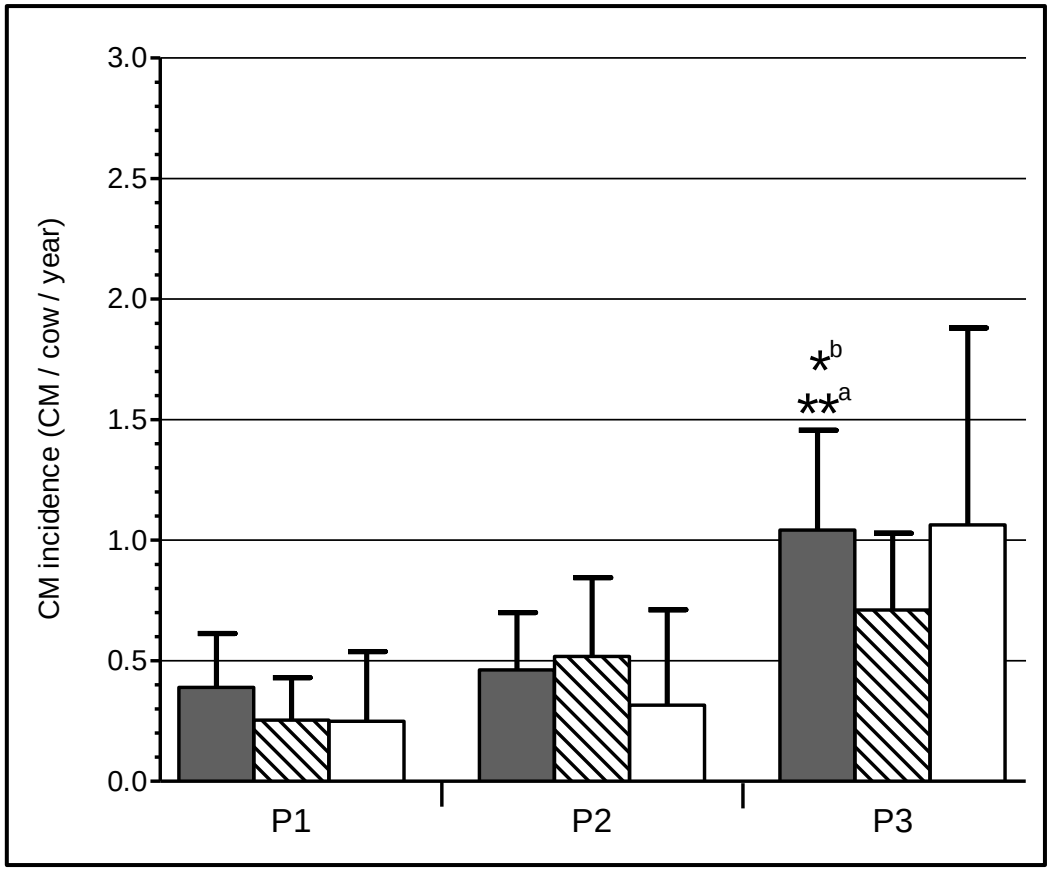


Figure 5.8 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CD14* -5C>T SNP variant populations: CC, CT and TT

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – CC animals (*n* =64), hatched bars – CT animals (*n* =52) and open bars – TT animals (*n* =15). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Genotypic means differ significantly at the following levels: **P*<0.05, ***P*<0.01, by ANOVA and Tukey's multiple comparisons.

5.2.4.4 Association analysis of boTLR5 tSNP -311G>A

Association analysis of the bo*TLR5* -311G>A tSNP with CM revealed no significant differences between genotypes over the first three lactations. The data trends revealed all three genotypes to have similar CM incidences (0.57-0.58 cases/cow/year) (Table 5.3). Analysis for SNP association with CM per individual parity revealed no significant differences between genotypes (Table 5.4). The highest CM incidence for each genotype was observed at P3 (Figure 5.9). However, only GG and AA populations showed a significantly increased P3 incidence when compared with their respective P1 and P2 incidences ($P<0.01$). Analysis of production traits revealed no significant differences between genotypes for milk SCC, yield, fat and protein concentrations (Table 5.3).

5.2.5 In-silico analysis for putative transcription binding sites

The AliBaba2.1 (TRANSFAC 4.0 sites) software programme was used to search for putative TBS located immediately upstream of the ATG start codon for bo*TLR5*. The 5' upstream SNPs identified in this study were then analysed for any potential impact on predicted TBS. Bo*TLR5* SNPS, -311G>C, -277T>C and –235T>C, and an in/del at -211, are surrounded by several putative TBS, including matched sites for the transcription enhancing factor Specificity Protein 1 (Sp1), and gene up-regulators GATA-binding factor-1 (GATA-1) and Activator Protein-1 (AP-1) (Figure 5.10). However, both published and alternate allele sequences suggest these polymorphisms have no obvious impact on the presence or absence of TBS. Due to both allele sequences having identical predictions of surrounding TBS, only the published allele sequence is shown (Figure 5.10).

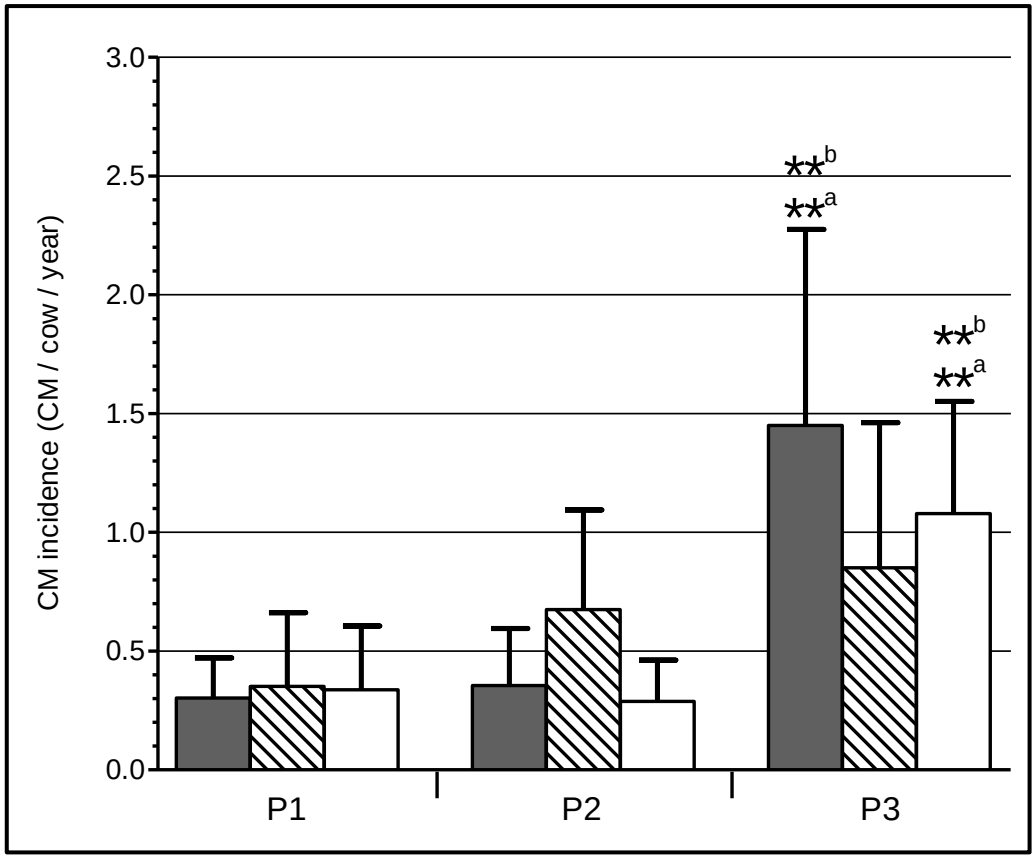


Figure 5.9 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*TLR5* -311G>A variant populations: GG, GA and AA

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – GG animals (*n* =40), hatched bars – GA animals (*n* =23) and open bars – AA animals (*n* =43). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. **Genotypic means differ significantly ($P<0.01$) by ANOVA and Tukey's multiple comparisons.

*Bos_taurus*_UMD_3.1 Primary Assembly Sequence

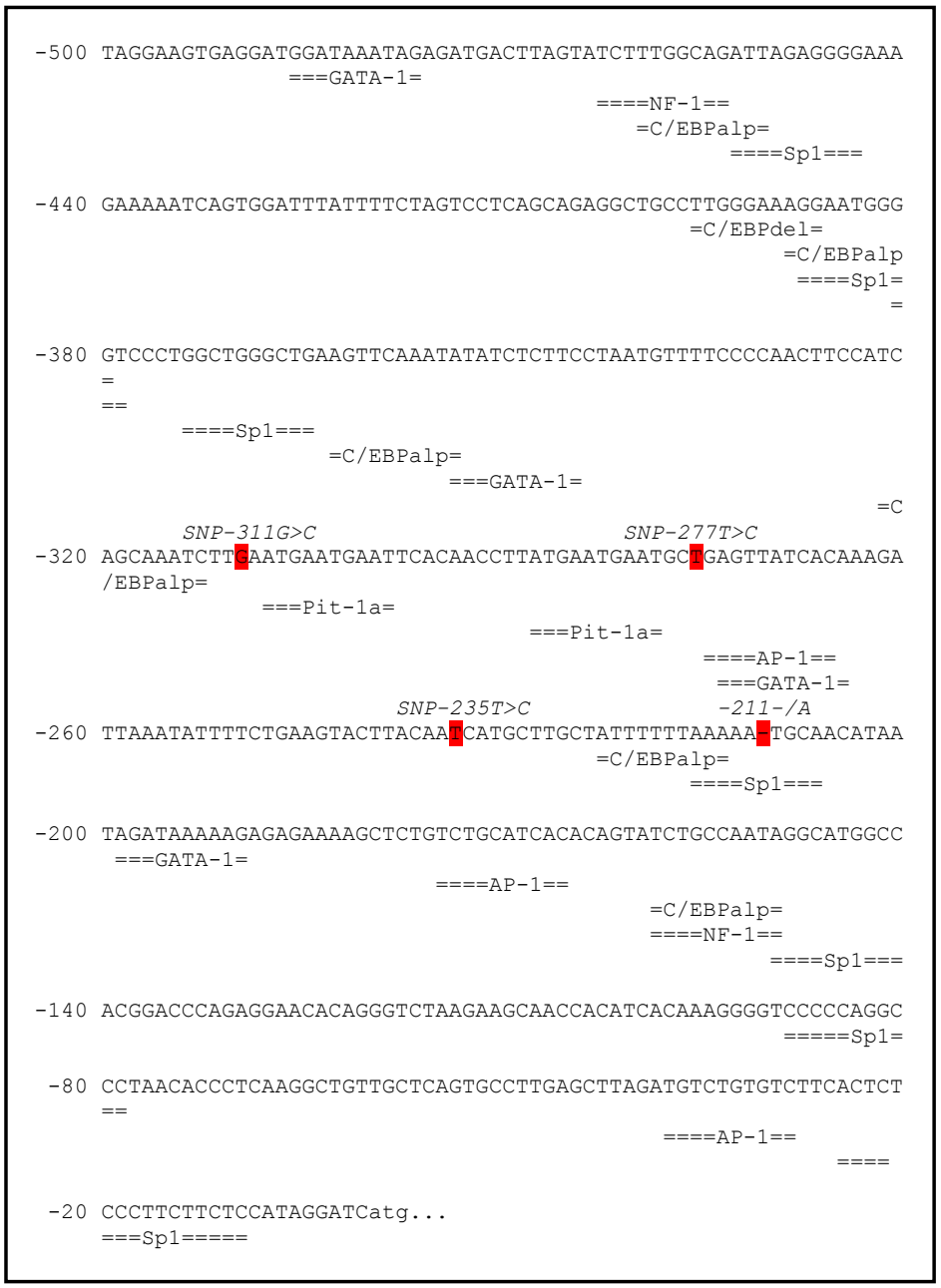


Figure 5.10 - Location of upstream bo*TLR5* SNPs in relation to putative transcription binding sites for the published allele sequence

The AliBaba 2.1 software programme was used to predict (75% match threshold) transcription factor binding sites (TBS) in the bo*TLR5* upstream region. Upstream nucleotide sequence shown in upper case, ATG start codon shown by lower case. Numbering indicates nucleotide position from the start codon. SNP positions are highlighted in red, with SNP names above. Positions of putative TBS are aligned below the nucleotide sequence. TBS abbreviations: Sp1 = Specificity Protein 1, GATA-1 = GATA-binding factor-1, AP-1 = Activator Protein-1, NF-1 = Nuclear Factor-1, C/EBP = CCAAT-Enhancer-Binding Protein, Pit-1a = POU class 1 homeobox-1.

5.2.6 Q-PCR analysis of basal boTLR4 and boTLR5 mRNA abundance

Milk somatic cells from cows representing bo*TLR4* and bo*TLR5* genotypes were assessed for differences in basal *boTLR4* and *boTLR5* expression. All contributing animals were of similar age and stage of lactation and none had any known health issues or recorded any incidence of mastitis two weeks preceding/following the collection. Q-PCR assays were performed using RNA from 15 animals, five from each bo*TLR4/5* SNP genotype. Analysis of *boTLR4* expression by representative SNP -226G>C genotypes revealed no significant differences or trends in basal expression levels between the three genotypes (Figure 5.11). Bo*TLR5* basal expression levels in mammary somatic cells was generally much lower in comparison to *boTLR4* expression, and further revealed no significant differences in expression levels between the three bo*TLR5* tSNP -311G>A variant populations: GG, GA and AA. A marginal trend in the data suggests lowered expression in somatic cells from AA cows when compared with cells from GG animals.

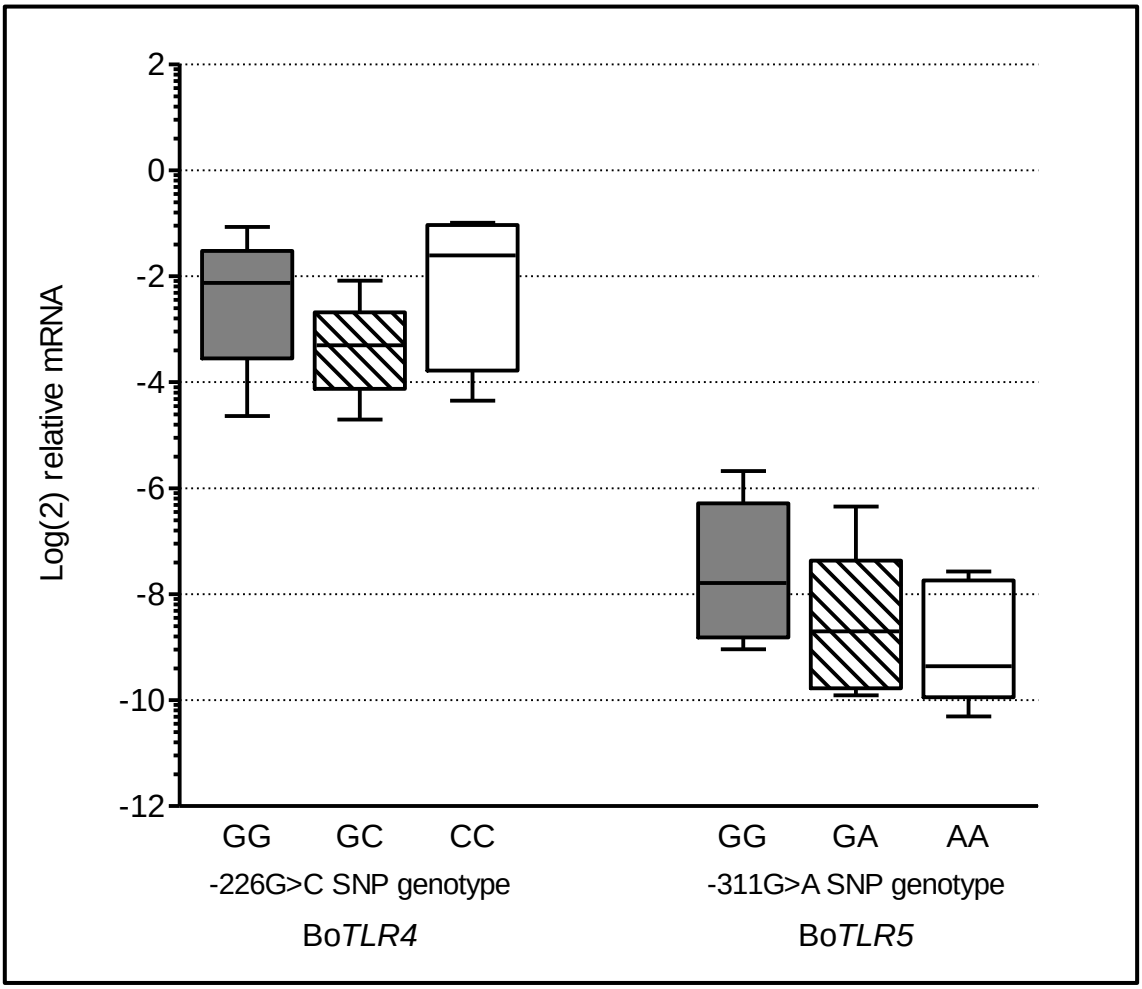


Figure 5.11 - Q-PCR data showing basal mRNA abundance levels of *boTLR4* and *boTLR5* in milk somatic cells grouped into their respective SNP variant populations

The three bo*TLR4* -226G>C SNP variant populations, GG, GC and CC, are indicated, as are the three bo*TLR5* -311G>A SNP variant populations, GG, GA and AA. Data from each variant population (n = 5) is presented as a box and whisker plot, with the ends of the whiskers representing the minimum and maximum data values. Genotypic means do not differ significantly (P>0.05 by ANOVA and Tukey's multiple comparisons).

5.3 Discussion

The importance of the boTLR4 and boCD14 complex to mediate inflammatory responses during mammary infection has been highlighted previously (De Schepper, De Ketelaere et al. 2008; Buitenhuis, Rontved et al. 2011). Preliminary studies have so far linked the presence of bo*TLR4* and bo*CD14* SNPs with SCC (Sharma, Leyva et al. 2006; Wang, Xu et al. 2007; Ibeagha-Awemu, Lee et al. 2008). However, some of these SNP associations lacked significance when studied in other herds and breeds (Beecher, Daly et al. 2010), highlighting the need for further work to clarify their potential as a marker for CM. In this study, bo*TLR4*, bo*CD14* and bo*TLR5* were analysed for SNPs within their 5' and 3' regions, allowing assessment of non-exonic and exonic UTR and CDS SNPs. Identified SNPs were then investigated as markers for dairy health using actual recorded CM and production data.

Sequencing pooled DNA from Holstein-Friesian cows of high and low CM incidence histories revealed thirteen SNPs between the candidate genes. Seven SNPs (one coding) and one in/del polymorphism were identified within bo*TLR5*, all previously reported (Seabury, Cargill et al. 2007). Complete linkage was observed within the herd between the three bo*TLR5* 5' upstream SNPs, defined into a single haploblock, and represented by tSNP -311G>A. LD between -311G>A and the remaining coding +2459G>A SNP and three 3' downstream SNPs, was low. Due to low animal numbers for both the CDS mutation (a synonymous change unlikely to have functional impact) and for the 3' downstream SNPs, association analysis was not performed. However, the 3' SNPs may provide a future target for study. Likewise the bo*TLR4* SNP +4620A>G, one of three bo*TLR4* SNPs identified within the herd, requires greater animal numbers for association analysis. This SNP has recently been associated with IBK infection rates in American cattle (Kataria, Tait et al. 2011), and may also provide a future target for study. The 5'UTR bo*CD14* SNP -5C>T, and the 3' UTR SNPs +1305A>G and +1325G>T reported in this study have previously been

found to be in complete linkage with one another (Ibeagha-Awemu, Lee et al. 2008a). Therefore, only the bo*CD14*-5C>T SNP was genotyped extensively. The observed genotypic frequencies for SNPs bo*TLR4* +96C>T and bo*TLR5* -311G>A were not in agreement with the Hardy-Weinberg equilibrium, indicating potential for false-positive genotyping (Xu, Turner et al. 2002). However, these two SNPs were still put forward, along with the bo*TLR4* SNP -226G>C and the bo*CD14* SNP -5C>T, for association analysis.

No associations for CM or milk production were found for any of the SNPs studied. The overall CM trend demonstrates increased incidence for cows in their third lactation for most of the SNP genotypes studied, demonstrating the positive correlation between age and CM. Significantly increased CM rates were observed for all bo*TLR4* SNP -226G>C genotypes during P3, however, there are no genotypic variation or trends. This upstream bo*TLR4* SNP is located within a putative c-Ets-1 DNA-binding site, and further surrounded by GATA-like sites (Sharma, Leyva et al. 2006). In previous studies, the -226G>C mutation impacted bo*TLR4* mRNA levels, with the GG genotype demonstrating increased expression following LPS exposure (Sharma, Leyva et al. 2006; Sharma, Mount et al. 2008). This study did not observe any significant differences or trends between -226G>C genotypes for basal bo*TLR4* mRNA levels in mammary somatic cells. It is therefore likely that this mutation affects inducible expression levels only. In terms of impact on disease, the GG population has been suggested as being more disease-resistant owing to better lactation persistency (Sharma, Leyva et al. 2006), although a recent study has associated the G allele with increased susceptibility to paratuberculosis (Ruiz-Larranaga, Manzano et al. 2011). Trends from this study suggest GG cows to have lowered CM incidence when compared to CC cows but no significance was detected, potentially due to low numbers of animals of the latter genotype. In agreement with Sharma *et al* (2006), no associations with SCC were observed. As a potential functional SNP impacting inducible bo*TLR4* levels, -226G>C warrants further

study. In addition, the bo*TLR4* SNP +96C>T will benefit from further work to improve numbers for the rare T allele, which will then reveal whether it has potential as a marker for dairy disease.

Variation in inducible bo*TLR4* expression could be the result of polymorphic influence from additional proteins important for TLR4 signalling, for example variants of the TLR4-associating protein MD-2. A rare MD-2 SNP in humans affecting gene promoter activity impedes *MD-2* and cytokine expression levels following LPS exposure, and was linked to sepsis in patients (Gu, Shan et al. 2007). Similarly, polymorphic variation within the human LBP protein has been linked to disease susceptibility (Chien, Boeckh et al. 2008). Bovine *MD-2* and *LBP* could therefore provide additional marker candidates for impaired TLR4 function and disease association.

The TLR4 co-receptor CD14 is important for complete LPS signalling and has previously demonstrated polymorphic variation in CD14 expression. The bo*CD14* SNP -5C>T (reported as 1291C>T) has been associated with reduced CD14 protein expression by bovine monocytes from TT variant cows (Ibeagha-Awemu, Lee et al. 2008a). CD14 expression correlates with apoptosis in cells from bovine mammary glands (Sladek and Rysanek 2011), suggesting reduced expression for this receptor could impact mammary cell survival as well as TLR4 function. Although no significance was detected, trends for the TT population in this study show a lowered CM rate, but a higher herd percentage with CM when compared to CC cows. More genotyped animals could reveal genotypic differences if they exist and/or improve interpretation of the trends and accuracy of the association analysis. In addition, basal bo*CD14* mRNA levels between the -5C>T genotypes will be useful to compare with previously observed variation in protein expression levels. The -5C>T SNP may be linked with further upstream promoter SNPs yet to be identified. Analysis for putative DNA binding sites within a 1.2kb promoter region immediately 5' of the bo*CD14*

start codon has been performed (Ibeagha-Awemu, Lee et al. 2008a), revealing several species-conserved predicted binding sites. However, the -5C>T SNP had no impact on any of these. Extended sequencing into this promoter region may identify additional SNPs. In the human system SNPs located in a *CD14* promoter region, shown to enhance transcription, have links with heart disease (Shimada, Watanabe et al. 2000).

This study did not observe any significant differences or trends between bo*TLR5* -311G>A genotypes for basal bo*TLR5* mRNA levels in mammary somatic cells. Searches for putative DNA binding sites within a 500bp genomic region immediately upstream of the start codon revealed that the presence or absence of the -311G>A SNP, the two tagged SNPs and an in/del polymorphism had no obvious impact on the predicted binding sites. The overall level of bo*TLR5* expression when compared to bo*TLR4* was very low, agreeing with observations from another study analysing bovine mammary cells exposed to *E. coli* (Porcherie, Cunha et al. 2012). These mammary cells further demonstrated an inability to initiate inflammatory responses to the TLR5 ligand flagellin. Whilst a role for boTLR5 during mastitis has yet to be fully determined, this study did not find any associations or trends for CM with bo*TLR5* SNPs. Verification of mastitic pathogens that are flagellated inside the mammary gland, and whether flagella is highly immunogenic in milk, will improve our understanding of the role of boTLR5, which currently demonstrates no significant activity in the early stages of mastitis. In addition, a number of previously reported NS SNPs (Seabury, Cargill et al. 2007) could provide markers for protein function variation and susceptibility to other diseases.

In conclusion, no associations for CM or milk production were found for any of the selected SNP markers within the bovine genes bo*TLR4*, bo*CD14* and bo*TLR5*. However, these findings would benefit from larger sample populations and extensive coverage and analysis of gene regions may reveal disease-associated SNPs missed by this study. Due to the

importance of boTLR4 signalling during mastitis, bo*TLR4* SNPs remain potential candidates for investigations into CM susceptibility in other herds/breeds, and warrant further study which could extend to markers within bo*CD14* and the genes for the accessory molecules MD-2 and LBP. Combined analysis of SNPs from these TLR4-related genes could then yield greater accuracy when associating with polygenic diseases such as mastitis, an approach that has associated the presence of both a *TLR4* and a *CD14* SNP, instead of them individually, with human atherosclerotic disease (Vainas, Stassen et al. 2006). Furthermore, investigating CM incidence by the causative pathogen may reveal significant associations between bo*TLR4* SNPs and susceptibility to *E. coli* mastitis.

Chapter 6.0 - Identification of bovine *CXCR1* and *CXCR2* SNPs - association with mastitis and production traits in cattle

6.1 Introduction

6.1.1 Chemokine Receptors CXCR1 and CXCR2

The chemokine receptors CXCR1 and CXCR2 have pivotal roles in leukocyte motility as they both recognise the chemokine CXCL8, the most potent chemoattractant for neutrophils (Murdoch and Finn 2000). Increased transcript and protein levels of CXCL8 have been demonstrated during mastitis (Lee, Bannerman et al. 2006). The receptors' immunological function of activating and directing effector cells to regions of increased CXCL8 are vital in cell-mediated resolution of IMI and mastitis. CXCR1 activity is strongly correlated with inflammatory responses towards both Gram-negative and Gram-positive infection. TLR4 binding of *E. coli* LPS, for example, led to the up-regulation of bo*CXCR1*, giving increased cell sensitivity to CXCL8 and improved migratory efficiency (Olson and Ley 2002). Another study however, demonstrated that *S. aureus* may inhibit neutrophil responsiveness to CXCL8 via TNF-α-mediated (induced by the bacteria) down-regulation of CXCR1 and CXCR2 (Tikhonov, Doroshenko et al. 2001).

Both CXCR1 and CXCR2 share a similar structure and function with all other chemokine receptors, whereby an extracellular NH_2-terminus, an intracellular COOH-terminus and seven transmembrane domains are connected by three extracellular loops (ECLs) and three intracellular loops (ICLs), as illustrated in Figure 6.1. The NH_2-terminal region and ECLs, each containing a conserved and structurally important cysteine residue, interact with the chemokine ligand. In a human CXCR1 study the NH_2-terminal amino acids 1-38 were mainly responsible for binding CXCL8, with ECLs 1 and 2 stabilising this interaction (Park, Casagrande et al. 2011). The study demonstrated CXCL8 binding signals from a truncated receptor lacking ECLs, but no signal from protein lacking an NH_2-terminal. Chemokine receptors are G protein-coupled receptors, whereby the COOH-terminus and ICLs associate with inactivated intracellular G protein components (α/βγ) within the cell.

Following ligand binding, the receptor activates the G protein subunits by facilitating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in the Gα subunit, leading to its dissociation from the Gβγ subunit. Subsequently this then activates complex and partially characterised pathways leading to inactivation and internalisation of the receptor protein (via phosphorylation of COOH-terminal serine and threonine residues), and activation of cell mechanisms for cell motility, degranulation and release of superoxide anions (Murdoch and Finn 2000; Allen, Crown et al. 2007) (Figure 6.1). Internalisation of activated CXCR1/CXCR2 for 'desensitisation' before their recycling to the cell surface is important to maintain a cell's responsiveness and chemotactic efficiency (Borroni, Mantovani et al. 2010).

High homology (84% identity) exists between bovine CXCR1 and CXCR2 proteins, with each sharing >70% identity to their respective human homologues. However, boCXCR1 and boCXCR2 share 100% identity at the COOH-terminus, which differ in the human system and other species (Lahouassa, Rainard et al. 2008). Although close identity between boCXCR1 and boCXCR2 is reflected in their shared ability to detect chemokines CXCL6, CXCL7 and CXCL8, boCXCR2 has the added capacity to recognise a further four CXCL chemokines (Table 1.3). High homology has led to confusion in previous studies, with the misidentification of genes bo*CXCR1* (previously known as *IL8RA*) and bo*CXCR2* (previously known as *IL8RB*) (Pighetti and Rambeaud 2006). Both genes are of similar size; the CDS for bo*CXCR1* is 1080bp in length, corresponding to a 360aa chain, while bo*CXCR2* is 1089bp, corresponding to a 363aa chain. Both genes are located in close proximity, approximately 90.3cM on chromosome two (Bta 2), with bo*CXCR1* (reading antisense) and bo*CXCR2* (reading sense) separated by 22.6kb. Both receptors are similarly expressed on human neutrophils (Chuntharapai, Lee et al. 1994), and expressed at variable levels in bovine MØ,

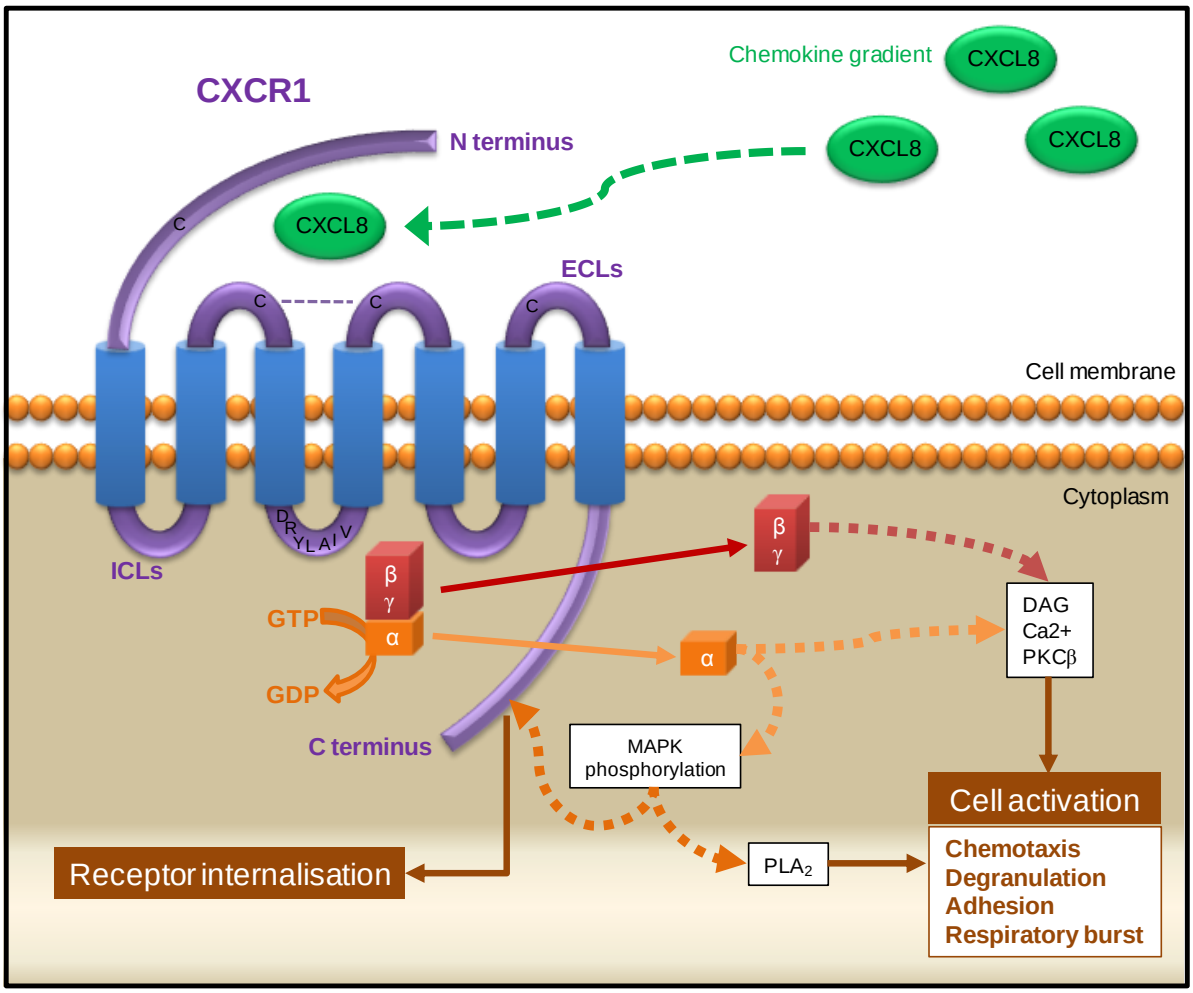


Figure 6.1 - Schematic of CXCR1 structure, activation and signalling

The basic tertiary structure of CXCR1 is outlined by the seven transmembrane domains (in blue), connecting three intracellular loops (ICLs) and three extracellular loops (ECLs) with N and C termini. The conserved cysteine residues (C) and a DRYLAIV motif important for signalling are highlighted. The N terminus and ECLs interact with the ligand (CXCL8), whilst the C terminus and ICLs associate with inactivated G proteins comprised of an α (orange) subunit and a βγ (red) subunit. Receptor/ligand interaction results in the replacement of GDP with GTP within Gα, dissociating the G-protein into the two active Gα and Gβγ components to initiate a complex series of intracellular signalling events (generalised as dashed arrows). Both Gα and Gβγ direct calcium mobilisation (Ca^{2+}), diacyl-glycerol (DAG) formation and activation of the enzyme phospholipase C-β (PKCβ). Gα further directs MAPK phosphorylation of C terminus residues leading to receptor internalisation and recycling (desensitisation). MAPK activity leads to phospholipase A_2 (PLA_2) activation. PLA_2, Ca^{2+}, DAG and PKCβ are all important for specific cell activation mechanisms.

monocytes and dendritic cells (Widdison, Siddiqui et al. 2010). Polymorphic variation impacting CXCR1 and CXCR2 expression in neutrophils has been linked to human disease (Qiu, Zhu et al. 2003; Kormann, Hector et al. 2011), and due to the importance of the neutrophil response in mammary defence, the presence of SNPs within bo*CXCR1* and bo*CXCR2* has been implicated in dairy health and disease.

6.1.2 Association of boCXCR1 SNPs with health traits in dairy cattle

There have been a number of disease association studies linking SNPs within bo*CXCR1* with mastitis and health. An initial study from the USA associated a previously reported and misidentified SNP within bo*CXCR2* (Grosse, Kappes et al. 1999; Youngerman, Saxton et al. 2004b) with significant differences in the incidence of subclinical mastitis (Youngerman, Saxton et al. 2004a). This SNP, which has now been correctly annotated within bo*CXCR1* causing a coding G>C base substitution at position +735bp relative to the ATG start codon (SNP position previously reported as +777bp relative to the study's sequenced transcript) results in a NS glutamine to histidine amino acid change. Cows expressing the CC polymorphism were observed to have a significant (P<0.05) increase in subclinical mastitis incidence as determined by microbial status, a slight decrease in CM and a greater milk yield. However, these associations may strengthen (or weaken) with a larger sample size, since only 4 of the 37 Holsteins genotyped expressed the CC allele.

The bo*CXCR1* +735G>C NS SNP is located in a structural region important for receptor signalling, as demonstrated in a follow-up study where carriers of the C base (CC and GC genotype) had significantly lower neutrophil migration towards human recombinant CXCL8 than those with the GG genotype (Rambeaud and Pighetti 2005). In addition, neutrophils from homozygous CC cows demonstrated decreases in receptor numbers, calcium

signalling, generation of reactive oxygen species and adhesion molecule expression, yet increased neutrophil survival (Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Rambeaud and Pighetti 2007). The influence of the bo*CXCR1*+735 SNP may be polygenic, as another initial study focusing on the UTRs identified three novel polymorphisms in a 2.1kb upstream region of bo*CXCR1* (Leyva-Baca, Schenkel et al. 2008). The SNP bo*CXCR1* -1768T>A substitution significantly decreased EBV for SCC by a fifth for the first and second lactations. The Canadian dairy industry currently select against bulls with EBV for increased SCC. The -1768T>A SNP site was surrounded by a host of potential transcription binding sites, including NF-κB, and may therefore be *cis*-acting; further work will be required to verify this. The findings from the above studies involving bo*CXCR1* SNPs -1768T>A and +735G>C are sound evidence to support the additional investigations as detailed in this chapter, as they provide an existing immunological mechanism to explain potential genetic differences in mastitis susceptibility.

Since the initial associative reporting of bo*CXCR1* SNPs -1768T>A and +735G>C by Leyva-Baca *et al.* (2008) and Youngerman *et al.* (2004a) respectively, further studies analysing these and additional bo*CXCR1* SNPs have had conflicting results. Whilst the Leyva-Baca *et al.* (2008) study observed no significant association with SCC for the NS +735G>C SNP, another study found that SNP +735G>C tended to associate with SCC in a Holstein-Friesian dataset (Beecher, Daly et al. 2010). A large scale German Holstein study revealed no significant associations for -1768T>A and +735G>C SNPs with SCC on a population-wide basis using a suitably adjusted dataset (Goertz, Baes et al. 2009). A study analysing -1768T>A and +735G>C SNPs in Black Japanese cattle (n = 150) for association with clinical intestinal or respiratory disease revealed no significance. However, there were significant differences between -1768T>A genotypes for white blood cell counts (Tsuchida, Yamad et al. 2010). Another study analysing Chinese Holstein cattle (n = 610) associated the

upstream -1768T>A SNP, as well as two additional SNPs (-1830A>G and -344T>C), with SCC (Chen, Yang et al. 2011a). The +735G>C SNP, however, was not detected within their sample herd.

Discrepancies for -1768T>A and +735G>C SNP associations are likely due to the differences in sampling methods and criteria for measuring phenotype. With regard to +735G>C SNP associations, Youngerman *et al*. (2004) sampled a small cohort and used both CM and SCC recordings, Leyva-Baca *et al*. (2008) sampled one large herd and measured SCC, whilst Goertz *et al*. (2009) sampled many herds and measured SCC. Additional differences in breeds, causative pathogen/s (undetermined in many/previous studies) and herd management must also be taken into consideration. Furthermore there are limitations when relying on SCC data, as arguably the use of current SCC recordings as a proxy for IMI is not absolutely correlated with bacterial presence (Heringstad, Gianola et al. 2006; Koeck, Miglior et al. 2012). The use of actual recorded CM data may therefore be more accurate. Analysis of 350 cows from up to 23 commercial Holstein dairy farms in New York State, USA, revealed the SNP +735G>C associated with CM incidence, as measured by recorded clinical observations (Galvao, Pighetti et al. 2011). Interestingly cows of CC and GC genotypes, previously associated with lowered neutrophil migration, demonstrated a significant decrease in CM when compared with GG cows. The GG cows also had significantly lowered milk yields.

The association of two bo*CXCR1* SNPs with impaired neutrophil function and potential mastitis susceptibility demonstrates the potential of bo*CXCR1* (and bo*CXCR2*) SNPs as markers for disease in cattle.

6.1.3 Rationale for research

Although a large body of work exists regarding bo*CXCR1* polymorphisms, these findings provide scope and potential for further work. Existing bo*CXCR1* SNP associations can be expanded upon and extended into the study of SNPs yet to be identified in the coding or non-coding regions. The influence of *CXCR1* SNPs +735G>C and -1768T>A may be polygenic, linked with additional SNPs and inherited as a haplotype. QTL evidence supports the potential of both CXCR genes as suitable markers for CM. A genome-wide association study to identify QTL associated with CM in first lactating cows in Norwegian Red cattle observed potential significant associations from a genomic region of ~8.5 Mb on chromosome 2 (Bta2) that contains bo*CXCR1* and bo*CXCR2* (Sodeland, Kent et al. 2011).

The aims for this part of the research were to analyse the impact of existing and novel bo*CXCR1* and bo*CXCR2* SNPs on CM incidence and production traits in the Mayfield Holstein-Friesian herd. The two candidate genes were investigated for SNPs within coding and non-coding regions including introns, 5'UTR and 3'UTR, and adjacent genomic upstream/downstream regions. In addition, potential differences in relative mRNA levels between observed genotypes were quantified. These differences could influence CXCR activity and support existing evidence of boCXCR1 functional variation.

6.2 Results

6.2.1 Characterisation of boCXCR1 and boCXCR2 SNPs

The genomic organisation of bo*CXCR1* and bo*CXCR2* was confirmed using internal cDNA sequencing and NCBI EST data, mapped to the Ensembl prediction build Bos_taurus_UMD_3.1. Both full length bo*CXCR1* and bo*CXCR2* mRNA transcripts comprise two exons with an intron dividing their 5'UTR; however, a 3'UTR has yet to be identified in bo*CXCR2*. Sequencing 5' and 3' regions of both candidate genes within a Holstein-Friesian herd (*n* = 186) revealed a total of 21 SNPs, of which three are novel (Table 6.1). Bo*CXCR1* was found to be more polymorphic, with 19 SNPs identified compared to only two within bo*CXCR2*. 16 of the 19 bo*CXCR1* SNPs were exonic; 10 were located in the CDS including six NS changes, while the remaining six were located in the non-coding 3'UTR. The three non-exonic SNPs were identified within the intron of the 5' UTR (Figure 6.2). The two identified bo*CXCR2* SNPs are upstream of the 5' UTR (Figure 6.3).

6.2.2 Analysis of LD patterns in boCXCR1 for tSNP selection

All 19 SNPs within the bo*CXCR1* region analysed were included for LD analysis and tSNP selection. A graphical representation of the LD between these SNPs is shown in Figure 6.4. All 19 SNPs were defined as one haploblock, with three linkage groups identified. SNPs 3, 4, 5, 6, 7, 9, 13, 14, 15, 16, 17 and 19 are in perfect LD (r^2=1) with each other. This linkage group of 12 SNPs includes four NS SNPs: +37A>T, +38T>A, +68G>A, and the well-documented +735G>C. As a result, the +735G>C marker was selected as the tSNP for these 12 SNPs. The SNPs 2, 10, 11 and 18, are all in complete LD (r^2=1) with each other. This linkage group of four includes the remaining two NS SNPs, +980A>G and +995A>G. The marker -181T>C was selected as the tSNP to represent these four SNPs. SNPs 8 and 12 are in complete LD (r^2=1) with one another, and the marker +816C>A was selected as the tSNP for this linkage group (Table 6.2). A singleton marker, -205C>T, was also identified.

LD between tSNPs t+735G>C, t-181T>C and t+816C>A was low (LD~0.3). The lowest LD was observed between t-181T>C and t+816C>A (LD~0.15). LD between the three tSNPs and the one singleton SNP -205C>T was low to moderate. Consequently, t-181T>C, t+735G>C, t+816C>A and singleton SNP -205C>T were used for association analysis and haplotype reconstruction. No LD was observed between bo*CXCR2* and bo*CXCR1* SNPs. Both bo*CXCR2* SNPs were used for association analysis but excluded from bo*CXCR1* haplotypes.

6.2.3 Analysis of genotypic and allelic frequencies of markers selected for association analysis

The four boCXC*R1* SNPs, -205C>T, t-181T>C, t+735G>C and t+816C>A, and two bo*CXCR2* SNPs, -512T>C and -496G>A, were analysed within the herd (*n* = 139, 182, 183, 182, 129 and 132 respectively). The genotypic and allelic frequencies for all six selected markers are shown in Table 6.3. The bo*CXCR1* SNP marker t+735G>C identifies heterozygotes as the most abundant genotype, with G and C alleles of near equal frequency (Table 6.3). The remaining SNPs all display a common homozygous population whereby one allele is by far the most abundant (frequency ranges 0.71- 0.81) compared to the rarer allele (frequency ranges 0.19-0.29). With the exception of the bo*CXCR2* SNP -496G>A, all SNPs were found to be in agreement with the Hardy-Weinberg equilibrium. Despite this, the bo*CXCR2* SNP -496G>A was still put forward for association analysis.

Table 6.1 - Identified SNPs within bo*CXCR1* and bo*CXCR2* from Holstein-Friesians

Gene	SNP	Location	Genomic (Bta2) position	Amino acid position	Amino acid	Previously reported	dbSNP ID
Bo*CXCR1*	-205C>T	Intron1	106938784	-	-	Yes[3]	-
	-181T>C	Intron1	106938760	-	-	Yes[2]	-
	-41A>C	Intron1	106938620	-	-	No	-
	+37A>T	Exon2	106938542	13	Ile-Phe	Yes	rs110296731
	+38T>A	Exon2	106938541	13	Ile-Asn	Yes	rs134770474
	+68G>A	Exon2	106938511	23	Gly-Glu	Yes	rs133273369
	+735G>C	Exon2	106937845	245	Glu-His	Yes[1, 4]	rs137681722
	+816C>A	Exon2	106937764	272	Ile-Ile	Yes[1]	-
	+819G>A	Exon2	106937761	273	Ala-Ala	Yes[1]	rs134348371
	+980A>G	Exon2	106937600	327	Lys-Arg	Yes[3]	rs136740306
	+995A>G	Exon2	106937585	332	His-Arg	Yes[3]	rs135131046
	+1008C>T	Exon2	106937572	336	Ser-Ser	Yes[3]	rs43323014
	+1068G>A	Exon2	106937512	356	Thr-Thr	Yes[3]	rs132989373
	+1089C>A	3'UTR	106937491	-	-	Yes[3]	-
	+1090A>C	3'UTR	106937490	-	-	Yes[3]	-
	+1093C>T	3'UTR	106937487	-	-	Yes[3]	-
	+1098C>T	3'UTR	106937482	-	-	Yes[3]	-
	+1180G>A	3'UTR	106937400	-	-	Yes[3]	rs134970823
	+1282G>A	3'UTR	106937298	-	-	Yes[3]	rs137732683
Bo*CXCR2*	-512T>C	5' to gene	106904491	-	-	No	-
	-496G>A	5' to gene	106904507	-	-	No	-

Abbreviations: Bta2 = *Bos taurus* chromosome 2, dbSNP = Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/projects/SNP). All sequences were aligned with the Bos_taurus_UMD_3.1 assembly AC_000159.1 (underlined allele denotes the published sequence). Light shading - synonymous coding SNPs, dark shading - non-synonymous coding SNP. All SNP offsets are given relative to their position (bp) from the A nucleotide of the first ATG codon. Prior publication for reported SNPs /or SNP equivalent: [1]Youngerman, Saxton et al. 2004b, [2]Leyva-Baca, Schenkel et al. 2008, [3]Pighetti, Kojima et al. 2012, [4]Grosse, Kappes et al. 1999.

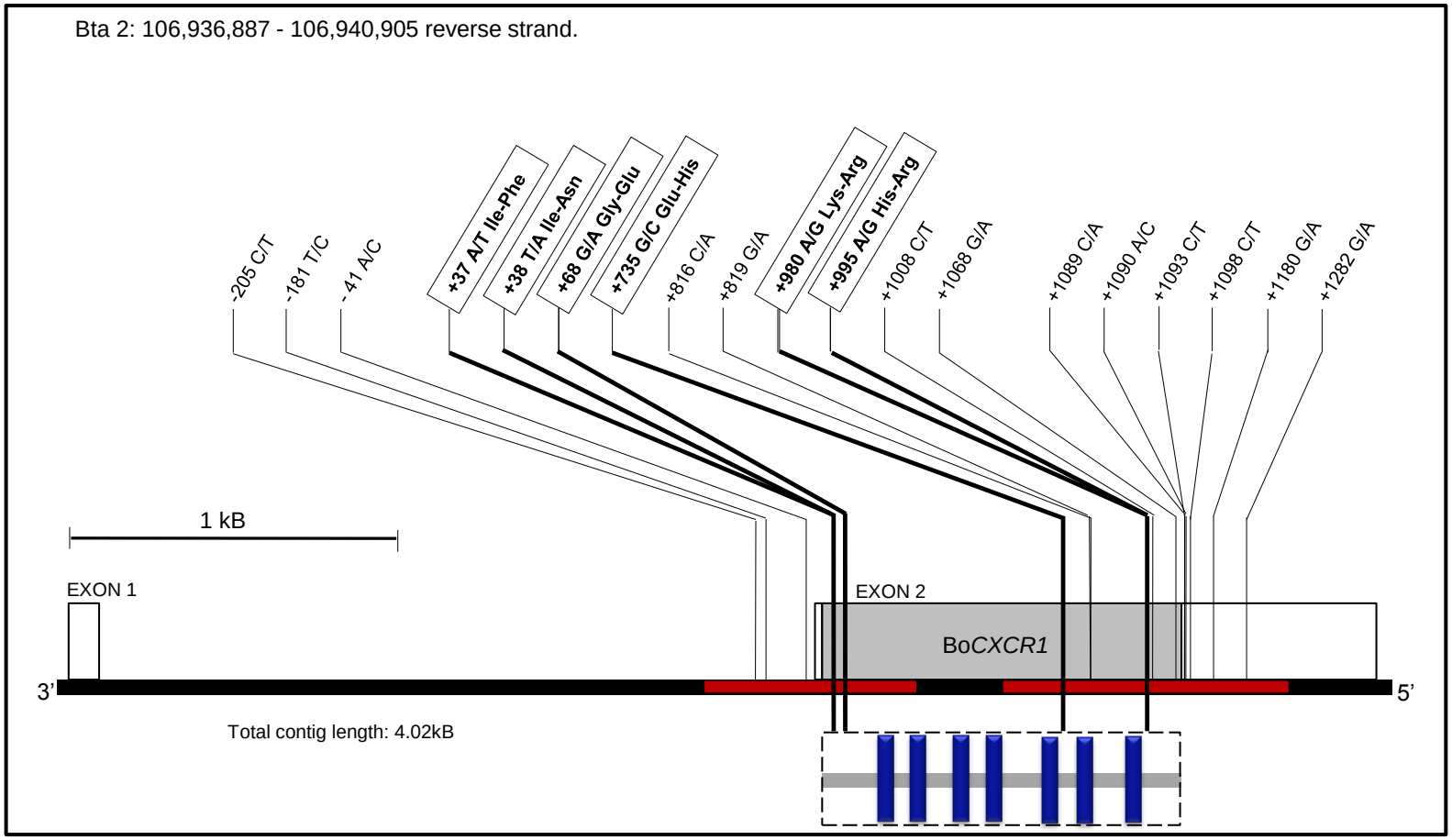


Figure 6.2 - Schematic showing the positions of identified SNPs within bo*CXCR1*

Grey box represents coding exon (CDS = 1080bp), open boxes represent exonic non-coding untranslated regions. A CXCR1 protein domain architecture diagram (designed using http://smart.embl-heidelberg.de/) is illustrated below the CDS, showing the seven transmembrane domains (vertical blue bars). All NS SNPs are boxed in bold. Red lines represent sequencing coverage (total = 1500bp). Sequence and alignment from Bos_taurus_UMD_3.1 Primary Assembly (AC_000159.1).

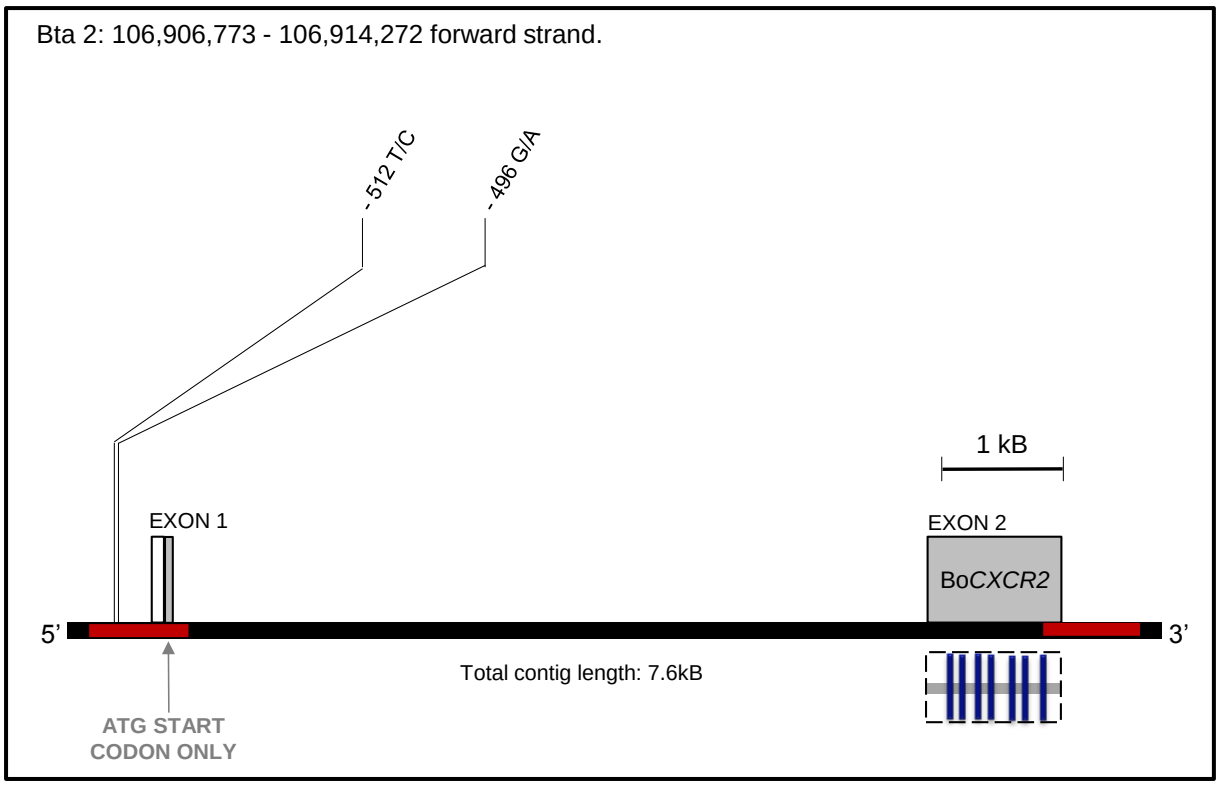


Figure 6.3 - Schematic showing the positions of identified SNPs within bo*CXCR2*

Grey box represents coding exon (CDS = 1089bp), open boxes represent exonic non-coding untranslated regions (UTR). Coding portion of exon1 = ATG start codon only. A CXCR2 protein domain architecture diagram (designed using http://smart.embl-heidelberg.de/) is illustrated below the CDS, showing the seven transmembrane domains (vertical blue bars). Red lines represent sequencing coverage (total = 1557bp). Sequence and alignment from Bos_taurus_UMD_3.1 Primary Assembly (AC_000159.1).

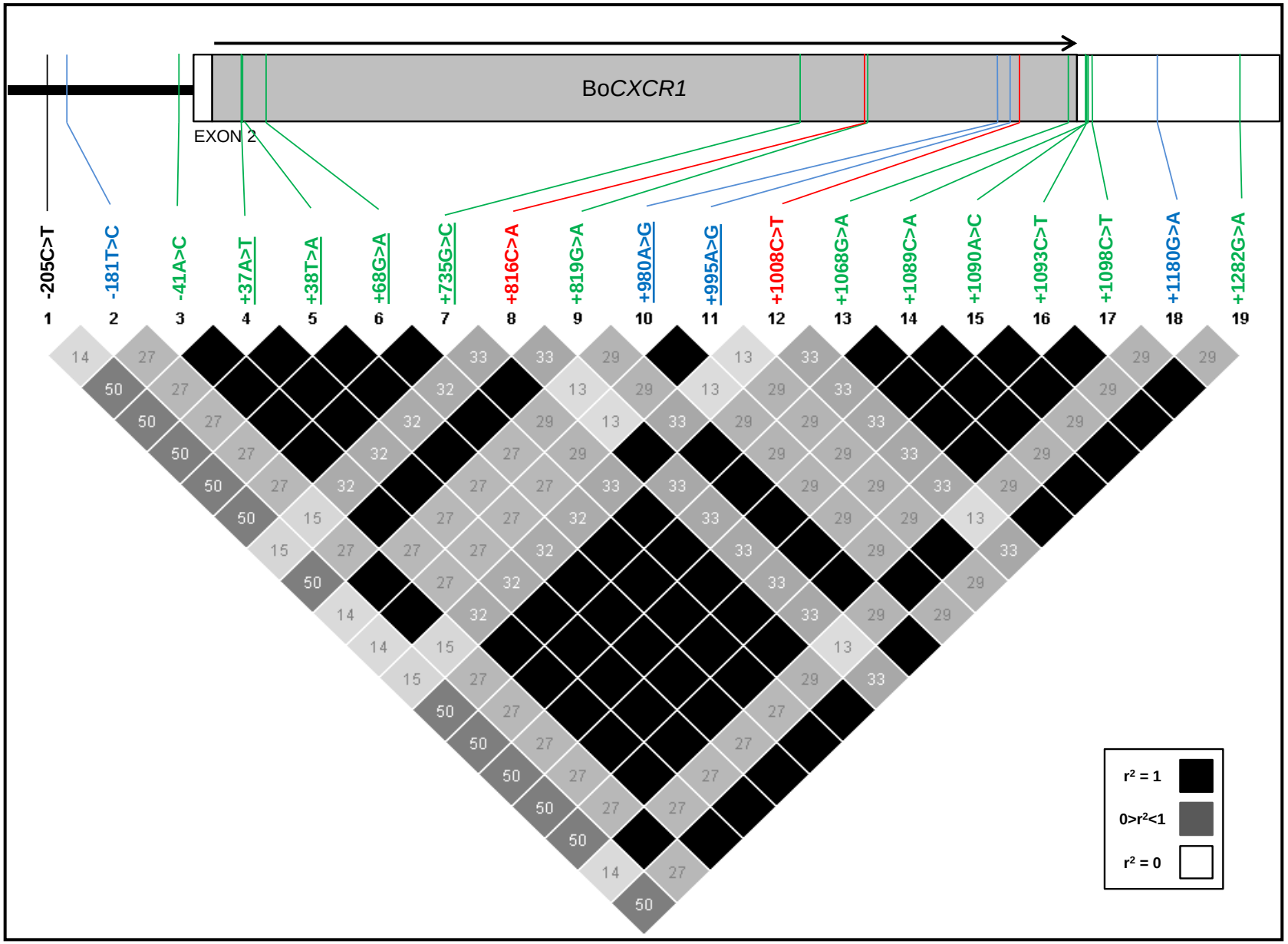

BoCXCR1
EXON 2
-205C>T
-181T>C
-41A>C
+37A>T
+38T>A
+68G>A
+735G>C
+816C>A
+819G>A
+980A>G
+995A>G
+1008C>T
+1068G>A
+1089C>A
+1090A>C
+1093C>T
+1098C>T
+1180G>A
+1282G>A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
r² = 1
0>r²<1
r² = 0

Figure 6.4 - Linkage disequilibrium (LD) plot for genotyped SNPs in relation to the bo*CXCR1* gene model

Grey box represents bo*CXCR1* coding exon, open boxes represent non-coding UTR exonic regions. The six NS SNPs are underlined. The graphical representation, generated by Haploview, of the r^2 LD relationship between each SNP is expressed on a grey-scale gradient; where black = perfect LD (r^2 = 1.0) and white = very low LD (<0.1). The r^2 value for each pair-wise comparison is shown, where no value is indicated r^2 = 1. SNP positions highlighted as blue, green or red, to show patterns of perfect LD (r^2 = 1) between matching colours, represent the three linkage groups. All 19 SNPs were defined as one haploblock.

Table 6.2 - Summary of tSNP markers for bo*CXCR1* linkage groups

Linkage group	tSNP marker	Tagged SNPS
1	-181T>C	-181T>C +980A>G +995A>G +1180G>A
2	+735G>C	-41A>C +37A>T +38T>A +68G>A +735G>C +819G>A +1068G>A +1089C>A +1090A>C +1093C>T +1098C>T +1282G>A
3	+816C>A	+816C>A +1008C>T

Table 6.3 - Genotype and allele frequencies including significance from Hardy-Weinberg equilibrium for bo*CXCR1* and bo*CXCR2* SNPs selected for trait association

Bovine Gene	SNP	Genotype	*n* =	Genotype Frequency	HWE	Allele	Allele Frequency
CXCR1	-205C>T	CC	74	0.53	2.09	C	0.71
		CT	50	0.36		T	0.29
		TT	15	0.11			
		Total	139				
	t-181T>C	TT	8	0.04	0.02	T	0.21
		TC	59	0.32		C	0.79
		CC	115	0.64			
		Total	182				
	t+735G>C	GG	50	0.27	0.66	G	0.51
		GC	86	0.47		C	0.49
		CC	47	0.26			
		Total	183				
	t+816C>A	CC	93	0.51	0.01	C	0.71
		CA	74	0.41		A	0.29
		AA	15	0.08			
		Total	182				
CXCR2	-512T>C	TT	8	0.06	2.7	T	0.19
		TC	35	0.27		C	0.81
		CC	86	0.67			
		Total	129				
	-496G>A	GG	91	0.69	13.13*	G	0.80
		GA	29	0.22		A	0.20
		AA	12	0.09			
		Total	132				

*Hardy-Weinberg equilibrium (HWE) significant at 1 degree of freedom (P>0.001).

6.2.4 Association of boCXCR1 and boCXCR2 SNPs with CM and milk production traits

The four bo*CXCR1* SNPs, -205C>T, t-181T>C, t+735G>C and t+816C>A, and the two bo*CXCR2* SNPs, -512T>C and -496G>A, were analysed for association with CM and milk production traits during the first three lactations, as illustrated in Table 6.4. The six SNPs were further tested for association with CM per individual parity (P1, P2 and P3), as illustrated in Table 6.5.

6.2.4.1. Association analysis of boCXCR1 SNP -205C>T

Analysis of the singleton SNP -205C>T revealed the heterozygote CT genotype significantly associated (P<0.05) with an increased rate of CM (0.69 cases/cow/year) compared with the most abundant CC genotype (0.39 cases/cow/year), as illustrated in Table 6.4. A CM incidence of 0.41 cases/cow/year was detected in the rarer TT genotype. The proportion of animals within each genotyped population having any CM during their first three lactations was significantly greater in the CT genotype (68%) compared with CC and TT genotypes (both 47%).

Analysis of CM incidence per individual parity revealed the higher CM rate genotype, CT, significantly associated with increased CM incidence for both second (P2) and third (P3) parities (P<0.05) when compared to the common homozygous CC genotype, as illustrated in Table 6.5. CM incidence between the three genotype populations was similar for the first parity (P1), with rates of 0.32, 0.27 and 0.26, cases/cow/year for CC, CT and TT cows respectively. However by P2, CT cows exhibited a significant increase in CM incidence, to 0.52 cases/cow/year (P<0.05), when compared to their P1 data. By contrast only a slight increase in incidence for TT cows (0.38), and a moderate but non-significant decrease for CC cows (0.20) was detected. Significance was detected between genotypes for the proportion of animals within each population having any CM during P2; with the highest in the CT genotype (35%) compared with CC (16%) and TT (27%).

By P3, CT cows continued to demonstrate an increase in CM incidence, to 1.42 cases/cow/year, in comparison to CT incidence data at P1 ($P<0.001$) and P2 ($P<0.01$) (Figure 6.5). Over half of the genotyped CT population (56.5%) had at least one CM event during P3, however only near significance was detected ($P<0.1$) when compared to the CC and TT genotypes (37% and 29% respectively). CC cows also display a significant increase in CM incidence, to 0.80 cases/cow/year (Table 6.5), in comparison to CC incidence data from P1 ($P<0.05$) and P2 ($P<0.01$). The rare TT genotype shows a steady increase in CM incidence at P3 (0.52 cases/cow/year) however this was not significant (Figure 6.5).

Analysis of the production traits revealed that heterozygous CT animals, characterised by higher CM rates, associated significantly with lowered milk fat concentration ($P<0.05$) and highly significantly with increased milk yields ($P<0.001$) when compared to the CC genotype (Table 6.4). Lowered milk yield and fat percentage was shown in TT animals when compared with CC, but this was not significant. The CC genotype tended to associate with increased SCC when compared to TT cows ($P<0.1$). However, no significant differences were observed between all three genotypes despite the demonstrated association with CM incidence from heterozygotes. No significant differences between genotypes for milk protein concentrations were detected.

Table 6.4 – Association of bo*CXCR1* and bo*CXCR2* SNP genotypes with CM and milk production traits over the first three lactations

SNP	Gen.	*n* =	P % with CM[1]	Mean CM rate[2]	*P* =[3]	Mean SCC[4]	*P* =[3]	Mean 305d milk yield[4]	*P* =[3]	Mean milk fat (%)[4]	*P* =[3]	Mean milk protein (%)[4]	*P* =[3]
Bo*CXCR1*	CC	70	47	0.39 (±0.17)		2.97 (±0.71)		7961 (±1238)		4.14 (±0.46)		3.23 (±0.19)	
-205C>T	CT	48	68*	0.69 (±0.20)*	<0.05	2.74 (±0.85)	-	8577 (±1220)**	<0.01	3.93 (±0.38)*	<0.05	3.18 (±0.10)	-
	TT	15	47	0.41 (±0.31)	-	2.54 (±0.71)	<0.1	7583 (±1292)	-	3.93 (±0.32)	-	3.22 (±0.16)	-
	TT	8	50	0.37 (±0.37)	-	2.53 (±0.69)	-	8223 (±1130)	-	3.92 (±0.59)	-	3.16 (±0.13)	-
t-181T>C	TC	59	56	0.62 (±0.22)	-	3.01 (±0.78)	<0.1	7983 (±1204)	-	4.07 (±0.43)	-	3.24 (±0.20)	-
	CC	115	57	0.54 (±0.13)		2.76 (±0.76)		8135 (±1299)		4.06 (±0.43)		3.21 (±0.17)	
	GG	50	60	0.54 (±0.16)		2.72 (±0.79)		8195 (±1319)		4.02 (±0.39)		3.24 (±0.18)	
t+735G>C	GC	86	57	0.64 (±0.19)	-	2.80 (±0.80)	-	8091 (±1202)	-	4.07 (±0.42)	-	3.21 (±0.18)	-
	CC	47	47	0.57 (±0.28)	-	2.98 (±0.69)	-	7937 (±1275)	-	4.08 (±0.51)	-	3.21 (±0.19)	-
	CC	93	60	0.54 (±0.12)		2.76 (±0.79)		8225 (±1207)		4.01 (±0.41)		3.23 (±0.19)	
t+816C>A	CA	74	55	0.62 (±0.20)	-	2.91 (±0.79)	-	7858 (±1303)	-	4.12 (±0.43)	-	3.23 (±0.18)	-
	AA	15	40	0.41 (±0.35)	-	2.87 (±0.54)	-	8329 (±1261)	-	4.06 (±0.40)	-	3.14 (±0.16)	-
Bo*CXCR2*	TT	8	75	0.65 (±0.80)	-	2.94 (±1.06)	-	8422 (±1295)	-	4.07 (±0.45)	-	3.23 (±0.20)	-
-512T>C	TC	35	49	0.53 (±0.34)	-	2.86 (±0.71)	-	8222 (±1173)	-	4.00 (±0.30)	-	3.24 (±0.17)	-
	CC	86	60	0.72 (±0.15)		2.73 (±0.71)		8269 (±1183)		4.08 (±0.47)		3.21 (±0.19)	
	GG	91	60	0.62 (±0.21)		2.81 (±0.74)		8210 (±1149)		4.07 (±0.37)		3.23 (±0.17)	
-496G>A	GA	29	55	0.74 (±0.50)	-	2.77 (±0.65)	-	8371 (±1139)	-	4.06 (±0.43)	-	3.20 (±0.21)	-
	AA	12	42	0.44 (±0.40)	-	2.77 (±0.80)	-	7950 (±1611)	-	4.00 (±0.60)	-	3.16 (±0.22)	-

Abbreviations: Gen. = genotype, P % = population percentage, CM = clinical mastitis, SCC = somatic cell count.[1]Column presence of * represents detection of significance between genotypes by Pearson Chi^2 odds ratio test ($P<0.05$), with positioning of * alongside the genotype contributing most to the Chi^2. [2]Mean±95% confidence interval given in column parentheses. [3]*P* values analysed by ANOVA and Tukey's multiple comparisons, showing only results tested against the underlined (most abundant) homozygous genotype; – (dash) denotes no significance.

Table 6.5 - Association of bo*CXCR1* and bo*CXCR2* SNP genotypes with CM at first (P1) second (P2) and third (P3) parity

SNP	Gen.	*n* =	P1 P % with CM[1]	P1 Mean CM rate[2]	*P* =[3]	P2 P % with CM[1]	P2 Mean CM rate[2]	*P* =[3]	P3 P % with CM[1]	P3 Mean CM rate[2]	*P* =[3]
Bo*CXCR1*	CC	70	20	0.32 (±0.18)		16	0.20 (±0.12)		37	0.80 (±0.33)	
-205C>T	CT	48	19	0.27 (±0.20)	-	35*	0.52 (±0.22)*	<0.05	56†	1.42 (±0.51)*	<0.05
	TT	15	13	0.26 (±0.42)	-	27	0.38 (±0.37)	-	29	0.58 (±0.61)	-
	TT	8	12	0.18 (±0.43)	-	12	0.10 (±0.23)	-	43	1.08 (±1.51)	-
t-181T>C	TC	59	22	0.38 (±0.21)	-	24	0.61 (±0.43)	-	44	1.09 (±0.45)	-
	CC	115	18	0.31 (±0.13)		30	0.42 (±0.14)		43	1.18 (±0.42)	
	GG	50	14	0.18 (±0.15)		39†	0.54 (±0.22)		42	0.96 (±0.41)	
t+735G>C	GC	86	20	0.34 (±0.16)	-	25	0.43 (±0.23)	-	49	1.45 (±0.55)	-
	CC	47	23	0.44 (±0.28)	-	19	0.45 (±0.45)	-	31	0.76 (±0.42)	-
	CC	93	18	0.26 (±0.13)		31	0.51 (±0.20)		44	1.06 (±0.32)	
t+816C>A	CA	74	20	0.32 (±0.17)	-	23	0.46 (±0.32)	-	47	1.43 (±0.61)	-
	AA	15	33	0.71 (±0.70)	-	20	0.26 (±0.29)	-	14†	0.28 (±0.45)	-
Bo*CXCR2*	TT	8	50*	0.51 (±0.63)	-	27	0.23 (±0.59)	-	41	1.74 (±2.5)	-
-512T>C	TC	35	11	0.35 (±0.38)	-	24	0.41 (±0.43)	-	32	0.82 (±0.51)	-
	CC	86	24	0.40 (±0.25)		17	0.58 (±0.31)		60	1.56 (±0.99)	
	GG	91	21	0.45 (±0.26)		24	0.33 (±0.16)		38	0.97 (±0.43)	
-496G>A	GA	29	22	0.41 (±0.40)	-	34	0.91 (±0.76)	<0.1	43	0.87 (±0.68)	-
	AA	12	25	0.50 (±0.71)	-	18	0.19 (±0.29)	-	18	0.61 (±0.93)	-

Abbreviations: Gen. = genotype, P % = population percentage, CM = clinical mastitis, P1 = parity 1, P2 = parity 2, P3 = parity 3. [1]Column presence of * represents detection of significance between genotypes by Pearson Chi^2 odds ratio test ($P<0.05$), with positioning of * alongside the genotype contributing most to the Chi^2. Column presence of † represents detection of near significance between genotypes ($P<0.1$), with positioning of † alongside the genotype contributing most to the Chi^2. [2]Mean±95% confidence interval given in column parentheses. [3]*P* values analysed by ANOVA and Tukey's multiple comparisons, showing only results tested against the underlined (most abundant) homozygous genotype; – (dash) denotes no significance.

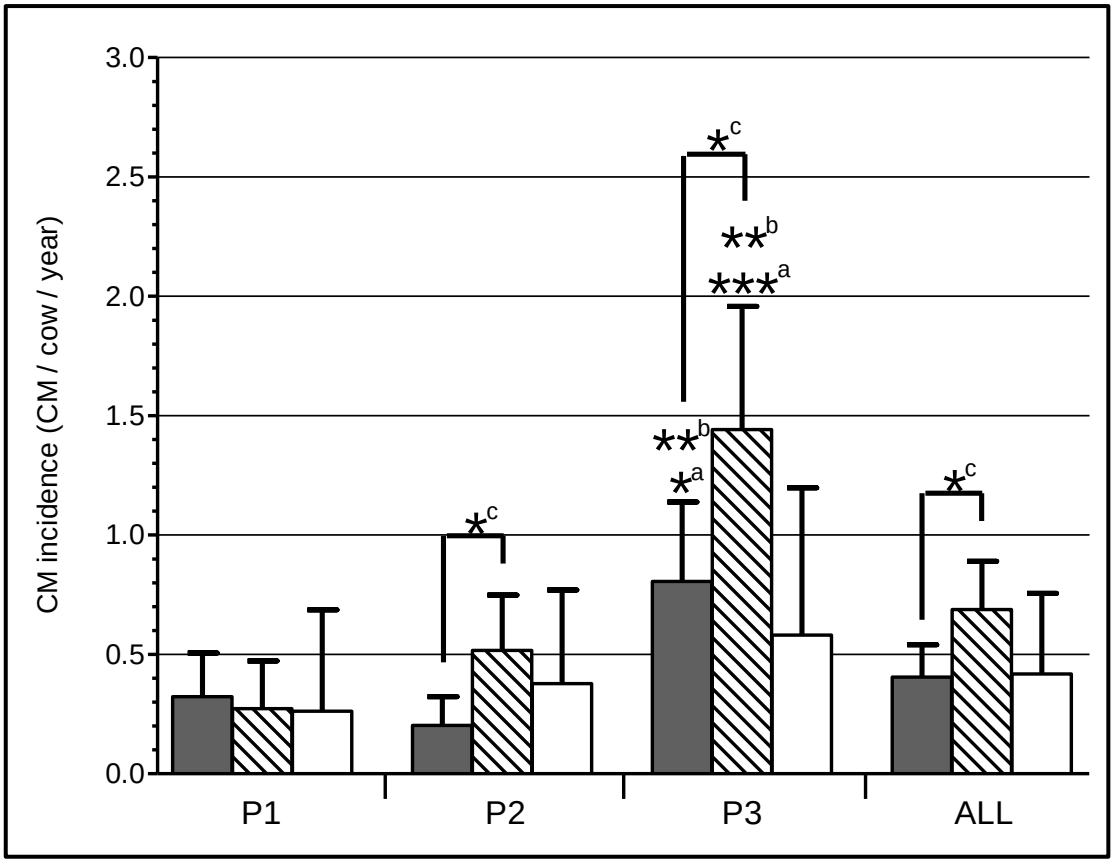


Figure 6.5 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CXCR1* -205C>T SNP variant populations: CC, CT and TT

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – CC animals (*n* = 70), hatched bars – CT animals (*n* = 48) and open bars – TT animals (*n* = 15). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. [c] = tested between genotypes indicated below capped line. Genotypic means differ significantly at the following levels: **P*<0.05, ***P*<0.01 ****P*<0.001, by ANOVA and Tukey's multiple comparisons.

6.2.4.2 Association analysis of boCXCR1 tSNP -181T>C

Association analysis of the -181T>C tSNP with CM revealed no significant differences between genotypes over the first three lactations. The data trend showed the more frequent CC and TC animals to have higher CM incidence (0.54 and 0.62 cases/cow/year, respectively) when compared with the rarer TT population (0.37 cases/cow/year) (Table 6.4). The proportion of animals within each genotyped population having any CM during their first three lactations was marginally higher in the CC and TC genotypes (57% and 56% respectively) compared with TT genotypes (50%).

Analysis for SNP association with CM per parity also revealed no significant differences between genotypes (Table 6.5). The CC population did show a significant increase in P3 incidence when compared with P1 and P2 ($P<0.001$), whereas the heterozygote TC population showed a significant increase in P3 incidence compared with P1 ($P<0.05$) only (Figure 6.6).

Analysis of production traits revealed the TC genotype tended to associate with increased SCC when compared to CC cows ($P<0.1$). No significant differences between genotypes for milk yield, fat and protein concentrations were detected (Table 6.4).

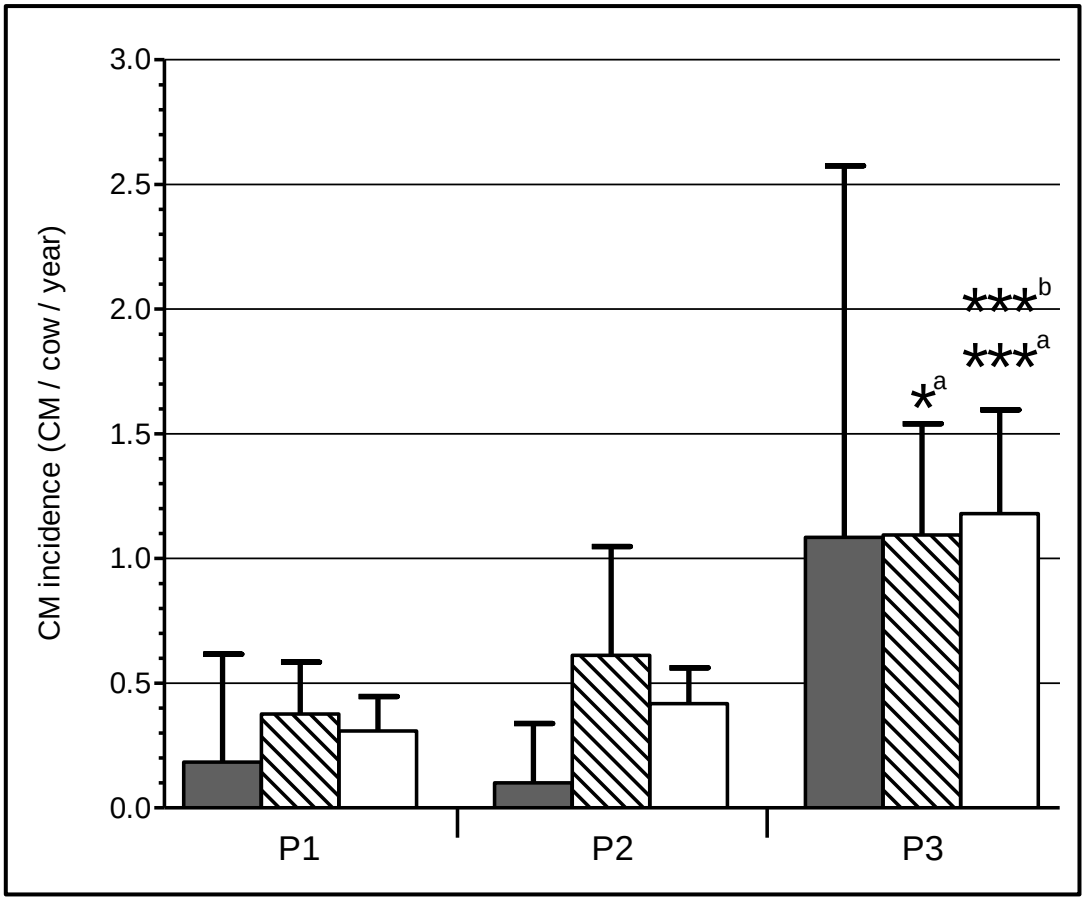


Figure 6.6 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CXCR1* -181T>C SNP variant populations: TT, TC and CC

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – TT animals (*n* = 8), hatched bars – TC animals (*n* = 59) and open bars – CC animals (*n* = 115). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Genotypic means differ significantly at the following levels: **P*<0.05, ****P*<0.001, by ANOVA and Tukey's multiple comparisons.

6.2.4.3 Association analysis of boCXCR1 tSNP +735G>C

Association analysis of the NS bo*CXCR1* +735G>C tSNP demonstrated heterozygote GC animals had higher rates of CM (0.74 cases/cow/year, respectively) across three lactations when compared with GG and CC animals (0.54 and 0.57 cases/cow/year respectively), although these differences were not significant (Table 6.4). The proportion of animals within each genotyped population having any CM during their first three lactations was higher for the GG and GC genotypes (60% and 57% respectively) compared with the CC genotype (47%).

Analysis of CM data per individual parity identified no significant differences for CM incidence between genotypes at P1, P2 and P3 (Table 6.5). The data trend showed the highest CM incidence for each genotype occurs at P3 compared with P1 and P2. For the GG population, CM incidence significantly increased from 0.18 cases/cow/year at P1, to 0.54 by P2 ($P<0.05$) and to 0.96 by P3 ($P<0.001$). The heterozygous GC animals display significantly increased CM incidence ($P<0.001$) of 1.45 cases/cow/year at P3 when compared with incidences at P1 and P2 (0.34 and 0.42 respectively). CC cows displayed a steady rise in incidence per parity; however no significance was detected (Figure 6.7). A higher percentage of animals having CM during P2 was demonstrated by the GG population (39%) when compared with GC and CC populations (25% and 19% respectively), with near significance ($P<0.1$) detected (Table 6.5). Analysis of the production traits revealed no significance between genotypes for milk yield, SCC, fat and protein concentrations (Table 6.4).

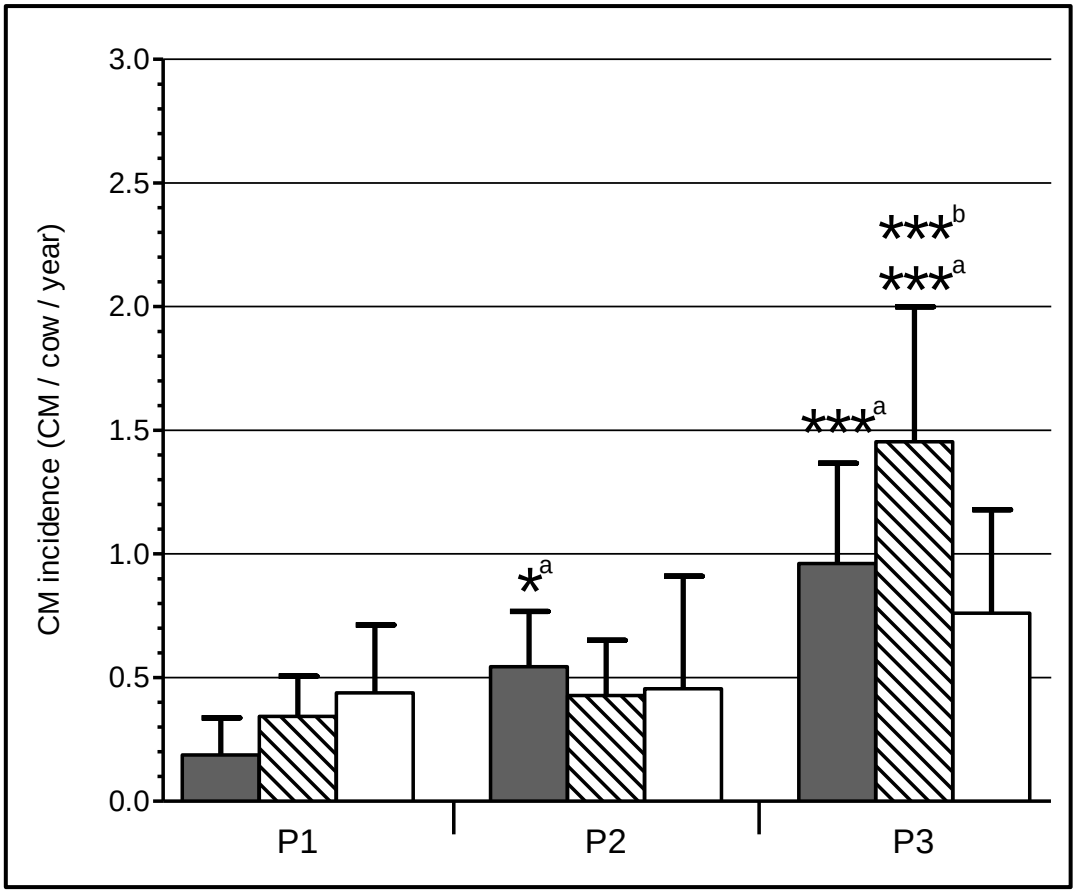


Figure 6.7 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CXCR1* +735G>C SNP variant populations: GG, GC and CC

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – GG animals (n = 50), hatched bars – GC animals (n = 86) and open bars – CC animals (n = 47). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Genotypic means differ significantly at the following levels: *P<0.05, ***P<0.001, by ANOVA and Tukey's multiple comparisons.

6.2.4.4 Association analysis of boCXCR1 tSNP +816C>A

Association analysis of the +816C>A tSNP with CM revealed no significant differences between genotypes over the first three lactations. The data trend suggests heterozygous CA cows have a higher CM incidence (0.62 cases/cow/year), than CC and the rarer AA populations (0.54 cases and 0.41 cases respectively) (Table 6.4). The proportion of animals within each genotyped population having any CM during their first three lactations was higher for the CC and CA populations (60% and 55% respectively) compared with AA (40%).

Analysis of CM data per individual parity revealed no significant differences between genotypes at P1, P2 and P3 (Table 6.5). The data trend demonstrates high CM incidence for genotypes CC and CA occurring at P3 compared with P1 and P2, whereas AA population incidence declines at P3 compared to P1 (Figure 6.8). CM incidence for CC cows significantly increases to 1.06 cases/cow/year at P3, compared with 0.26 at P1 ($P<0.001$); and 0.51 at P2 ($P<0.05$). Heterozygous CA animals display significantly increased CM incidence of 1.45 cases/cow/year at P3, when compared with incidences of 0.34 and 0.42 at P1 and P2 respectively ($P<0.001$) (Figure 6.8). Analysis of the production traits revealed no significance between genotypes for milk yield, SCC, fat and protein concentrations (Table 6.4).

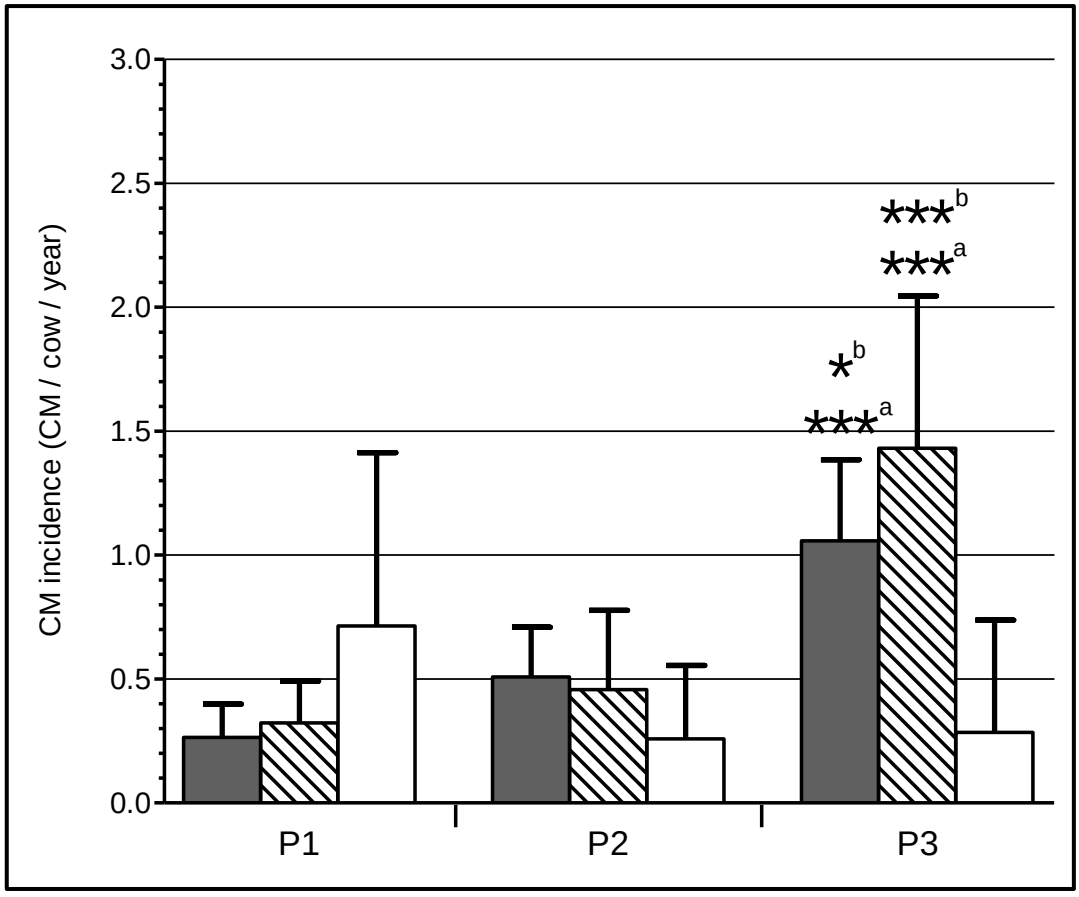


Figure 6.8 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CXCR1* +816C>A SNP variant populations: CC, CA and AA

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – CC animals (*n* = 93), hatched bars – CA animals (*n* = 74) and open bars – AA animals (*n* = 15). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Means differ significantly at the following levels: **P*<0.05, ****P*<0.001, by ANOVA and Tukey's multiple comparisons.

6.2.4.5 Association analysis of boCXCR2 SNP -512T>C

Association analysis of the bo*CXCR2* -512T>C SNP with CM revealed no significant differences between genotypes over the first three lactations. The data trend suggests the most abundant genotype population CC had higher rates of CM (0.72 cases/cow/year) across three lactations when compared with TC animals (0.53 cases/cow/year), and the rare TT population (0.65 cases/cow/year) (Table 6.4). The proportion of animals within each genotyped population having any CM during their first three lactations was highest for the rare TT genotype (75%), intermediate for the abundant CC genotype (60%) and lower for the heterozygous TC genotype (49%).

Analysis of CM data per individual parity revealed no significant differences for CM incidence between genotypes at P1, P2 and P3 (Table 6.5). CM incidence is increased for each genotype at P3 compared with P1 and P2. For the CC population, CM incidence is significantly increased at P3 to 1.56 cases/cow/year when compared to a P1 incidence of 0.40 (P<0.05) and a P2 incidence of 0.58 (P<0.05). The heterozygous TC animals display marginal increases of CM incidence per parity, whilst TT cows display a steady rise in incidence per parity, with large variation at P3; however, no significance was detected (Figure 6.9). Significant differences were detected between the proportion of animals within each genotyped population having any CM during P1 (P<0.05), with the TT population displaying a greater percentage having CM (50%) compared with the TC and abundant CC populations (11% and 24% respectively) (Table 6.5). Analysis of the production traits revealed no significance between genotypes for milk yield, SCC, fat and protein concentrations (Table 6.4).

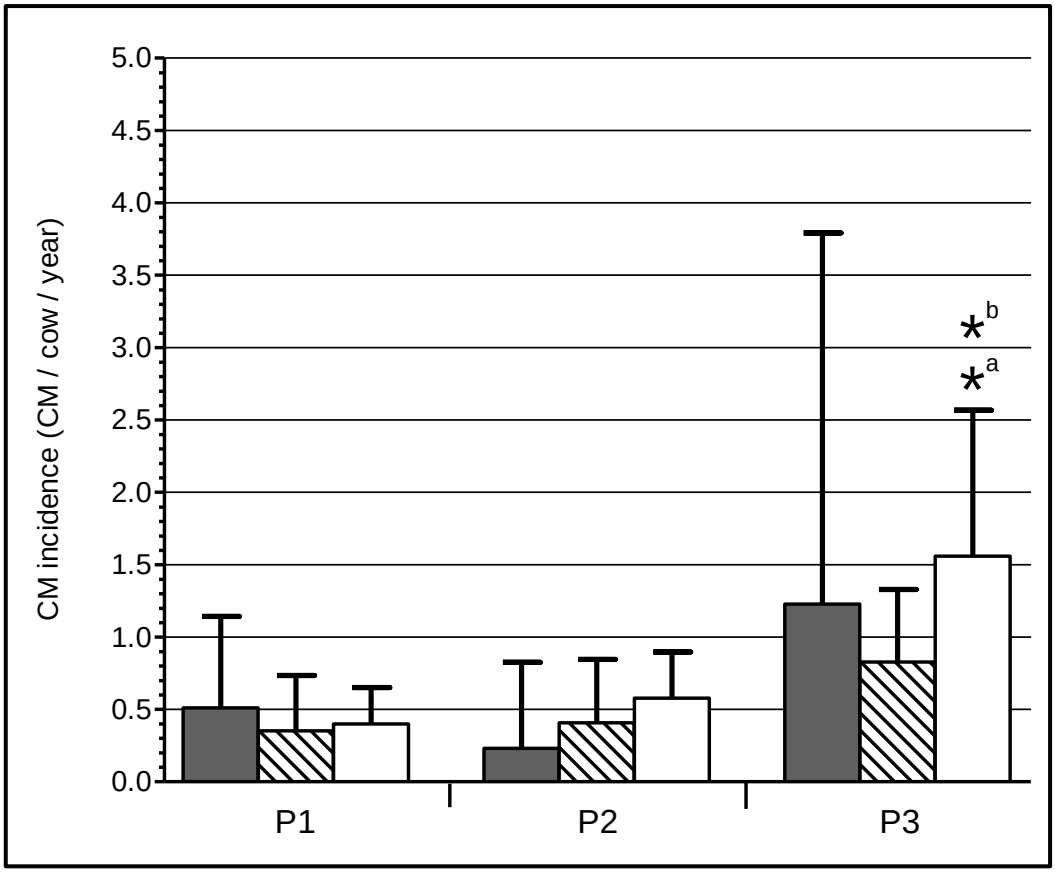


Figure 6.9 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CXCR2* -512T>C SNP variant populations: TT, TC and CC

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – TT animals (n = 8), hatched bars – TC animals (n = 35) and open bars – CC animals (n = 86). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. *Means differ significantly (P<0.05) by ANOVA and Tukey's multiple comparisons.

6.2.4.6 Association analysis of boCXCR2 SNP -496G>A

Association analysis of the bo*CXCR2* -496G>A SNP with CM revealed no significant differences between genotypes over the first three lactations. The data trend demonstrates that the heterozygous genotype population GA have an increased rate of CM, 0.74 cases, /cow/year, when compared with the most abundant genotype population GG (0.62 cases) and the rare AA population (0.44 cases) (Table 6.4). The proportion of animals within each genotyped population having any CM over three lactations were similar for the abundant GG (60%) and GA (55%) genotypes; although both were higher when compared with the rare AA genotype population (42%).

Analysis of CM data per individual parity revealed no significant differences for CM incidence between genotypes at P1, P2 and P3, however, at P2 the GA genotype tended to associate with increased CM when compared to GG cows ($P<0.1$) (Table 6.5). Trends suggest a decrease in CM incidence for GG and AA genotypes at P2 compared with P1; however, incidence increases and peaks at P3. For the GG population, P3 CM incidence significantly increased to 0.97 cases/cow/year when compared to a P1 incidence of 0.45 ($P<0.05$) and a P2 incidence of 0.33 ($P<0.01$). The heterozygous GA animals display a large increase of CM incidence at P2 compared to P1 and a marginal decrease at P3 compared with P2 (Figure 6.10). Analysis of the production traits revealed no significant differences between genotypes for milk yield, SCC, fat and protein concentrations (Table 6.4).

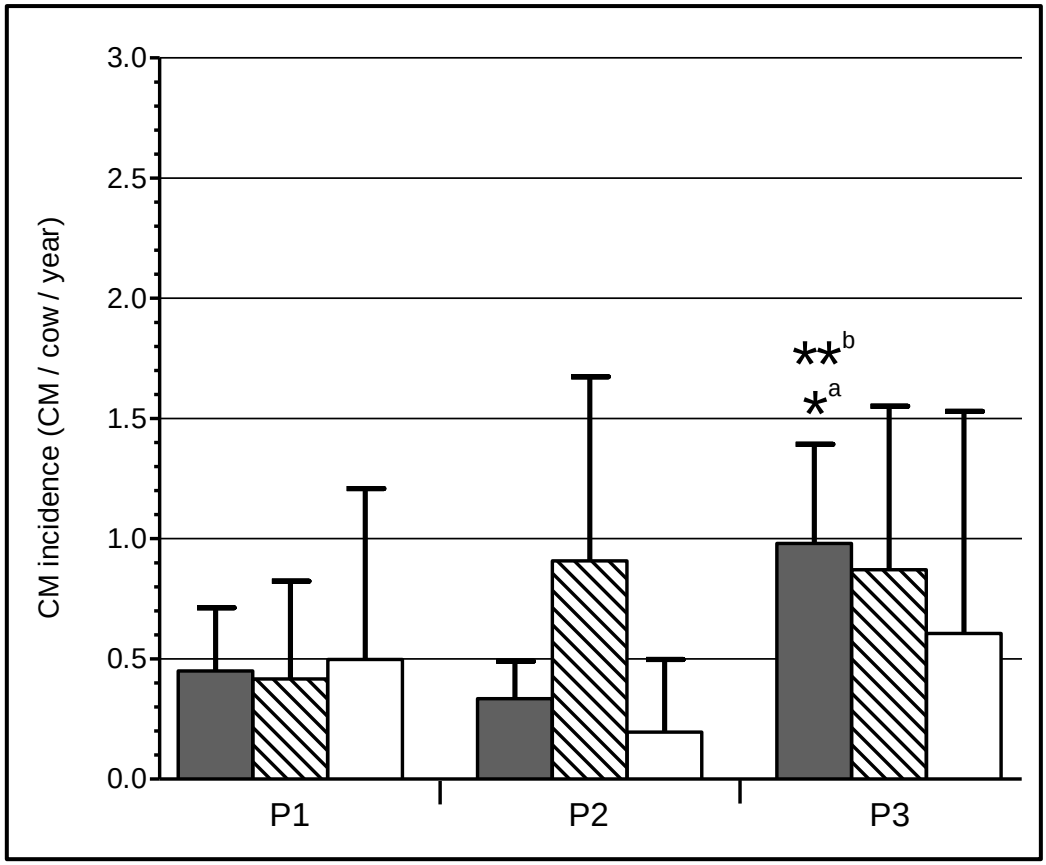


Figure 6.10 - Mean incidence of clinical mastitis across three parities for cows grouped into the three bo*CXCR2* -496G>A SNP variant populations: GG, GA and AA

Data from each variant population are presented as a bar, with the positive whiskers representing 95% confidence interval of the mean. Grey bars – GG animals (*n* = 91), hatched bars – GA animals (*n* = 29) and open bars – AA animals (*n* = 12). P1 = first parity, P2 = second parity, and P3 = third parity. [a] = tested against P1 data for same genotype. [b] = tested against P2 data for same genotype. Means differ significantly at the following levels: **P*<0.05, ***P*<0.01, by ANOVA and Tukey's multiple comparisons.

6.2.5 Multiple comparisons

Correlation coefficient analysis was performed revealing a weak positive correlation between CM and SCC (Pearson coefficient = 0.198) and weak negative correlations between CM and milk fat and protein percentages (Pearson coefficient = -0.2 and -0.1 respectively). All significant associations between individual SNP genotypes and CM were found to survive adjustments for each factor using a GLM.

6.2.6 BoCXCR1 haplotype analysis

The four bo*CXCR1* SNPs, -205C>T, t-181T>C, t+735G>C and t+816C>A, were used for haplotype reconstruction to reveal four major haplotypes with their frequencies displayed in Table 6.6, along with the genotypes of all tagged bo*CXCR1* SNPs. The most abundant haplotype, carrying alleles CCCA, was designated Haplo1 and had a frequency of 0.3. Haplo2, carrying alleles TGCA, had a marginally lower frequency of 0.27, Haplo3, carrying alleles CTCC, had frequency 0.27, Haplo4, the rarer haplotype carrying alleles CCGC, had a frequency of 0.16. Owing to low genotyped numbers for each haplotype, association analysis was not performed.

Table 6.6 - Bo*CXCR1* haplotype frequencies in the Mayfield Holstein-Friesian herd

	Bo*CXCR1* SNP																		
Haplotype	**-205C>T**	**-181T>C**	-41A>C	+37A>T	+38T>A	+68G>A	**+735G>C**	**+816C>A**	+818G>A	+980A>G	+995A>G	+1008C>T	+1068G>A	+1089C>A	+1090A>C	+1093C>T	+1098C>T	+1180G>A	+1282G>A	Frequency
Haplo1	**C**	**C**	A	A	T	G	**C**	**A**	G	G	G	T	G	C	A	C	C	A	G	0.30
Haplo2	**T**	**C**	C	T	A	A	**G**	**C**	A	G	G	C	A	A	C	T	T	A	A	0.27
Haplo3	**C**	**T**	A	A	T	G	**C**	**C**	G	A	A	C	G	C	A	C	C	G	G	0.27
Haplo4	**C**	**C**	C	T	A	A	**G**	**C**	A	G	G	C	A	A	C	T	T	A	A	0.16

Prediction of phased haplotypes reconstructed using Haploview 4.2 (2008) (http://www.broadinstitute.org/haploview/haploview) (Barrett, Fry et al. 2005). Underlined SNPs are tSNPs from genotypic associations. SNP positions are colour coded as blue, green or red, to represent the three linkage groups.

6.2.7 Q-PCR analysis of basal boCXCR1 and boCXCR2 mRNA abundance

Milk somatic cells from cows with representative bo*CXCR1* and bo*CXCR2* genotypes were assessed for differences in basal *boCXCR1* and *boCXCR2* expression. All contributing animals were of similar age and stage of lactation and none had any known health issues and did not record any incidence of mastitis two weeks preceding/following the collection. Q-PCR assays were performed using RNA from animals of each bo*CXCR1/2* SNP genotype.

Analysis of bo*CXCR1* expression by representative SNP+735G>C genotypes revealed significant differences in expression levels. Cows with the CC genotype demonstrated reduced basal *boCXCR1* mRNA expression ($P<0.05$) compared with either the GG or GC genotypes ($n = 6$). Due to close homology and the potential for co-expression, the abundance of *boCXCR2* mRNA was also measured and revealed no significant differences (Figure 6.11).

Q-PCR data, segregated by representative SNP-205C>T genotypes ($n = 5$), revealed significant differences in expression levels, with cows from the CC variant population having lowered ($P<0.01$) *boCXCR1* mRNA expression compared with TC cows (Figure 6.12). The absence of data for the TT variant population is due to the low frequency of TT cows (0.11) within the herd. *BoCXCR2* mRNA was also measured and revealed no significant differences or trends.

Analysis of bo*CXCR2* mRNA levels between representative bo*CXCR2* SNP-496G>A genotypes ($n = 6$) indicates lowered bo*CXCR2* expression in the AA cow when compared with GG and GA animals; however, results were affected by the low frequency of the AA cows (Figure 6.13).

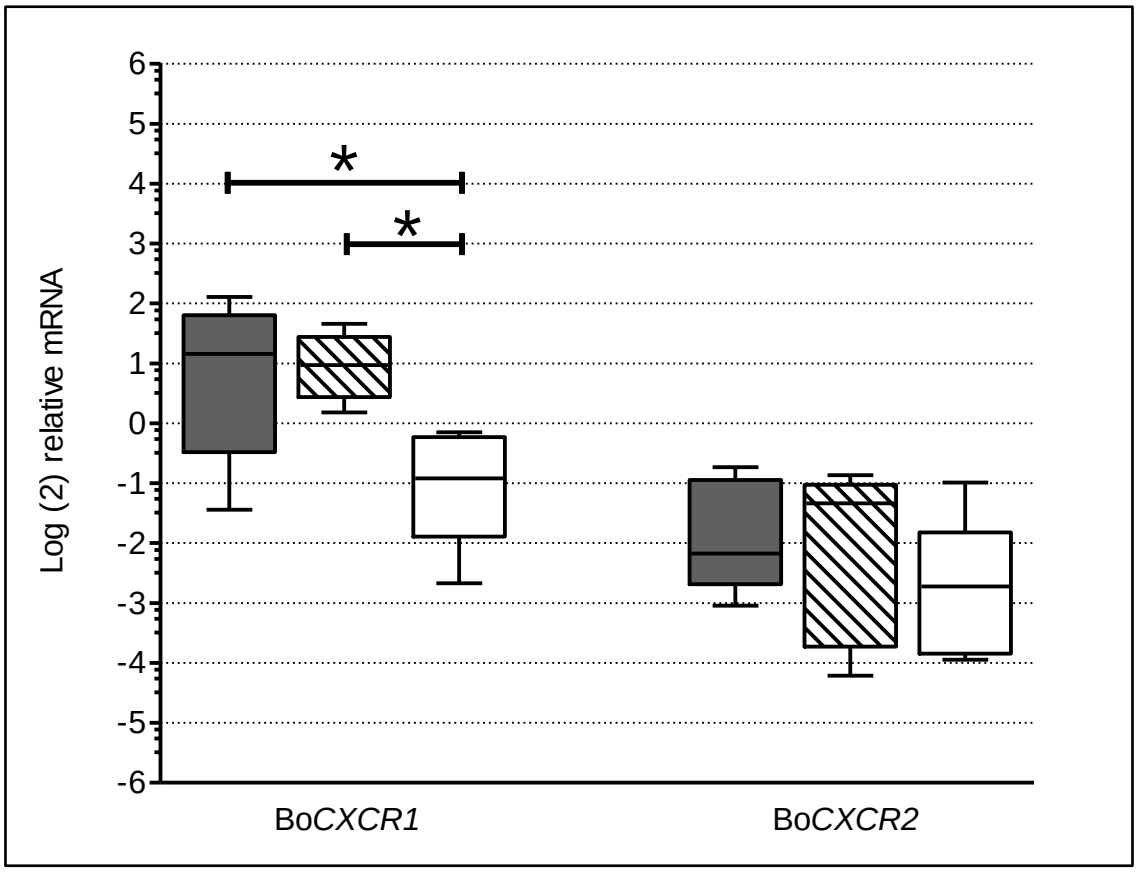


Figure 6.11 - Q-PCR data showing basal mRNA abundance levels of *boCXCR1* and *boCXCR2* in milk somatic cells grouped into the three bo*CXCR1* +735G>C SNP variant populations: GG, GC and CC

Data from each variant population (n = 6) are presented as a box and whisker plot, with the ends of the whiskers representing the minimum and maximum data values. Grey box plot – GG animals, hatched box plot – GC animals, and open box plot – CC animals. *Genotypic means differ significantly (P<0.05 by ANOVA and Tukey's multiple comparisons).

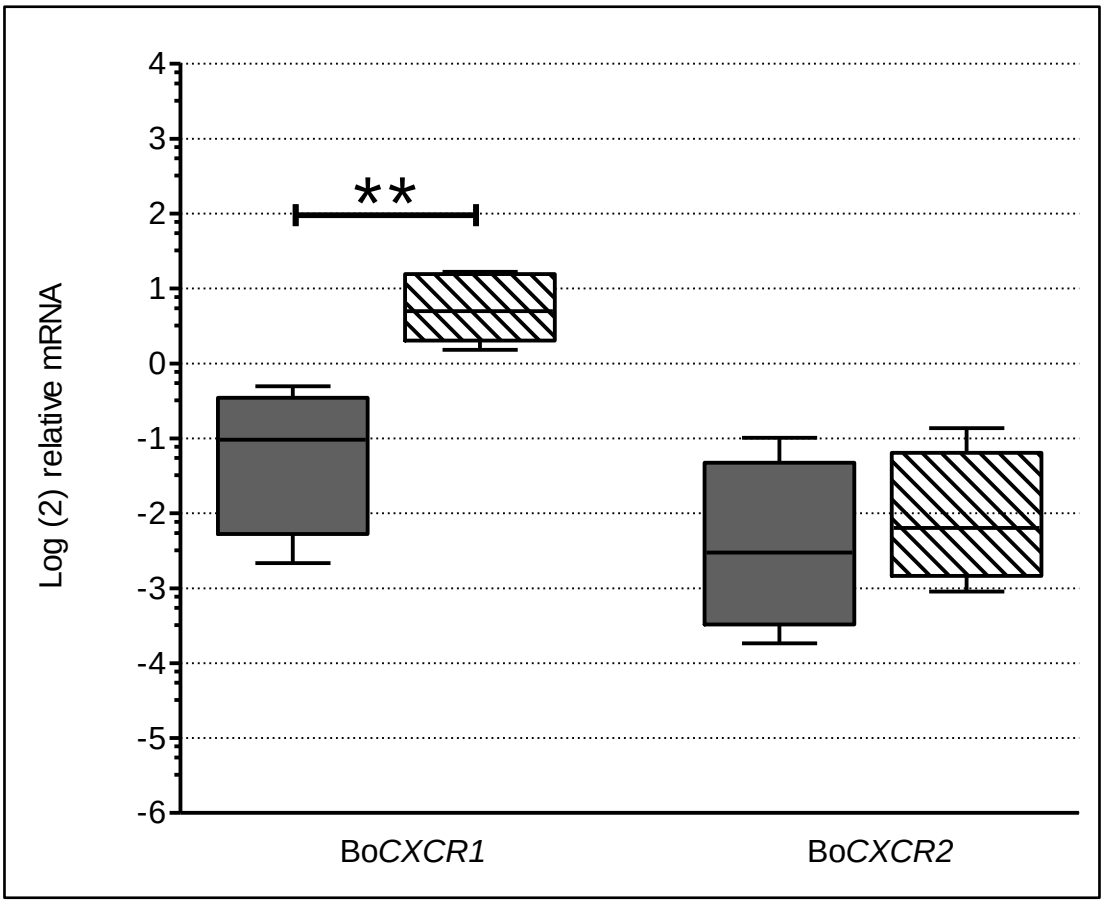


Figure 6.12 - Q-PCR data showing basal mRNA abundance levels of *boCXCR1* and *boCXCR2* in milk somatic cells grouped into the two bo*CXCR1* -205C>T SNP variant populations: CC and CT

Data from each variant population (n = 5) are presented as a box and whisker plot, with the ends of the whiskers representing the minimum and maximum data values. Grey box plot – CC animals, hatched box plot – CT animals. **Genotypic means differ significantly (P<0.01) by unpaired t test.

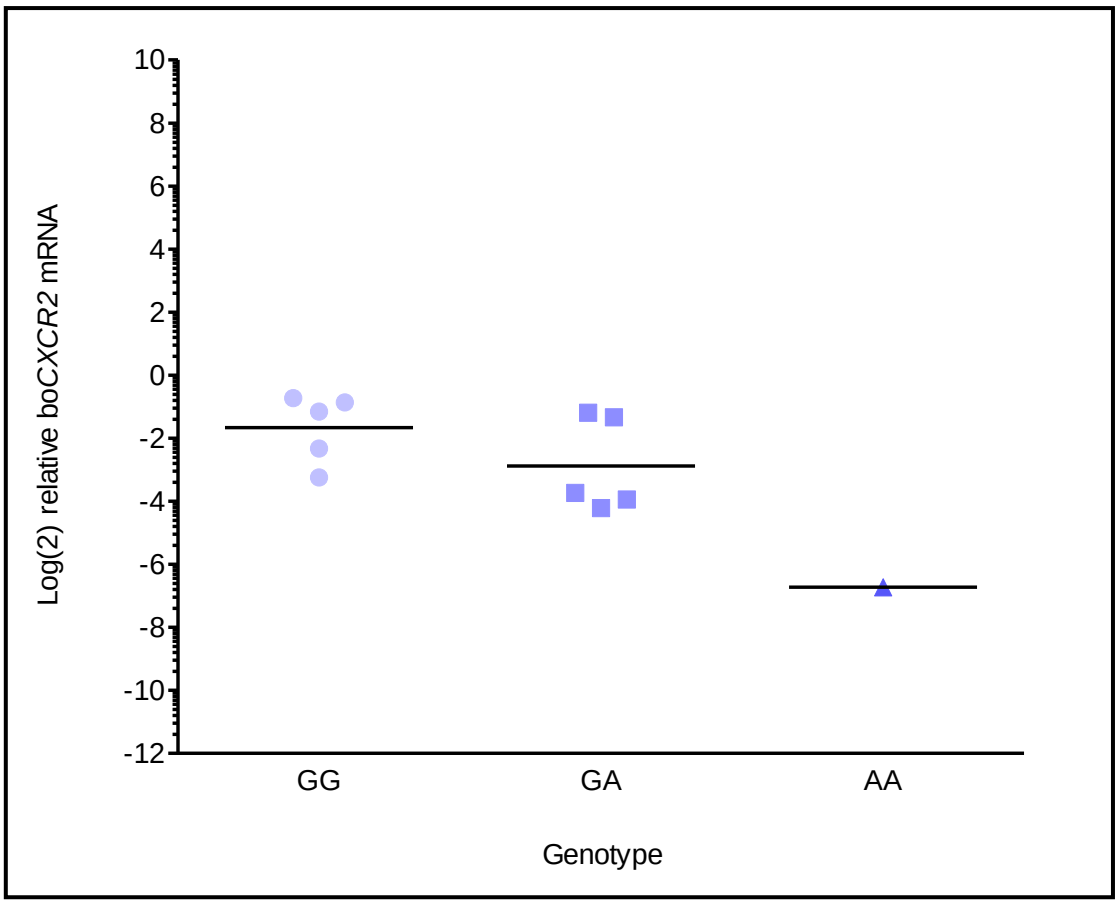


Figure 6.13 - Q-PCR data showing basal mRNA abundance levels of *boCXCR2* in milk somatic cells grouped into the three bo*CXCR2* SNP-496G>A variant populations: GG, GA and AA

Each point represents data from one animal, with a horizontal line to indicate genotype mean.

6.2.8 In silico analysis for putative transcription binding sites

The AliBaba2.1 (TRANSFAC 4.0 sites) software programme was used to search for putative TBS located immediately upstream of the ATG start codon for both bo*CXCR1* and bo*CXCR2*. The upstream SNPs identified in this region from each gene were then analysed for any potential impact on predicted TBS.

The three upstream bo*CXCR1* SNPS, -205T>C, -181C>T and -41A>C, are surrounded by several putative TBS, including two binding sites for the transcription enhancing factor, Specificity Protein 1 (Sp1), and single matched sites for gene up-regulators GATA-binding factor-1 (GATA-1) and Activator Protein-1 (AP-1) (Figure 6.14A). Importantly, the bo*CXCR1* SNP-205T>C determines the presence (C allele) or absence (T allele) of one of the Sp1 binding sites. Further, the bo*CXCR1* -41A>C SNP, in complete LD with the tagging NS SNP +735G>C, repositions an AP-1 binding site (Figure 6.14B).

The two upstream bo*CXCR2* SNPs, -512T>C and -496G>A, are surrounded by many putative TBS, including several binding sites for the Sp-1 protein (Figure 6.15). However, both published and alternate allele sequences suggest bo*CXCR2* SNPs -512T>C and -496G>A have no obvious impact on the presence or absence of TBS. Due to both allele sequences having identical predictions of surrounding TBS, only the published allele sequence is shown (Figure 6.15).

A

*Bos_taurus*_UMD_3.1 Primary Assembly Sequence

```
            SNP-205C>T              SNP-181T>C
-215 ATTTAGGCCTCGGGGTTCTGGAAAGCTGACAACATGATGAAGCTGATCCTTAAAGCAGCC
          ====Sp1===

-155 TGTGACAGAGGTGAAGGAAGGGCTCTTCAGTTACCAAGCTAGCAGATAAGCACAAGATAA
           ===Elk-1==
                 ====Sp1===
                                                ===GATA-1=
                                                  ===Erg-1==
                                                           SNP-41A>C
 -95 CCTAGTAACAGTTATCACATCCTTGATGAGAGTGATTTGGATCTGAGTTACTAAATCACC
                                                   ====AP-1==

 -35 TTTCTTTCCTTCTAGTCTTTCAACCAGATCACACCatg...
```

B

Alternate Allele Sequence

```
            SNP-205C>T              SNP-181T>C
-215 ATTTAGGCCTTGGGGTTCTGGAAAGCTGACAACACGATGAAGCTGATCCTTAAAGCAGCC


-155 TGTGACAGAGGTGAAGGAAGGGCTCTTCAGTTACCAAGCTAGCAGATAAGCACAAGATAA
           ===Elk-1==
                 ====Sp1===
                                                ===GATA-1=
                                                  ===Erg-1==
                                                           SNP-41A>C
 -95 CCTAGTAACAGTTATCACATCCTTGATGAGAGTGATTTGGATCTGAGTTACTAACTCACC
                                                           ====AP-1==

 -35 TTTCTTTCCTTCTAGTCTTTCAACCAGATCACACCatg...
```

Figure 6.14 - Location and impact of upstream bo*CXCR1* SNPs in relation to putative transcription binding sites for both published allele (A) and alternate allele (B) sequences

The AliBaba 2.1 software programme was used for the prediction (75% match threshold) of transcription factor binding sites (TBS) in the bo*CXCR1* upstream region. Upstream nucleotide sequence shown in CAPS, purple text represents 5'UTR. ATG start codon shown by lower case. Numbering indicates nucleotide position from the start codon. SNP positions are highlighted in red, with SNP names above colour coded according to their LD group (Figure 6.3). Positions of putative TBS are aligned below the nucleotide sequence. TBS abbreviations: Sp1 = Specificity Protein 1, Elk-1 = E twenty-six (ETS)-like transcription factor-1, GATA-1 = GATA-binding factor-1, Erg-1 = ETS Related Gene-1, AP-1 = Activator Protein-1.

*Bos_taurus*_UMD_3.1 Primary Assembly Sequence

Figure 6.15 - Location of upstream bo*CXCR2* SNPs in relation to putative transcription binding sites for the published allele sequence

The AliBaba 2.1 software programme was used for the prediction (75% match threshold) of transcription factor binding sites (TBS) in the bo*CXCR2* upstream region. Alternate SNP allele sequence had no impact upon the presence of further TBS (sequence not shown). Upstream nucleotide sequence shown in CAPS, purple text represents 5'UTR. ATG start codon shown by small caps. Numbering indicates position of the nucleotide from the start codon. SNP positions are highlighted in red with the SNP identification above. Positions of putative TBS are aligned below the nucleotide sequence. TBS abbreviations: PR = Progesterone Receptor, RAP1 = Ras-related Protein 1, Sp1 = Specificity Protein 1, C/EBP = CCAAT-Enhancer-Binding Protein, USF = Upstream transcription Factor, NF-kappa = Nuclear Factor-kappa, TEC-1 = TEA/ATTS domain protein, NF-1 = Nuclear Factor 1, ICSBP = Interferon Consensus Sequence-Binding Protein, IRF-1 = Interferon Regulatory Factor-1, Pit-1a = POU class 1 homeobox-1, MIG1 = Regulatory protein MIG1, PU.1 = PU-box (purine-rich sequence).

6.2.9 Evaluating the influence of existing NS SNP mutations within bovine CXCR1

All NS bo*CXCR1* SNPs are illustrated in an amino acid model of boCXCR1 in Figure 6.16. The W206X mutation encoded by the bo*CXCR1* SNP +621G>A introduces a stop codon mutation. This SNP was not detected in this study although it is potentially linked to the 5' intronic SNP -205C>T (Pighetti, Kojima et al. 2012), which has been associated with CM. Although a truncated protein would contain a DRYLAIV motif important for signalling (start of the second ICL), lack of a COOH terminus may severely impact function. Mutation Q245H = +735G>C SNP, linked with CM and impaired neutrophil signalling/migration in prior studies, belongs to a separate linkage group to SNP -205C>T. Q245H is in linkage with the three NH_2 terminus mutations I13F, I13N, and E23G. COOH terminus mutations K327R and H332R are inherited as a pair belonging to another linkage group. Although no associations with CM were detected for these mutations, any functional consequence has yet to be evaluated.

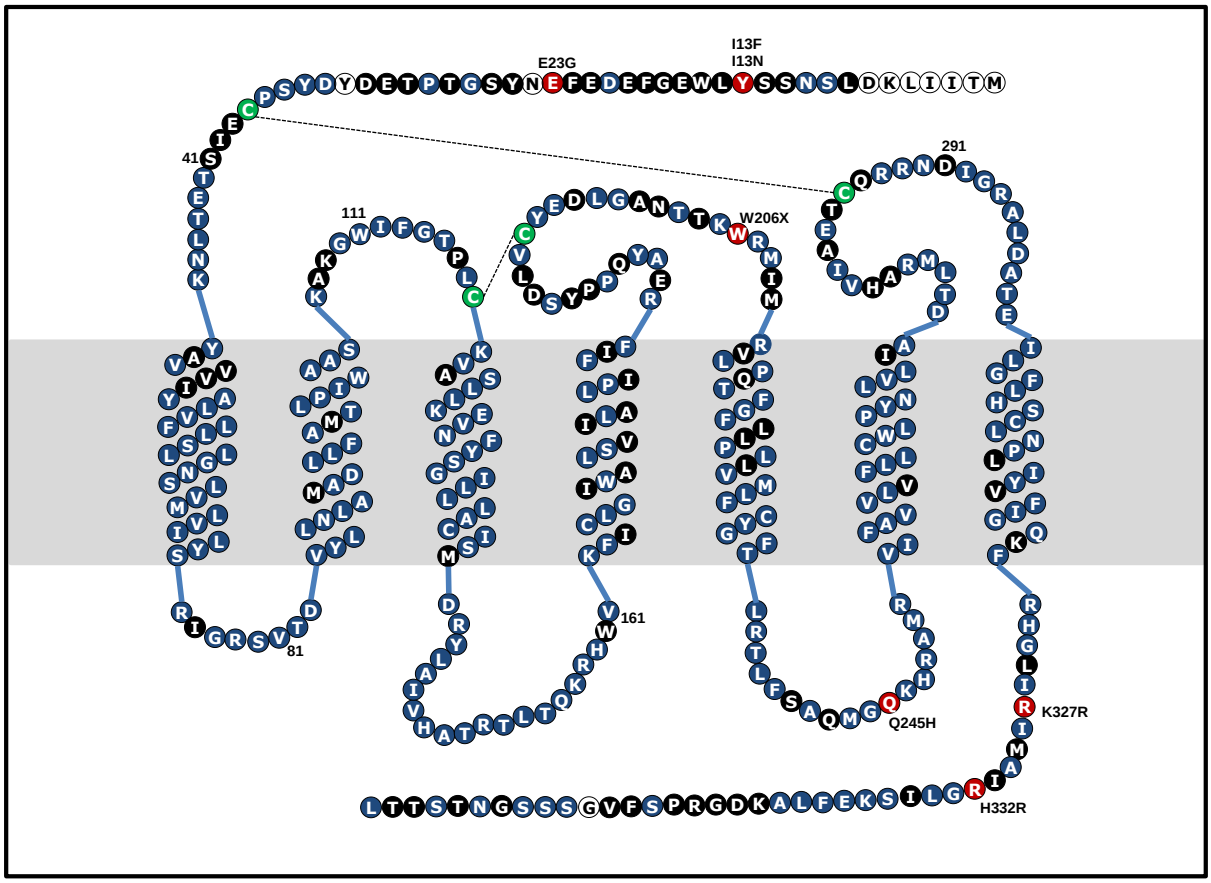


Figure 6.16 - Schematic of an amino acid model for boCXCR1

Blue circles represent conserved amino acid residues between cow and human, black circles represent non-conserved residues, open circles represent additional residues in cow, green circles represent cysteine residues important to structure, red circles highlight NS SNP mutation with amino acid change at AA position indicated. Mutation W206X = +621G>A SNP that introduces a stop codon mutation was not detected in this study. The DRYLAIV motif that is important for intracellular signalling can be observed at the start of the second ICL loop.

6.3 Discussion

The importance of the chemokine receptors CXCR1 and CXCR2 as mediators of the inflammatory response has been highlighted in several disease studies linking SNP presence to susceptibility and severity. Analyses of bo*CXCR1* SNPs have already shown associations with impaired neutrophil migration and susceptibility to mastitis (Youngerman, Saxton et al. 2004a; Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Galvao, Pighetti et al. 2011). However, these SNP associations lacked significance when studied in other herds (Goertz, Baes et al. 2009) underlining the need for further work to clarify their reliability as a marker for CM. The inconsistencies of sampling methods coupled with the use of differing phenotypic measures (CM and SCC data) may have contributed towards these discrepancies.

In this study, both bo*CXCR1* and bo*CXCR2* were analysed for SNPs at their 5' and 3' ends, allowing assessment of non-exonic SNPs and exonic UTR and CDS SNPs as markers for mastitis using actual recorded CM and potential impact on production data. Bo*CXCR1*, encoding the less promiscuous receptor, was found to be more polymorphic than bo*CXCR2*, with nineteen reported SNPs compared to two respectively. Bo*CXCR1* has previously been reported as a highly polymorphic gene (Pighetti, Kojima et al. 2012), possibly due to a high degree of evolutionary pressure from the environment and pathogens; CXCR1 is evolving rapidly in the human system due to Darwinian positive selection (Liu, Yang et al. 2005). A high degree of linkage was observed within the herd between bo*CXCR1* SNPs, defined into a single haploblock. LD patterning revealed three distinct bo*CXCR1* linkage groups; group 1 contained four SNPs and was represented by the tSNP -181T>C, group 2 contained twelve SNPs and was represented by the well documented NS tSNP +735G>C, and group 3 contained two SNPs and was represented by the tSNP +816C>A. No linkage was observed between any of the bo*CXCR1* and bo*CXCR2* SNPs. The three bo*CXCR1* tSNPs, a remaining

singleton bo*CXCR1* SNP -205C>T, and the two bo*CXCR2* SNPs were each used for association analysis.

The singleton bo*CXCR1* SNP -205C>T was found to be the only marker to associate significantly with CM susceptibility, and to the author's knowledge this is the first study to report this. Rates of CM for CC genotyped cows were significantly lower than heterozygous CT cows over the first three lactations. Analysis of the individual parities revealed rates of CM for CC cows were significantly lowered for the second and third lactations. This is important as overall CM incidence usually rises per successive parity, as observed for all SNP genotypes. In addition, the percentage of the CC genotyped population having any CM within the first three lactations was significantly lower (47%) than the CT genotyped population (68%). Recurrence rates for CT and CC populations are similar (~23% of CM cases requiring repeated treatment), demonstrating little difference in chronic mastitis rates between both genotypes. This pattern therefore suggests that CT population incidence is impacted more highly by environmental causes of acute mammary infection; further evidence comes from a preliminary observation revealing a larger ratio of *E. coli* cases reported in the CT population.

Dairy cows are subjected to intense selection pressures for higher milk yields and reduced SCC that may unintentionally increase CM incidence (Suriyasathaporn, Schukken et al. 2000). CT cows strongly associated with increased milk yields, and high CM may be a consequence of this. CT cows further associated with lowered milk fat percentages, suggesting breeding for this marker may impact milk quality and production. Although no significance was detected between genotypes for SCC, the trend shows this trait is increased in CC cows despite a lowered incidence of CM. This finding continues to demonstrate the dichotomy that the surrogate measure of mastitis is also the presence of the effector cell which can at times eliminate infection in the absence of overt clinical signs. Due to their low

herd frequency (11%), it is difficult to ascertain a confident CM and production trend for TT cows from a limited sample population (n =15).

In addition to the reporting of bo*CXCR1* SNP -205C>T associations with CM susceptibility, this study may be the first to report a functional consequence. In this study animals segregated by -205C>T SNP genotypes exhibited significantly lower basal bo*CXCR1* mRNA levels in milk somatic cells from CC animals when compared with CT (P<0.05). The absence of TT cow data may strengthen or weaken this difference, which appeared to be specific for bo*CXCR1* as it was not seen for bo*CXCR2*, indicating potential SNP interference with bo*CXCR1* transcription. Preliminary *in silico* analysis for causal mutations using the TRANSFAC 4.0 programme AliBaba2.1 (www.gene-regulation.com/pub/programs.htmL) highlighted the -205C>T SNP as part of a Sp1 motif, where a C allele confers presence. Sp1 sites within a 300bp region immediately 5' to *CXCR1* start codons appear to be conserved across several species. Further *in silico* analysis matched Sp1 motifs within similar regions from pig, dog, mouse and human, with the latter matching a Sp1 site -208bp from the ATG start site, almost identical to the aforementioned bovine -205C>T SNP affected site.

Sp1 sites regulate transcription by either repressing or boosting transcript production. SNPs adjacent to a repressive Sp1 site in the human system affect IL-10 promoter function by impacting Sp1 interactions with additional regulatory proteins (Steinke, Barekzi et al. 2004), enhancing transcription (Larsson, Johansson et al. 2009). Presence of the T allele (-205C>T SNP) may relieve the repressive role of Sp1, increasing bo*CXCR1* expression as observed in T allele-carrying CT cows. Impairment of CXCR1 expression and activity suppresses the immune response. This has been demonstrated in humans where reduced expression of CXCR1 causes larger aggregates of cells adhering to vessel membranes, impeding migration efficiency (Hu, Westra et al. 2011). By contrast, over-expression of CXCR1 has also been linked to disease severity, for example in human glomerular diseases (Segerer, Henger et al.

2006). In terms of the impact of the -205C>T SNP on disease, data in this study showed CC cows with reduced mRNA levels have less CM. Whether CC cows have decreased CXCR1 protein expression requires verification. Although speculative, excessive CXCR1 due to impaired Sp1 regulation may contribute towards an excessive or aggravated mammary response to pathogen, leading to more inflammatory tissue damage to the udder and increased susceptibility to CM.

Whilst this poses an attractive hypothesis, further work is required to elucidate any causative impact of SNP -205C>T. It may also be possible that additional SNPs in linkage with -205C>T have a contributing role. Evidence of this may come from a very recent study characterising bo*CXCR1* SNPs (Pighetti, Kojima et al. 2012). The authors report the introduction of an early stop codon at the expense of a tryptophan residue at amino acid 206 (T206X mutation) with the presence of the A allele of the SNP +621G>A. The T206X mutation is located within the protein's second extracellular loop (Figure 6.16). SNP +621G>A is reported to be in complete LD with the singleton marker -205C>T, providing an additional fourth linkage group to the three shown in this study (Pighetti, Kojima et al. 2012). The +621G>A SNP was not detected in this study due to incomplete sequencing coverage of the bo*CXCR1* CDS, therefore both its presence and inheritance with the T allele (-205C>T) requires verification. Whether a shortened CXCR1 protein can effectively signal through its second ICL (containing the DRYLAIV motif), or whether expression is simply non-functional remains to be determined. The measure of downstream boCXCR1 signalling events, such as activation of MAPK, Gα and Gβγ subunits, phospholipase C-β or calcium mobilisation (Figure 6.1), could indicate whether a truncated boCXCR1 variant can signal. Lack of a COOH terminus will ultimately impede receptor internalisation (desensitisation), resulting in repeated receptor exposure to ligand. This may lead to the breakdown of potential feedback loops and up-regulation of CXCR1 to compensate, resulting in increased mRNA

levels of bo*CXCR1*. Impaired CXCR1 protein expression and function due to the inheritance of a stop codon may therefore underlie the increased mRNA levels of bo*CXCR1* and higher CM rates associated with the T allele carrying CT cows (-205C>T). These preliminary findings present an immunological mechanism for the differences identified in CM rates, and further present bo*CXCR1* as a suitable marker for susceptibility to other diseases.

The bo*CXCR1* SNP +735G>C, identified in this study, and employed as a tSNP for bo*CXCR1* linkage group 2, has been previously demonstrated to impact neutrophil function and has been analysed for association with intestinal, respiratory, uterine and mammary health. The CC genotype displayed reduced migration of neutrophils responding to ligand *in vitro* (Rambeaud and Pighetti 2005). Taqman data from this study shows lowered basal mRNA levels in milk somatic cells from CC cows, potentially complementing the above evidence. However, this study did not identify any significant associations between SNP +735G>C and traits for CM incidence or milk production. Previous studies (Youngerman, Saxton et al. 2004a; Galvao, Pighetti et al. 2011) have associated CC genotyped cows with lowered CM when compared with GC and GG genotyped animals. In this study, the incidence between CC and GG genotypes was similar. However, the CC population do display a lower percentage of animals experiencing at least one CM event across the three lactations, with near significance detected at P2, when compared with the GG population. Furthermore, CC cows were the only genotype to show no significant increases in CM rate from P1 through to P3. Data from lactations post P3 may strengthen any potential trend of lowered CM in CC cows. Galvao *et al* (2011) failed to specify exact lactation details for each genotyped (+735G>C) population, only that they contain a mixture of primiparous and multiparous animals. Furthermore, their study measured population incidence as number of cases per 10,000 days at risk, whereas this study measured the population mean for individual incidence as number of cases per year (365days) at risk, over the first three lactations.

The influence of SNP -205T>C may contribute to associations of +735G>C with CM. In this study, LD between the markers -205T>C and +735G>C was moderate (0.5), but may be sufficient to influence any CM associations with the +735G>C SNP in other herds. Reconstruction of bo*CXCR1* haplotypes using linkage groups in this study and the work of Pighetti *et al* (2012) show the T allele of -205T>C SNP (potentially linked with an early stop codon) to be present in haplotypes containing the G allele of +735G>C. Both the T allele of -205T>C SNP and the G allele of +735G>C have been associated with increased bo*CXCR1* mRNA levels in this study. Further work will be required to substantiate the influence of the -205T>C SNP, and comprehensive sequencing of bo*CXCR1* and bo*CXCR2* may identify other causative candidates. The additive effect from additional bo*CXCR1* SNPs and SNPs from other linked genes should also be considered. For example, the bovine gene encoding the ligand CXCL8 is highly polymorphic, with promoter and coding SNPs impacting relative mRNA levels and transcriptional responsiveness to ligand stimulation (Chen, Yang et al. 2011a; Meade, O'Gorman et al. 2012). Bo*CXCL8* SNPs are further strongly associated with milk yield, protein and SCC within a large Chinese Holstein (*n* =610) cohort (Chen, Yang et al. 2011). Bo*CXCL8* variation may therefore influence bo*CXCR1* genotype associations.

No significant differences between +735G>C genotypes for SCC were detected within the herd, agreeing with previous findings (Leyva-Baca, Schenkel et al. 2008; Goertz, Baes et al. 2009; Beecher, Daly et al. 2010; Galvao, Pighetti et al. 2011). Whilst increased milk yield from GG cows had been observed (Youngerman, Saxton et al. 2004a; Galvao, Pighetti et al. 2011), no significant differences in milking yield between +735G>C genotypes were detected within this herd. The continued discrepancy between findings for SNP +735G>C from this and previous studies may reflect breed and herd variation, sampling method and pathogen-specific causes of disease. Although not detected, the bo*CXCR1* SNP -1768T>A, identified and associated with SCC in previous studies (Leyva-Baca, Schenkel et

al. 2008; Chen, Yang et al. 2011), was still represented due to its linkage with SNP +980A>G (Pighetti, Kojima et al. 2012), a mutation represented by tSNP -181T>C in linkage group 1 for this study. Data from this study indicated no association between SNP -1768T>A (t-181T>C) and CM, or SCC, as demonstrated by Goertz *et al* (2009). The two bo*CXCR2 5*' upstream SNPs identified in this study did not associate with CM or milk production traits, and did not appear to impact any putative TBS. Whilst data potentially revealed lowered bo*CXCR2* expression in AA cows segregated by their -496G>A genotype, this is speculative since only one AA individual was analysed.

In conclusion, the findings presented here demonstrate an association between the bo*CXCR1* SNP -205C>T and CM susceptibility. Furthermore, evidence suggests that this SNP may contribute towards functional variation by influencing Sp1-regulated transcription. In addition, this marker may be inherited with the introduction of a stop codon determined by the bo*CXCR1* SNP +621G>A, a marker shown to be in linkage with -205C>T in a separate study. Any causative impact from SNPs -205C>T and +621G>A on transcription and receptor expression/activity warrants further investigation, along with the additive impact of the +735G>C SNP that has been associated with impaired neutrophil migration. Figure 6.16 illustrates the location and impact of mutations within the CXCR1 structure. Rapid but controlled influx of neutrophils into the mammary gland to kill organisms often determines the outcome of bacterial infection. However, data from this and other studies show positive correlation between CM, bo*CXCR1* levels and neutrophil migration rate, suggesting increased protein expression may influence excessive inflammation, greater risk of permanent tissue damage and increased susceptibility to infection. Taken together, bo*CXCR1* SNPs -205C>T (+621G>A) and +735G>C can be used in a tagging SNP haplotype approach to identify cattle more susceptible to CM, similar to a human study analysing CXCR1/2 genetic variation and influence in Cystic Fibrosis (Kormann, Hector et al. 2011).

The favourable bo*CXCR1* SNP -205 CC variant could reduce detrimental clinical manifestations of diseases via an efficient yet controlled influx of inflammatory cells to neutralise pathogens and control infection, particularly during *E. coli* mastitis whereby rapidly multiplying bacteria demand neutrophil control. The complexity of mastitis infection suggests a polygenic and multi-factorial immune response comprising many different proteins which will in turn interact with one another. The ability to detect significant associations with CM for a single gene indicates the importance of the CXCR1-related response in the control of bacterial disease in cattle. A bovine population with impaired bo*CXCR1* expression may also be more susceptible to other diseases and health issues. The potential use of SNPs within bo*CXCR1* to act as genetic markers for altered susceptibility to mastitis warrants further investigation. However, the non-favourable linkage of lowered CM with decreased milk yield and fat concentrations demonstrated here indicates selection for lowered CM using this marker would be detrimental to milk quality, as previously suggested in studies linking SNPs with increased SCC as a marker for CM susceptibility.

Chapter 7.0 - General Discussion and Conclusions

7.1 Discussion

Bovine mastitis is recognised worldwide as the most important and costly disease affecting dairy cattle. Widespread measures to improve hygiene and disease control have had little impact on UK mastitis rates over the past 20 years. The latest UK figures suggest CM incidence is on the rise, and a contributing factor could be the consequence of socioeconomic pressure on UK dairy producers to maximise profitability from animal performance. Many herds demonstrate limited genetic diversity on the basis of promoting favourable production traits that may unintentionally be detrimental to animal health. Holstein-Friesians are the commercially favoured crossbreed; however, a predominance of Holstein genetics in artificial insemination (AI) breeding programmes has led to the overuse of sires from a limited sample population, and may be contributing to increased inbreeding and the risks associated with it (Kearney, Wall et al. 2004). Comprehensive genome sequencing of a particular Holstein bull and leading dairy sire revealed a total of 3.7 million SNP mutations when referenced against the Hereford sequence, including >11k NS SNPs and many early stop codons which may have phenotypic effects (Stothard, Choi et al. 2011). The introduction and spread of traits have resulted in decreased fertility, reproduction, health and longevity, traits not sustainable genetically or financially in any dairy breeding strategy. It is therefore important to balance herd genetics so that both health and performance may be improved. This could involve the expanded use of crossbreeding, although increased heterozygosity can promote genetic recombination that, via changes in the genetic effects of dominance and epistasis, could impact these health and production traits. Balancing outbreeding with the monitoring of disease-associated markers within a herd will be of great benefit to dairy producers, and could be a viable option with suitable SNP chip technologies. These represent a fast and cost effective approach to genotyping many (>60k) SNP markers, including some of known

disease importance, in cattle, providing a robust platform for mapping disease genes and QTLs (Matukumalli, Lawley et al. 2009).

The identification of SNPs linked to dysfunction of proteins vital for the initiation, regulation and progression of an immune response may provide suitable genetic risk factors for CM, and aid bovine SNP chip development. By selecting animals with favourable mutations, herd mastitis rates could be reduced. In addition to the considerable economic costs of mastitis, there is an ethical precedence for a reduction in antibiotic usage and the improvement of animal welfare. Another concern is the widespread use of waste milk from mastitis cows as cost effective feed for calves on UK farms (Brunton, Duncan et al. 2012). Waste milk containing antimicrobial products is a biosecurity risk to the calf and may promote the development of antibiotic resistance. Lowering the overall use and reliance on antibiotics to control mastitis will reduce treatment costs and limit any potential for the selection of antibiotic resistance in strains of bacteria important for mastitis.

By correlating to actual CM data, the research reported in this thesis has identified SNPs within key immune genes that associate with reduced CM susceptibility without compromising milk yield or quality, and this may improve accuracy of breeding for these favourable phenotypes.

7.1.1 Polymorphisms common to boTLR1 and boCXCR1 could be used for marker-assisted selection of cattle less susceptible to mastitis and other diseases

This is the first report that the bo*TLR1* tSNP -79T>G, bo*TLR1* 3'UTR SNP +2463C>T, and the bo*CXCR1* SNP -205C>T were each found to associate significantly with CM susceptibility. The differences in CM incidence observed between the defined genotypes would have a significant impact on both animal welfare and farm profitability. For example, bo*TLR1* -79 TT animals recorded, on average, 40 CM cases/100 cows/year, below the

historic national average for England and Wales of 47 cases (Bradley, Leach et al. 2007). However, GG and TG animals recorded 75 and 73 cases respectively, substantially more than the national average. As a simple demonstration of potential impact, if estimated average costs for a clinical case are £175 (Kossaibati 2000), potential losses per 100 cows equate to £6,125 and £5,775 for TG and GG animals respectively (when compared to TT animals). Likewise animals with the bo*CXCR1* SNP -205 CC genotype recorded on average 39 CM cases/100 cows/year while animals within the CT genotype had 70 cases, equating to potential losses per 100 cows of £5,425 for the CT animals (Table 7.1). The estimation of average case costs for CM is composed of direct costs, which include antimicrobials, farm-associated time and discarded milk, and indirect costs, which include those associated with reduced yield, increased culling and any fatality due to mastitis (Kossaibati 2000). A more recent study reviewing US dairy production calculated average losses per case of CM at $179, of which $115 (64%) was due to milk yield losses, $50 (28%) to treatment-associated costs and $14 (8%) to increased mortality (Bar, Tauer et al. 2008). Milk losses are therefore a major contributory factor to mastitis costs and, as a consequence, estimated CM cost figures will depend and vary on the current wholesale price of milk.

Table 7.1 - Comparison of economic losses between genotype populations of CM associated SNP markers

CM Marker	Genotype	CM Incidence (CM/100 cows/year)	Cost (£)	Losses (£)
Bo*TLR1* t -79T>G	TT*	40	7,000	-
	TG	73	12,775	5,775
	GG	75	13,125	6,125
Bo*TLR1* +2463C>T	CC	73	12,775	6,475
	CT	74	12,950	6,650
	TT*	36	6,300	-
Bo*CXCR1* -205C>T	CC*	39	6,825	-
	CT	70	12,250	5,425

*****Lower CM genotype.

The favourable association of reduced CM with increased milk fat and protein, a financially rewarded premium, was observed for both bo*TLR1* SNPs, indicating selection for these markers would be beneficial to milk quality. Selection for lowered CM using the bo*CXCR1* SNP -205C>T may also increase milk fat; it remains unclear whether a biological link between reduced CM and increased milk fat exists. Reduced milk fat compositions have been observed previously in mastitic milk when compared to 'healthy' milk (Randolph and Erwin 1974). Reduced fat content is therefore more likely a consequence, as opposed to a contributory factor, of IMI. Whilst bo*TLR1* SNPs had no impact on milk yields, analysis of the bo*CXCR1* SNP -205C>T revealed significantly increased milk yields from CT variants that associated with CM susceptibility. These findings potentially demonstrate the concerns that selecting for optimal milk production can lead to the introduction of mutations in other genes detrimental to animal health. Whilst increased milk production from animals with the bo*CXCR1* SNP -205 CT offsets any associated milk losses from increased CM in the Mayfield herd, there remains the unfavourable concern of reduced animal welfare and increased antibiotic usage.

All significant CM genotype associations survived adjustments for other milking factors (mean logged SCC, milk yield, fat and protein percentages) using a GLM. In a separate analysis, the year of CM data recording was shown to not significantly impact yearly incidence between variant populations, with genotype CM trends fluctuating with herd average trends (Appendixes II and III).

7.1.2 DNA pools proved successful for initial screening for disease-associated SNPs

Sequencing of PCR amplicons from a DNA pool comprising Holstein-Friesians with high (greater than six lifetime CM cases) or no mastitis case histories revealed 46 SNPs, nine of which are novel, within candidate genes bo*TLR1*, bo*TLR4*, bo*TLR5*, bo*CD14*, bo*CXCR1* and bo*CXCR2*. Although this pooling method may miss rare SNPs, and did not identify SNPs

within bo*TLR2* and bo*TLR6* UTR regions, it proved effective for the rapid detection of three CM-associated SNPs common to the Mayfield herd. Extended genotyping of individuals did not uncover any additional SNPs indicating a high level of sensitivity in the DNA pooling approach. Despite no CM or milk production associations for any of the selected SNP markers within bo*TLR4*, bo*TLR5* bo*CD14* and bo*CXCR2*, this was a small study and these findings would benefit from larger sample populations. Comprehensive analyses of all genes in this project may identify other SNPs of interest with regards to disease susceptibility. Due to the importance of boTLR4 for the detection of mastitis-causing pathogens, bo*TLR4* remains a candidate for additional association studies with CM susceptibility in other herds/breeds. Furthermore, the expanded analysis of bo*TLR1* and bo*CXCR1* CM-associated SNPs from this project into other herds/breeds is highly recommended. These SNPs may, for example, exhibit a more deleterious effect within a single herd under the influence of inbreeding. Whilst studying one herd can limit variability in CM recordings, reliability and accuracy of CM incidence data from the Mayfield herd, and other herds when expanding SNP analysis, is crucial for the precise identification and verification of disease-associated SNPs in cattle populations.

7.1.3 SNPs found to associate with CM may contribute towards an underlying immunological mechanism for disease susceptibility

This is the first report that the bo*TLR1* and bo*CXCR1* SNPs found to associate with CM further demonstrate potential consequences at the gene/protein level, and therefore possibly underlie an immunological mechanism for disease susceptibility. Both genes display a large degree of polymorphism and high pattern of LD that could influence variation in immune responses between the genotype populations and subsequently influence the outcome of infection. Published research documenting mutations in bo*CXCR1*, a receptor critical to the

recruitment of neutrophils, have demonstrated association with disease, impair cell migration and impact on immune responses (Youngerman, Saxton et al. 2004a; Rambeaud and Pighetti 2005; Rambeaud, Clift et al. 2006; Galvao, Pighetti et al. 2011). Evidence presented in this thesis (Chapter 6.0) proposes that the bo*CXCR1* SNP -205C>T may contribute towards functional variation by influencing Sp1-regulated transcription. Animals with the CC genotype, associated with lowered CM, had significantly lower bo*CXCR1* expression in milk somatic cells when compared with CT animals. In addition this marker may be inherited with the introduction of a stop codon determined by the bo*CXCR1* SNP +621G>A, shown to be in linkage with -205C>T in a separate study (Pighetti, Kojima et al. 2012). Cows with the -205 T allele may express a truncated and dysfunctional boCXCR1 protein with severe implications for health, particularly during transient *E. coli* infections where an efficient neutrophil defence is vital to prevent progression of infection and toxic death. A population of animals expressing a truncated boCXCR1 protein may therefore demonstrate reduced longevity and sustainability, potentially supported by the rare frequency of -205 homozygous TT cows in the Mayfield herd, which may have resulted from premature culling due to mastitis and other diseases.

SNPs common to bo*TLR1* may underpin functional variation in boTLR1, an important cell membrane PRR for the detection of bacterial PAMPs, specifically triacylated lipoproteins. This is the first report that bo*TLR1* SNPs that were significantly associated with CM demonstrate impaired bo*TLR1* expression, possibly via interference of Oct-1 binding (Chapter 3.0), and subsequent dampening in cytokine responses from bovine whole blood and enriched cell populations when exposed to PAM_3 or HK bacteria (Chapter 4.0). An appropriate immune response, conferred by the favourable bo*TLR1* tSNP -79 TT variant, could reduce clinical manifestations of disease via an efficient and regulated influx of inflammatory cells to neutralise pathogens and control infection. These findings further

emphasise the importance of boTLR1-related responses in the control of bacterial disease in cattle, with particular implications for *S. aureus* mastitis. Functional data (Chapter 4.0) indicate that animals of a -79 GG genotype may be more susceptible to *S. aureus* infection. However, preliminary analysis of Mayfield herd data revealed little difference between the variant populations in the percentage of CM cases caused by *S. aureus*. This may in part be due to the lack of comprehensive data on the causative agent of each CM case, as only ~32% of Mayfield CM cases resulted in the successful isolation of a pathogen. A separate study observed an increase in the frequency of *S. aureus* isolation from older cows that were into their fourth, fifth and sixth parities (De Vliegher, Fox et al. 2012). Analysis of cow data post parity three, coupled with comprehensive pathogen data for each CM and subclinical case, may strengthen associations between bo*TLR1* SNP -79 GG variants with susceptibility to mastitis caused by *S. aureus*.

A bovine population with impaired boTLR1 and boCXCR1 transcript expression and protein function may also be more susceptible to other diseases such as bovine TB (Sun, Song et al. 2012). This warrants further investigation to determine the suitability of the SNPs identified in this thesis as genetic markers of disease. These could then be incorporated into bovine SNP chips with the potential to screen currently used and unproven sires that are selected for AI programmes, to improve herd fitness and disease resistance in cattle. As a multifactoral condition, CM may be influenced by the collective influence of many causal SNPs in different genes, which on their own may contribute to a low percentage of cases in other herds. In the Mayfield herd, bo*TLR1* and bo*CXCR1* SNPs appear to significantly influence the immune response and incidence of CM.

7.1.4 Mean log SCC as a proxy for CM

Where possible the careful use of accurate CM data, as demonstrated in this thesis, may allow the identification of significant disease associations. In most countries selection of a reduced

mean lactation SCC (often logged) is used as a surrogate for CM. However, its reliability as a marker for disease continues to be questioned. A weak positive correlation of 0.03 was initially detected between CM incidence and SCC for Mayfield herd data recorded during the years 2001-2010 (Chapter 2.0). Analysis of suitably adjusted datasets (logged SCC and adjusted CM incidence) from SNP association analysis did strengthen this to a positive correlation of 0.2 (Table 7.2). However, no associations were detected for a raised SCC in variants associated with CM at a herd level. The use of individual cow means for log SCC may not be the most appropriate disease indicator since it is the measure of all four quarters, which will dilute a raised SCC from an infected quarter. Alternative and potentially more accurate SCC approaches for determining and monitoring disease status may include the use of quarter SCC data or the proportion of high SCC (>200,000 cells/mL milk) events per lactation (Bradley and Green 2005). A significant negative correlation (-0.2) was detected between CM incidence and milk fat percentage (Table 7.2) following SNP association analysis, with all genotypes associating with CM demonstrating significantly reduced milk fat content at the herd level. Additional study re-evaluating the potential use of milk fat compositions as an indirect measure of infection status could be considered (Randolph and Erwin 1974; Kitchen 1981).

Table 7.2 - Correlation coefficients between CM and production traits for adjusted cow data used for SNP association analyses

	CM	**SCC**	**Milk yield**	**Protein%**	**Fat%**
CM RATE		**0.003**	**0.331**	**0.126**	**0.003**
SCC	0.198		**0.108**	**0.168**	**0.901**
Milk yield	-0.066	-0.109		**0**	**0.004**
Protein%	-0.1	0.094	-0.3		**0**
fat%	-0.2	0.008	-0.2	0.6	

P values in bold.

7.1.5 Improving heritability and accuracy of CM markers

Widespread availability of reliable herd CM data is essential for accurate analyses and interpretation of SNP data and subsequent associations. Furthermore, knowing the causative pathogen for each mastitis case may promote identification of pathogen-specific disease markers; bo*TLR4*/bo*CD14* SNPs are likely to associate with *E. coli* mastitis for example. The current disadvantage of using CM resistance as a breeding trait is its low heritability (Rupp and Boichard 1999), in part due to the variability of causative agents and subsequent immune responses during disease. Categorising CM QTLs by a predominant causative pathogen may improve accuracy for selective breeding for such traits. This could also address an additional problem with CM QTLs, in that they cover large regions of chromosome with wide confidence intervals, within which many candidate genes are located. Disease-associated SNPs from this research are within bo*TLR1* and bo*CXCR1*, genes already mapped and highlighted as strong candidates for underlying QTL regions (Bta 6 and 2 respectively) for resistance to diseases such as mastitis (Jann, King et al. 2009; Sodeland, Kent et al. 2011). Where bo*TLR1* markers may underlie a CM QTL by influencing responses to *S. aureus* for example, it is important to understand that weakened CM associations and heritability may be demonstrated in other herds where contagious spread is controlled.

7.1.6 PMNs may contribute to acute cytokine responses during mastitis

The cytokines produced from PMNs in response to various ligands (Chapter 4.0) not only represent stimulation of TLR-induced genes, but also others known to be induced during mastitis. The PMN 'granulocyte' populations used in this study contained a high proportion of neutrophils, short lived phagocytes which undergo apoptosis usually within 6-10 h (Savill, Wyllie et al. 1989). However, neutrophil activation, due to CXCR and TLR stimulation for example, will prolong their lifespan enabling them to perform antimicrobial effector functions important in the early stages of bacterial IMI. PMNs demonstrate greater immune

potential through the synthesis of cytokines that influence their own activity and survival, recruit additional immune cells and modulate the immune response (Hayashi, Means et al. 2003; Paape, Bannerman et al. 2003; Parker, Whyte et al. 2005; Kobayashi 2008). Data in this thesis provide further evidence for PMN involvement in directing the mammary immune response, although this work would benefit from additional studies with highly purified neutrophil populations.

7.2 Conclusions and Future work

- Marker-assisted selection involving SNPs within bo*TLR1* and bo*CXCR1*, and other genes of immune importance, if well implemented into breeding programmes, may play a role in addressing the current trends for increased CM and reduced fertility and profitability in UK dairy herds. All SNP associations in the research presented here will benefit from expanded analysis in other herds/breeds.
- Reduced milk fat content may potentially indicate herd problems with IMI. Future work to evaluate any breeding and diagnostic potential, for example fat composition thresholds of infection and levels of fatty acids in milk during clinical and subclinical mastitis, is required.
- Non-coding SNPs bo*TLR1*-79T>G and bo*CXCR1* -205C>T may interfere with Oct-1 and Sp1 DNA binding respectively. To verify these DNA-protein interactions, DNA-protein binding assays on relevant upstream genomic DNA sequences will be needed. Methods for detecting DNA-protein interactions can be performed *in vitro* via DNA electrophoretic mobility shift assays (EMSA), or *in vivo* via a chromatin immunoprecipitation (ChIP) assay, which allows analysis of DNA-protein interactions in living cells that can be quantified if coupled with Q-PCR. Assay readouts could further reveal differences in binding potential between SNP genotypes.

- To validate the *cis*-acting potential of non-coding SNPs bo*TLR1*-79T>G and bo*CXCR1*-205C>T, DNA constructs, involving insertion of a 5' genomic/reporter 3' DNA template into a suitable expression vector, will be required. The DNA template will specifically comprise 1-2 kB of upstream genomic sequence (containing the SNP) preceding a reporter gene encoding the enzyme luciferase. Constructs will be transfected into a cell-line and the resulting cellular expression and activity of luciferase quantified. These measurements could reveal differences between the genotypes (SNP variant constructs) indicating that SNP presence, upstream of the reporter gene, has an impact on the transcription of luciferase; further demonstrating that non-coding SNPs bo*TLR1*-79T>G and bo*CXCR1*-205C>T could interfere with Oct-1 / Sp1 regulation of bo*TLR1* / bo*CXCR1* transcription *in vivo*.
- The presence and inheritance of the bo*CXCR1* SNP +621G>A (stop codon mutation) with the T allele (-205C>T) requires verification. Functional assays to determine whether a truncated boCXCR1 protein can effectively signal through its second intracellular loop (containing the DRYLAIV motif), or whether the protein is non-functional, will be required. These may involve investigations into the downstream signalling molecules induced by boCXCR1 chemokine interactions, for example utilising RNA-Seq, an approach to transcriptome profiling using deep sequencing-based technologies (Wang, Gerstein et al. 2009). Cell migration assays can also be performed and could reveal impaired migration towards a CXCL8 gradient from neutrophils expressing truncated CXCR1.
- Expanding boTLR1 variant assays, by stimulating with live pathogen and measuring additional cytokine output, may further demonstrate the importance of boTLR1-directed immune responses during mastitis and other diseases. The use of microarrays or RNA-Seq to evaluate pathway analysis from variants could reveal and detail

downstream transcriptome differences that may correlate with genotype differences in the cytokine response. Assays to measure NF-κB activity induced by TLR can also be performed.

- Extending the analysis of boTLR1 in T cell activation, a cell response observed during *S. aureus* mastitis (Rivas, Schwager et al. 2007), could be of particular interest and further our understanding of disease. Assays to measure TLR1-induced levels of immune factors important in T cell recruitment, for example a panel of Th1/Th2/Th17-related cytokines, can be performed.

References

"Leucine-rich Repeat finder (LRRfinder) web-based tool." from http://www.lrrfinder.com/.

"National Center for Biotechnology Information: SNP Database." from http://www.ncbi.nlm.nih.gov/projects/SNP.

"SMART: Simple Modular Architecture Research Tool." from http://smart.embl-heidelberg.de/

Allen, S. J., S. E. Crown, et al. (2007). "Chemokine: receptor structure, interactions, and antagonism." Annu Rev Immunol **25**: 787-820.

Anderson, J. C. (1977). "Experimental staphylococcal mastitis in the mouse: the induction of chronic mastitis and its response to antibiotic therapy." J Comp Pathol **87**(4): 611-621.

Asanuma, M., K. Kurokawa, et al. (2011). "Structural evidence of alpha-aminoacylated lipoproteins of Staphylococcus aureus." FEBS J **278**(5): 716-728.

Babu, M. M., M. L. Priya, et al. (2006). "A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins." J Bacteriol **188**(8): 2761-2773.

Bannerman, D. D. (2009). "Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows." J Anim Sci **87**(13 Suppl): 10-25.

Bannerman, D. D., M. J. Paape, et al. (2004). "Innate immune response to intramammary infection with Serratia marcescens and Streptococcus uberis." Vet Res **35**(6): 681-700.

Bar, D., L. W. Tauer, et al. (2008). "The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming." J Dairy Sci **91**(6): 2205-2214.

Barkema, H. W., Y. H. Schukken, et al. (1999). "Management practices associated with the incidence rate of clinical mastitis." J Dairy Sci **82**(8): 1643-1654.

Barkema, H. W., Y. H. Schukken, et al. (1998). "Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts." J Dairy Sci **81**(2): 411-419.

Barrett, J. C., B. Fry, et al. (2005). "Haploview: analysis and visualization of LD and haplotype maps." Bioinformatics **21**(2): 263-265.

Beecher, C., M. Daly, et al. (2010). "Polymorphisms in bovine immune genes and their associations with somatic cell count and milk production in dairy cattle." BMC Genet **11**: 99.

Bell, J. K., I. Botos, et al. (2005). "The molecular structure of the Toll-like receptor 3 ligand-binding domain." Proc Natl Acad Sci U S A **102**(31): 10976-10980.

Bonecchi, R., M. Locati, et al. (2011). "Chemokines and cancer: a fatal attraction." Cancer Cell **19**(4): 434-435.

Borroni, E. M., A. Mantovani, et al. (2010). "Chemokine receptors intracellular trafficking." Pharmacol Ther **127**(1): 1-8.

Botos, I., D. M. Segal, et al. (2011). "The structural biology of Toll-like receptors." Structure **19**(4): 447-459.

Bougarn, S., P. Cunha, et al. (2011). "Technical note: Validation of candidate reference genes for normalization of quantitative PCR in bovine mammary epithelial cells responding to inflammatory stimuli." J Dairy Sci **94**(5): 2425-2430.

Bradley, A. (2002). "Bovine mastitis: an evolving disease." Vet J **164**(2): 116-128.

Bradley, A. and M. Green (2005). "Use and interpretation of somatic cell count data in dairy cows." In Practice **27**: 310-315.

Bradley, A. J., K. A. Leach, et al. (2007). "Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales." Vet Rec **160**(8): 253-257.

BramLey, A. J. (1976). "Variations in the susceptibility of lactating and non-lactating bovine udders to infection when infused with Escherichia coli." J Dairy Res **43**(2): 205-211.

Brookes, A. J. (1999). "The essence of SNPs." Gene **234**(2): 177-186.

Brunton, L. A., D. Duncan, et al. (2012). "A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales." Vet Rec. **171**(12): 296.

Buitenhuis, B., C. M. Rontved, et al. (2011). "In depth analysis of genes and pathways of the mammary gland involved in the pathogenesis of bovine Escherichia coli-mastitis." BMC Genomics **12**: 130.

Cargill, E. J. and J. E. Womack (2007). "Detection of polymorphisms in bovine toll-like receptors 3, 7, 8, and 9." Genomics **89**(6): 745-755.
Carpenter, S. and L. A. O'Neill (2009). "Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins." Biochem J **422**(1): 1-10.
Chen, R., Z. Yang, et al. (2011). "Polymorphisms of the IL8 gene correlate with milking traits, SCS and mRNA level in Chinese Holstein." Mol Biol Rep **38**(6): 4083-4088.
Chen, R., Z. Yang, et al. (2011a). "SNPs of CXCR1 gene and its associations with somatic cell score in Chinese Holstein cattle." Anim Biotechnol **22**(3): 133-142.
Chien, J. W., M. J. Boeckh, et al. (2008). "Lipopolysaccharide binding protein promoter variants influence the risk for Gram-negative bacteremia and mortality after allogeneic hematopoietic cell transplantation." Blood **111**(4): 2462-2469.
Chow, J. C., D. W. Young, et al. (1999). "Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction." J Biol Chem **274**(16): 10689-10692.
Chuntharapai, A., J. Lee, et al. (1994). "Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes." J Immunol **153**(12): 5682-5688.
Collins, R. A., C. J. Howard, et al. (1999). "Bovine interleukin-12 and modulation of IFNgamma production." Vet Immunol Immunopathol **68**(2-4): 193-207.
Craven, N. and M. R. Williams (1985). "Defences of the bovine mammary gland against infection and prospects for their enhancement." Vet Immunol Immunopathol **10**(1): 71-127.
De Schepper, S., A. De Ketelaere, et al. (2008). "The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of Escherichia coli mastitis in dairy cattle." Vet Res **39**(1): 5.
De Vliegher, S., L. K. Fox, et al. (2012). "Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control." J Dairy Sci **95**(3): 1025-1040.
Edmondson, P. and B. Blowey (1998). "Record analysis in mastitis investigation." In Practice**:** 297-303.
Egan, S. A., D. Kurian, et al. (2010). "Identification of sortase A (SrtA) substrates in Streptococcus uberis: evidence for an additional hexapeptide (LPXXXD) sorting motif." J Proteome Res **9**(2): 1088-1095.
Ericsson Unnerstad, H., A. Lindberg, et al. (2009). "Microbial aetiology of acute clinical mastitis and agent-specific risk factors." Vet Microbiol **137**(1-2): 90-97.
Faldt, J., C. Dahlgren, et al. (2002). "Difference in neutrophil cytokine production induced by pathogenic and non-pathogenic mycobacteria." APMIS **110**(9): 593-600.
Farhat, K., S. Riekenberg, et al. (2010). "Identification of full length bovine TLR1 and functional characterization of lipopeptide recognition by bovine TLR2/1 heterodimer." Vet Res **41**(3): 34.
Farhat, K., K. S. Sauter, et al. (2008). "The response of HEK293 cells transfected with bovine TLR2 to established pathogen-associated molecular patterns and to bacteria causing mastitis in cattle." Vet Immunol Immunopathol **125**(3-4): 326-336.
Field, T. R., P. N. Ward, et al. (2003). "The hyaluronic acid capsule of Streptococcus uberis is not required for the development of infection and clinical mastitis." Infect Immun **71**(1): 132-139.
Figueroa, L., Y. Xiong, et al. (2012). "The Asp299Gly polymorphism alters TLR4 signaling by interfering with recruitment of MyD88 and TRIF." J Immunol **188**(9): 4506-4515.
Fitzgerald, K. A., D. C. Rowe, et al. (2003). "LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF." J Exp Med **198**(7): 1043-1055.
Flo, T. H., L. Ryan, et al. (2002). "Involvement of toll-like receptor (TLR) 2 and TLR4 in cell activation by mannuronic acid polymers." J Biol Chem **277**(38): 35489-35495.
Francois, S., J. El Benna, et al. (2005). "Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad." J Immunol **174**(6): 3633-3642.
Galvao, K. N., G. M. Pighetti, et al. (2011). "Association between interleukin-8 receptor-alpha (CXCR1) polymorphism and disease incidence, production, reproduction, and survival in Holstein cows." J Dairy Sci **94**(4): 2083-2091.

Gantner, B. N., R. M. Simmons, et al. (2003). "Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2." J Exp Med **197**(9): 1107-1117.
Gibbs, R. A., J. F. Taylor, et al. (2009). "Genome-wide survey of SNP variation uncovers the genetic structure of cattle breeds." Science **324**(5926): 528-532.
Goertz, I., C. Baes, et al. (2009). "Association between single nucleotide polymorphisms in the CXCR1 gene and somatic cell score in Holstein dairy cattle." J Dairy Sci **92**(8): 4018-4022.
Goldammer, T., H. Zerbe, et al. (2004). "Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle." Clin Diagn Lab Immunol **11**(1): 174-185.
Goni, P., Y. Vergara, et al. (2004). "Antibiotic resistance and epidemiological typing of Staphylococcus aureus strains from ovine and rabbit mastitis." Int J Antimicrob Agents **23**(3): 268-272.
Graham, G. J., M. Locati, et al. (2012). "The biochemistry and biology of the atypical chemokine receptors." Immunol Lett **145**(1-2): 30-38.
Grosse, W. M., S. M. Kappes, et al. (1999). "Single nucleotide polymorphism (SNP) discovery and linkage mapping of bovine cytokine genes." Mamm Genome **10**(11): 1062-1069.
Gu, W., Y. A. Shan, et al. (2007). "Functional significance of gene polymorphisms in the promoter of myeloid differentiation-2." Ann Surg **246**(1): 151-158.
Guan, Y., D. R. Ranoa, et al. (2010). "Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling." J Immunol **184**(9): 5094-5103.
Guo, Y., G. Zhao, et al. (2009). "Differential responses between monocytes and monocyte-derived macrophages for lipopolysaccharide stimulation of calves." Cell Mol Immunol **6**(3): 223-229.
Hajishengallis, G. and J. D. Lambris (2010). "Crosstalk pathways between Toll-like receptors and the complement system." Trends Immunol **31**(4): 154-163.
Hasan, U., C. Chaffois, et al. (2005). "Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88." J Immunol **174**(5): 2942-2950.
Hawn, T. R., E. A. Misch, et al. (2007). "A common human TLR1 polymorphism regulates the innate immune response to lipopeptides." Eur J Immunol **37**(8): 2280-2289.
Hawn, T. R., A. Verbon, et al. (2003). "A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease." J Exp Med **198**(10): 1563-1572.
Hawn, T. R., H. Wu, et al. (2005). "A stop codon polymorphism of Toll-like receptor 5 is associated with resistance to systemic lupus erythematosus." Proc Natl Acad Sci U S A **102**(30): 10593-10597.
Hayashi, F., T. K. Means, et al. (2003). "Toll-like receptors stimulate human neutrophil function." Blood **102**(7): 2660-2669.
He, Y., Q. Chu, et al. (2011). "Association of bovine CD4 and STAT5b single nucleotide polymorphisms with somatic cell scores and milk production traits in Chinese Holsteins." J Dairy Res: 1-8.
Hebert, A., K. Sayasith, et al. (2000). "Demonstration of intracellular Staphylococcus aureus in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk." FEMS Microbiol Lett **193**(1): 57-62.
Heringstad, B., D. Gianola, et al. (2006). "Genetic associations between clinical mastitis and somatic cell score in early first-lactation cows." J Dairy Sci **89**(6): 2236-2244.
Hill, A. W. (1988). "Pathogenicity of two strains of Streptococcus uberis infused into lactating and non-lactating bovine mammary glands." Res Vet Sci **45**(3): 400-404.
Hu, N., J. Westra, et al. (2011). "Decreased CXCR1 and CXCR2 expression on neutrophils in anti-neutrophil cytoplasmic autoantibody-associated vasculitides potentially increases neutrophil adhesion and impairs migration." Arthritis Res Ther **13**(6): R201.
Hull, J., K. Rowlands, et al. (2003). "Variants of the chemokine receptor CCR5 are associated with severe bronchiolitis caused by respiratory syncytial virus." J Infect Dis **188**(6): 904-907.
Huxley, J. N., M. J. Green, et al. (2002). "Evaluation of the efficacy of an internal teat sealer during the dry period." J Dairy Sci **85**(3): 551-561.

Ibeagha-Awemu, E. M., P. Kgwatalala, et al. (2008). "A critical analysis of disease-associated DNA polymorphisms in the genes of cattle, goat, sheep, and pig." Mamm Genome **19**(4): 226-245.
Ibeagha-Awemu, E. M., J. W. Lee, et al. (2008). "Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells." Vet Res **39**(2): 11.
Ibeagha-Awemu, E. M., J. W. Lee, et al. (2008a). "Bovine CD14 gene characterization and relationship between polymorphisms and surface expression on monocytes and polymorphonuclear neutrophils." BMC Genet **9**: 50.
Jann, O. C., A. King, et al. (2009). "Comparative genomics of Toll-like receptor signalling in five species." BMC Genomics **10**: 216.
Jin, M. S., S. E. Kim, et al. (2007). "Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide." Cell **130**(6): 1071-1082.
Jin, M. S. and J. O. Lee (2008). "Structures of the toll-like receptor family and its ligand complexes." Immunity **29**(2): 182-191.
Johnson, C. M., E. A. Lyle, et al. (2007). "Cutting edge: A common polymorphism impairs cell surface trafficking and functional responses of TLR1 but protects against leprosy." J Immunol **178**(12): 7520-7524.
Kang, J. Y., X. Nan, et al. (2009). "Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer." Immunity **31**(6): 873-884.
Kantanen, J., I. Olsaker, et al. (2000). "Genetic diversity and population structure of 20 North European cattle breeds." J Hered **91**(6): 446-457.
Kataria, R. S., R. G. Tait, Jr., et al. (2011). "Association of toll-like receptor four single nucleotide polymorphisms with incidence of infectious bovine keratoconjunctivitis (IBK) in cattle." Immunogenetics **63**(2): 115-119.
Kawai, T. and S. Akira (2005). "Pathogen recognition with Toll-like receptors." Curr Opin Immunol **17**(4): 338-344.
Kawai, T. and S. Akira (2007). "TLR signaling." Semin Immunol **19**(1): 24-32.
Kawai, T. and S. Akira (2011). "Toll-like receptors and their crosstalk with other innate receptors in infection and immunity." Immunity **34**(5): 637-650.
Kearney, J. F., E. Wall, et al. (2004). "Inbreeding trends and application of optimized selection in the UK Holstein population." J Dairy Sci **87**(10): 3503-3509.
Kingwill, R. G., F. K. Neave, et al. (1970). "The effect of a mastitis control system on levels of subclinical and clinical mastitis in two years." Vet Rec **87**(4): 94-100.
Kitchen, B. J. (1981). "Review of the progress of dairy science: bovine mastitis: milk compositional changes and related diagnostic tests." J Dairy Res **48**(1): 167-188.
Klungland, H., A. Sabry, et al. (2001). "Quantitative trait loci affecting clinical mastitis and somatic cell count in dairy cattle." Mamm Genome **12**(11): 837-842.
Kobayashi, Y. (2008). "The role of chemokines in neutrophil biology." Front Biosci **13**: 2400-2407.
Kobe, B. and J. Deisenhofer (1995). "Proteins with leucine-rich repeats." Curr Opin Struct Biol **5**(3): 409-416.
Kobe, B. and A. V. Kajava (2001). "The leucine-rich repeat as a protein recognition motif." Curr Opin Struct Biol **11**(6): 725-732.
Koeck, A., F. Miglior, et al. (2012). "Alternative somatic cell count traits to improve mastitis resistance in Canadian Holsteins." J Dairy Sci **95**(1): 432-439.
Kormann, M. S., A. Hector, et al. (2011). "CXCR1 and CXCR2 haplotypes synergistically modulate cystic fibrosis lung disease." Eur Respir J.
Kossaibati, M. A. (2000). "The costs of clinical mastitis in UK dairy herds." Cattle Practice **8**: 323-328.
Kossaibati, M. A., M. Hovi, et al. (1998). "Incidence of clinical mastitis in dairy herds in England." Vet Rec **143**(24): 649-653.
Kovach, N. L., E. Yee, et al. (1990). "Lipid IVA inhibits synthesis and release of tumor necrosis factor induced by lipopolysaccharide in human whole blood ex vivo." J Exp Med **172**(1): 77-84.
Kovacs-Simon, A., R. W. Titball, et al. (2011). "Lipoproteins of bacterial pathogens." Infect Immun **79**(2): 548-561.

Kurokawa, K., H. Lee, et al. (2009). "The Triacylated ATP Binding Cluster Transporter Substrate-binding Lipoprotein of Staphylococcus aureus Functions as a Native Ligand for Toll-like Receptor 2." J Biol Chem **284**(13): 8406-8411.
Kurt-Jones, E. A., L. Popova, et al. (2000). "Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus." Nat Immunol **1**(5): 398-401.
Kwong, L. S., J. C. Hope, et al. (2002). "Development of an ELISA for bovine IL-10." Vet Immunol Immunopathol **85**(3-4): 213-223.
Lahouassa, H., P. Rainard, et al. (2008). "Identification and characterization of a new interleukin-8 receptor in bovine species." Mol Immunol **45**(4): 1153-1164.
Larsson, L., P. Johansson, et al. (2009). "The Sp1 transcription factor binds to the G-allele of the -1087 IL-10 gene polymorphism and enhances transcriptional activation." Genes Immun **10**(3): 280-284.
Lazarus, R., B. A. Raby, et al. (2004). "TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples." Am J Respir Crit Care Med **170**(6): 594-600.
Lee, C. S., F. B. Wooding, et al. (1980). "Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum and milk from normal cows." J Dairy Res **47**(1): 39-50.
Lee, J. W., D. D. Bannerman, et al. (2006). "Characterization of cytokine expression in milk somatic cells during intramammary infections with Escherichia coli or Staphylococcus aureus by real-time PCR." Vet Res **37**(2): 219-229.
Leigh, J. A. (1999). "Streptococcus uberis: a permanent barrier to the control of bovine mastitis?" Vet J **157**(3): 225-238.
Leigh, J. A., P. N. Ward, et al. (2004). "The exploitation of the genome in the search for determinants of virulence in Streptococcus uberis." Vet Immunol Immunopathol **100**(3-4): 145-149.
Lemaitre, B., E. Kromer-Metzger, et al. (1995). "A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense." Proc Natl Acad Sci U S A **92**(21): 9465-9469.
Leyva-Baca, I., F. Schenkel, et al. (2008). "Polymorphisms in the 5' upstream region of the CXCR1 chemokine receptor gene, and their association with somatic cell score in Holstein cattle in Canada." J Dairy Sci **91**(1): 407-417.
Li, C., W. Shi, et al. (2009). "Polymorphisms of *TLR1* Gene and Their Relationship with Somatic Cell Score in Holstein Cows." Scientia Agricultura Sinica **42 (6)**: 2118-2125.
Liang, Y., Y. Zhou, et al. (2004). "NF-kappaB and its regulation on the immune system." Cell Mol Immunol **1**(5): 343-350.
Liu, L., I. Botos, et al. (2008). "Structural basis of toll-like receptor 3 signaling with double-stranded RNA." Science **320**(5874): 379-381.
Liu, Y., X. Qin, et al. (2009). "Bos taurus genome assembly." BMC Genomics **10**: 180.
Liu, Y., S. Yang, et al. (2005). "Molecular evolution of CXCR1, a G protein-coupled receptor involved in signal transduction of neutrophils." J Mol Evol **61**(5): 691-696.
Lopez-Benavides, M. G., J. H. Williamson, et al. (2007). "Field observations on the variation of Streptococcus uberis populations in a pasture-based dairy farm." J Dairy Sci **90**(12): 5558-5566.
Manavalan, B., S. Basith, et al. (2011). "Similar Structures but Different Roles - An Updated Perspective on TLR Structures." Front Physiol **2**: 41.
Mantovani, A. (1999). "The chemokine system: redundancy for robust outputs." Immunol Today **20**(6): 254-257.
Mantovani, A., B. Savino, et al. (2010). "The chemokine system in cancer biology and therapy." Cytokine Growth Factor Rev **21**(1): 27-39.
Matsushima, N., T. Tanaka, et al. (2007). "Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors." BMC Genomics **8**: 124.
Matukumalli, L. K., C. T. Lawley, et al. (2009). "Development and characterization of a high density SNP genotyping assay for cattle." PLoS One **4**(4): e5350.
McGuire, K., M. Jones, et al. (2006). "Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors." Anim Genet **37**(1): 47-50.

Meade, K. G., G. M. O'Gorman, et al. (2012). "Functional characterisation of bovine interleukin 8 promoter haplotypes in vitro." Mol Immunol **50**(1-2): 108-116.
Medzhitov, R. (2001). "Toll-like receptors and innate immunity." Nat Rev Immunol **1**(2): 135-145.
Medzhitov, R., P. Preston-Hurlburt, et al. (1997). "A human homologue of the Drosophila Toll protein signals activation of adaptive immunity." Nature **388**(6640): 394-397.
Menzies, M. and A. Ingham (2006). "Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues." Vet Immunol Immunopathol **109**(1-2): 23-30.
Misch, E. A. and T. R. Hawn (2008). "Toll-like receptor polymorphisms and susceptibility to human disease." Clin Sci (Lond) **114**(5): 347-360.
Misch, E. A., M. Macdonald, et al. (2008). "Human TLR1 deficiency is associated with impaired mycobacterial signaling and protection from leprosy reversal reaction." PLoS Negl Trop Dis **2**(5): e231.
Mockenhaupt, F. P., J. P. Cramer, et al. (2006). "Toll-like receptor (TLR) polymorphisms in African children: Common TLR-4 variants predispose to severe malaria." Proc Natl Acad Sci U S A **103**(1): 177-182.
Moser, B. and L. Ebert (2003). "Lymphocyte traffic control by chemokines: follicular B helper T cells." Immunol Lett **85**(2): 105-112.
Moser, B. and K. Willimann (2004). "Chemokines: role in inflammation and immune surveillance." Ann Rheum Dis **63 Suppl 2**: ii84-ii89.
Moyes, K. M., J. K. Drackley, et al. (2010). "Greater expression of TLR2, TLR4, and IL6 due to negative energy balance is associated with lower expression of HLA-DRA and HLA-A in bovine blood neutrophils after intramammary mastitis challenge with Streptococcus uberis." Funct Integr Genomics **10**(1): 53-61.
Moyes, K. M., J. K. Drackley, et al. (2010). "Mammary gene expression profiles during an intramammary challenge reveal potential mechanisms linking negative energy balance with impaired immune response." Physiol Genomics **41**(2): 161-170.
Mucha, R., M. R. Bhide, et al. (2009). "Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to Mycobacterium avium subsp. paratuberculosis infection in cattle." Vet Immunol Immunopathol **128**(4): 381-388.
Murdoch, C. and A. Finn (2000). "Chemokine receptors and their role in inflammation and infectious diseases." Blood **95**(10): 3032-3043.
Murphy, M. A., M. R. Shariflou, et al. (2002). "High quality genomic DNA extraction from large milk samples." J Dairy Res **69**(4): 645-649.
Ogorevc, J., T. Kunej, et al. (2009). "Database of cattle candidate genes and genetic markers for milk production and mastitis." Anim Genet **40**(6): 832-851.
Oliver, S. P., B. M. Jayarao, et al. (2005). "Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications." Foodborne Pathog Dis **2**(2): 115-129.
Olson, T. S. and K. Ley (2002). "Chemokines and chemokine receptors in leukocyte trafficking." Am J Physiol Regul Integr Comp Physiol **283**(1): R7-28.
Omueti, K. O., J. M. Beyer, et al. (2005). "Domain exchange between human toll-like receptors 1 and 6 reveals a region required for lipopeptide discrimination." J Biol Chem **280**(44): 36616-36625.
Opsal, M. A., S. Lien, et al. (2008). "Association analysis of the constructed linkage maps covering TLR2 and TLR4 with clinical mastitis in Norwegian Red cattle." J Anim Breed Genet **125**(2): 110-118.
Opsal, M. A., D. I. Vage, et al. (2006). "Genomic organization and transcript profiling of the bovine toll-like receptor gene cluster TLR6-TLR1-TLR10." Gene **384**: 45-50.
Oviedo-Boyso, J., J. J. Valdez-Alarcon, et al. (2007). "Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis." J Infect **54**(4): 399-409.
Paape, M. J., D. D. Bannerman, et al. (2003). "The bovine neutrophil: Structure and function in blood and milk." Vet Res **34**(5): 597-627.
Pal, A., A. Sharma, et al. (2011). "Molecular Characterization and SNP Detection of CD14 Gene of Crossbred Cattle." Mol Biol Int **2011**: 507346.

Pant, S. D., F. S. Schenkel, et al. (2008). "Identification of polymorphisms in bovine TLR2 and CARD15,associations between CARD15 polymorphisms and milk somatic cell score in Canadian Holsteins, and functional relevance of SNP c.3020A>T." Dev Biol (Basel) **132**: 247-253.
Park, B. S., D. H. Song, et al. (2009). "The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex." Nature **458**(7242): 1191-1195.
Park, S. H., F. Casagrande, et al. (2011). "Interactions of interleukin-8 with the human chemokine receptor CXCR1 in phospholipid bilayers by NMR spectroscopy." J Mol Biol **414**(2): 194-203.
Parker, L. C., M. K. Whyte, et al. (2005). "The expression and roles of Toll-like receptors in the biology of the human neutrophil." J Leukoc Biol **77**(6): 886-892.
Partipilo, G., P. D'Addabbo, et al. (2011). "Refinement of Bos taurus sequence assembly based on BAC-FISH experiments." BMC Genomics **12**: 639.
Pasare, C. and R. Medzhitov (2005). "Toll-like receptors: linking innate and adaptive immunity." Adv Exp Med Biol **560**: 11-18.
Paulrud, C. O. (2005). "Basic concepts of the bovine teat canal." Vet Res Commun **29**(3): 215-245.
Petrkova, J., Z. Cermakova, et al. (2003). "CC chemokine receptor (CCR)2 polymorphism in Czech patients with myocardial infarction." Immunol Lett **88**(1): 53-55.
Pighetti, G. M., C. J. Kojima, et al. (2012). "The bovine CXCR1 gene is highly polymorphic." Vet Immunol Immunopathol **145**(1-2): 464-470.
Pighetti, G. M. and M. Rambeaud (2006). "Genome conservation between the bovine and human interleukin-8 receptor complex: improper annotation of bovine interleukin-8 receptor b identified." Vet Immunol Immunopathol **114**(3-4): 335-340.
Plantinga, T. S., W. J. van der Velden, et al. (2009). "Early stop polymorphism in human DECTIN-1 is associated with increased candida colonization in hematopoietic stem cell transplant recipients." Clin Infect Dis **49**(5): 724-732.
Porcherie, A., P. Cunha, et al. (2012). "Repertoire of Escherichia coli agonists sensed by innate immunity receptors of the bovine udder and mammary epithelial cells." Vet Res **43**(1): 14.
Prenafeta, A., R. March, et al. (2010). "Study of the humoral immunological response after vaccination with a Staphylococcus aureus biofilm-embedded bacterin in dairy cows: possible role of the exopolysaccharide specific antibody production in the protection from Staphylococcus aureus induced mastitis." Vet Immunol Immunopathol **134**(3-4): 208-217.
Qiu, Y., J. Zhu, et al. (2003). "Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease." Am J Respir Crit Care Med **168**(8): 968-975.
Rainard, P. and C. Riollet (2006). "Innate immunity of the bovine mammary gland." Vet Res **37**(3): 369-400.
Rambeaud, M., R. Clift, et al. (2006). "Association of a bovine CXCR2 gene polymorphism with neutrophil survival and killing ability." Vet Immunol Immunopathol **111**(3-4): 231-238.
Rambeaud, M. and G. M. Pighetti (2005). "Impaired neutrophil migration associated with specific bovine CXCR2 genotypes." Infect Immun **73**(8): 4955-4959.
Rambeaud, M. and G. M. Pighetti (2007). "Differential calcium signaling in dairy cows with specific CXCR1 genotypes potentially related to interleukin-8 receptor functionality." Immunogenetics **59**(1): 53-58.
Randhawa, A. K., M. S. Shey, et al. (2011). "Association of human TLR1 and TLR6 deficiency with altered immune responses to BCG vaccination in South African infants." PLoS Pathog **7**(8): e1002174.
Randolph, H. E. and R. E. Erwin (1974). "Influence of mastitis on properties of milk. X. Fatty acid composition." J Dairy Sci **57**(8): 865-868.
Rinaldi, M., R. W. Li, et al. (2010). "A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to E. coli mastitis." Funct Integr Genomics **10**(1): 21-38.
Rinaldi, M., R. W. Li, et al. (2010a). "Mastitis associated transcriptomic disruptions in cattle." Vet Immunol Immunopathol **138**(4): 267-279.

Riollet, C., P. Rainard, et al. (2001). "Cell subpopulations and cytokine expression in cow milk in response to chronic Staphylococcus aureus infection." J Dairy Sci **84**(5): 1077-1084.
Ritter, M., D. Mennerich, et al. (2005). "Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response." J Inflamm (Lond) **2**: 16.
Rivas, A. L., S. J. Schwager, et al. (2007). "Multifactorial relationships between intramammary invasion by Staphylococcus aureus and bovine leukocyte markers." Can J Vet Res **71**(2): 135-144.
Roger, T., I. Miconnet, et al. (2005). "Critical role for Ets, AP-1 and GATA-like transcription factors in regulating mouse Toll-like receptor 4 (Tlr4) gene expression." Biochem J **387**(2): 355-365.
Rosetto, M., Y. Engstrom, et al. (1995). "Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line." Biochem Biophys Res Commun **209**(1): 111-116.
Rozen, S. and H. Skaletsky (2000). "Primer3 on the WWW for general users and for biologist programmers." Methods Mol Biol **132**: 365-386.
Ruiz-Larranaga, O., C. Manzano, et al. (2011). "Genetic variation of toll-like receptor genes and infection by Mycobacterium avium ssp. paratuberculosis in Holstein-Friesian cattle." J Dairy Sci **94**(7): 3635-3641.
Rupp, R. and D. Boichard (1999). "Genetic parameters for clinical mastitis, somatic cell score, production, udder type traits, and milking ease in first lactation Holsteins." J Dairy Sci **82**(10): 2198-2204.
Russell, C. D., S. Widdison, et al. (2012). "Identification of single nucleotide polymorphisms in the bovine Toll-like receptor 1 gene and association with health traits in cattle." Vet Res **43**(1): 17.
Samarasinghe, R., P. Tailor, et al. (2006). "Induction of an anti-inflammatory cytokine, IL-10, in dendritic cells after toll-like receptor signaling." J Interferon Cytokine Res **26**(12): 893-900.
Savill, J. S., A. H. Wyllie, et al. (1989). "Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages." J Clin Invest **83**(3): 865-875.
Schepers, A. J., T. J. Lam, et al. (1997). "Estimation of variance components for somatic cell counts to determine thresholds for uninfected quarters." J Dairy Sci **80**(8): 1833-1840.
Schroder, N. W. and R. R. Schumann (2005). "Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease." Lancet Infect Dis **5**(3): 156-164.
Schultze, W. D. and S. C. Bright (1983). "Changes in penetrability of bovine papillary duct to endotoxin after milking." Am J Vet Res **44**(12): 2373-2375.
Seabury, C. M., E. J. Cargill, et al. (2007). "Sequence variability and protein domain architectures for bovine Toll-like receptors 1, 5, and 10." Genomics **90**(4): 502-515.
Seabury, C. M. and J. E. Womack (2008). "Analysis of sequence variability and protein domain architectures for bovine peptidoglycan recognition protein 1 and Toll-like receptors 2 and 6." Genomics **92**(4): 235-245.
Segerer, S., A. Henger, et al. (2006). "Expression of the chemokine receptor CXCR1 in human glomerular diseases." Kidney Int **69**(10): 1765-1773.
Seykora, A. J. and B. T. McDaniel (1985). "Udder and teat morphology related to mastitis resistance: a review." J Dairy Sci **68**(8): 2087-2093.
Sharma, B. S., I. Leyva, et al. (2006). "Association of toll-like receptor 4 polymorphisms with somatic cell score and lactation persistency in Holstein bulls." J Dairy Sci **89**(9): 3626-3635.
Sharma, B. S., J. Mount, et al. (2008). "Functional characterization of a single nucleotide polymorphism in the 5' UTR region of the bovine toll-like receptor 4 gene." Dev Biol (Basel) **132**: 331-336.
Shimada, K., Y. Watanabe, et al. (2000). "Common polymorphism in the promoter of the CD14 monocyte receptor gene is associated with acute myocardial infarction in Japanese men." Am J Cardiol **86**(6): 682-684, A688.
Shimazu, R., S. Akashi, et al. (1999). "MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4." J Exp Med **189**(11): 1777-1782.

Shook, G. E. (2006). "Major advances in determining appropriate selection goals." J Dairy Sci **89**(4): 1349-1361.
Simianer, H., H. Solbu, et al. (1991). "Estimated genetic correlations between disease and yield traits in dairy cattle." J Dairy Sci **74**(12): 4358-4365.
Skelding, A., F. S. Schenkel, et al. (2010). "Identification of single nucleotide polymorphisms in the bovine interleukin-12 and interleukin-23 receptor genes and their associations with health and production traits in Holstein cows." J Dairy Sci **93**(10): 4860-4871.
Sladek, Z. and D. Rysanek (2011). "Cell death and CD14 expression in resident and inflammatory polymorphonuclear leukocytes from virgin bovine mammary gland." Res Vet Sci **90**(2): 226-234.
Smit, L. A., V. Siroux, et al. (2009). "CD14 and toll-like receptor gene polymorphisms, country living, and asthma in adults." Am J Respir Crit Care Med **179**(5): 363-368.
Sodeland, M., M. P. Kent, et al. (2011). "Quantitative trait loci for clinical mastitis on chromosomes 2, 6, 14 and 20 in Norwegian Red cattle." Anim Genet **42**(5): 457-465.
Soltys, J. and M. T. Quinn (1999). "Selective recruitment of T-cell subsets to the udder during staphylococcal and streptococcal mastitis: analysis of lymphocyte subsets and adhesion molecule expression." Infect Immun **67**(12): 6293-6302.
Song, C., L. Z. Chen, et al. (2006). "Functional variant in the 3'-untranslated region of Toll-like receptor 4 is associated with nasopharyngeal carcinoma risk." Cancer Biol Ther **5**(10): 1285-1291.
Sordillo, L. M., K. Shafer-Weaver, et al. (1997). "Immunobiology of the mammary gland." J Dairy Sci **80**(8): 1851-1865.
Spagnolo, P., E. A. Renzoni, et al. (2003). "C-C chemokine receptor 2 and sarcoidosis: association with Lofgren's syndrome." Am J Respir Crit Care Med **168**(10): 1162-1166.
Steinke, J. W., E. Barekzi, et al. (2004). "Functional analysis of -571 IL-10 promoter polymorphism reveals a repressor element controlled by sp1." J Immunol **173**(5): 3215-3222.
Stothard, P., J. W. Choi, et al. (2011). "Whole genome resequencing of black Angus and Holstein cattle for SNP and CNV discovery." BMC Genomics **12**: 559.
Su, G., B. Guldbrandtsen, et al. (2010). "Preliminary investigation on reliability of genomic estimated breeding values in the Danish Holstein population." J Dairy Sci **93**(3): 1175-1183.
Sun, J., F. Wiklund, et al. (2005). "Sequence variants in Toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk." J Natl Cancer Inst **97**(7): 525-532.
Sun, L., Y. Song, et al. (2012). "Polymorphisms in toll-like receptor 1 and 9 genes and their association with tuberculosis susceptibility in Chinese Holstein cattle." Vet Immunol Immunopathol **147**(3-4): 195-201.
Suriyasathaporn, W., Y. H. Schukken, et al. (2000). "Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd." J Dairy Sci **83**(6): 1248-1255.
Swanson, K. M., K. Stelwagen, et al. (2009). "Transcriptome profiling of Streptococcus uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model." J Dairy Sci **92**(1): 117-129.
Tabachnick, B. G. and L. S. Fidell (2007). Using multivariate statistics (5th ed.) Boston, Allyn and Bacon.
Takeuchi, O., S. Sato, et al. (2002). "Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins." J Immunol **169**(1): 10-14.
Tanimura, N., S. Saitoh, et al. (2008). "Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling." Biochem Biophys Res Commun **368**(1): 94-99.
Tenhagen, B. A., I. Hansen, et al. (2009). "Prevalence of pathogens in milk samples of dairy cows with clinical mastitis and in heifers at first parturition." J Dairy Res **76**(2): 179-187.
Threadgill, D. W. and J. E. Womack (1990). "Genomic analysis of the major bovine milk protein genes." Nucleic Acids Res **18**(23): 6935-6942.
Tikhonov, I., T. Doroshenko, et al. (2001). "Down-regulation of CXCR1 and CXCR2 expression on human neutrophils upon activation of whole blood by S. aureus is mediated by TNF-alpha." Clin Exp Immunol **125**(3): 414-422.

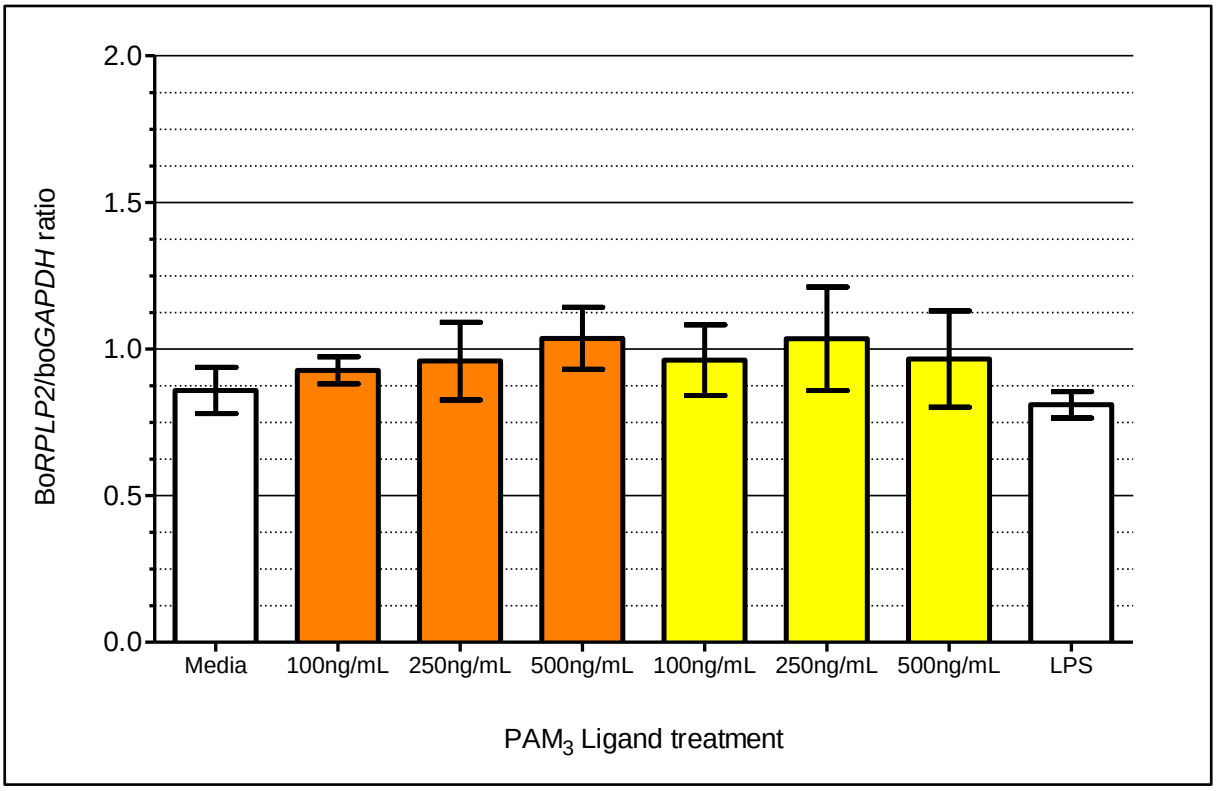
Tsuchida, S., Y. Yamad, et al. (2010). "Distribution of single nucleotide polymorphisms in the CXCR1 gene and association with calf diseases in Japanese Black cattle." J Vet Med Sci **72**(12): 1609-1614.
Vainas, T., F. R. Stassen, et al. (2006). "Synergistic effect of Toll-like receptor 4 and CD14 polymorphisms on the total atherosclerosis burden in patients with peripheral arterial disease." J Vasc Surg **44**(2): 326-332.
Vanderhaeghen, W., T. Cerpentier, et al. (2010). "Methicillin-resistant Staphylococcus aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows." Vet Microbiol **144**(1-2): 166-171.
Verschoor, C. P., S. D. Pant, et al. (2009). "SNPs in the bovine IL-10 receptor are associated with somatic cell score in Canadian dairy bulls." Mamm Genome **20**(7): 447-454.
Viguier, C., S. Arora, et al. (2009). "Mastitis detection: current trends and future perspectives." Trends Biotechnol **27**(8): 486-493.
Waage, S., S. Sviland, et al. (1998). "Identification of risk factors for clinical mastitis in dairy heifers." J Dairy Sci **81**(5): 1275-1284.
Wang, X., S. Xu, et al. (2007). "Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle." J Genet Genomics **34**(5): 406-412.
Wang, Z., M. Gerstein, et al. (2009). "RNA-Seq: a revolutionary tool for transcriptomics." Nat Rev Genet **10**(1): 57-63.
Watts, J. L. (1988). "Etiological agents of bovine mastitis." Vet Microbiol **16**(1): 41-66.
Werling, D., R. A. Collins, et al. (2002). "Cytokine responses of bovine dendritic cells and T cells following exposure to live or inactivated bovine respiratory syncytial virus." J Leukoc Biol **72**(2): 297-304.
Werling, D., O. C. Jann, et al. (2009). "Variation matters: TLR structure and species-specific pathogen recognition." Trends Immunol **30**(3): 124-130.
Werling, D. and T. W. Jungi (2003). "TOLL-like receptors linking innate and adaptive immune response." Vet Immunol Immunopathol **91**(1): 1-12.
White, S. N., K. H. Taylor, et al. (2003). "Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain." Proc Natl Acad Sci U S A **100**(18): 10364-10369.
Widdison, S. and T. J. Coffey (2011). "Cattle and chemokines: evidence for species-specific evolution of the bovine chemokine system." Anim Genet **42**(4): 341-353.
Widdison, S., N. Siddiqui, et al. (2010). "The bovine chemokine receptors and their mRNA abundance in mononuclear phagocytes." BMC Genomics **11**: 439.
Widdison, S., M. Watson, et al. (2009). "Correlation between lymph node pathology and chemokine expression during bovine tuberculosis." Tuberculosis (Edinb) **89**(6): 417-422.
Widdison, S., M. Watson, et al. (2011). "Early response of bovine alveolar macrophages to infection with live and heat-killed Mycobacterium bovis." Developmental and Comparative Immunology **35**(5): 580-591.
Widdison, S., M. Watson, et al. (2008). "Granulocyte chemotactic properties of M. tuberculosis versus M. bovis-infected bovine alveolar macrophages." Mol Immunol **45**(3): 740-749.
Wurfel, M. M., A. C. Gordon, et al. (2008). "Toll-like receptor 1 polymorphisms affect innate immune responses and outcomes in sepsis." Am J Respir Crit Care Med **178**(7): 710-720.
Xu, J., A. Turner, et al. (2002). "Positive results in association studies are associated with departure from Hardy-Weinberg equilibrium: hint for genotyping error?" Hum Genet **111**(6): 573-574.
Yang, W., H. Zerbe, et al. (2008). "Bovine TLR2 and TLR4 properly transduce signals from Staphylococcus aureus and E. coli, but S. aureus fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (CXCL8) expression in the udder." Mol Immunol **45**(5): 1385-1397.
Youngerman, S. M., A. M. Saxton, et al. (2004a). "Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle." J Dairy Sci **87**(8): 2442-2448.
Youngerman, S. M., A. M. Saxton, et al. (2004b). "Novel single nucleotide polymorphisms and haplotypes within the bovine CXCR2 gene." Immunogenetics **56**(5): 355-359.
Zanoni, I., R. Ostuni, et al. (2011). "CD14 controls the LPS-induced endocytosis of Toll-like receptor 4." Cell **147**(4): 868-880.

Zenger, K. R., M. S. Khatkar, et al. (2007). "Genome-wide genetic diversity of Holstein Friesian cattle reveals new insights into Australian and global population variability, including impact of selection." Anim Genet **38**(1): 7-14.

Zhao, X. and P. Lacasse (2008). "Mammary tissue damage during bovine mastitis: causes and control." J Anim Sci **86**(13 Suppl): 57-65.

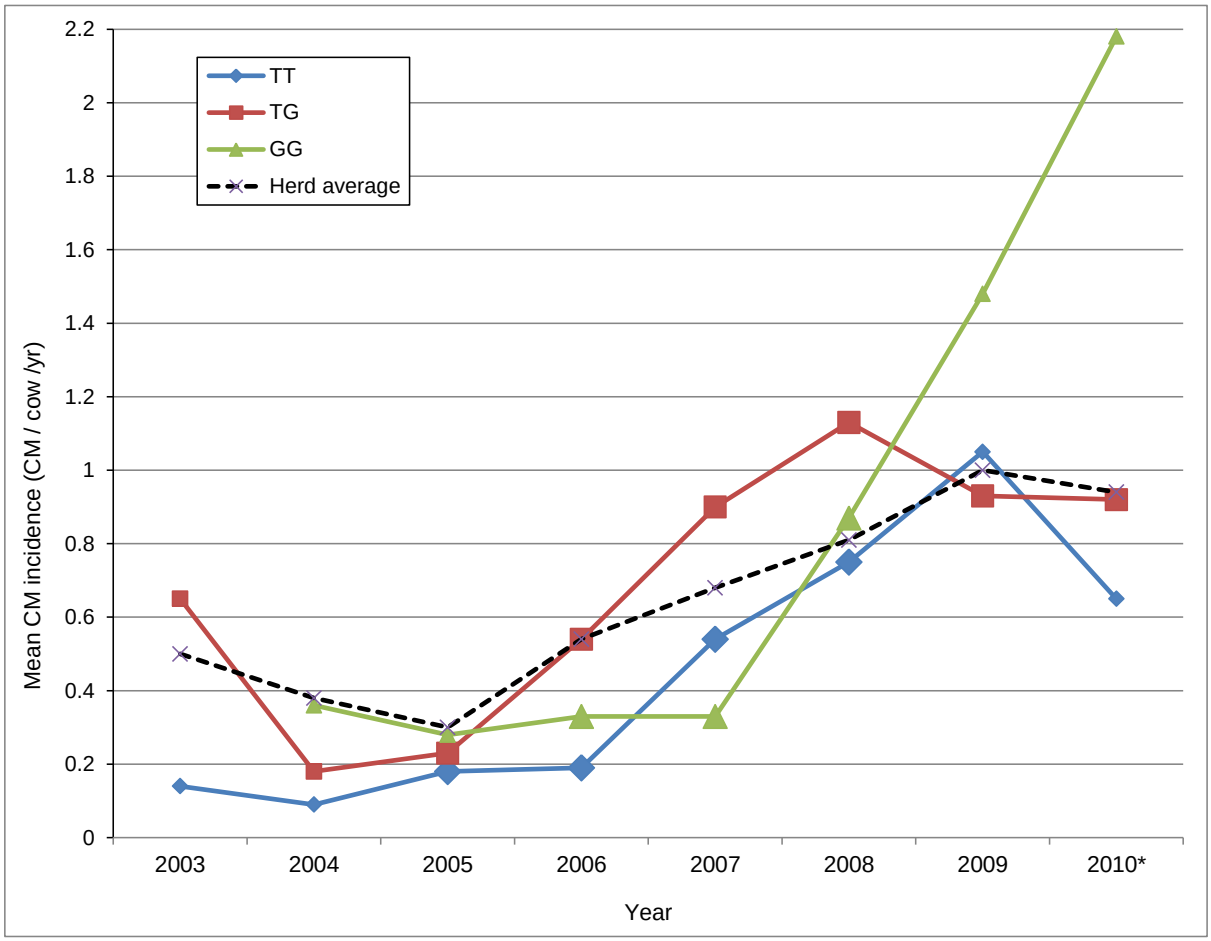
Ziegler-Heitbrock, L., P. Ancuta, et al. (2010). "Nomenclature of monocytes and dendritic cells in blood." Blood **116**(16): e74-80.

Appendix I - Stability of bo*RPLP2*/bo*GAPDH* expression ratios in PMNs exposed to TLR ligand



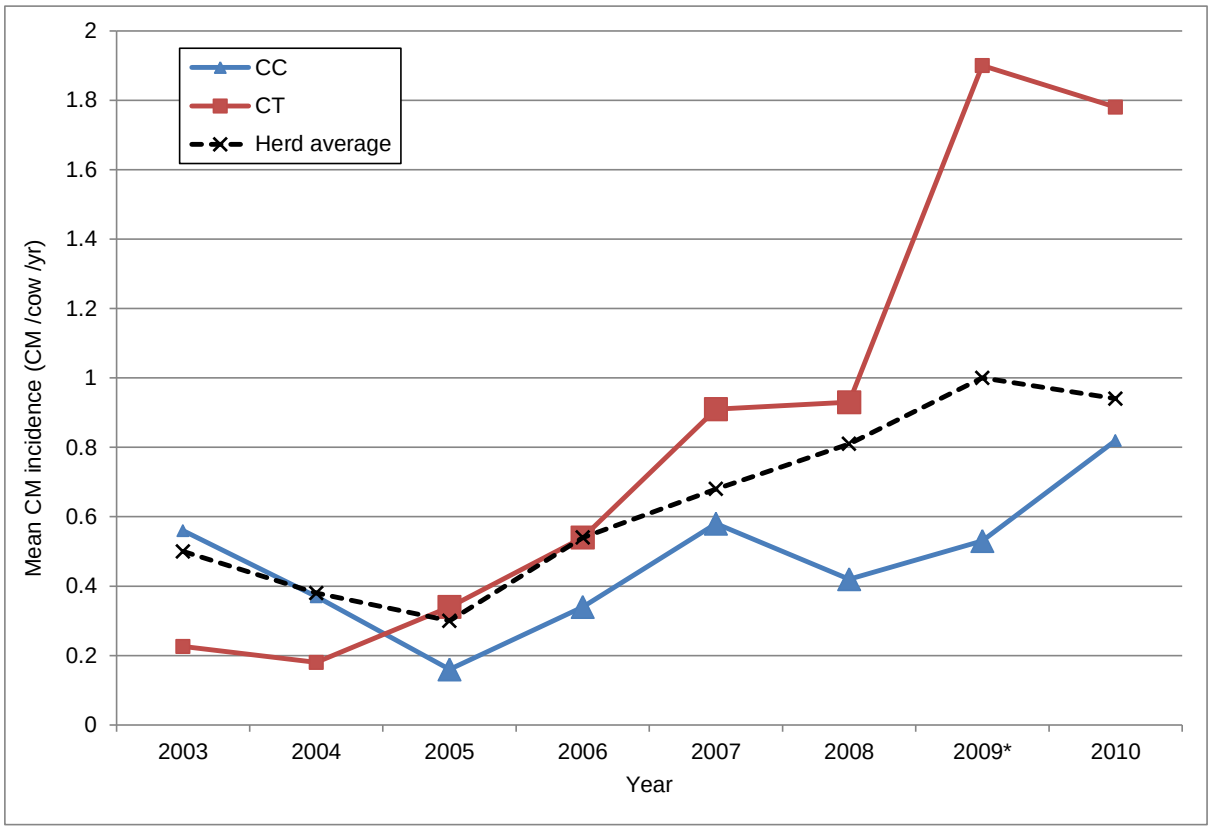
Each column represents the data mean for bo*RPLP2*/bo*GAPDH* gene expression ratios from animal samples (*n* =12) used for Q-PCR assays. Orange columns = 4 h post-stimulation data, yellow columns = 8 h post-stimulation data. Error bars = SEM. No significant differences between columns detected using one-way ANOVA with Tukey's post-test.

Appendix II - Genotype trends for yearly CM incidence (2003-2010) between bo*TLR1* - 79T>G TT, TG and GG cows



Large marker points represent data mean from >30 lactations.*Significance detected (P<0.05 by ANOVA and Tukey's multiple comparisons) for that year between GG cows tested against both TG and TT cows.

Appendix III - Genotype trends for yearly CM incidence (2003-2010) between bo*CXCR1* -205C>T CC and CT cows



Large marker points represent data mean from >20 lactations.*Significance detected ($P<0.05$ by unpaired t test) for that year between genotypes cows.