Polymorphism screening and association study of CXCR1 (IL8RA) with udder health and milk production of dairy heifers

by

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Research project in the context of the master thesis
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ABSTRACT

Mastitis is considered as the most important disease in dairy and occurs both in heifers and pluriparous cows. Selection against mastitis susceptible animals might be an elegant way of controlling it. Therefore, genetic markers associated with udder health are searched for. Innate immune responses and neutrophil migration from peripheral blood to the mammary gland, in specific, form the first line of defence against invading pathogens. Interleukin 8 (IL8) is the main chemoattractant in this process and binds on two receptors, namely CXCR1 and CXCR2. Polymorphisms in CXCR1 have been described making it a potential candidate gene for selection. The objective of this research was to identify polymorphisms in the whole coding region of CXCR1 in a Belgian dairy heifers population and to verify whether or not they were associated with udder health and milk production during first lactation. In total, 140 heifers from 20 dairy herds were genotyped. Data on intramammary infection (IMI) status in early lactation, test-day SCC and milk production were available. Almost 75% of the heifers had and IMI in at least one quarter with coagulase-negative staphylococci (CNS) causing the majority of the infections.

Twenty single nucleotide polymorphisms (SNP) were identified from which 15 have not been described in literature. Half of these mutations cause a change in amino acid and are non-synonymous polymorphisms. A significant association between the SNP at position 735 relative to the start codon and subclinical mastitis had already been described and therefore focus was put on this SNP in the statistical analysis. No associations were detected between the SNP and natural log of the SCC (LnSCC) and milk production. Heifers expressing genotype GG were, however, at a lower risk of having IMI (OR = 0.196, 95% CI = 0.03-1.09) around calving compared to heifers expressing the genotype GC or CC. Differences were borderline non-significant (P = 0.063,). The results suggest a pathogen-specificity in the altered mastitis susceptibility caused by this mutation. Small differences were present in prevalence of IMI caused by major pathogens between heifers expressing the different genotype whereas a trend (P = 0.055) towards lower odds of IMI caused by CNS was detected in heifers expressing the genotype GG compared to heifers expressing genotype GC or CC. It looks as of heifers of the GG genotype are less susceptible to CNS, known as minor pathogens inducing only moderate changes in SCC. This might explain the difficulties in finding an association between SNP 735 and SCC. More research is required to confirm this hypothesis.
I. INTRODUCTION

Worldwide, on average 46 kg of milk is consumed per capita per year whereas this is 55 kg per capita/year in Belgium and Luxemburg, (Speedy, 2003). In order to reach those required large amounts of milk, many efforts have been done over the last decades to increase the average daily milk yield per cow. The eventually higher milk yields per cow were realized through intense genetic selection as well as improved cow nutrition and management. Of course, to meet those higher requirements the cow should dispose of a healthy and well-developed udder.

Still, one of the most common diseases in dairy cattle is mastitis. The inflammatory reaction primarily occurs in response to intramammary infections (IMI). These IMI are mostly caused by bacteria and can largely affect milk yield (Seegers et al., 2003). Besides causing production losses, mastitis also leads to a higher culling rate, additional costs for the veterinarian and drugs, necessity of discarding milk, and extra labour complicating farming in general. The total economic cost of mastitis was recently estimated at € 65-182/cow per year on an average Dutch dairy farm (Huijps et al., 2008). For at least a century research has been done on bovine mastitis (e.g. Delépine, 1910) and many articles were published on the subject. Unfortunately, focus was entirely put on multiparous cows, and udder health of heifers (primiparous cows) has long been neglected. Since 1980, many prevalence studies on heifer mastitis have been conducted all concluding that not only older cows but a high proportion of heifers as well suffer from mastitis, either clinical or subclinical, at first calving (Meaney, 1981; Oliver et al., 1983; Boddie et al., 1987; Fox et al., 1995; Nickerson et al., 1995). This was in sharp contradiction with the common believe that heifers’ udders were sterile before first milking. Remarkably, milk production losses are not seen in all mastitis cases and heifers infected with coagulase-negative staphylococci (CNS) in early lactation were even found to out-produce healthy heifers (Piepers et al., 2010).

Mastitis is a multi-factorial disease and under equal management conditions, some heifers suffer from it while others remain healthy. Genetic make-up is considered as one of the main causes of this variance. Therefore, many research groups have tried to identify candidate genes with polymorphisms associated with mastitis resistance (Ogorevc et al., 2009). Most of these genes encode for proteins involved in the innate immunity. This is because innate immune responses lead to migration of large amount of neutrophils towards the infection site forming the first line of cellular defence against invading pathogens (Paape et al., 2000). The most crucial molecule for this migration from the peripheral blood to the mammary gland is interleukin 8 (IL8). Interleukin 8 has proven to be a strong chemoattractant for neutrophils both in vitro and in vivo (Caswell et al., 1999). Single nucleotide polymorphisms (SNP) were identified in the gene coding for CXCR1, one of the two receptors binding IL8 (Grosse et al., 1999; Youngerman et al., 2004; Leyva-Baca et al., 2008b). One SNP in the coding region (Youngerman et al., 2004) and one SNP in the 5’ upstream region (Leyva-Baca et al., 2004) of CXCR1 were found to be associated with mastitis susceptibility. However, these associations have been questioned (Goertz et al., 2009) and so far no papers have been published which confirm these findings in European cattle.

This study had two objectives. The first was to identify polymorphisms in the complete coding region of CXCR1 in Belgian dairy heifers. The second objective was to identify associations between these polymorphisms and a number of phenotypic parameters related to udder health and milk yield.
II. REVIEW OF LITERATURE

A. MASTITIS IN DAIRY HEIFERS

1. Intramammary infections in dairy heifers

In the previous century, many studies were performed on the prevalence of mastitis in lactating and dry cows and on how to prevent and control it. Until 1980 little was known on the prevalence and incidence of IMI in heifers. Heifers were thought to be free from the disease because their teats had not been challenged yet by the milking process which is considered one of the principal risk factors of mastitis (Fox, 2009). Nevertheless, dairy heifers can suffer from mastitis. From the 1980ies on, research made clear that both subclinical and clinical mastitis (CM) occurred more often in dairy heifers than previously assumed (Meaney, 1981; Oliver et al., 1983; Boddie et al., 1987; Fox et al., 1995; Nickerson et al., 1995). Several studies showed that the prevalence of heifer mastitis ranges between 28.9%-74.6% positive quarters prepartum, and 12.3%-45.5% positive quarters at parturition (Fox, 2009). In a study of De Vliegher et al. (2004) more than 27% of almost 15,000 Belgian dairy heifers had an elevated SCC in early lactation suggesting the presence of IMI in these heifers at calving.

Somatic cell count is often used to evaluate the udder health of both pluriparous cows and heifers. In case of mastitis, an increase in cells in milk, mainly consisting of leucocytes, is observed (Sarikaya et al., 2006). This elevation is correlated with an increased probability of presence of IMI (Dohoo et al., 1991). The threshold value of 100,000 cells/ml has been suggested to determine whether a quarter is healthy or not (Hillerton, 1999; Krömker et al., 2001). Because of practical and economical reasons, often composite milk samples are taken instead of quarter milk samples. In this case the threshold of 200,000 cells/ml, having a sensitivity of 73-89% and a specificity of 75-85% (Ruegg et al., 2002), is commonly used. After detecting an elevated SCC, confirmation of IMI and species identification can be performed through bacteriological culturing as described in the NMC laboratory handbook on bovine mastitis (Hogan et al., 1999).

The relevance of heifer mastitis was recently studied. De Vliegher et al. (2005a) found that dairy heifers with an elevated test-day SCC early in lactation (SCCel) had a significant loss in milk production in their first lactation. The risk of being culled was increased as well (De Vliegher et al., 2005b). In case of a high prevalence of heifer mastitis on his farm, the farmer will suffer severe economical losses caused by the decreased milk production, the higher rate of culling, additional costs for veterinarian and drugs, discarding milk during treatment period and waiting days and extra labour. Recently, Huijps et al. (2009) estimated the cost of heifer mastitis per heifer present on a farm in the Dutch/Belgian dairy sector at € 31, ranging from € 0 to € 220. However, it was recently suggested that the negative impact of heifer mastitis in early lactation for the heifers’ future performances depended on the pathogen that was involved (Kirk et al., 1996; Piepers et al., 2010).

Remarkably, clinical mastitis (CM) in early lactation occurs more often in heifers than in older cows (Barkema et al., 1998). Heifers suffering from clinical mastitis in early lactation can have high production losses (Gröhn et al., 2004). Furthermore, the risk of being culled for these heifers is highly elevated (Waage et al., 2000) and therefore production losses might be underestimated (Piepers et al., 2009).
2. Pathogens causing heifer mastitis

Intramammary infections in heifers are basically caused by the same pathogens as IMI in older cows (Fox, 2009). Mastitis causing pathogens are often grouped as major and minor pathogens (Table 1.). Major pathogens are considered to be more virulent, are more likely to cause clinical mastitis, and result in more pronounced milk yield losses (Timms and Schultz, 1987). Mastitis pathogens can also be classified as contagious (or “adapted”) or environmental (or “opportunistic”) pathogens, depending on their epidemiological behaviour.

<table>
<thead>
<tr>
<th>Minor pathogens</th>
<th>Major pathogens</th>
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<tbody>
<tr>
<td>Coagulase-negative staphylococci (CNS)</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Corynebacterium bovis</td>
<td>Streptococcus dysgalactiae</td>
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<td></td>
<td>Streptococcus agalactiae</td>
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<td></td>
<td>Streptococcus uberis</td>
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<td></td>
<td>Escherichia coli</td>
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<td></td>
<td>Klebsiella pneumoniae</td>
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<td>Arcanobacterium pyogenes</td>
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<td></td>
<td>Peptococcus indolicus</td>
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<td>Mycoplasma spp.</td>
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</table>

Table 1. Pathogens causing intramammary infections in heifers

(Reviewed in: Fox et al., 2009)

Coagulase-negative staphylococci (CNS) are the most frequently isolated pathogens from dairy heifers suffering from subclinical mastitis (Fox et al., 1995; Aarestrup and Jensen, 1997; Piepers et al., 2010). In many countries, they cause as a group the majority of IMI (Piepers et al., 2007; Schukken et al., 2009). Coagulase-negative staphylococci consist of a large number of species with up till now new species being discovered (Suprè et al., 2010). They are considered to be minor pathogens, inducing only a moderate increase in SCC and often causing transient infections (Timms and Schultz, 1987). Still, opinions are divided on their importance for bovine udder health. Recent studies even found higher milk yield in CNS-infected cows than in culture-negative cows (Schukken et al., 2009). Taponen et al. (2006) on the contrary claimed that CNS infections might be more harmful than assumed and that certain species can persist for a long time causing severe damage to the infected quarter.

Corynebacterium bovis is classified as a contagious pathogen (Fox et al., 1995) and is often isolated from cases of subclinical mastitis (Fox et al., 1995; Parker et al., 2007a). As C. bovis rarely causes clinical mastitis, has a low impact on SCC and usually cures spontaneously, it is considered as a minor pathogen (Honkanen-Buzalski et al., 1984). Some studies have reported protective effects of IMI caused by minor pathogens against IMI with major pathogens (Lam et al., 1997).

Unlike CNS and C. bovis, Staphylococcus aureus IMI often lead to strongly elevated SCC or clinical mastitis, even in dairy heifers (Waage et al., 1999). The milking process is considered to be the most important route of spreading from cow to cow (Bramley et al., 1984). Milk of infected cows and heifer body sites are believed to be the major sources for S. aureus IMI in heifers (Roberson et al., 1998).
Streptococcus dysgalactiae spreads from cow to cow but the environment can just as well be the source of infection. In a Norwegian study, primiparous cows (heifers) with an IMI caused by S. dysgalactiae produced 1.1 kg of milk per day less than culture-negative animals of the same age (Whist et al., 2007). In contrast to S. dysgalactiae which prevalence is even growing in some countries (Whist et al., 2007), Streptococcus agalactiae is nowadays rarely isolated from milk samples in Belgium and the Netherlands (Piepers et al., 2007; Sampimon et al., 2009). Due to the development and implementation of proper contagious mastitis control programs, S. agalactiae forms no longer a threat for the udder health of dairy cattle (Barkema et al., 2009).

Streptococcus uberis is a common major mastitis pathogen and although cow-to-cow transmission is described (Zadoks et al., 2003), the environment is likely to be the major infective source (McDougall et al., 2004).

Coliforms can cause severe clinical mastitis in heifers. In a Norwegian study performed by Waage et al. (1999) 6.7% of milk samples collected from quarters with clinical signs from heifers were positive for coliforms. Coliforms are considered to be environmental pathogens. Two of the more important members are Escherichia coli and Klebsiella pneumoniae (Hogan et al., 2003).

Arcanobacterium pyogenes and Peptococcus indolicus are frequently isolated from cases of “summer mastitis” (Shearer and Harmon, 1993). The latter pathogens can cause severe clinical mastitis in both dry cows and heifers on pasture and are spread by the fly Hydrotaea irritans (Yeomen and Warren, 1984). In the past, heifer mastitis and summer mastitis were often wrongly used as synonyms (Seno and Azuma, 1983).

Heifers as well as pluriparous cows can also suffer from mastitis caused by Mycoplasma species. The prevalence of Mycoplasma mastitis seems to increase in several countries (Fox et al., 2005). Furthermore, Mycoplasma mastitis might be under-diagnosed because identification of this group of pathogens requires 10 days of incubation under highly specific conditions (Fox et al., 2005). Diagnosing as well as successfully treating IMI caused by Mycoplasma is difficult. Mycoplasma spp. are hardly sensitive to antibiotics and therefore treatment is from an economical point of view not feasible (Bushnell, 1984). Mycoplama bovis is likely to be the most prevalent Mycoplasma species causing IMI and is highly contagious (González and Wilson, 2003).

3. Prevention of heifer mastitis

Heifer mastitis is a multi-factorial disease and studies have been conducted on how to prevent it. Several authors suggested prepartum antibiotic treatment as a way of controlling it. Oliver et al. (2003) showed that heifers treated with a lactating cow product prepartum had a higher milk production and a lower SCC than untreated heifers and claimed therefore that the economic benefit cannot be doubted. Sampimon et al. (2009) proved similar positive effects by using a dry cow product and concluded that prepartum treatment of dairy heifers can offer a temporarily solution on farms with heifer mastitis problems. However, the results on the positive effect of prepartum antibiotic treatment of heifers before calving and the heifers' milk production and udder health during first lactation are not conclusive yet. E.g., Borm et al. (2005) observed no significant effect of treatment on milk production or SCC and questioned the usefulness of prophylactic antibiotic therapy as a control measurement. One should also be aware of the disadvantages of prophylactic pre-calving treatment with antibiotics. Appropriate withholding times are not known and the risk of antibiotic residues in food for human consumption is elevated (Compton and McDougall, 2008). Additionally,
antimicrobial resistance can develop due to the latter practice (Rajala-Schultz et al., 2004). Besides antibiotic treatment, other treatments were suggested as well. For example, in a study in New Zealand, heifers under pastoral conditions sprayed with a commercial iodine-based teat sanitizer in the prepartum period were less likely to freshen with \textit{S. uberis} IMI in the peri-partum period compared to control animals (Lopez-Benavides et al., 2009). Parker et al. (2007) found a decreased prevalence of both subclinical and clinical mastitis in the first 2 weeks postpartum when a bismuth subnitrate teat-canal sealant was administered before calving. The efficacy of commercial vaccines with a potential protective effect against IMI with \textit{Staphylococcus aureus} and CNS is still under debate (Middleton et al., 2009).

All the latter measurements to prevent heifer mastitis require individual treatment and can be costly. Changes in heifer management to prevent IMI are to be preferred above tools to cure existing IMI. Dystocia and udder oedema are risk factors for heifer mastitis and therefore minimizing the incidence of these through optimization of the feeding and housing management can aid in the prevention of heifer mastitis (Svensson et al., 2006; Compton et al., 2007b). Flies may act as vectors for several mastitis pathogens (Chirico et al., 1997; Gillespie et al., 1999). Hence, fly control can reduce the incidence of mastitis in heifers and pluriparous cows (Nickerson et al., 1995; Edwards et al., 2000). Mastitis pathogens can spread from older cattle to heifers making a physical separation between both age groups advisable (Barkema et al., 1999; Parker et al., 2007b). Furthermore, heifers with poor udder hygiene have a higher risk of IMI (Compton et al., 2007a). Farms where clean calving pens are present and cubicles are cleaned more than twice daily are more likely to have a lower bulk milk SCC (Barkema et al., 1999). Therefore, hygiene should be optimal on farms to prevent heifer mastitis problems and mastitis problems in general.

Finally, a lower incidence of IMI in early lactating heifers can also be achieved by increasing the genetic resistance against mastitis pathogens through selection. Several selection criteria are possible. Because of its correlation with mastitis, SCC is often used as an indirect selection criterion (Ødegård et al., 2005). In Nordic countries, data on CM cases are registered and can be used to select directly against mastitis (Ødegård et al., 2003). Because data on CM are not readily available in Belgium and the Netherlands, Ouweltjes et al. (2008) suggested using bacteriological culture data to improve genetic resistance against subclinical and clinical mastitis of dairy cattle. Although dairy cattle is a typically outbred population complicating qualitative genetic research, several research groups have searched for candidate genes which might be of interest in the selection towards a lower mastitis susceptibility. Most of these genes are related to one or more components of the innate immunity and certain mutations in these genes have already been shown to be associated with mastitis susceptibility (Ogorevc et al., 2009).

4. Milk production of heifers infected with coagulase-negative staphylococci at calving

Piepers et al. (2010) performed a study on heifer mastitis in Flanders (Belgium). Milk samples were collected from 191 heifers in early lactation from 20 herds for bacteriological culture. Similar to other studies, a high prevalence of IMI in early lactation was found. Almost 80% of the heifers had at least one culture-positive quarter. The vast majority (72%) of isolated pathogens were CNS. Data on milk production, SCC and CM cases were available. As expected, SCC of CNS infected heifers (84,000 cells/ml) was only slightly higher than healthy heifers (53,000 cells/ml; Fig. 1). Milk production was much lower in heifers infected with major
pathogens than in non-infected heifers. Surprisingly, CNS-infected heifers had significantly higher milk productions than their healthy herd mates (Fig. 2).

**Fig. 1**: Natural log of the SCC (LnSCC) during the first 285 days of the first lactation for non-infected heifers, heifers infected with coagulase-negative staphylococci (CNS) and heifers infected with a major pathogen (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and environmental streptococci) in early lactation. (From: Piepers et al., 2010).
Using survival analysis, it was shown that significantly less CM cases occurred in the group of heifers infected with CNS compared with the non-infected heifers. Previously published papers already suggested protective effects of minor pathogen IMI against major pathogen IMI (Lam et al. 1997). The findings of Piepers et al. (2010) reinforce the latter hypothesis. The higher milk production in CNS-infected heifers might at least partly be explained by the lower incidence of CM in the CNS-infected heifers. Clinical mastitis has indeed a detrimental effect on both udder health and milk yield (Wilson et al., 2008). Nevertheless, the authors concluded that further research is needed to confirm these remarkable findings and to elucidate the mechanism behind it.

Fig. 2 : Milk production (kg milk/day) during the first 285 days of the first lactation for non-infected heifers, heifers infected with coagulase-negative staphylococci (CNS) and heifers infected with a major pathogen (Staphylococcus aureus, Streptococcus dysgalactatiae, Streptococcus agalactiae and environmental streptococci) in early lactation. (From: Piepers et al., 2010).
B. INNATE IMMUNITY OF THE UDDER

Mastitis occurs once mastitis bacteria have penetrated the teat orifice and have succeeded in multiplying in the mammary gland (MG) causing inflammation. Luckily, immune defence mechanisms are present, providing protection against this process and the resistance to new IMI depends on their efficiency. The immunity of the MG is composed of two forms, namely the innate and the acquired immunity which are in close interaction (Sordillo et al., 1997). Acquired immunity relies on the recognition of antigens by antibodies formed after previous contact, while innate immunity cells recognize molecular patterns on pathogens even at first encounter (Rainard and Riollet, 2006; Oviedo-Boyso et al., 2007). Innate immunity is the predominant form of defence in the early stage of infection and contains a broad diversity of defence mechanisms including anatomical, cellular and soluble factors (Sordillo et al., 1997).

1. Anatomical factors

Intramammary infection requires penetration of the teat canal and therefore the teat canal barrier is considered as the actual first line of defence (Sordillo et al., 1997; Rainard and Riollet, 2006; Oviedo-Boyso et al., 2007). The teat canal, being the connection between environment and the inside of the udder, is closed by contraction of the sphincter muscle in the teat end and presence of a keratin plug. Damage to the epithelium of the teat orifice and removal of the keratin plug have both been shown to increase the susceptibility towards mastitis (Capuco et al., 1992; Myllys et al., 1994). The keratin plug consists of a waxy material derived from the stratified epithelium of the teat canal and forms a physical barrier preventing access to the MG cistern (Rainard and Riollet, 2006). At the beginning of the milking process, the keratin plug is washed out and the sphincter muscle relaxes. Sufficient contraction and thus closure of the teat canal takes place approximately two hours after milking (Schultze and Bright, 1983). Zecconi et al. (2000) showed that machine milking can induce stress on the teat tissue and on its circulation causing malfunction of the teat canal barrier and even modification of the immune defences of the teat end.

2. Cellular factors

In milk of perfectly healthy quarters, the SCC does not exceed $10^5$/ml with macrophages being the predominant cell type (Lee et al., 1980). After having penetrated the teat canal, mastitis pathogens encounter macrophages and other resident immune cells, resulting in a release of chemoattractants (Hoeben et al., 2000). This immune response leads to a rapid increase in SCC, caused by immigration of mainly neutrophils from blood to milk (Sordillo and Streicher, 2002). Hill (1981) showed that severity of mastitis can depend on the speed of this mobilisation.

The vast majority of cells in the milk during the early stages of inflammation caused by IMI consists of neutrophils (Paape et al., 2000), which are attracted to the place of infection by different inflammatory mediators such as cytokines, complement and prostaglandins (Persson et al., 1993). The migrated neutrophils are able to phagocytose and kill mastitis pathogens. The bactericidal effect during phagocytosis relies on a respiratory burst which produces hydroxyl and oxygen radicals and on exposure to oxygen-independent reactants such as peroxidase, lysozyme and lactoferrin (Sordillo et al., 1997).
Additionally, neutrophils are capable of killing mastitis pathogens by producing small antibacterial peptides called defensins (Selsted et al., 1993). Unfortunately, the inflammatory response through neutrophils does not only affect bacteria but also the mammary tissue. The released chemicals can damage secretory cells resulting in a decreased secretory activity and thus decreased milk production (Paape et al., 2000).

Macrophages in milk have some similar functions as neutrophils. Both cell types can ingest and eliminate mastitis pathogens. However, the efficiency of phagocytosis is remarkably higher in neutrophils than in macrophages (Mullan et al., 1985). Therefore, the main function of macrophages is believed to be recognition of bacteria and initiation of inflammation rather than phagocytosis (Sordillo and Streicher, 2002). Macrophages do not only activate the innate immune response but also the specific immune response. Antigens from pathogens are processed and presented in association with major histocompatibility complex (MHC) class II molecules by macrophages, stimulating T-cell proliferation (Politis et al., 1992).

The third cell type who plays an important role in the innate immunity of the mammary gland is the natural killer (NK) cell, a large granular lymphocyte. Pathogens can be killed in the udder by NK cells in two ways. First, NK cells produce bactericidal proteins belonging to the saposin-like protein family which are released after interleukin 2 (IL2) stimulation (Rainard and Riollet, 2006). Secondly, NK cells possess Fc receptors on their cell membrane enabling them to bind antibody-bound pathogens. After binding, NK cells degranulate and secrete perforin, causing membrane disruption of the pathogens (Sordillo et al., 1997; Sordillo and Streicher, 2002).

3. Soluble factors

Besides cells, different soluble substances are present in milk of healthy mammary glands which hinder invading bacteria to survive and multiply. A well known example of one of these soluble factors is lactoferrin (Lf). Bovine Lf is mainly secreted by mammary epithelial cells (MEC) and is capable of binding iron which is required for bacterial growth. Concentrations in bovine milk are rather low (20-200 µg/ml) compared with concentrations in human milk (1-2 mg/ml; Rainard and Riollet, 2006). Rainard (1986) proved that mastitis pathogens such as *E. coli* and *S. aureus* are susceptible to the bacteriostatic activity of Lf. In the same study, *S. uberis* and *S. agalactiae* were shown to be resistant. Not only a bacteriostatic activity of Lf, but also a bactericidal activity was found. Especially lactoferricin (Lfc), a peptide obtained from Lf by enzymatic cleavage has a direct bactericidal activity (Orsi, 2004). Transferrin is a second iron-binding protein present in milk of ruminants, and is contrary to Lf not synthesized in the udder (Sanchez et al., 1992), but enters the mammary gland by transcytosis from the blood (Ollivier-Bousquet, 1998).

Bovine milk contains two enzymes associated with the production of reactive oxygen species (ROS), namely lactoperoxidase (LPO) and xanthine oxidase (XO). The produced reactive oxygen species can cause bacteriostasis and death of bacteria and thus LPO and XO contribute to the innate immune response in the mammary gland against mastitis pathogens (Silnikov et al., 2005).

In milk of healthy quarters significant concentrations of several complement factors can be found. Due to lack of complement factor C1q in healthy quarters, the classical pathway is non-functional in the early stage of infection. The alternative pathway does not depend on the presence of C1q and can operate briefly after...
contact with invading pathogens. This results in opsonisation by deposition of C3b and C3bi on the bacteria and in the generation of C5a, a pro-inflammatory fragment (Rainard and Poutrel, 1995; Rainard et al., 1998).

Both the innate and the acquired immune responses are regulated by cytokines (Sordillo and Streicher, 2002). In milk from healthy cows, transcription of IL8, tumor necrosis factor alfa (TNF-α), interferon gamma (IFN-γ), granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin 12 (IL12) could be detected (Leutenegger et al., 2000). Transcription of cytokines such as interleukin 6 (IL6) are higher in infected than in healthy quarters (Taylor et al., 1997). The high potency of cytokines stimulated scientists to investigate possible applications in diagnosis and therapy of mastitis (Alluwaimi, 2004).

C. INTERLEUKIN 8 AND INTERLEUKIN 8 RECEPTORS

The bovine interleukin 8 gene (IL8, GenBank Acc. No. NC_007304) is located on chromosome 6 and is 3771 base pairs (bp) long. It codes for a mRNA fragment of 1485 bp long (GenBank Acc. No. NM_173925) which is translated into a precursor protein (GenBank Acc. No. NP_776350) of 101 amino acids. Hassfurther et al. (1994) were the first to isolate and characterize this protein. Morsey et al. (1996) showed that this precursor protein shares 87% and 76% amino acid homology with the porcine and human precursor protein of IL8, respectively. Interleukin 8 is a member of the group of chemokines. Chemokines are characterized by the presence of 4 conserved cysteines (C) which form two disulfide bonds (see Fig. 3). The disulfide bonds play an important role in the configuration of the protein and are essential for the biological activity. Chemokines are divided into two sub-groups, namely CXC chemokines and CC chemokines, based on the fact that the first two cysteines are separated by one amino acid or not. The two sub-groups activate different cell types, CXC chemokines mainly act on neutrophils while CC chemokines act on monocytes, basophils, eosinophils and T-lymphocytes (Baggiolini et al., 1994).

![Figure 3: Human interleukin 8 (From: Baggiolini et al., 1994). Sequence of the 72 amino acids of human interleukin 8. The two disulfide bonds clearly influence the configuration of the protein. IL8 is a member of the CXC chemokines in which the first two cysteines are separated by an amino acid. In the case of IL8, this is glutamine (Q).](image-url)
The Glu-Leu-Arg (ELR) sequence at the amino-terminus is common to all CXC chemokines and is necessary for receptor binding. This was proven by substituting the natural DLQ sequence with ELR in platelet factor 4 (PF4). The generated ELR-PF4 competed with IL8 for receptor binding and had effects on neutrophils that were similar to those of IL8 (Clark-Lewis et al., 1991). In contrast, the amino acids at the carboxyl-terminus are less essential for the function of IL8. Chemically synthesized IL8 lacking the whole C-terminus after the fourth cysteine showed decreased but still significant biological activity (Baggiolini et al., 1995). Interleukin 8 is not only produced by several types of leukocytes such as macrophages, monocytes, neutrophils and lymphocytes but also by epithelial cells (Baggiolini et al., 1994). The bovine MEC as well is capable of producing IL8 (Boudjellab et al., 1998).

Interleukin 1 and TNF are believed to be important IL8 inducers. Levels of these cytokines are elevated in case of infection and neutrophils are attracted to the infected tissue. Interleukin 8 is likely to be the main cause of this neutrophil influx (Baggiolini et al., 1995). The migration of neutrophils from peripheral blood to the infected tissue is called chemotaxis and IL8 is an important chemoattractant. Bovine IL8 as well, has been proven to induce selective chemotaxis on neutrophils in vivo and in vitro (Caswell et al., 1999). Barber and Yang (1998) showed that both nonmastitic and mastitic secretions are chemotactic for neutrophils in vitro. When anti-IL8 antibodies were added to the mastitic secretions, chemotactic activity was nearly reduced to 0%. This indicates that IL8 is crucial for chemotaxis during mastitis. Latter findings were confirmed by injection of recombinant bovine IL8 (rbIL8) into the skin of a healthy calf. Intravascular margination of neutrophils was seen shortly (15-60 s) after administration at the injection sites. The number of extravascular neutrophils in the injected tissues increased from 1 h to 18 h after injection (Caswell et al., 1999).

Migration of neutrophils into the infected mammary gland form the first line of cellular defence against the invaded pathogen (Paape et al., 2000). Therefore, IL8 which stimulates this migration can be considered as a major important cytokine in the immunity of the udder.

Besides chemotaxis, several other effects of IL8 were found. Kettritz et al. (1998) showed that human IL8 does not only causes migration of neutrophils but also prolongs their lifetime by delaying spontaneous apoptosis and inhibiting apoptosis induced by TNF-α. Mitchell et al. (2003) demonstrated that bovine IL8 enhances the activity, and more specific the oxidative burst and phagocytic responses of neutrophils both in vivo and in vitro. In a study performed by Watanabe et al. (2008), injection of rbIL8 in bovine mammary glands affected the integrity of the blood-milk barrier. Concentrations of albumine, immunoglobulin G1 and immunoglobulin G2, which are typical serum proteins, were significantly elevated in milk from 24h after administration. Furthermore, secretion of milk-specific protein such as α-casein (α-CN) and β-casein (β-CN) was decreased from 24 h until 720 h after injection. Because CN has a negative effect on bactericidal activity of bovine neutrophils (Cooray, 1996), decreased concentrations might be an advantage in the opposition against the pathogen (Watanabe at al., 2008).

Interleukin 8 carries out its function by binding to two receptors named CXCR1 (alias IL8RA) and CXCR2 (alias IL8RB). Human CXCR1 (Holmes et al.) and human CXCR2 (Murphy and Tiffany) were first cloned in 1991. Comparison between these human genes and the bovine genome showed that the two interleukin 8 receptor genes are also present in the bovine genome. The two genes lay on the second chromosome approximately 20 kb separated from each other and on opposite strands. The gene located more centromeric encodes for CXCR1, the gene located more telomeric encodes for CXCR2 (Pighetti and
Rambeaud, 2006). Unfortunately, prior to this comparison, misannotation occurred and several articles were published on CXCR2 while actually dealing with CXCR1 (Youngerman et al., 2004a,b; Rambeaud and Pighetti, 2005; Rambeaud et al., 2006). CXCR1, CXCR2 and all other known receptors binding CXC chemokines are members of a large family of receptors called rhodopsin-type receptors, characterized by a seven-transmembrane configuration (see Fig. 4; Baggiolini et al., 1994).

**Fig. 4**: Human CXCR1 (Interleukin 8 receptor A; from: Baggiolini et al., 1994)

The amino sequence and seven-transmembrane configuration of human CXCR1

After binding of IL8, the signal is transmitted inside the cell by activation of a G-protein complex. These guanosine triphosphate (GTP) binding proteins are associated with both CXCR1 and CXCR2 (Wu et al., 1993). Stillie et al. (2009) proposed following signalling pathway after binding of the ligand (Fig. 5). First, the guanosine diphosphate (GDP) which is bound to the subunit Gα in the inactive state dissociates and becomes replaced by GTP. At the same time, the Gβγ subunit dissociates from Gα allowing phosphorylation of the carboxyl-terminus of the receptor by G-protein receptor kinase (GRK). Phosphorylation of the cytoplasmic tail of CXCR1 was shown to be required for chemotaxis (Richardson et al., 1998). The unbound Gβγ subunit activates phosphoinositide 3-kinase gamma (PI3K-γ). This enzyme causes phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP2). The generated phosphatidylinositol-3,4,5-triphosphate (PIP3) activates both protein kinase-B (PKB) and GTP-ases, resulting in directed cell migration.
Amino acid sequences of human CXCR1 and CXCR2 are 78% identical but yet they differ largely from each other in the selectivity of binding chemokines. CXCR1 only binds IL8 with high affinity, whereas CXCR2 binds other CXC chemokines such as growth-regulated oncogen alpha (GROα), GROβ, GROγ, neutrophil-activating protein 2 (NAP-2) and epithelial cell-derived neutrophil-activating peptide 78 (ENA-78) with similar affinity as IL8 (Ahuja and Murphy, 1996). Other functional differences were suggested as well. Activation of CXCR1 is believed to result in a wider array of antimicrobial processes than activation of CXCR2, but CXCR2 might respond to a lower concentration of ligand (Stillie et al., 2009).
D. CXCR1 POLYMORPHISMS AND THE ASSOCIATION WITH MASTITIS SUSCEPTIBILITY

The 1703 bp long bovine CXCR1 gene (GenBank Acc. No. NC_007300) is located on chromosome 2 and contains no introns. The coding region is 1083 bp long and is translated into the CXCR1 protein (GenBank Acc. No. NP_776785) of 360 amino acids. Several research groups identified SNPs in this gene. Research performed on bovine cytokine loci in a beef cattle population revealed 4 SNPs in a 523-bp amplified product from the coding region of the gene located from 456 bp to 978 bp relative to the start codon. Primers for this product were designed to bind selectively on one gene but it was assumed to contain multiple IL8R-like loci (Grosse et al., 1999). Youngerman et al. (2004a) genotyped 37 Holstein and 42 Jersey cows and identified the same 4 SNPs as Grosse et al. (1999) found. One additional SNP was found as well. The polymorphisms were located at position +570, +642, +735, +816 and +819 relative to the start codon (with A = 1). None of them affect the amino sequence of the protein (= synonymous) except for the SNP at +735 which causes a glutamine to histidine substitution at amino acid 245. Because this amino acid is located in the third intracellular loop of the receptor (see Fig. 4), an important region in the signal transduction after binding IL-8, Youngerman et al. (2004a) claimed that CXCR1 is an excellent potential candidate marker in the selection for mastitis resistance. Analysis of the genotype frequencies showed that some mutations are linked 100%. In total 10 haplotypes were identified. The frequencies of these haplotypes differed between the two breeds (Youngerman et al., 2004b; Table 2.).

<table>
<thead>
<tr>
<th>Haplotype label</th>
<th>570</th>
<th>642</th>
<th>735</th>
<th>816</th>
<th>819</th>
<th>Frequency in Jersey group (in %)</th>
<th>Frequency in Holstein group (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>48</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>1</td>
<td>1</td>
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<td>5</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>1</td>
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<td>A</td>
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<td>C</td>
<td>C</td>
<td>A</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

(From: Youngerman et al., 2004b)

Data on milk production and somatic cell score (SCS) were available from the genotyped cows. Somatic cell score is a scoring system for SCC developed by Dairy Herd Improvement (DHI) which categorizes SCC into 10 classes. Bacteriological examination was done on foremilk samples and cases of CM were noted. The relationship between the genotype at position 735 and each of these parameters was evaluated. Holstein cows with genotype GC were found to have significantly higher SCS compared to Holstein cows with genotype CC. A difference between Holstein cows with genotype GG and those with CC was also observed but was not significant.
Holstein cows with genotype CC had a significantly higher prevalence of subclinical mastitis than the other Holstein cows. However, Youngerman et al. (2004a) remarked that cows with the CC genotype tended to have lower clinical mastitis incidence. Surprisingly, significantly lower 305-d milk yields were measured in the group of Holstein cows expressing the GG genotype. Because polymorphism in CXCR1 was found to be associated with subclinical mastitis, Youngerman et al. (2004a) concluded that selection against the disadvantageous genotype might lead to a more mastitis resistant dairy population. Nevertheless, the authors noted that the association study was done on a relatively small number of animals and that further research was required to confirm the findings.

The same research group recently attempted to elucidate why cows with genotype CC have more subclinical mastitis. Rambeaud and Pighetti (2005) studied neutrophils from cows with different genotype in vitro, and found that significantly less neutrophils from cows with the CC or GC genotype migrate to recombinant human interleukin 8 (rhIL8) compared to neutrophils from cows with genotype GG. Additionally, adhesion molecule (CD18 and CD11b) up-regulation was significantly lower in neutrophils from cows with the CC or GC genotype after incubation with rhIL8 than in neutrophils from cows with the other genotype. These findings indicated that the mutation at position 735 has an effect on the functionality of the receptor (Rambeaud and Pighetti, 2005). Further in vitro research showed that differences exist in the capacity of generating ROS between cows with different genotype (Rambeaud et al., 2006). Neutrophils from cows with the CC genotype produced significantly less ROS after stimulation than neutrophils from cows with the GG genotype. However, equivalence in bactericidal activity could not be rejected. Against expectations, a longer survival time was observed in neutrophils from cows with CC genotype. Stimulation of neutrophils with an optimal dose of IL8 led to a lower intracellular Ca\(^{2+}\) release in neutrophils from cows with the CC genotype than in other neutrophils, indicating that intracellular signalling is less functional (Rambeaud and Pighetti, 2007). Furthermore, the number of CXCR1 receptors per neutrophil was found to be significantly lower in cows with the CC genotype compared to cows with the GG genotype (Rambeaud and Pighetti, 2007).

Other research groups as well investigated polymorphism in CXCR1 in Holstein dairy. Leyva-Baca et al. (2008b) identified 3 new SNPs in the 5’ upstream region of the gene which in included in a shotgun sequence from the NCBI GenBank with Acc. No. AC150887.4. Twenty Canadian Holstein bulls with high estimated breeding values (EBV) for SCS and twenty Canadian Holstein bulls with low EBV for SCS were genotyped. Analysis revealed that one newly found SNP at position -1828 was associated with EBV for SCS. The sequence of the surrounding region was studied and potential binding sites for gene regulating factors such as nuclear factor-κB (NF-κB), GATA-binding factor 1 (GATA-1) and Barbie transcription-factors were found (Leyva-Baca et al., 2008b). A study using neutrophils from cows with different genotypes at position -1828 was performed. Expression of CXCR1 in neutrophils from cows with genotype AA was found to be significantly greater compared to neutrophils from cows with other genotypes both before and after lipopolysaccharide challenge (Leyva-Baca et al., 2008a). In a recent German study using a large data set from the national German bovine genome mapping, neither an association between the SNP at +735 and SCC nor an association between the SNP at -1828 and SCC could be found (Goertz et al., 2009).
III. RESEARCH PROJECT

A. MATERIALS AND METHODS

1. Phenotypic data

a. Herds and animals

The database for the current research consisted of data gathered by Piepers et al. (2010) combined with genetic information on DNA released from blood samples taken from the tail vein and stored in EDTA vacuum tubes (Vacuette® 4 ml EDTA K3) at -20°C. In total, 140 heifers from 20 farms were enrolled in the study. All visited farms participated in the dairy herd improvement (DHI) program in Flanders of the Flemish Cattle Breeding Association (CRV, Oosterzele, Belgium) and were located within a radius of 30 km of the Faculty of Veterinary Medicine (Merelbeke, Belgium). On average, 7 heifers per farm were included, ranging between 3 and 10. Databases of the CRV (Oosterzele, Belgium) were consulted to analyse relationship and the name of the sire of each heifer.

b. Intramammary infection status at calving

Materials and methods for IMI status are described in Piepers et al. (2010). In short, collection of quarter milk samples in early lactation was done aseptically twice for each heifer; the first time between 1 and 4 days in milk (DIM), the second time between 5 and 8 DIM. An interval of at least 3 days between both samplings was respected. Samples were taken before morning milking. Teats were disinfected and the first streams of milk were discarded. In order to analyse the IMI status, bacteriological culture was performed as described by Piepers et al. (2007). For each quarter, 10 µl of milk was spread on blood-esculin agar and on a MacConkey’s agar. In order to grow bacteria, plates were incubated aerobically for 24-48 h at 36-38°C. Identification of bacteria was done by Gram-staining and inspection of the colony morphology. Differentiation of Gram-positive cocci in catalase-positive or catalase-negative cocci was done by performing catalase tests. *Staphylococci* were identified as CNS or *S. aureus* by colony morphology, hemolysis patterns, and DNase tests. *Streptococci* were differentiated in esculine-positive (*S. uberis*) and esculine-negative streptococci (*S. agalactiae* and *S. dysgalactiae*). *Streptococcus agalactiae* and *S. dysgalactiae* were distinguished using the Christie, Atkins, Munch-Petersen (CAMP) test. *Corynebacterium bovis* and CNS were categorised as minor pathogens, while *S. aureus*, esculine-positive streptococci, *S. dysgalactiae*, and *S. agalactiae* were categorised as major pathogens. Heifers were divided in 8 classes (heifer IMI status) using the IMI statuses of their quarters (Table 3).
Table 3. Definitions of intramammary infection (IMI) status of a heifer

<table>
<thead>
<tr>
<th>IMI status</th>
<th>Culture result between 1 and 4 DIM(^1) (S1)</th>
<th>Culture result between 5 and 8 DIM (S2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non-infected all quarters culture-negative</td>
<td>all quarters culture-negative</td>
</tr>
<tr>
<td>1</td>
<td>S1 CNS-positive at least one quarter CNS-positive and no quarters major pathogen-positive</td>
<td>all quarters culture-negative</td>
</tr>
<tr>
<td>2</td>
<td>S2 CNS-positive all quarters culture-negative</td>
<td>at least one quarter CNS-positive and no quarters major pathogen-positive</td>
</tr>
<tr>
<td>3</td>
<td>Infected with CNS at least one quarter CNS-positive and no quarters major pathogen-positive same quarter CNS-positive as in S1 and no quarters major pathogen-positive</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>S1 major pathogen(^2)-positive at least one quarter major pathogen-positive</td>
<td>all quarters culture-negative or minor pathogen(^3)-positive</td>
</tr>
<tr>
<td>5</td>
<td>S2 major pathogen-negative all quarters culture-negative or minor pathogen(^3)-positive Minor pathogen-positive</td>
<td>at least one quarter major pathogen-positive</td>
</tr>
<tr>
<td>6</td>
<td>Infected with major pathogen at least one quarter major pathogen-positive same quarter major pathogen-positive as in S1 with the same pathogen</td>
<td></td>
</tr>
<tr>
<td>9999</td>
<td>Contaminated at least one quarter with a contaminated culture result</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Days in milk

\(^2\) *Staphylococcus aureus*, esculin-positive streptococci, *Streptococcus agalactiae, Streptococcus dysgalactiae*

\(^3\) *Corynebacterium bovis* or coagulase-negative staphylococci (CNS)

c. Somatic cell count and milk production in first lactation

Composite milk SCC and daily milk production at test-day were determined as a part of the DHI program. Data on these parameters from 14 until 285 days lactation were used.
2. Genotyping

DNA was released from each blood sample and used as a template to amplify simultaneously both copies of the complete CXCR1 coding region (1236 bp) in a single polymerase chain reaction (PCR). The PCR product was sequenced by direct sequencing using the primers designed for the PCR as sequencing primer. Cloning was performed on samples from which the haplotypes could not be determined.

a. Products

- 0.8% Agarose gel + EtBr
  Made by dissolving 3.2 g agarose (Gentaur®, Brussels, Belgium) in 400 ml 0.5x TBE buffer and adding 40 µl 10 mg/ml EtBr

- 1Kb Plus DNA ladder™ working solution
  Made by diluting 50µl 1 µg/µl 1Kb Plus DNA ladder™ stock solution (Life Technologies™, Merelbeke, Belgium) in 475µl 6x loading buffer and 475µl 10mM Tris-HCl (pH 8).

- 5x SEQ buffer
  200mM Tris-HCl (pH 8) and 5mM MgCl₂

- 10x EcoRI restriction buffer
  New England BioLabs®, Frankfurt am main, Germany

- 10x FastStart™ Taq DNA Polymerase Buffer
  Roche Applied Science®, Mannheim, Germany

- 10x ligation buffer
  Invitrogen, Merelbeke, Belgium

- 10x TBE buffer
  0.9 M Tris-borat and 0.02 M EDTA, pH 8 (0.5x working solution made by diluting 0.25 l 10x TBE with 4.75 l H₂O)

- Ampicilline
  Made by diluting 50 mg Ampicilline sodium salt (Invitrogen, Merelbeke, Belgium) in 1 ml distilled water
  Sterilized through filtration using 0.2 µm Acrodisc® Syringe filter (Pall Corporation, Zaventem, Belgium)

- BigDye® Terminator v3.1 Cycle Sequencing Kit
  Contains BigDye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems™, Halle, Belgium) containing ddNTPs, dNTPs and DNA Polymerase.

- EcoRI restriction endonuclease
  20 U/µl stored at -20°C (New England BioLabs®, Frankfurt am main, Germany)

- dNTP Mix
  Made by diluting 25mM each, pH 7.0 (BIOLINE®, Luckenwalde, Germany) with distilled water till 10mM each

- Ethidium bromide (EtBr; 10 mg/ml)
  Made by diluting 100 mg EtBr (USB Corporation, Staufen, Germany) in 10 ml distilled water
  Handled with extreme care, EtBr is a potential mutagen, carcinogen and teratogen

- EXO-AP solution
  4U Exonuclease I and 2U Antarctic phosphatase per 0.6 µl (both obtained from New England BioLabs®, Frankfurt am main, Germany)

- Forward Primer (BtauCXCR1+1, 100µM working solution)
  Made by dissolving 30.4 nMoles lyophilized content (Integrated DNA Technologies IDT®, Leuven, Belgium) in 304 µl PCR-grade water.

- Geneclean® II Kit
  Contains TBE modifier, NaI, New wash concentrate and Glassmilk® (QBiogene, Illkirch Cedex, France)
- **Glycerol**
  USB Corporation, Staufen, Germany

- **LB medium**
  Made by dissolving 25g Luria Broth Base (Invitrogen, Merelbeke, Belgium) in 1l distilled water
  Sterilised in autoclave

- **LB plate**
  Made by pouring 15ml of a solution containing 1.5 g Select Agar powder (Invitrogen, Merelbeke, Belgium) in 100 ml LB medium on sterile plates, sterilised in autoclave

- **loading buffer**
  0.25% bromophenol blue, 0.25% xylene cyanol and 15% ficoll

- **Lysebuffer**
  10mM Tris-HCl (pH 8.3), 50mM KCl, 0.5% Tween 20

- **CleanSeq magnetic beads**
  Agencourt®, Grenoble, France

- **P1 buffer**
  Made by diluting 5 ml 1M Tris-HCl (pH 8.0), 2 ml 0.5M EDTA (pH 8.0) and 1 µl 10 mg/ml RNase A with distilled water until 100 ml.

- **P2 buffer**
  Made, briefly before usage by dissolving 100 µl 10% SDS (Invitrogen, Merelbeke, Belgium), 40 µl 5M NaOH and 860 µl distilled water.

- **P3 buffer**
  Made by dissolving 29.5 g Potassium acetate (VWR international, Leuven, Belgium) in 88.5 ml distilled water and adding 11.5 ml acetic acid (VWR international, Leuven, Belgium).

- **PCR-grade water**
  Roche Applied Science®, Mannheim, Germany

- **Proteinase K**
  Made by dissolving 5 µl stock solution (20 mg/ml; Roche Applied Science®, Mannheim, Germany) in 1 ml 10 mM Tris-HCl (pH 7.5)

- **Reverse Primer (BtauCXCR1-1, 100 µM working solution)**
  Made by dissolving 38.5 nMoles lyophilized content (Integrated DNA Technologies IDT®, Leuven, Belgium) in 385 µl PCR-grade water.

- **RNase A**
  20 mg RNase A/ml in 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA (purchased from Invitrogen, Merelbeke, Belgium)

- **Subcloning Efficiency™ DH5α™ Competent Cells**
  Invitrogen, Merelbeke, Belgium

- **TA Cloning® Kit**
  Contains 10x ligation buffer, pCR® 2.1 vector (25ng/µl) and T4 DNA ligase (4.0 Weiss units) (Invitrogen, Merelbeke, Belgium)

- **Taq DNA Polymerase 5U/µl**
  Roche Applied Science®, Mannheim, Germany

- **TBE buffer**
  45mM Tris-borate and 1mM EDTA

- **TE buffer**
  10 mM Tris-HCl and 1 mM EDTA, pH 8.0

- **Vacuum blood tubes: Vacuette® 4 ml EDTA K3**
  Greiner Bio-One B.V., Alphen aan den Rein, The Netherlands
• X-gal solution (20 mg/ml)
  Made by diluting 20 g X-gal (Fermentas life sciences, St. Leon Rot, Germany) in 1 ml dimethylformamide
  Stored at -20°C, protected from light (light sensitive)

b. Devices

• 96-caryllar 3730x/ DNA Analyzer
  Applied Biosystems™, Halle, Belgium

• Chemidoc XRS Gel Documentation System
  Bio-Rad®, Hercules, USA

• Gel electrophoresis apparatus
  My-Run (Westburg, Leusden, The Netherlands)

• Gradient PCR device
  Eppendorf Gradient Mastercycler® Thermal Cyclers (Eppendorf, Hamburg, Germany)

• Kötterman® 2737 Incubator
  Kötterman® GmbH & Co, Uetze-Hänigsen, Germany

• Microcentrifuge
  Eppendorf Microcentrifuge Model 5415D (Eppendorf, Hamburg, Germany)

• Orbital shaker
  Queue System Orbital Shaker Model #4710 (Abbott sa/nv, Louvain-la-Neuve, Belgium)

• PCR devices
  T3 Thermocycler (Biometra®, Goettingen, Germany)
  Eppendorf Mastercycler® Thermal Cyclers (Eppendorf, Hamburg, Germany)

• Refrigerated microcentrifuge
  Eppendorf Refrigerated Microcentrifuge Model 5415R (Eppendorf, Hamburg, Germany)

• Spectrophotometer
  ND-1000 spectrophotometer, NanoDrop®, Wilmington, USA

c. DNA release

Each blood sample was thawed at room temperature. In order to roughly wash away everything except for
the white blood cells (WBC), 500 µl of TE buffer was added to 100 µl of whole blood in a 1.5 ml vial and
mixed using a vortex mixer. The mix was centrifugated at 13,200 rpm for 30 s to pellet the WBC. The
supernatant was removed and this washing step was repeated at least twice until the pellet was white. The
WBC were resuspended in 100 µl of Lysebuffer containing 10 µg Proteinase K, and lysed at 56°C for 45
min, followed by inactivation of the Proteinase K at 95°C for 10 min. Finally, the crude lysate was centrifuged
at 13,200 rpm for 1 min in order to pellet the cell debris. The supernatant, containing the released DNA, was
placed in a new vial and stored at 4°C upon use (the DNA can be stored at -20°C for longer periods).
Quantification of DNA was performed using a spectrophotometer (ND-1000 spectrophotometer). DNA and
protein absorbs UV light with an absorption peak at 260 nm and 280 nm wave length, respectively. The
higher the concentration of DNA in a solution is, the higher the amount of absorption of 260 nm UV light will
be, which can be measured with the spectrophotometer. Samples with a high level of protein contamination
have a low 260:280 ratio (Sambrook et al., 2001).
The spectrophotometer was cleaned with distilled water and blanked with Lysebuffer. Approximately 1.5 µl of sample was loaded and the DNA concentration was estimated. Pure DNA possesses a 260:280 ratio of around 2. However, with this quick and cheap procedure, DNA was not extracted but only released from the cells in the presence of a lot of other cell components, such as proteins. As a result, the values of the 260:280 ratio are much lower. Experience in the laboratory showed that values down to 0.8 are sufficient for downstream applications such as PCR and sequencing. Only if the measurement was lower than 50 ng/µl or if the 260:280 ratio was lower than 0.8, DNA release was repeated.

d. Primer design

In order to design primers which bind specifically at our place of interest (close before the start codon and close after the stop codon of bovine CXCR1), the gene database from the National Centre for Biotechnology Information (NCBI) Entrez Gene was consulted to view the reference sequence of CXCR1. To avoid binding on the CXCR2 gene, the Basic Local Alignment Search Tool (BLAST) from NCBI was used to compare the sequence of CXCR1 and CXCR2. Locations with a low percentage of homology between both genes are preferred. To ensure binding on all genotypes, locations where SNPs are described in literature need to be avoided. Because primers can not bind on hairpin structures, locations with these secondary structures were searched using DNA Mfold (Zuker, 2003). To obtain a good and genome specific bond between the PCR primers and the DNA, primers with a size of 18-25 bp and a percentage of 40-60% C+G are preferred (overall but also specifically in the last 5 bp of the primers). Primers which easily form secondary structures with itself or the other primer or which contain 4 or more following G's (...GGGG...) have difficulties to bind on DNA and should not be chosen. Keeping the latter in mind, primers with a similar annealing temperature (less than 1°C difference) were picked using the software program Primer3plus (Untergasser et al., 2007).

The specificity of binding at the place of interest was tested by applying BLAST between the primers and sequences from all GenBank databases. The lyophilized primers (Integrated DNA Technologies, Leuven, Belgium) were dissolved in PCR-grade water at a concentration of 100 µM and stored at -80°C

e. PCR

To find out at which annealing temperature the primers generate the specific PCR product with the highest efficiency, identical PCRs were first performed in a gradient PCR device with different annealing temperatures.

- 1.0 µl sample as DNA template
- 1.0 µl 10x FastStart™ Taq DNA Polymerase Buffer
- 0.5 µl 5µM forward primer
- 0.5 µl 5µM reverse primer
- 0.2 µl dNTP Mix (10mM each)
- 0.1 µl Taq DNA Polymerase (5 U/µl)
- 6.7 µl PCR-grade water
The following reaction mix in a total volume of 10 µl was made for the PCR of the 1236 bp amplicon;

1. Initiation step: 5 min at 95°C
2. Denaturation step: 30 s at 95°C
3. Annealing step: 30 s at 56°C, 58°C, 62°C, 64°C or 66°C
4. Extension step: 1 min 15 s at 72°C
5. 30x step 2-3-4
6. Final elongation: 4 min at 72°C
7. Final hold: 10°C

A negative control, containing 1 µl PCR water instead of DNA, was added with each PCR run. If a band was visible, contamination between cups was suspected and the PCR was repeated. The multiplication of the DNA fragment was examined by performing agarose gel electrophoresis using a 0.8% agarose gel containing ethidium bromide (EtBr). A gel of approximately 0.5 cm height was poured, cooled and placed in the gel electrophoresis apparatus filled with 0.5x TBE buffer. First, 5 µl 1 Kb Plus DNA ladder™ solution (250 ng ladder) was loaded into a well. Secondly, 2 µl of the negative control was mixed with 8 µl PCR water and 5 µl loading buffer and loaded into a well. The next wells were loaded with 2 µl PCR product of samples mixed with 8 µL PCR water and 5 µl loading buffer.

An electric field of 150 V was applied for approximately 20 min. The DNA fragments are negatively charged and migrate toward the positive anode. Speed of migration depends on the size of the fragments, the more bp the shorter distance of migration will be after a giving time in a gel with a given concentration. Ethidium bromide intercalates with DNA and can be visualized by lighting the gel with an UV lamp (Sambrook et al., 2001). Digital pictures of each gel were taken with a Chemidoc XRS Gel Documentation System.

f. EXO-AP

Primers and nucleotides were enzymatically inactivated and destructed from the remaining 8 µl of PCR product by respectively phosphatase and exonuclease to avoid interference during the following sequencing reaction. Therefore, 0.6 µl EXO-AP solution (4 U exonuclease I and 2 U antartic phosphatase) was added to the PCR products. The cups were placed in a PCR device with following program;

1. Exonuclease and phosphatase treatment: 30 min at 37°C
2. Inactivation of the enzymes: 15 min at 80°C
g. Sequencing

After this PCR clean-up, the sequence reaction was initiated. The BigDye® Terminator v3.1 Cycle Sequencing Kit was used for sequencing. For each sample, two mixes (one with each primer) were prepared on ice:

- 3.0 µl Post-PCR solution after EXO-AP
- 2.0 µl 5x SEQ buffer
- 1.5 µl 2 µM primer
- 0.5 µl Ready reaction mix
- 3.0 µl PCR-grade water

If a high concentration of DNA (> 15 ng/µl) was suspected by observing brightness of the band in the gel electrophoresis, more PCR water and less Post-PCR solution after EXO-AP was added. The cups were placed in a PCR device with following sequencing program:

1. Initiation step: 2 min at 95°C
2. Denaturation step: 20 s at 95°C
3. Annealing step: 10 s at 55°C
4. Extension step: 4 min at 60°C
5. 25x step 2-3-4
6. Final hold: 4°C

Because of the presence of ddNTPs, lacking a 3'-OH group and acting as chain-terminating nucleotides in the reaction, DNA fragments of different size are formed (Sanger et al., 1977). ddATP, ddGTP, ddCTP and ddTTP are each labeled with fluorescent dyes with different wavelengths of fluorescence and emission, which permits sequencing in a single reaction. This method is called dye-terminator sequencing (Rosenthal et al., 1992). First, purification was performed with CleanSeq magnetic beads to improve the quality of the sequences, and secondly separation by electrophoresis and detection of the fluorescent sequences on a 96-capillary 3730xl DNA Analyzer was performed.

h. SNP screening

The DNA sequences were verified manually by checking the chromatograms with the software program BioEdit Sequence Alignment Editor (Hall, 1999). The two sequences obtained using the forward primer and the reverse primer were merged with the Contig Assembly Program (CAP©; Xiaoqui, 1991). Sequences of the coding region of CXCR1 of all samples were compared with the reference sequence used in the primer design and with each other. Differences indicate the presence of a SNP, named as “SNP x ” where the “x” stands for the position relative to the start codon. Four different haplotypes could be identified based on 4 different homozygous genotypes, present in a number of animals. All other different genotypes found (6), could be characterised as a combination of 2
different haplotypes mentioned above, except for 2 genotypes. The amplicons of those 2 animals were cloned to sequence the haplotypes.

i. Cloning

A visual representation on the principle of cloning is given in Fig. 6.

**Fig. 6**: Cloning: the multiplied DNA fragment containing the coding region of **CXCR1** was ligated into a plasmid. This plasmid was transformed into competent cells. These competent cells grow on an agar plate, multiply the plasmid and form colonies. The colonies were inoculated in Luria Broth (LB) medium and plasmid DNA was extracted from these bacterial cultures. Because only one DNA fragment can be ligated into a vector, the culture will only contain DNA of one haplotype.

Blood samples of heifers from which the haplotypes could not be deducted from their genotype were thawed on room temperature. DNA release and PCR were performed exactly similar as previously described. PCR products were loaded on gel and electrophoresis was performed as previously described. The fluorescent band was excised on an UV lamp. Geneclean® II kit was used to purify the multiplied DNA fragment from agarose gel. The piece of gel containing the DNA fragment was placed in a vial with 450 µl of NaI and 50 µl of TBE modifier. This vial was warmed at 55°C for at least 1 min in order to melt and dissolve the gel in the solution.

Five µl of Glassmilk® was added and the vial was incubated for 10 min at room temperature to allow the DNA to bind to the silica of the Glassmilk®. During this period, the vial was stirred gently to ensure that Glassmilk® stayed in solution. Next, the solution was centrifuged at 13,200 rpm for 10 s to pellet the DNA bound to the...
silica. The supernatant was discarded and 500 µl of New Wash solution was added, in which the silica was gently resuspended using a pipet, followed by centrifugation for 10 s at 13,200 rpm and removal of supernatant. This washing step was repeated to properly wash the silica. In order to remove residual ethanol, the vial was centrifuged one more time at 13,200 rpm for 10 s and supernatant was removed with a pipet followed by a drying period for 5 min at room temperature with the cup open.

To unbind DNA and silica matrix, 8 µl of TE buffer was added and mixed in the vial by gently pipetting up and down. Centrifugation for 30 s at 13,200 rpm was applied and 7 µl supernatant containing DNA was pipeted in a new cup. This 7 µl was centrifuged a second time for 30 s at 13,200 rpm, 6 µl supernatant was pipeted in a new cup. The residual contents in these two step containing silica matrix were discarded.

Next, ligation was performed using the TA Cloning® Kit. The 6 µl of PCR product cleaned with Geneclean® II kit was mixed by tapping with finger with 1 µl 10x ligation buffer, 2 µl of pCR® 2.1 vector (25 ng/µl) and 1 µl of T4 DNA ligase (4.0 Weiss units). Ligation of the PCR product with the vector was allowed by incubating this mix for approximately 24 h at 14°C. Transformation was done in Subcloning Efficiency™ DH5α™ Competent Cells. These E. coli bacteria were thawed on ice and 50 µl was added in a tube with 2 µl of the ligation reaction. Cells and vectors were mixed by gently tapping this tube.

To allow DNA to approach the cells, the tube was incubated for 30 min on ice. Next, cells were heat-shocked for 30 s at 37°C to stimulate uptake of the vector. After this heat-shock, the vial was placed on ice for 2 min to allow the bacteria to recover and 950 µl of LB medium (without antibiotics) was added in which the bacteria were shaken for 1 h at 180 rpm in an orbital shaker at 37°C for expression. The tubes were centrifuged at 13,200 rpm for 10 s, supernatant was removed and the pellet was gently resuspended in the remaining fluid using a pipet.

All of latter solution was spread on a 15 ml LB plate with 100 µg/ml ampicillin and 50 µg/ml X-gal. In order to avoid contamination, this action was performed close to the flame of a Bunsen burner. Plates were incubated overnight at 37°C. The vector used contains the ampicillin resistance gene, competent cells which took up the vector can grow and multiply, other competent cells can not. The place where the insert can be ligated is positioned in the LacZ gene of the vector, a gene which is necessary for the production of β-galactosidase in the competent cells. This enzyme cleaves X-gal into galactose and 5-bromo-4-chloro-3-hydroxyindole which oxidizes into an insoluble blue product. Ligation causes interruption of the reading frame of LacZ. In this case, β-galactosidase will not be produced, X-gal will not be cleaved and colonies will remain white (Vieira and Messing, 1991).

After incubation, plates were inspected. Four white colonies per plate were picked up with a pipet tip close to the flame of a Bunsen burner to avoid contamination. Each pipet tip was released in a falcon tube with 5 ml LB medium with 100 µg/ml ampicillin. The falcon tubes were incubated at 37°C overnight and shaken at 180 rpm in an orbital shaker to supply the bacteria of sufficient oxygen. The next day, slants were made by mixing 500 µl of each bacterial culture using the vortex mixer with 250 µl sterile glycerol and stored at -80°C. Bacteria can survive for a long time in these slants.
Two ml of bacterial culture was pipetted into a 2 ml vial and centrifuged at 13,200 rpm for 1 min to pellet the cells. Supernatant was removed. Another two ml of bacterial culture was added to the pellet and centrifuged at 13,200 rpm for 1 min to double the amount of pellet. Supernatant was removed. This pellet was resuspended in 100 µl P1 buffer using a vortex mixer. Lysis of the cells was performed by mixing the dissolved pellet gently with 200 µl P2 buffer. After incubation for 5 min at room temperature, plasmid DNA was extracted by several centrifugation steps with different media. First, 150 µl of P3 buffer was added and the vial was inverted 5 times to mix. After 5 min incubation on ice, during which the bacterial chromosomal DNA, the cellular debris and the SDS from the lysis buffer were precipitated, the vial was centrifuged at 13,200 rpm for 15 min. The supernatant was pipetted into a new vial containing 450 µl phenol/chloroform solution (50% each), mixed using a vortex mixer in order to extract the plasmid DNA from the proteins, and centrifuged for 5 min at 13,200 rpm. The supernatant was pipetted into a new vial containing 400 µl chloroform, mixed using a vortex mixer in order to clear all remaining phenol and centrifuged for 2 min at 13,200 rpm. The supernatant was pipetted into a third vial containing 350 µl isopropanol, mixed using a vortex mixer, incubated for 30 min at -20°C to allow precipitation of the plasmid DNA, and pelleted by centrifugation for 10 min at 13,200 rpm. The supernatant was removed and the pellet was washed by adding 500 µl of 70% ethanol, mixing by tapping, centrifuging for 5 min at 13,200 rpm and carefully removing the supernatant using a pipet. The precipitate was resuspended in 100 µl TE buffer supplemented with 2 µl of 1 µg/ml RNase to degrade present RNA.

Restriction fragment length polymorphism (RFLP) with EcoRI was used to control whether plasmid DNA contained the insert or not.

Following mix was made in a PCR cup:

- 1.0 µl solution after Plasmid DNA extraction
- 0.5 µl 20 U/µl EcoRI
- 1.0 µl 10x EcoRI restriction buffer
- 7.5 µl PCR water

This mix was incubated for 2 h at 37°C to allow the restriction enzyme to cleave the DNA. After incubation, gel electrophoresis was performed as previously described. Banding patterns of plasmid DNA from different bacterial cultures were compared on an UV lamp and digital pictures were taken with a Chemidoc XRS Gel Documentation System. Sequencing was performed similar as previously described using the same primers on insert containing plasmid DNA. The amount added depended on the brightness of bands after the electrophoresis of the cleaved DNA. The obtained chromatograms were analyzed similar as previously described. The sequence was noted and the sequence of the other haplotype was deducted from the sequence of this haplotype and the sequence of the genotype.
3. Statistical analysis

Associations between polymorphisms at position 735 in \textit{CXCR1} and IMI status, SCC and milk production were analysed, respectively. In case that several heifers had the same sire, one heifer per sire was randomly selected. All other heifers were excluded from the analysis. This resulted in a dataset containing information for 86 heifers.

a. Association between polymorphism and intramammary infection status at calving

The association between heifer IMI status at calving (0 = non-infected, 1 = CNS infected, 2 = major pathogen-infected) and genotype at position 735 (0 = CC or GC, 1 = GG) was tested using logistic mixed regression models. These models were fit using first order penalized quasi-likelihood algorithms (MlwiN 2.02, Centre for Multilevel Modeling, Bristol, UK). Herd was included as random effect to correct for clustering of heifers within herd. The association was tested separately for the binary outcome variables, first for all IMI (“all IMI”) at heifer level (0 = non-infected versus 1 = infected with CNS or \textit{Staphylococcus aureus}, \textit{Streptococcus agalactiae}, \textit{Streptococcus dysgalactiae} and environmental streptococci), and secondly for IMI specifically caused by CNS (“CNS IMI”; 0 = non-infected versus 1 = infected with CNS). Statistical significance was assessed at P < 0.05.

b. Association between polymorphism and somatic cell count in first lactation

The association between the heifers’ genotype at position 735 (0 = CC or GC, 1 = GG) and test-day SCC was determined using a linear mixed model with herd and heifer within herd as random effects (PROC MIXED, SAS 9.2, SAS Institute Inc., NC, USA). A first order autoregressive correlation structure was used to account for the clustering of repeated milk recordings within heifer. A natural logarithmic transformation of SCC (LnSCC) was performed to obtain a normal distribution. The model with LnSCC as outcome variable included days in milk (DIM) as continuous predictor variables, and the heifers’ genotype (2 levels, 0 = CC or GC, 1 = GG; main predictor of interest) as categorical predictor variables. The quadratic term for DIM and the interaction between DIM and genotype were tested as well.

c. Association between polymorphism and milk production in first lactation

The association between the heifers’ genotype at position 735 (0 = CC or GC, 1 = GG) and test-day milk yield was determined similarly as for latter association with milk yield as outcome variable instead of LnSCC.
RESULTS

1. Phenotypic data

Results on IMI status, SCC and milk production were already described somewhere else (Piepers et al., 2010). Still, a subpopulation of those heifers were enrolled (140 out of 191 heifers) in the present study. Furthermore, only heifers with different sires were included (86 out of 140 heifers) in the analysis. Therefore, phenotypic results on these 86 heifers were recalculated.

a. Intramammary infection status at calving

Intramammary infection status at calving of heifers are shown in Fig. 7.

*Fig. 7*: Intramammary infection status of 86 dairy heifers divided over 20 dairy herds

Milk samples of all quarters were collected at two periods (S1: between 1 and 4 days after calving, S2: between 5 and 8 days after calving). Heifers were considered non-infected if all quarters tested culture-negative in both S1 and S2. Heifers were considered coagulase-negative staphylococci (CNS)-infected if at least one quarter tested CNS-positive at both periods and if no quarters tested major pathogen-positive. Heifers were considered major pathogen-infected if at least one quarter tested positive on the same major pathogen (Staphylococcus aureus, esculin-positive streptococci, Streptococcus agalactiae, Streptococcus dysgalactiae) at both periods.
b. Somatic cell count and milk production in first lactation

Results for average SCC and daily milk yield were recalculated and shown in Fig. 8.

Fig. 8 : Milk production (kg milk/day) and logarithmic-transformed SCC (LnSCC) of 86 heifers during the first 285 days of lactation

2. Genotypic data

In order to design the primers, a shotgun sequence (110615593-110618295) from locus NC_007300.3 on the *Bos taurus* chromosome 2 was selected. This sequence contains the coding region of *CXCR1* (mRNA: NM_174360.2) and was compared to the sequence of *CXCR2* (mRNA: NM_001101285.1) and sequences gathered from all GenBank databases using the Basic Local Alignment Search Tool (BLAST) from NCBI. Keeping in mind the restrictions mentioned in the section “materials and methods”, following primers (Table 4.) were chosen:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length (in bp)</th>
<th>G+C (in %)</th>
<th>Tm (in °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-TCCTTGATGAGAGTGATTTGGA-3'</td>
<td>22</td>
<td>40.9</td>
<td>59.7</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TTGACATGGGACTGTGAACG-3'</td>
<td>20</td>
<td>50.0</td>
<td>60.6</td>
</tr>
</tbody>
</table>

Tm stands for melting temperature.

Table 4. Forward and reverse primer and their characteristics.
After ordering, 30.4 nMoles and 38.5 nMoles lyophilized content of respectively forward and reverse primer were delivered. The contents were dissolved in respectively 304µl and 385µl PCR-grade water so primer suspensions with a concentration of 100µM were obtained. Gel electrophoresis of the products after PCR in the gradient PCR device resulted in 5 bright bands.

The brightest obtained band was those of the product from the PCR with annealing temperature 62°C. This temperature was further used during the research. A photo of a gel after gel electrophoresis is shown in Fig. 9. For the vast majority of the samples, 3 µl PCR product was used for sequencing.

Fig 9. : Digital picture taken from a gel after electrophoresis of PCR products. In well 1, 2, 3 and 4 respectively 1Kb Plus DNA ladder™, the PCR product of a negative control and two PCR products containing a 1236 bp amplified DNA fragment of samples were loaded. The bands of lane 3 and 4 are clearly visible and migrated to the expected location (between 1000 and 1650bp).
In total, 20 SNP were identified, among them the 4 described by Grosse et al. (1999) and the additional SNP described by Youngerman et al. (2004a).

Fig. 10: Single nucleotide polymorphism at position 777. Chromatograms of 3 samples are shown. From left to right: Genotype CC, genotype GC and genotype GG.

Ten SNPs were found to be non-synonymous polymorphisms (see Table 5), the other 10 (at position 291, 333, 337, 570, 642, 714, 816, 819, 1008 and 1068) synonymous polymorphisms.

Table 5. Non-synonymous single nucleotide polymorphisms (SNP) identified

<table>
<thead>
<tr>
<th>Position</th>
<th>37</th>
<th>38</th>
<th>68</th>
<th>163</th>
<th>313</th>
<th>365</th>
<th>475</th>
<th>735</th>
<th>980</th>
<th>995</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>AT→TA</td>
<td>G→A</td>
<td>G→T</td>
<td>A→G</td>
<td>T→C</td>
<td>T→C</td>
<td>C→G</td>
<td>A→G</td>
<td>A→G</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>I→Y</td>
<td>G→E</td>
<td>D→Y</td>
<td>T→A</td>
<td>V→A</td>
<td>W→R</td>
<td>H→Q</td>
<td>K→R</td>
<td>H→R</td>
<td></td>
</tr>
<tr>
<td>Charge²</td>
<td>N→P</td>
<td>N→A</td>
<td>A→P</td>
<td>P→N</td>
<td>N→N</td>
<td>N→C</td>
<td>C→P</td>
<td>C→C</td>
<td>C→C</td>
<td></td>
</tr>
</tbody>
</table>

¹ SNP at position 37 and 38 were found to be 100% linked. Position are relative to the start codon.
² A, C, N and P stand respectively for anionic, cationic, non-polar and polar

In case of non-synonymous polymorphism, there are two possibilities. The mutation can affect the charge at the position of the translated amino acid or not (Table 5). Change of charge can have an effect on the configuration of the protein and thus also on the functionality. Charge of each amino acid is shown in appendix A. On the next page, a visual representation of the sequence and structure of bovine CXCR1 is given (Fig. 11). Locations where SNP were detected are marked.
Fig. 11: Visual representation of bovine CXCR1. The positions of the identified single nucleotide polymorphisms in the DNA sequence are noted. The structure is based on publications on human CXCR1 (Baggiolini et al., 1994; Damaj et al., 1996).
The number of heifers per genotype per SNP is given in Table 7.

Table 6. The 20 identified single nucleotide polymorphisms (SNPs) and the number of heifers per genotype

<table>
<thead>
<tr>
<th></th>
<th>AA$^2$</th>
<th>36</th>
<th>AA</th>
<th>0</th>
<th>GG</th>
<th>35</th>
<th>GG</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>37$^1$</td>
<td>AT</td>
<td>67</td>
<td>313</td>
<td>AG</td>
<td>0</td>
<td>570</td>
<td>GA</td>
<td>67</td>
</tr>
<tr>
<td>TT</td>
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<td>GG</td>
<td>140</td>
<td>AA</td>
<td>38</td>
<td>AA</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>36</td>
<td>TT</td>
<td>3</td>
<td>GG</td>
<td>66</td>
<td>AA</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>TA</td>
<td>67</td>
<td>333</td>
<td>TC</td>
<td>42</td>
<td>642</td>
<td>GA</td>
<td>64</td>
</tr>
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<td>AA</td>
<td>10</td>
<td>GG</td>
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</tr>
<tr>
<td>GG</td>
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<td>GG</td>
<td>3</td>
<td>AA</td>
<td>0</td>
<td>AA</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>GA</td>
<td>67</td>
<td>337</td>
<td>GA</td>
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<td>714</td>
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<td>0</td>
</tr>
<tr>
<td>AA</td>
<td>37</td>
<td>AA</td>
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<td>GG</td>
<td>140</td>
<td>GG</td>
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</tr>
<tr>
<td>GG</td>
<td>0</td>
<td>TT</td>
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<td>CC</td>
<td>36</td>
<td>CC</td>
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</tr>
<tr>
<td>163</td>
<td>GT</td>
<td>0</td>
<td>365</td>
<td>TC</td>
<td>67</td>
<td>735</td>
<td>CG</td>
<td>67</td>
</tr>
<tr>
<td>TT</td>
<td>140</td>
<td>CC</td>
<td>38</td>
<td>GG</td>
<td>37</td>
<td>TT</td>
<td>14</td>
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</tr>
<tr>
<td>CC</td>
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<td>TT</td>
<td>0</td>
<td>CC</td>
<td>64</td>
<td>GG</td>
<td>35</td>
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</tr>
<tr>
<td>291</td>
<td>CT</td>
<td>63</td>
<td>475</td>
<td>TC</td>
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<td>CA</td>
<td>63</td>
</tr>
<tr>
<td>TT</td>
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<td>CC</td>
<td>140</td>
<td>AA</td>
<td>13</td>
<td>AA</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

$^1$These numbers indicate the position of the SNP in the DNA sequence relative to the start codon

$^2$The first genotype noted per SNP is the genotype having nucleobases homozygous equal to the reference

Sequencing showed that the investigated heifers belong to 12 different genotype groups. Four of these genotypes contained no heterozygosity, enabling the deduction of 4 haplotypes which were named haplotype 1, 2, 3 and 4. Six other genotypes could be explained by combination of 2 haplotypes. In total, 138 of the 140 heifers belonged to these 10 genotype groups. Cloning of the sample of one heifer belonging to another genotype group was successful. The obtained sequence was equal to the sequence of haplotype 3. The sequence of the other haplotype could be deducted from the sequence of the genotype and haplotype 3. This newly found haplotype was named haplotype 5. The genotype of the remaining heifer was found to be a combination of haplotype 2 and haplotype 5. An overview of the 12 genotypes and their haplotypes is given in Table 7.
Table 7. Haplotypes found in 140 dairy heifers and the genotypes their combinations form

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H4</th>
<th>H5</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>G1</td>
<td>G5</td>
<td>G6</td>
<td>G7</td>
<td>/</td>
</tr>
<tr>
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<td>G2</td>
<td>G8</td>
<td>G9</td>
<td>G11</td>
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<tr>
<td>H5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“H” and “G” are abbreviations for respectively haplotype and genotype. “/” indicates that this genotype might occur but that it was not found in this research.

Fig. 12 shows the frequency of each genotype and haplotype in the investigated population.

![Fig. 12](image)

Fig. 12 : Genotype (left) and haplotype (right) frequencies in 140 dairy heifers

The nucleotides of the reference sequence and of the 5 haplotypes at the positions of the SNPs are given in Table 8.

Table 8. Nucleotides of the reference sequence and of 5 haplotypes found in a population of 140 dairy heifers at the position of the single nucleotide polymorphisms (SNPs)

<table>
<thead>
<tr>
<th></th>
<th>37</th>
<th>38</th>
<th>68</th>
<th>163</th>
<th>291</th>
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</tbody>
</table>

R stands for reference (GenBank Accesion No. NC_007300), H for haplotype. Numbers indicate positions of SNP relative to the start codon.
3. Associations

Because of prior results from literature suggest the importance of the SNP at position 735, it was decided to focus on the latter (Youngerman et al., 2004a).

a. Association between polymorphism and intramammary infection status at calving

Data revealed that a smaller percentage of heifers with genotype GG at position 735 were infected in early lactation compared to heifers with the other genotypes. Especially CNS infection occurred more frequently in the group of heifers with genotype CC and GC (see Fig. 13).

**Fig. 13**: The number of non-infected heifers, heifers infected with coagulase-negative staphylococci (CNS) and heifers infected with a major pathogen (*Staphylococcus aureus*, esculin-positive streptococci, *Streptococcus agalactiae* or *Streptococcus dysgalactiae*), with genotype CC or GC and genotype GG, respectively. Only heifers with different sires were included in the analysis (n = 86).
Statistical analysis showed a borderline non-significant association between SNP 735 and risk of IMI at calving. Heifers with genotype GG seem to be at higher risk of IMI and especially of IMI caused by CNS (Table 9.).

**Table 9.** Statistical analysis of the association between a heifers’ genotype at position 735 and intramammary infection status using logistic mixed regression models.

<table>
<thead>
<tr>
<th>Binary outcome variable</th>
<th>Predictor variable</th>
<th>n&lt;sup&gt;1&lt;/sup&gt;</th>
<th>β&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SE&lt;sup&gt;3&lt;/sup&gt;</th>
<th>OR&lt;sup&gt;4&lt;/sup&gt;</th>
<th>95% CI&lt;sup&gt;5&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;6&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Total IMI</td>
<td>Constant</td>
<td>1.872</td>
<td>0.551</td>
<td></td>
<td></td>
<td></td>
<td>0.063</td>
</tr>
<tr>
<td>Genotype CC + GC</td>
<td>31 ref.</td>
<td>-1.627</td>
<td>0.876</td>
<td>0.196</td>
<td>0.03 - 1.09</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Genotype GG</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMI CNS&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Constant</td>
<td>1.746</td>
<td>0.553</td>
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<td></td>
<td>0.055&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>Genotype CC + GC</td>
<td>27 ref.</td>
<td>-1.730</td>
<td>0.903</td>
<td>0.177</td>
<td>0.03 - 1.04</td>
<td>0.055</td>
<td></td>
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<tr>
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<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only heifers with different sires were included in the analysis. (n = 86).

Milk samples of all quarters were collected at two periods (S1: between 1 and 4 days after calving, S2: between 5 and 8 days after calving). Heifers were considered to have an IMI if the same pathogen was cultured from the 2 consecutive samples of at least one quarter. Heifers were considered to have an IMI caused by CNS if CNS was cultured from the 2 consecutive samples of at least one quarter and if no major pathogen (*Staphylococcus aureus*, esculin-positive streptococci, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*) was cultured at S1 or S2.

<sup>1</sup>Number of heifers per genotype

<sup>2</sup>Regression coefficients

<sup>3</sup>Standard error

<sup>4</sup>Odds ratio = $e^\beta$

<sup>5</sup>95% confidence interval = $e^{(\beta \pm 1.96 \times SE)}$

<sup>6</sup>P-value for genotype effect

<sup>7</sup>Reference, values of other genotypes are compared with the value of this genotype

<sup>8</sup>Coagulase-negative staphylococci
b. Association between polymorphism and log somatic cell count in first lactation

Overall, no differences in LnSCC were seen between heifers with genotype CC or GC and heifers with genotype GG (Fig. 14).

![Graph showing average logarithmic-transformed SCC (LnSCC) during the first 285 days of the first lactation for heifers with genotype CC or GC and heifers with genotype GG at position 735, respectively. Only heifers with different sires were included in the analysis (n = 86).](image)

No significant association (P = 0.72) was observed between SNP 735 and LnSCC (Table 10.).

Table 10. Statistical analysis of the association between a heifers’ genotype at position 735 and logarithmic-transformed SCC (LnSCC) using linear mixed regression models

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>n</th>
<th>β</th>
<th>SE</th>
<th>P-value</th>
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<tr>
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<td>...</td>
<td>4.58</td>
<td>0.22</td>
<td>&lt; 0.001</td>
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<tr>
<td>Days in milk</td>
<td>...</td>
<td>-0.004</td>
<td>0.0026</td>
<td>0.14</td>
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<tr>
<td>Days in milk x days in milk</td>
<td>...</td>
<td>0.0002</td>
<td>0.000</td>
<td>0.04</td>
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<tr>
<td>Genotype</td>
<td></td>
<td>0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype CC + GC</td>
<td>62</td>
<td>ref.¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype GG</td>
<td>24</td>
<td>-0.07</td>
<td>0.18</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Only heifers with different sires as father were included in the analysis (n = 86)

¹Reference group
²Number of heifers per genotype
³Regression coefficients
⁴Standard error
c. Association between polymorphism and milk production in first lactation

Overall, no differences were seen in daily milk yield between heifers with genotype CC or GC and heifers with genotype GG (Fig. 15).

**Fig. 15**: Average daily milk yield (kg/day) of 86 dairy heifers during the first 285 days of the first lactation for heifers with genotype CC or GC and heifers with genotype GG, respectively. Only heifers with different sires were included in the analysis ($n = 86$).

No significant association ($P = 0.48$) was observed between SNP 735 and milk production (Table 11.).

<table>
<thead>
<tr>
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<th>SE²</th>
<th>P-value</th>
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<td>&lt; 0.001</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Genotype CC + GC</td>
<td>62</td>
<td>ref.</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Genotype GG</td>
<td>24</td>
<td>0.76</td>
<td>1.09</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Only data of heifers with different sires were included.

¹Reference group
²Number of heifers per genotype
³Regression coefficients
⁴Standard error

Table 1. Statistical analysis of the association between a heifers' genotype at position 735 and daily milk yield (kg/day) using linear mixed regression models
For long, mammary glands of heifers were thought to be sterile prior to the first milking. From 1980 on, studies showed that, contrary to latter common believe, heifers are as well at risk of IMI (Meaney, 1981; Oliver et al., 1983; Boddie et al., 1987; Fox et al., 1995; Nickerson et al., 1995). Intramammary infections can lead to mastitis and heifer mastitis was found to range between 28.9%-74.6% prepartum and between 12.3%-45.5% at parturition (Fox, 2009). From almost 75% of the heifers in the present research, a pathogen could be cultured from at least one quarter.

Not all heifers in a herd become infected and while IMI in early lactation is only transient in some heifers, it can persist in other heifers (Taponen, 2007). Differences in pathogens were observed. Intramammary infections caused by major pathogens are more likely to persist, inducing high SCC and causing CM and severe production losses compared to IMI caused by minor pathogens such as CNS and C. bovis (Timms and Schultz, 1987). Fortunately for farmers, most IMI in heifers are caused by CNS (Fox et al., 1995; Aarestrup and Jensen, 1997; Schukken et al., 2009; Piepers et al., 2010). In the data that were used in this study, CNS were the most frequently isolated pathogens (70% of all culture positive samples).

Mastitis is a multi-factorial disease and not only pathogen-specific but host-specific factors as well determine whether or not a heifer becomes infected and whether or not this infection persists. In the early stage of infection, innate immune responses provide the predominant form of defence against pathogens (Sordillo et al., 1997). One of the key components in this innate immunity is the migration of neutrophils from the blood to the mammary gland. The speed of this migration can be decisive in whether or not pathogens persist (Paape et al., 2000). Neutrophils are attracted to the udder by chemoattractants from which IL8 is considered as the most crucial one (Caswell et al., 1999). Binding IL8 causes migration, prolonged lifetime (Kettriz et al., 1998), and activation (Mitchell et al., 2003) of neutrophils. Two receptors were found to bind IL8, namely CXCR1 (alias IL8RA) and CXCR2 (alias IL8RB).

In total, 5 SNPs are described in the coding region of the CXCR1 gene at position 570, 642, 735, 816 and 819 relative to the start codon (Grosse et al., 1999; Youngerman et al., 2004b). An association between polymorphism at position 735 and subclinical mastitis was shown (Youngerman et al. 2004a). The mutation at position 735 causes a glutamine (Q) to histidine (H) substitution at amino acid 245 in the third intracellular loop, proven to be an important region in the signal transduction which might explain the differences observed in mastitis phenotype (Youngerman et al., 2004a). Our research differed from research performed by Grosse et al. (1999) and Youngerman et al. (2004b) as not a segment of the coding region of CXCR1 but the whole coding region was sequenced. This resulted in the discovery of 15 SNPs that have not been described in literature before. The 5 previously identified SNPs were found as well.

In total, 10 of these mutations do not cause a change in amino acid and are synonymous polymorphisms. The other 10 do cause a change and are non-synonymous polymorphisms. The mutation described by Youngerman et al. (2004a) was found to be 100% linked with 4 other mutations (SNP 37, 38, 68 and 819). In total, these mutations cause 3 changes in amino acids (position 13, 23 and 245). Furthermore, all 3 changes in amino acid affect the charge of the protein at their position. This might influence the configuration and thus also the functionality of the protein at one or more locations. Latter findings motivate further research on either
one of these 5 SNPs. Nevertheless, other SNPs, especially the non-synonymous polymorphisms might affect the phenotype as well and could be studied.

Compared to the relative high number of identified SNP (20), only a small number of haplotypes (5) were found. Four of these haplotypes were common in the study population, one was rarer. In total, 140 dairy heifers from 20 different herds and from 80 sires were enrolled in this study. Genotype and haplotype frequencies found are likely to approximate frequencies of the whole Flemish dairy population. Interestingly, the 4 most common haplotypes in this research were similar to the 4 most common haplotypes in Holstein cows in the research performed by Youngerman et al. (2004b). Haplotype frequencies were comparable as well. This is not unlikely, as the Holstein breed is used worldwide and selection in it is becoming more and more an international matter.

The association between SNP 735 and mastitis phenotype was analysed. Focus was put on this mutation because it potentially affects the phenotype (see above) and because an association was previously described (Youngerman et al., 2004a). We opted not to compare all 3 genotypes to obtain sufficient power using the limited dataset. Genotype GG was described as advantageous for mastitis resistance (Youngerman et al., 2004a) and therefore heifers expressing this genotype were compared to heifers expressing genotype GC and CC. Because IMI in early lactation caused by CNS was shown to affect heifers’ performances during lactation positively (Piepers et al., 2010), risk of IMI caused by CNS in specific was tested separately besides risk of IMI in general.

Heifers with genotype GG were found to have a borderline non-significant lower odds of IMI at calving. However, similar to other studies (Youngerman et al., 2004a, Levy-Baca et al., 2008b; Goertz et al., 2009), lower SCC or higher milk yield during lactation could not be demonstrated in animals expressing this genotype. Youngerman et al. (2004a) suggested that absence of increased SCC in animals expressing genotype CC might be explained by malfunctioning of one the biochemical processes responsible for neutrophil migration from peripheral blood to the mammary gland. We would like to propose an alternative explanation. Intramammary infection can be caused by a variety of pathogens and some are more likely to persist and to induce profound inflammation, milk production losses and SCC elevation (Timms and Schultz, 1987). The association between SNP 735 and mastitis susceptibility could be pathogen specific, as suggested by our results. Therefore animals with genotype GG might be more resistant to CNS which are less likely to induce high SCC and severe production losses during lactation.
D. CONCLUSIONS

In conclusion, this research shows that polymorphisms in the coding region of *CXCR1* are highly present in Flemish dairy heifers. The 5 SNPs described in literature were all observed and 15 additional SNPs were identified. Ten SNPs were found to cause a change in amino acids and are non-synonymous polymorphisms. A borderline non-significant association between SNP 735 and odds of IMI in early lactation was found. Heifers expressing genotype GG seem to be less susceptible to IMI compared to heifers with genotype CC or GC. This susceptibility appears to be pathogen-specific as well. Still, polymorphism at position 735 did neither affect the heifers’ somatic cell count nor the average daily milk yield during first lactation. Pathogen-specific susceptibility IMI is suggested. However, more research is required to further elucidate the role of polymorphism at position 735 and other SNPs and their relevance for susceptibility of heifers against pathogen-specific IMI, SCC and milk traits in first lactation.
IV. ACKNOWLEDGEMENTS

Tijdens het afgelopen jaar heb ik veel werk in deze thesis gestoken maar nooit heb ik het gevoel gehad dat ik volledig op mijzelf aangewezen was. Ik heb van heel wat personen veel hulp gekregen en zou hen hier graag voor bedanken.

Beste prof. dr. Sarne De Vliegher,
Ik ben nog altijd heel tevreden dat ik jou vorig jaar vanuit Jimma gemaild heb met de vraag of je mij wat tips kon geven hoe ik de uiergezondheid van de koeien daar best onderzocht. De dag zelf nog kreeg ik antwoord en was ik geholpen. Ook dit jaar stond jouw deur steeds voor mij open en was je zelf op momenten waarop je het heel druk had steeds bereid naar mijn vragen te luisteren en mij te helpen. Toen ik vorige zomer je vroeg of ik onderzoek mocht doen op het CXCR1 (cursief! ☺) gen in plaats van op het gen voorgesteld door u en prof. Peelman heeft u niet lang getwijfeld. Ik kreeg onmiddellijk goedkeuring van u. Ik heb steeds het gevoel gehad dat je volledig achter mij stond. Dit heeft mij zeer gemotiveerd en ik ben je hier dan ook zeer dankbaar voor. Ik heb veel bewondering voor jou als onderzoeker en als persoon. Je bent als lesgever recht ongetwijfeld niet de meest populaire prof onder de studenten maar toch kan ik mij moeilijk een promotor voorstellen waarmee ik liever had samen gewerkt.

Beste prof. dr. Luc Peelman,
Tijdens het derde jaar ben ik graag naar jouw lessen geweest en door deze lessen is genetica mij erg gaan boeien. Ik was dan ook heel enthousiast toen ik in het begin van het jaar te horen kreeg dat ik op de genetische component van mastitis mocht werken. Af en toe kwam je in het labo kijken en vroeg je me hoe alles ging. Je verplichtte me nooit iets te doen maar vertelde mij eerder wat ik kon doen of wat jouw het beste leek. Net als prof. De Vliegher heb je mij dit jaar veel vertrouwen gegeven. Bij problemen of vragen kon ik steeds rekenen op jouw hulp. Ik vond het heel aangenaam om op jouw dienst te werken. Heel erg bedankt!

Beste dr. Mario Van Poucke,
Tijdens de eerste vijf jaar van mijn opleiding heb ik helaas niet veel geleerd over hoe je labowerk uitvoert. Toen ik in september begon is dat jou waarschijnlijk niet ontgaan. Telkens heb je mij echter heel duidelijk uitgelegd hoe een techniek in zijn werk gaat en hoe je de dingen best aanpakt. Je toonde me elke handeling voor maar liet mij vrij snel alles zelfstandig uitvoeren. Heel vaak heb ik op je deur geklopt en gevraagd hoe ik best bepaalde dingen aanpakte of vroeg ik je gewoon een epje van een product die ik niet had of opgebruikt was in mijn frigo. Elke keer opnieuw maakte je tijd voor mij vrij en deed je er alles aan om mij zo goed mogelijk verder te helpen. Hoewel je eigenlijk zelf geen diploma diergeneeskunde hebt bent u toch één van de personen van wie ik het meest geleerd heb en naar opgekeken heb tijdens mijn opleiding. Muchas gracias por todo!
Beste dr. Sofie Piepers,
Zonder stalen, geen onderzoek… Ik had het geluk dit jaar dat ik vanaf dag 1 al over 140 bloedstalen en een hele dataset kon beschikken. Ik kon mij onmiddellijk toe spitsen op de genetica en wist dat ik later alle gegevens die ik verzamelde kon vergelijken met de vele dagen (en nachten?) werk die jij reeds had geleverd. De passie, enthousiasme en volharding waarmee jij je werk beoefent werken heel motiverend voor een jonge onderzoeker als ik. De dag nadat je de verdediging voor jouw doctoraat had afgelegd stond je reeds klaar om mij te helpen met de statistische analyse. Op onze faculteit loopt er heel wat personeel rond maar eigenlijk kan ik niemand vergelijken met jou. En dat bedoel ik enkel positief. Na een jaartje veel rond genen gewerkt te hebben vermoed ik dan ook dat een uitzonderlijke mutatie bij jou opgetreden is waardoor je in staat bent om de job van praktijkdierenarts, toponderzoekster en M-teamster te combineren. En bovendien ben je ook nog heel aangenaam om mee samen te werken.
V. REFERENCES


APPENDIX A: THE GENETIC CODE AND AMINO ACID ABBREVIATIONS

The genetic code

<table>
<thead>
<tr>
<th>Codon</th>
<th>1-letter code</th>
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<td>Ala</td>
<td>Alanine</td>
<td>nonpolar</td>
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1-codon
2 1-letter code of the translated amino acid
3 3-letter code of the translated amino acid
4 * stands for initiation

Amino acids abbreviations

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<thead>
<tr>
<th>1-letter code</th>
<th>3-letter code</th>
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<th>Charge</th>
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(From NCBI, 2010)