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Association of *TLR4* and *CARD15/NOD2* polymorphisms with SCC in Holstein–Friesian cattle

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Abstract. *Mastitis* is one of the most important dairy cattle diseases which results in economic losses in dairy production. *Mastitis* cases can be classified as subclinical or clinical. All forms of *mastitis* lead to changes in milk composition and induce an increase in somatic cell count (SCC). SCC is a very important and basic indicator of udder health. An increase in SCC is usually caused by the immune response to the invasion of pathogens contributing to *mastitis*. The aim of this study was to investigate associations between the polymorphisms of selected genes (*TLR4* and *CARD15/NOD2*) whose products are involved in the identification of pathogen-associated molecular patterns (PAMPs) during the innate immune response to infection, and immunity to *mastitis* expressed as SCC. The genes under study were also examined for epistatic effects as well as effects of interactions with parity and stages of lactation. In all the studied classes, allele G of *TLR4* had a favourable additive effect with negative values, contributing to a lower lnSCC. Allele A of *CARD15/NOD2* had a desirable additive effect which varied with time and the changing internal environment during lactation. With regard to the dominance effect, allele A of *CARD15/NOD2* was found to be significantly associated with a higher SCC in milk in the first lactation and in the third stage of each single lactation. Moreover, statistically significant epistatic effects were found, in particular additive–additive and dominance–additive interactions were favourably associated with SCC which was lower than expected in the case of no epistasis.

1 Introduction

Mastitis is the most devastating cattle disease in terms of economic losses occurring worldwide (Kumar et al., 2010). Due to the involvement of multiple etiological agents, it has always been a challenge to veterinarians throughout the world (Vashney et al., 2012). *Mastitis* is the consequence of the adverse effect on the mammary gland and the cow's immune system of many factors – environmental (both external and internal), genetic and the interactions between them (Rinaldi et al., 2010; Schukken et al., 2011).

The bovine mammary gland is an organ which is able to produce several dozen litres of milk per day. *Mastitis* dramatically affects the profitability of milk production not only due to decreased milk yield, but also lower milk quality, high cost of drugs, risk of culling and sometimes animal death (Seegers et al., 2003; Halasa et al., 2007; Nielsen, 2009). *Mastitis* can be classified as subclinical or clinical, depending on whether clinical signs are present or not. In dairy practice, this inflammatory process in the dairy cow's mammary gland is generally detected by measuring somatic cell count, SCC (Schukken et al., 2003). SCC has a higher heritability than clinical *mastitis* (CM), allowing more effective genetic progress (Heringstad et al., 2003). Due to the absence of readily available and reliable data on the clinical *mastitis* phenotype, many countries around the world have included SCC in their breeding programmes as a way to improve resistance to intramammary infections (Rupp and Boichard, 2003; Beecher et al., 2010).

The host response to *mastitis* is complex and involves many different genes and cellular pathways (Oviedo-Boyso et al., 2007). Unsurprisingly, quantitative trait loci (QTL) for *mastitis* and/or SCC have been identified in nearly all chromosomes of the bovine genome (Rupp et al., 2009). The genes that are strong potential markers for resis-

tance/susceptibility to udder inflammation include, inter alia, genes encoding PAMP-recognition receptors (PRRs) (Sharma et al., 2006). Examples of such genes include toll-like receptor 4 (*TLR4*) and caspase recruitment domain 15 (*CARD15*), also known as *NOD2* (nucleotide oligomerization domain 2) (Beecher et al., 2010).

TLR4 protein recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and binds to LPS using the extracellular LRR (leucine-rich repeat) domain, which leads to intracellular activation of NF-κB (nuclear factor kappa-B). TLR4 is also able to recognize cell wall components of other important bacteria and fungi such as *Mycobacterium tuberculosis*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Candida albicans*, as well as cellular components, such as heat shock proteins (HPS) and fibrinogen (Lien and Ingalls, 2002). The bovine *TLR4* gene coding region is 2526 bp long, consists of 3 exons and is located on chromosome BTA 8. Exon 1 includes coding base pairs 1–95, exon 2 consists of base pairs 96–260 and exon 3 comprises base pairs 261–2526 (Mitra et al., 2012). More than 40 SNPs (single nucleotide polymorphisms) of the bovine *TLR4* gene have been found to date, which means an average of 1 SNP per 90 bp (Wang et al., 2008).

The *CARD15/NOD2* is a member of the NOD-like receptor gene family characterized by a central nucleotide-binding oligomerization domain (NOD), as well as the N-terminal effector binding domain (CARD) and C-terminal ligand recognition domain (comprised of leucine-rich repeats LRR) (Rosenstiel et al., 2006; Taylor et al., 2006). *CARD15/NOD2* gene is expressed in a variety of cell types which are involved in the inflammation process following pathogen recognition based on PAMP (Pant et al., 2007). The CARD15/NOD2 is a cytosolic protein which is a sensor of the smallest fragment of peptidoglycan (PG, a component of the bacterial cell wall) known to induce an immune response (Pinedo, 2009; Taylor et al., 2006). The natural product of the bacterial cell wall is muramyl dipeptide (MDP), which activates CARD15/NOD2 and induces the proinflammatory signalling pathway NF-κB (Mogensen, 2009). The bovine *CARD15/NOD2* gene is located on chromosome 18, its transcript is 5105 bp long and the protein comprises 1013 amino acids (Pinedo et al., 2009; Pighetti and Elliot, 2011). Thirty-six SNPs of *CARD15/NOD2* were identified so far (Taylor et al., 2006).

The TLR and NOD pathways have many similarities, for example they both activate mitogen-activated protein kinases (MAPKs) or NF-κB (Van Limbergen et al., 2007; Abraham and Cho, 2006). Therefore, it can be assumed that *TLR4* and *CARD15/NOD2* pathways may cooperate and modulate immunological responses, which may indicate epistatic effects of these genes.

The aim of this study was to investigate associations between the polymorphisms of the selected genes (*TLR4* and *CARD15/NOD2*) and SCC in milk of Holstein–Friesian cows. The genes were also examined for epistatic effects as well as effects of interactions with parity and stages of lactation.

2 Material and methods

This study included 534 Polish Holstein–Friesian dairy cows (Black and White variety) kept in one herd on a farm located in the north-western region of Poland. The animals were milked in their stalls, watered *ad libitum* and fed standard rations. The cows were examined under a milk performance monitoring system based on the A4 milk recording method, and data on their pedigree background and milk performance was collected in the SYMLEK (Automatic Cow Breeding Evaluation) animal recording system. The data concerning SCC was derived from the results of monthly test milkings and SCC in milk was determined using Fossomatic™ (optical somatic cell counting method). A total of 8521 test milkings were performed. The animals selected for analyses were those with lactations lasting a minimum of 265 and a maximum of 365 days. The study excluded data collected during non-standard lactations, that is, those which lasted too long or too short. The cows were daughters of 58 different bulls (the cows in the first lactation descended from 43 bulls, in the second lactation – from 42 bulls, and in the third – from 39 bulls), the average number of daughters per one bull being 10.96. The cows were characterized by different levels of expression of the Holstein–Friesian genes, varying from 37.5 to 100 %.

Peripheral blood samples were collected from all the animals under study using test tubes containing EDTA as an anticoagulant. Genomic DNA was isolated with Master Pure™ DNA Purification Kit for Blond (Epicentre, USA). The SNPs of the selected genes were identified by the tetra-primer amplification refractory mutation – PCR procedure (ARMS – PCR) (Ye et al., 2001). It is a reliable, inexpensive and easy to use method based on a single PCR reaction. ARMS – PCR uses four primers to amplify a fragment of a DNA sequence containing a given SNP producing amplicons representing each of the two allelic forms. The sets of primers used to genotype *TLR4* and *CARD15/NOD2* have been designed by Sharma et al. (2006) and Pant et al. (2007), respectively. The sequences of these primers are shown in Table 1. The *TLR4* SNP is located at the 226 bp position in the putative promoter region (P-226). SNP in *CARD15/NOD2* (c.2886-14A>G, rs43710287) is located in intron 10.

The PCR reactions were performed in a total volume of 10 µL, containing 30 ng of template DNA, 10 pmol of each of the inner primers, 1 pmol of each of the outer primers, 200 µM deoxyribonucleotide triphosphates, 2.5 mM $MgCl_2$, 1 × PCR buffer and 1 U of Taq DNA polymerase. To increase the specificity of the reaction, a touchdown profile was applied: the temperature of annealing was 5 °C higher in the first PCR cycle, and in the subsequent five cycles the temperature was decreased by 1 °C per cycle and then main-

Table 1. Primers and conditions applied to identify selected SNPs of *TLR4* and *CARD15/NOD2*.

Gene	Primers	Sequence, $5' \rightarrow 3'$	T_m	Amplicon size
*TLR4**	Forward inner	TTTCTTCTTCTTCCTCTAACTTCCCCACG	55	G allele: 185 bp
	Reverse inner	TTTCTCTGATATTTATCTCCTCTGCCAGCG		C allele: 277 bp
	Forward outer	CCAGGGTATTTTGTTATGGCTGGAACAT		Two outer primers: 403 bp
	Reverse outer	CTCCGAGGCACTGTAGTGTCGTCTCT		
*CARD15/NOD2***	Forward inner	CAAGCATCCTCAAAGTTCACCATTATG	60	A allele: 300 bp
	Reverse inner	GTGGTTGTTAGACAGCCTAGAGAGGAATAT		G allele: 150 bp
	Forward outer	CCTGGCACTAAAATGCACGTATTTTTAT		Two outer primers: 405 bp
	Reverse outer	AGTTATACGTTGGAAAACTGAGGCTCAG		

* Primer sequences designed by Sharma et al. (2006)
* Primer sequences designed by Pant et al. (2007)

tained at the target level throughout the remaining 30 cycles for *TLR4* and 34 cycles for *CARD15/NOD2*. The PCR profiles were as follows: *TLR4* $\rightarrow$ 94 °C for 10 min, 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature (including initial touchdown cycles) and 30 s of extension at 72 °C, ending with 5 min at 72 °C; *CARD15/NOD2* $\rightarrow$ 95 °C for 8 min, 34 cycles of 30 s at 94 °C, 30 s at the annealing temperature (including initial touchdown cycles) and 30 s of extension at 72 °C, ending with 5 min at 72 °C. The products of amplification were observed on 2 % agarose gels which were then stained with ethidium bromide to visualize the genotypes.

The results were recorded and analysed statistically to examine the genetic structure of the population under study, i.e. to determine allele and genotype frequencies for the selected genes. Subsequently, a statistical analysis was conducted to search for associations between the polymorphisms of the selected genes and SCC in milk and daily milk yield as well as protein, fat, lactose and dry matter content in milk. SCC was transformed logarithmically to normalize the distribution of this trait. The calculations were made separately for parities I to VI (parity VI also including subsequent parities, if any) and separately for selected stages of lactations (stage I: days 5 to 100, stage II: days 101 to 200, stage III: beyond day 200 to the day of the last milking test), as well as for all parities and all stages in total. Full lactations were taken into account, and not only standard lactations of 305 days, as is often the case in similar studies. This procedure was chosen due to the fact that the last days of lactation, i.e. the dry-off period, is a critical moment associated with a higher incidence of *mastitis* and increased SCC in milk. It was important, therefore, to capture the last milking test before the dry-off.

To estimate any such associations, a mixed model (containing both random and fixed effects) was applied. Apart from genotypes, the following factors were included as sources of variation: standard factors such as year/season and day in milk (DIM), and the additive polygenic effect (genetic background effect). Additionally, in the case of traits that are repeatable over time, the model also included fixed environmental effect. The set of equations was computed in accordance with the principles of the best linear unbiased prediction (BLUP) method. The lnSCC were modelled using current goodness-of-fit criteria such as error variance or log–likelihood ratios. To estimate lnSCC parameters within the parity and stage classes, Wilmink polynomials were used. A homogeneous distribution of error variance was assumed for the consecutive days of lactation. It was also assumed that there were no correlations between the random effects of DIM. There were also no correlations between random error effects either within or between parities and stages. The adopted statistical model also included the dominant and epistatic effects (including all four types of interactions: additive–additive, additive–dominant, dominant–additive and dominant–dominant) between the selected genes, as estimated with the natural and orthogonal interactions (NOIA) model (Alvarez-Castro and Carlborg, 2007). To estimate the genetic effects of the examined loci, single-trait mixed multiple regression models were used. The overall formula for the selected models was as follows:

$$Y_{jklmnp} = \mu + \sum_{i=1}^{8} G_{ij} + \mathrm{HTD}_k + \mathrm{HYS}_{ml} + \mathrm{DIM}_{npl} + A_j + \mathrm{PE}_{jl} + e_{jklmnp}$$

where Y_{jklmnp} – lnSCC (matrix of observation); μ – mean value of the analysed trait in the population; G_{ij} – additive (a), dominance (d) and epistatic (aa, ad, da and dd) effects of *TLR4* (1) and *CARD15/NOD2*(2)loci:

$$\sum_{i=1}^{8} G_{ij} = a_1 + a_2 + d_1 + d_2 + a_1a_2 + a_1d_2 + d_1a_2 + d_1d_2.$$

HDT_k – effect of milking test year/month; HYS_{ml} – effect of birth year/season; DIM_{npl} – effect of DIM; A_j – random additive polygenic effect; PE_{jl} – random permanent environmental effect (in the case of traits that are repeatable over time); e_{jklmnp} – random residual effect.

Table 2. Frequency of *TLR4* and *CARD15/NOD2* genotypes and alleles.

Gene	Genotype	Number of cows	Genotype frequency	Allele	Allele frequency
TLR4	GG	151	0.2828	G	0.3820
	CG	277	0.5187	C	0.6180
	CC	106	0.1985	–	–
CARD15/NOD2	AA	298	0.5581	A	0.6039
	AG	187	0.3502	G	0.3961
	GG	49	0.0918	–	–

Table 3. Additive, dominance and epistatic effects of *TLR4* and *CARD15/NOD2* with regard to lnSCC in selected stages of lactation.

Effect	Estimate lnSCC							(SE)
	Stage I of lactation		Stage II of lactation		Stage III of lactation		Whole lactation	
a_{TLR4}	−0.383**	(0.043)	−0.366**	(0.038)	−0.421**	(0.028)	−0.389**	(0.020)
d_{TLR4}	−0.061	(0.058)	0.079	(0.052)	0.038	(0.039)	0.025	(0.028)
$a_{CARD15/NOD2}$	−0.048	(0.064)	−0.130*	(0.055)	−0.136**	(0.043)	−0.114**	(0.031)
$d_{CARD15/NOD2}$	−0.005	(0.079)	0.073	(0.069)	0.123*	(0.053)	0.047	(0.038)
$a_{TLR4} \cdot a_{CARD15/NOD2}$	−0.125	(0.079)	−0.149*	(0.068)	−0.103	(0.053)	−0.132**	(0.038)
$d_{TLR4} \cdot a_{CARD15/NOD2}$	−0.081	(0.126)	−0.177	0.108	−0.259**	(0.085)	−0.179**	(0.060)
$a_{TLR4} \cdot d_{CARD15/NOD2}$	0.048	(0.105)	0.232*	(0.092)	0.186**	(0.070)	0.173**	(0.050)
$d_{TLR4} \cdot d_{CARD15/NOD2}$	−0.272	(0.159)	−0.107	(0.137)	0.265*	(0.106)	−0.008	(0.076)
Mean value of lnSCC	5.449	(0.096)	5.449	(0.168)	5.206	(0.159)	5.283	(0.055)

Asterisks indicate statistical significance levels $*P \leq 0.05$, $**P \leq 0.01$.

3 Results

The laboratory analysis showed three genotypes of *TLR4* and also three genotypes of *CARD15/NOD2* (Table 2).

When investigating the associations between SCC in milk and allele G of *TLR4* and allele A of *CARD15/NOD2*, it was found that single additive effects of the genes were statistically significantly associated with a lower lnSCC in all the test milkings in total (Table 3). There were also statistically significant associations between the genes under study and a lower SCC in all single stages of lactation. There were no statistically significant dominance effects in the tested genes, except for *CARD15/NOD2* in the third stage of lactation, where it had a statistically significant undesirable dominance effect. When analysed, the effects of interactions between *TLR4* and *CARD15/NOD2* had both positive and negative values, and accordingly were reflected by a lower or higher SCC in the selected stages of lactation.

The following statistically significant epistatic effects with desirable negative values associated with a lower SCC were found in lactation stages: $a_{TLR4} \cdot a_{CARD15/NOD2}$ in the second stage of lactation and in the whole lactation; $d_{TLR4} \cdot a_{CARD15/NOD2}$ in the third stage of lactation and in the whole lactation. In the case of effects with the undesirable positive values in lactation stages associated with a higher SCC, the following epistatic effects were found to be statistically significant: $a_{TLR4} \cdot d_{CARD15/NOD2}$ in the second and third stages of lactation and in the whole lactation; $d_{TLR4} \cdot d_{CARD15/NOD2}$ in the third stage of lactation/ The effects of $d_{TLR4} \cdot d_{CARD15/NOD2}$ interactions did not have any significant impact on the phenotypic values of the SCC in the whole lactation.

The results of the additive, dominance and epistatic effects of *TLR4* and *CARD15/NOD2* with regard to SCC in parities are presented in Table 4. A desirable, statistically significant additive effect was found in the case of allele G of *TLR4*. Allele A of *CARD15/NOD2* also had a statistically significant additive effect, except it was only recorded in the first three parities. The dominance effects of the same alleles were statistically significant in some parity classes. In the second parity, allele G of *TLR4* gene showed positive values, which resulted in an increased SCC. Allele A of *CARD15/NOD2* was found to have an undesirable effect, but only in the first parity. In the sixth and subsequent parities, *CARD15/NOD2* had a desirable dominance effect, which means that the impact of the gene changed with parity.

The results of the study show desirable statistically significant epistatic effects in the following parities: $a_{TLR4} \cdot a_{CARD15/NOD2}$ – in parities I and II; $d_{TLR4} \cdot a_{CARD15/NOD2}$ – in parities I, II and V; $d_{TLR4} \cdot d_{CARD15/NOD2}$ – in parity VI (and subsequent, if any).

On the other hand, an undesirable epistatic effect $a_{TLR4} \cdot d_{CARD15/NOD2}$ was detected in parities I and II.

Table 4. Additive, dominance and epistatic effects of *TLR4* and *CARD15/NOD2* with regard to lnSCC in parities.

Effect	lnSCC											(SE)
	Parity I		Parity II		Parity III		Parity IV		Parity V		Parity ≥ VI	
a_{TLR4}	−0.421**	(0.042)	−0.261**	(0.040)	−0.286**	(0.046)	−0.430**	(0.051)	−0.740**	(0.064)	−0.557**	(0.089)
d_{TLR4}	0.049	(0.057)	0.198**	(0.054)	−0.065	(0.063)	−0.090	(0.071)	0.109	(0.087)	−0.156	(0.117)
$a_{CARD15/NOD2}$	−0.194**	(0.063)	−0.114*	(0.052)	−0.294**	(0.070)	−0.101	(0.088)	0.029	(0.106)	0.510	(0.234)
$d_{CARD15/NOD2}$	0.354**	(0.077)	0.092	(0.068)	0.135	(0.085)	−0.179	(0.105)	−0.101	(0.129)	−0.770**	(0.249)
$a_{TLR4} \cdot a_{CARD15/NOD2}$	−0.247**	(0.071)	−0.145*	(0.064)	0.052	(0.085)	−0.196	(0.119)	−0.212	(0.167)	−0.371	(0.351)
$d_{TLR4} \cdot a_{CARD15/NOD2}$	−0.571**	(0.124)	−0.331**	(0.104)	0.013	(0.135)	0.183	(0.176)	−0.629**	(0.216)	0.405	(0.435)
$a_{TLR4} \cdot d_{CARD15/NOD2}$	0.212*	(0.100)	0.325**	(0.093)	−0.008	(0.113)	0.149	(0.145)	0.038	(0.196)	0.580	(0.368)
$d_{TLR4} \cdot d_{CARD15/NOD2}$	0.237	(0.154)	0.214	(0.137)	0.095	(0.169)	−0.048	(0.211)	−0.388	(0.260)	−1.315**	(0.469)
Mean value of lnSCC	4.737	(0.071)	4.517	(0.064)	4.979	(0.102)	5.097	(0.102)	5.525	(0.137)	5.654	(0.210)

Asterisks indicate statistical significance levels $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

In conclusion, in the studied parities and lactation stages allele G of *TLR4* had a favourable additive effect with negative values, contributing to a lower lnSCC. However, no statistically significant dominance effects were found for this allele.

Allele A of *CARD15/NOD2* had a desirable additive effect which varied with parity and the changing internal environment during lactation. The effect is not that strong in heterozygous individuals due to the unfavourable dominance of the opposite allele G. As for the dominance effect, allele A of *CARD15/NOD2* was found to be significantly associated with a higher SCC in the first parity and in the third stage of each lactation. A positive effect of the allele reflected in a lower SCC was observed in the sixth and subsequent parities.

It is difficult to make a clear interpretation of the epistatic effects of *TLR4* and *CARD15/NOD2* genes as the effects of their interactions had both positive and negative values in different parities and stages of lactation. Statistically significant $a_{TLR4} \cdot a_{CARD15/NOD2}$ interactions in the first two parities and in the second stage of lactation had negative values, thereby being favourably associated with SCC which was lower than expected in the case of no epistasis. The $d_{TLR4} \cdot a_{CARD15/NOD2}$ effect on SCC was always favourable. Like with $a_{TLR4} \cdot a_{CARD15/NOD2}$, such a positive effect was observed in parities I, II and V as well as in all parities in total. As for individual parities, a favourable $d_{TLR4} \cdot a_{CARD15/NOD2}$ effect was detected in the third stage of lactation. On the other hand, the $a_{TLR4} \cdot d_{CARD15/NOD2}$ interaction had undesirable positive values and was statistically significantly associated with a higher SCC in the first two parities, in all parities in total, in the first and second stage of lactation and the whole lactation. The $d_{TLR4} \cdot d_{CARD15/NOD2}$ effect played a negative role only in the third stage of lactation, which is a critical moment before dry-off. Moreover, negative values were observed in the sixth and subsequent parities.

4 Discussion

The *TLR4* SNP identified in this study had already been detected by Sharma et al. (2006). It is located at the 226 bp position in the putative promoter region (P-226). In our research, the most frequent variant found at this locus was allele C (62 %). Similarly, Carvajal et al. (2013) found allele C as the predominant variant in Chilean dairy cattle. However, in a Canadian Holstein, the predominant allele was G (60 %) (Sharma et al., 2006).

Mutations in *TLR4* can compromise the host immune response to certain pathogens because *TLR4* is highly polymorphic in the bovine breeds (White et al., 2003) and its expression has been shown to be associated with intramammary infection (Sharma et al., 2006). Polymorphisms in the coding and promoter regions of *TLR4* can determine different host resistance or susceptibility patterns to various infectious diseases (Lorenz et al., 2002). Therefore, this gene may be a potential candidate for use in marker-assisted selection to enhance resistance to *mastitis* in dairy cattle. This hypothesis seems to be confirmed by the results of the present study in which *TLR4* polymorphism was found to be statistically significantly associated with SCC in Polish Holstein–Friesian cows. Such results were also obtained by other authors. For example, Sharma et al. (2006) found that the SNP P-226 was associated with estimated breeding values (EBVs) for lactation persistency and somatic cell score (SCS) in Canadian Holstein bulls. This SNP has also been implicated in the susceptibility to paratuberculosis (Ruiz-Larrañaga et al., 2011). Arbour et al. (2000) found another *TLR4* polymorphism (Asp299Gly – c.896A>G) which leads to altered recognition of LPS by the extracellular domain of *TLR4*. The same polymorphism was associated with Crohn's disease and ulcerative colitis in a Belgian study by Franchimont et al. (2004).

Besides *TLR4*, the present study included an SNP in *CARD15/NOD2* (c.2886-14A>G, rs43710287) which was found in intron 10, 14 bp upstream of exon 11. The same SNP in *CARD15/NOD2* was also identified by Pant et

al. (2007), who reported the following genotype frequencies: AA 67.5 %, AG 29.5 %, GG 3 %; and the following allele frequencies: A 82.2 %, G 17.8 %. Similar results for this SNP were obtained in the current study, but the difference between the frequencies of allele A and allele G was not so big.

Pant et al. (2007) also detected other three *CARD15/NOD2* SNPs in exon 12: c.3020A>T, c.4500A>C and c.4950C>T. The c.3020A>T (Q1007L) polymorphism was studied by Pinedo et al. (2009), who also found SNP E2-(32) G>A located in intron 1, positioned 32 bp upstream of the first base of the second exon, and SNP c.2197.T>C (C733R) located in the leucine-rich repeat domain.

So far, most studies have focused on other *CARD15/NOD2* polymorphisms, predominantly on SNP c.3020A>T (rs43710288) (Pant et al., 2007; Tanabe et al., 2004; Pinedo et al., 2009). For example, Pant et al. (2007) found that this SNP was linked to EBVs for SCS, udder depth, milk and protein yields. The same authors detected associations between SNP c.4500A>C and milk fat and protein yields. Beecher et al. (2010) reported that another *CARD15/NOD2* polymorphism, namely c.3168 A>C within exon 12, was associated with increased SCS values, which may be related to the incidence of *mastitis*.

However, no less important seems to be the c.2886-14A>G polymorphism identified by us in *CARD15/NOD2*. The results of the present study show that this SNP is associated with SCC in Holstein–Friesian cattle, which suggests that it might play a role in the host response against *mastitis*. Such association of this particular SNP has not been studied before by other researchers and further detailed analyses are needed to understand its functional mechanisms.

It should be noted, however, that although both *TLR4* and CARD15/*NOD2* turned out to be statistically significantly associated with SCC in milk, the effects of these genes varied considerably depending on the parity and lactation stage. Therefore, it is difficult to say whether the effects of the studied polymorphisms are desirable (or not) from the point of view of cow breeding programmes.

In our work, besides the interactions between the studied genes and the environment, the epistatic effects between these genes were also detected. Epistasis, defined as interactions between genes, is essentially important for understanding the structure and function of genetic pathways and evolutionary dynamics of complex genetic systems (Phillips, 2008). If the effect of one locus is altered or masked by the effect of another locus, the first locus is less likely to be detected and the joint effect of the two loci will be obscured by their interactions. This situation is likely to be further complicated if more than two loci are involved (Cordell, 2002). This might be the case with the *TLR4* and *CARD15/NOD2* SNPs identified in this study as many scientists have demonstrated a synergy effect between these genes. For example, Abraham and Cho (2006) showed in their work a synergistic relationship between *CARD15/NOD2* and *TLR*. Stimulation through CARD15/NOD2 results in activation of co-stimulatory molecules in human immature dendritic cells and murine B cells, in particular in synergy with TLR stimuli.

Van Limbergen et al. (2007), who studied inflammatory bowel disease (IBD) genes involved in the innate immune, proposed *TLR4* and *CARD15/NOD2* as candidate genes and noticed that intracellular negative regulators of TLR signalling include, inter alia, *CARD15/NOD2*. The interactions between *CARD15/NOD2* and *TLR* pathways in human cells were also studied by Netea et al. (2005). They investigated the modulation of TLR-induced cytokine production by NOD2 signals activated by muramyl dipeptide (MPD), which is a component of peptidoglycan (PGN). They demonstrated that both TLR2 and CARD15/NOD2 are required for stimulation of cells by commercial PGN, whereas purified PGN is only recognized by CARD/NOD2. Research carried out by Heel et al. (2005) showed a strong enhancement of TLR-induced cytokine responses by NOD1 activation. Synergistic effects were observed at the level of multiple cytokines and with multiple TLR ligands, suggesting a generalised effect of NOD1 activation on TLR/cytokine signalling. Moreover, the findings reported by Tada et al. (2005) strongly suggest that the combinatory stimulation of dendritic cells (DCs) via the NOD pathway and the TLR pathway synergistically promotes T-helper type 1 (Th1) immune responses. A further study by Netea et al. (2005) provided evidence for the complex regulation of TLR signalling through induction of both pro- and anti-inflammatory cytokines in response to CARD15/NOD2 activation. Watanabe et al. (2004) described how muramyl dipeptide (MDP) (the ligand of CARD15/NOD2) suppressed TLR2-ligand-induced Th1-cell responses in wild mice, but not in CARD15/NOD2-deficient mice. In contrast, two other reports did not support this inhibitory hypothesis (Maeda et al., 2005; Kobayashi et al., 2005).

Undoubtedly, there are statistically significant associations between *TLR4* and *CARD15/NOD* SNPs and resistance to *mastitis*, however the effects of these genes vary throughout the lactation period and depend on epistasis. This indicates a very complex and multifactorial nature of cooperation between these genes and requires further research to understand the functional mechanisms behind their interactions.

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