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Association between Single Nucleotide Polymorphism in RelA with Somatic Cell Count and Longevity Supports Importance of NF-κB Signalling in Cattle Health

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Abstract

Mastitis reduces milk production and causes culling. The NF-κB transcription factor RelA plays a central regulatory role in innate immunity. This study used a candidate gene approach to investigate associations between the synonymous C/G SNP rs48035703 in *RELA* with somatic cell count (SCC) and survival time. Blood samples were collected from 337 Holstein-Friesian heifers on 19 farms and genotyped by PCR-restriction fragment length polymorphism. Animals were monitored from 6 months until 2340 d of age. Pedigree, milk production and disease records were obtained. Genotype frequencies were CC 0.63, CG 0.30 and GG 0.06. The C allele had a favourable additive effect on survival: average longevities from birth were CC, 1872 d; CG, 1745 d and GG 1596 d ($P < 0.003$). Log transformed first lactation somatic cell count (SCC)data showed a significant association with this SNP using an allele substitution model (mean residuals ± SD: GG 0.30 ± 1.263; CG 0.22 ± 0.994, CC −0.04 ± 0.803, $P < 0.05$). More CC cows than expected were classified as intermediate and fewer as mastitic (30.4% v 45.9%) with respect to SCC class when categorised as 0 (unaffected), 1 (intermediate) and 2 (mastitic), whereas for CG heterozygotes fewer were intermediate and more were mastitic (12.1% v 60.3%) ($p = 0.05$). *RELA* rs48035703 CC genotype cows were therefore less likely to experience a high SCC and survived longer. These results support a role for RelA in combating mammary gland infection and warrant further studies in additional populations.

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Keywords

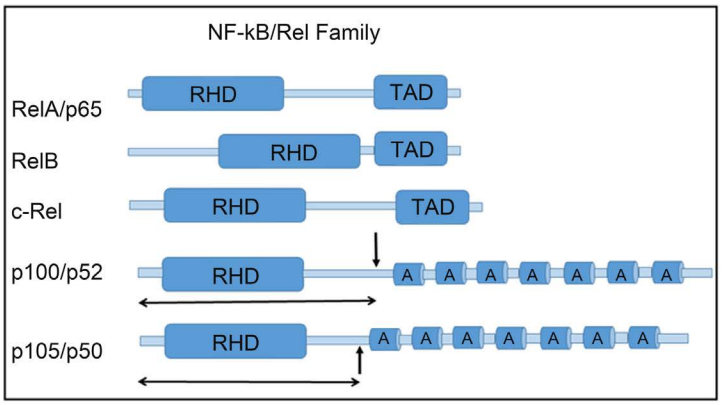
Mastitis, Longevity, RelA, NF-κB, Dairy Cow

1. Introduction

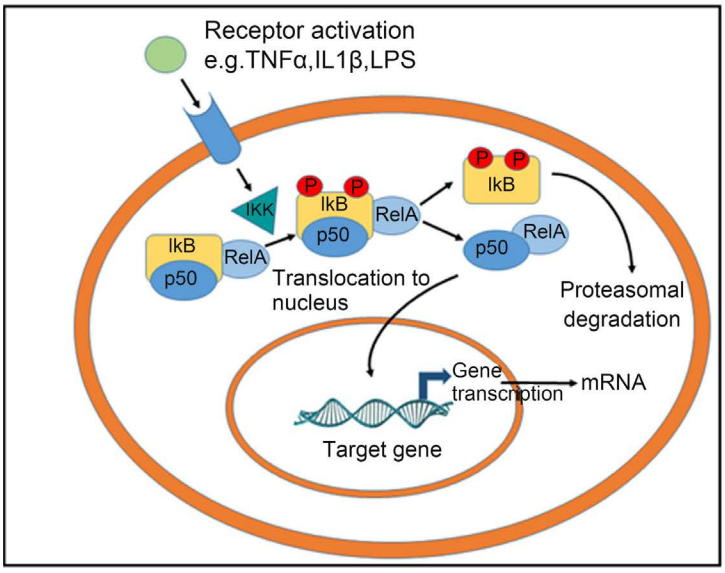
Mastitis is an inflammatory condition of the udder, most commonly due to bacterial infection. It causes major economic loss worldwide through reduced milk production, infertility and early culling and can be fatal in severe cases [1] [2] [3]. Environmental pathogens such as *Escherichia coli* and *Streptococcus uberis* can invade the gland, multiply, induce a rapid host response and are then generally eliminated [4] [5]. The inflammation is associated with a distinct acute phase response, which is accompanied by a reduction in milk yield, altered milk composition and damage to mammary tissue. Alternatively, contagious organisms, in particular *Staphyloccus aureus*, can survive in the udder and establish a chronic sub-clinical infection which manifests as an increase of somatic cells in the milk [6].

The different species of bacteria therefore elicit differential responses by the host. Gram negative bacteria, in particular *E. coli*, activate the TLR4 transduction pathway via lipopolysaccharide (LPS), causing inflammatory and apoptotic responses [6]. The severity of the ensuing disease is influenced mainly by the ability of the cow to control bacterial growth in the first few hours after infection [7], although the rapidity with which leukocytes are recruited into the affected quarter may also be important [2] [6]. The cow is less able to mount an effective immune response in the early postpartum period [8]. In contrast, *S. aureus* infection elicits a slower response and some key cytokines including TNF*α* and IL1 are barely up-regulated [9]. All toll-like receptor (TLR) signal transduction pathways eventually activate members of the NF-κB family of transcription factors [10]. Early phase NF-κB responses involve MyD88 (myeloid differentiation primary-response protein 88) dependent pathways, whereas later responses utilize MyD88-independent pathways. *S. aureus* probably acts through MyD88-independent NF-κB activation. NF-κB is known to be a central orchestrator of immune defence and many pro-inflammatory genes (e.g. *IL1B*, *IL6*, *IL8*, *TNFA*) have NF-κB attachment sites in their promoter.

The NF-κB family comprises five structurally homologous transcription factors p50 and p52 (which are activated by proteolysis of the larger precursors p105/NF-κB1 and p100/NF-κB2), RelA (also known as p65), RelB and c-rel. They all carry a Rel homology domain which contains a nuclear localization signal and they bind to κB enhancers as homo- or hetero-dimers [11]. The NF-κB members are usually present in an inactive form in the cytoplasm, complexed with members of a family of inhibitory IkB proteins. The IkB protein is degraded during pathway activation allowing the NF-κB complex to enter the nucleus to activate gene transcription [12] (**Figure 1**). The precursor proteins NF-κB1 and NF-κB2 also function as IκB-like molecules, which inhibit the nuclear translocation of associated NF-κB members [11].



(a)



(b)

Figure 1. Schematic diagram showing the role of RelA as part of transcriptional regulation by NFκB. Panel (a) illustrates the five different transcription factors which comprise the NFκB family. RHD, Rel homology domain; TAD, transcription activation domain; A, ankyrin repeat. Arrows show where the precursor proteins p100 and p105 are processed to derive p52 and p50 respectively. Panel (b) shows one potential signaling pathway. A variety of cytokines such as TNFα or IL1β or lipopolysaccaride (LPS) bind to their respective receptor on the cell membrane. This stimulates IKK (IκB kinase complex) to phosphorylate the inhibitor in the inhibitor/NFκB complex, causing dissociation of the inhibitor and activation of NFκB. This enters the nucleus to activate gene transcription. Note that the different NFκB family members can form a variety of homodimers and heterodimers which bind to a variety of target DNA sequences called κB sites to modulate gene transcription (see [12]).

The *RELA* gene drives pro-inflammatory responses by promoting the expression of inflammatory cytokines. It also maintains autocrine IFN-β signalling in uninfected cells, facilitates inflammatory and adaptive immune responses following infection, and promotes infected-cell survival during this process [13]. RelA is known to play a key role in mastitis. An elegant study by Notebaert *et al.* [14] utilized real time imaging of NF-κB activation in response to mammary gland

infection in transgenic mice. This experiment showed that *RELA* mRNA expression peaked at 6 - 8 h post infection with *E. coli*, returning to basal by 24 h. This expression was nearly all located in mammary epithelial cells as there was little contribution from neutrophils at this early stage. The evidence suggested induction of RelA homodimers rather than classic p65/p50 heterodimers. Maximal systemic NF-κB activation in liver also occurred at 6 - 8 h, together with increased circulating concentrations of serum amyloid A and TNF*α*. In cattle, Chen *et al.* [15] measured expression of NF-κB pathway genes in blood and a selection of tissues taken from Chinese Holstein cows and found higher *RELA* expression in animals with mastitis caused by *S. aureus*. Sun *et al.* [16] tested the ability of LPS binding protein to block LPS-induced apoptosis of bovine mammary epithelial cells. In this system TLR4 and RelA protein were both down-regulated and NF-κB transcription activity was weakened.

In the cow, the gene for *RELA* is located at 44.5 Mb on BTA29. It is highly polymorphic with 166 missense SNP, four nonsense SNP and one frameshift mutation reported (https://www.ncbi.nlm.nih.gov/snp). The frequency with which the majority of these SNP are present in cattle populations has not, however, been determined. Despite the central role for RelA in the immune response to mastitis, the majority of quantitative trait loci (QTL) and genome wide association studies (GWAS) have not identified a marker in this region associated with SCC, udder development or udder health ([17] [18] http://www.animalgenome.org/). Meredith *et al.* [19] did, however, find that a SNP rs110252785 at 44.6 Mb, was significantly associated with milk protein percent. More recently, Durán Aguilar *et al.* [20] undertook a GWAS for somatic cell score (SCS) in Holsteins using copy number variation (CNV) as marker. This approach yielded 18 candidate genes which included *RELA*. The CNVR_1640_P located on BTA29 extended from 44.41 to 44.50 Mb and was associated with eight genes including *RELA* and *MAP3K11*. The latter is part of the MAPK family, which are also key signalling molecules in innate immunity, controlling both pro- and anti-inflammatory processes including cytokine production in an orderly manner [21].

We undertook this study as part of a candidate gene approach focusing on genes which are of known importance in innate immunity. Their potential genetic associations with milk production, disease and survival times were investigated in a starting population of Holstein-Friesian cows in which extensive phenotypic data were obtained from birth until death or culling. Information on the animals and farms has been described previously [22] [23].

2. Material and Methods

2.1. Animals and Farms

All procedures involving animals were carried out under the Home Office Animals (Scientific Procedures) Act 1986 and approved by the Royal Veterinary College's (RVC) Ethics and Welfare Committee. Briefly, samples and data from Holstein-Friesian heifers born between August 2003 and October 2004 on 18 commercial UK dairy farms and one primarily research farm were used. Each

farm had a four-month enrolment period during which all consecutively live-born dairy heifers were recruited. The mean cohort size was 24 animals per farm, range 15 to 30. These farms provided a variety of management practices representative of those commonly encountered on dairy farms in the UK. Whole blood samples used in this study for SNP identification (10 ml into heparinized vacutainers, BD Vacutainer System, Devon, UK) were collected from 337 Holstein heifers at 6 months of age and stored at −20˚C. For each cow, pedigree information for the preceding three generations was collected from the Holstein UK website (www.ukcows.com), creating a total pedigree containing 2251 animals of which there were 93 sires and 181 maternal grandsires.

Once these animals had calved data were obtained through the herd management software on milk production traits (yield and SCC from monthly records provided by commercial milk recording services), fertility, health, and longevity. The number of records used in each analysis varied depending on the availability of information for each trait and the longevity of the individual cow. Animals were monitored until 2340 d (6.4 yr) or until the time of death or culling if that occurred earlier. Reasons for culling were obtained where possible and classified into the following categories: infertility, abortion, mastitis/high SCC, legs/feet/lameness, other illnesses (e.g. abscess, displaced abomasum), or no reason for culling given. Some animals were placed in more than one category, for example having a combination of poor fertility and high SCC [23].

2.2. SNP Selection and Genotyping

All reagents were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) unless otherwise stated. The method used for SNP identification has been described previously [24]. DNA from blood samples was extracted using a FlexiGene DNA Kit (Qiagen, North Manchester, UK) following the protocol supplied and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, USA). A DNA pooling sequence strategy was initially used to identify SNP in the bovine *RELA* gene by direct sequencing of PCR amplification products. Five primers were designed to sequence the exons and their flanking regions using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) based on the published gene sequence of *RELA* from the Ensembl database (ENSBTAG00000013895), with NCBI Genbank accession number NM_001080242.2. DNA samples (1 μl each of 50 ng·μL^{-1} DNA) from each of 10 British Holstein cows were used to construct the DNA pool. PCR was performed using a Qiagen Multiplex PCR kit as described previously [25]. Sequence files were assessed using the Chromas Pro software package (release 2.33; Technelysium Pty Ltd, South Brisbane, QLD 4101, Australia).

Based on the results generated in the above pooling sequence analysis, one synonymous SNP rs48035703 from *RELA* intron 5 was subsequently genotyped on all individual cow samples using PCR-restriction fragment length polymorphism (PCR-RFLP). Primers were designed as follows using Primer Premier 5.0:

Forward, 5'-TGTCAGCCAAAGTAAGGAGTG-3'; Reverse, 5'-TCTCCTTTGCCTCGTCTTT-3'. The appropriate enzyme was selected using the WatCut online tool (http://watcut.uwaterloo.ca/template.php?act=snp_new). PCR was then performed using a Qiagen multiplex PCR kit as described previously [25]. Thereafter, 10 µl PCR products were digested with 2 U restriction enzyme BsrBI (New England Biolabs Inc, Hitchin, Herts, UK) in a 20 µl reaction volume at 37˚C. The digested products were detected by electrophoresis with 1.5% agarose gel and alleles were indicated by bands of different sizes (**Figure 2**).

2.3. Data Analysis

Milk production traits were analyzed for each animal by measuring the total milk produced, 305 d milk yield and peak daily milk yield. Data on SCC from monthly milk analysis records were averaged over each lactation. Milk phenotype data for lactation 1 only and "all lactations" were calculated as residuals (L1) or cow effect (LAll) after fitting a mixed model to all herd data [25]. A trait to represent mastitis status was derived from the SCC data after Yoshida *et al.* [26]. This classified animals as unaffected (class 0, never had a SCC > 200,000 cells·mL^{-1}), affected (class 2 mastitic, cows with a SCC > 300,000 cells·mL^{-1} in two consecutive tests or in four non-consecutive tests, or with a SCC > 500,000 cells·mL^{-1} in any one test during any lactation period, regardless of parity) or intermediate between these two extremes (class 1).

The genotype frequency of the polymorphism was examined for deviations from Hardy-Weinberg equilibrium within the population using the χ^2 test. Association analyses were carried out for all traits except SCC, separately in each lactation using the mixed effect model in Equation (1).

$$Y_{ijkl} = \mu + H_i + \mathrm{SNP}_j + a_k + e_{ijkl} \quad (1)$$

with: Y = trait analyzed; μ = overall mean; H_i = fixed effect of the ith herd (i = 1 – 20); SNP_j = fixed effect of the jth genotype of the SNP (j = 1 – 3), a_k = the random animal term fitted using a pedigree file and e_{ijkl} = random residual error.

Significant differences between the genotypes within the SNP were tested using t-statistics and standard errors of differences were calculated for each pair of means

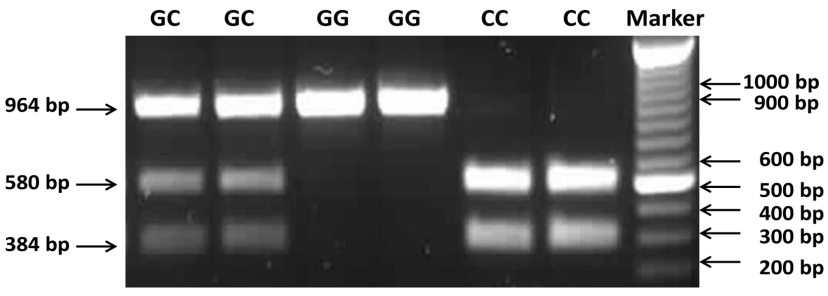


Figure 2. The PCR-RFLP analysis of the G/C polymorphism in intron 5 of the bovine *RELA* gene. Electrophoresis was performed using 1.5% agarose gels. Arrows on left hand side show the positions of the undigested 964 bp PCR product and the two PCR products digested with BsrBI restriction endonuclease. Examples of the heterozygous genotype GC, homozygous genotype GG and homozygous genotype CC are shown.

tested. SCC was analyzed using a trinomial generalized linear model with a logit link function. All analyses were carried out in ASReml (http://www.vsni.co.uk/). SCC as a tertiary trait was analyzed by Fisher's Exact Test in a 3 × 3 table of SCC class by genotype. Mean SCC for heifers and all lactations was also analyzed by an allele substitution model using the command-assoc in PLINK [27] with a quantitative trait.

Differences between genotypes in the number of animals that survived were measured by Kaplan-Meier survival analysis using the Statistical Package for the Social Sciences 17.0 (SPSS Inc., Chicago, IL, USA). Censored animals were those cows that survived > 2340 d (6.4 years). The proportions of cows censored were compared using the Cox proportional hazards regression model. The fixed effects of the herd and sire of the animal were initially included in the analyses but were found to have a non-significant effect on survival ($P > 0.20$) so were removed from the final model.

3. Results

The initial search in our population of British Holstein-Friesian cows identified a synonymous C/G SNP rs48035703 in *RELA* intron 5 on BTA29 at position 44,502,474 bp. This SNP was taken forward to genotyping 315 6-month old heifers using PCR-RFLP. Of these, there were 200 CC homozygotes, 95 CG heterozygotes and 20 GG homozygotes with respective genotype frequencies of 0.63, 0.30 and 0.06. These were distributed with a $\chi^2 = 0.18$ against the Hardy-Weinberg Equilibrium. This SNP in *RELA* was associated with survival time from birth to death or culling (**Figure 3**).

The C allele was favourable with an additive effect. The average survival times were: CC, 1872 d; CG, 1745 d and GG 1596 d. On average, the GG homozygotes therefore had lives that were about 4.9 and 9 months shorter than those of animals carrying one or two C alleles, respectively ($P < 0.003$). The survival curves showed that the genotypes did not start to diverge until after about 900 d of age (2.5 yr), by which time most cows had started their first lactation. For the 20 GG genotype animals, three were culled as heifers, three in lactation (L) 1, six in L2 and four in each of L3 and L4. Of these, 11/17 (65%) had at least one recorded episode of clinical mastitis. The reasons for culling were available for 13 animals with the rs48035703 GG genotype. The predominant reason given was mastitis ($n = 6$), followed by infertility ($n = 4$), with one each due to respiratory disease, abscess and injury.

The basic statistics of the milk traits analysed in this study (total milk produced, 305 d milk yield and peak daily milk yield in L1 and L2) are summarised in **Table 1**. None of these traits was significantly associated with the *RELA* SNP rs48035703.

The overall mean ± SD SCC data (log residuals) were 0.052 ± 0.894 in L1 and −0.00056 ± 0.250 when all lactations were included (log cow effect). These data were analysed separately using three different models. The first lactation only

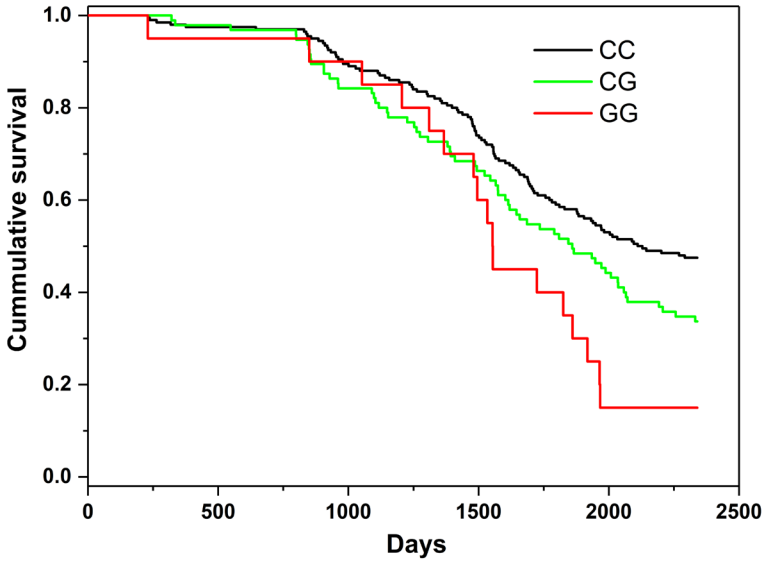


Figure 3. Kaplan-Meier analysis showing the proportion of animals surviving from birth through to 2340 d of age. A population of 315 Holstein Friesian cows were genotyped for the RELA SNP rs48035703. Survival time was significantly lower for animals with the GG genotype ($P < 0.003$).

Table 1. Basic statistics of the milk traits (in kg) analysed in this study.

Traits[a]	n	Mean	SD
Total milk yield_L1	307	9,303	3,957
305d milk yield_L1	200	8,723	1,542
Peak milk yield_L1	253	34	6
Total milk yield_L2	237	10,109	3144
305d milk yield_L2	160	9,983	1,675
Peak milk yield_L2	206	42	7

a: L1, lactation 1; L2, lactation 2.

data showed a significant association with SCC using an allele substitution model (Table 2). The mean log residuals ± SD by genotype were: GG 0.30 ± 1.263 (n = 13); CG 0.994 ± 0.994 (n = 59) and CC −0.039 ± 0.803 (n = 124; $P < 0.05$). Significance was lost when data from all lactations was included in the analysis ($P = 0.33$).

A Fisher's Exact Test categorical model was used to determine the cumulative probability of genotype affecting SCC categorised as Class 0 (unaffected), 1 (intermediate) and 2 (mastitic) (Table 3). This analysis showed a trend for more CC genotype cows than expected to be classified as intermediate (45/148, 30.4%) and fewer as mastitic (68/148, 45.9%), whereas for the heterozygote CG cows fewer were intermediate (7/58, 12.1%) and more were mastitic (35/58, 60.3%) ($P = 0.05$). Data were only available for 13 GG genotypes, so the frequency estimates are not reliable, but of these 15.4% were classed as intermediate and 53.8% were mastitic. The third model, using Equation (1), resulted in a non-significant

Table 2. Summary results from fitting an allele substitution model and a genotypic model to the analysis of SCC data with respect to SNP rs48035703 in *RELA* intron 5 on BTA29.

Trait	n	Allele substitution effect	SE	T	P: Allele substitution	P: Genotypic
SCCH[a]	191	0.224	0.10	2.14	0.03	0.09
SCCL	232	0.026	0.03	0.97	0.33	0.24

a: SCCH included data from the first lactation only whereas for SCCL all lactations were included.

Table 3. Summary results from fitting a Fisher's Exact Test categorical model to determine the cumulative probability of genotype for SNP rs48035703 in *RELA* intron 5 on BTA29 affecting SCC categorised as unaffected, intermediate or mastitic.

Genotype	Counts	Unaffected	Intermediate	Mastitic	Total
CC	Actual	35[a] (23.6%)	45[b] (30.4%)	68[a] (45.9%)	14
	Expected	37.2	36.5	74.3	
CG	Actual	16[a] (27.6%)	7[b] (12.1%)	35[a] (60.3%)	
	Expected	14.6	14.3	29.1	58
GG	Actual	4[a] (30.8%)	2[a] (15.4%)	7[a] (53.8%)	
	Expected	3.3	3.2	6.5	13
Total		55	54	110	

a & b: Proportions within rows differ from expected, $P < 0.05$.

effect of genotype on categorical SCC ($P = 0.09$). Together these data indicated that cows with the CC genotype at *RELA* rs48035703 were less likely than the heterozygotes to experience a high SCC of >200,000 cells·mL^{-1}, classified as mastitis.

4. Discussion

These results show that the *RELA* SNP rs48035703 was significantly associated with both survival time and SCC in a population of British Holstein Friesian cows. This region on BTA29 has not previously been associated with either productive life, SCC or SCS in traditional GWAS studies (http://www.animalgenome.org/). There are a number of possible reasons for this discrepancy. SCC is a complex multi-locus trait and involves a large number of variants with differing actions which together only explained 5.4% of the total genetic variance in a population of German Holstein cattle [18]. We, however, found convincing evidence that cows carrying the G allele of rs48035703 had a shorter survival time in the herd. In our data the significant association with SCC was detected when only the first lactation data were considered. If cows with the less favourable genotype have a shorter survival time, then fewer of these animals will have SCC data available in later lactations, which may impact on the results. The other trait analysed was survival time from birth. Others have previously reported significant associations of the trait "Length of productive life" (which starts when cows calve for the first time) with SNP in BTA29 [28] [29]. These were rs110734670, located at

48.4 Mbp, rs109761676 at 37.6 Mbp and rs109447102 at 37.7 Mbp. Although these SNP are at some distance away from *RELA*, they do flank the region of interest.

Durán Aguilar *et al.* [20] discovered recently that there is a CNV region on BTA29 with a length of 86,267 which stretches from 44.4 to 44.5 Mbp. CNVs are less frequent than SNP, but involve larger genomic regions that may affect gene structure and regulation [30]. As CNVs are heritable it is possible that they may be associated with animal health and production traits under recent selection [31]. When deletions occur there is only one, or occasionally no, copies of the gene present in the genome, which can result in dosage imbalance [32]. The particular CNVR in BTA29 reported by Durán Aguilar *et al.* [20] was associated with eight genes *SSSCA1, FAM89B, EHBP1L1, KCNK7, MAP3K11, PCNXL3, SIPA1* and *RELA*. This study compared Mexican Holstein cows with estimated breeding values for somatic cell score (SCS) which were ± 2 SD from the average SCS score, and included 140 high and 140 low phenotype samples. Of these 14 cows (5.8%) had this particular CNV loss. If the same CNV is also present in other Holstein populations, then it would clearly affect SNP genotyping results.

We focused on *RELA*, as this is a plausible candidate gene to influence an innate immune response involved in mastitis. The SNP analysed was located in intron 5, so is unlikely to be the causative mutation, although synonymous mutations have been shown to alter the functions of translated protein through a variety of mechanisms such as influencing splice sites, miRNA and exonic transcription factor binding sites, affecting mRNA stability and altering translational speed [33]. It could, however, have been in linkage with another functional SNP in either *RELA*, which is known to be highly polymorphic (https://www.ncbi.nlm.nih.gov/snp), or in adjacent genes. Of those mentioned above, *MAP3K*11 (also known as *MLK*3) is of most interest. This gene encodes a member of the serine/threonine kinase family which activates the NF-κB signalling pathway by directly phosphorylating and so activating inhibitory IkB proteins. It can also activate both MAPK8/JNK kinase and AMPK [34]. Other genes adjacent to *RELA* from 44.5 to 44.6 MBp are *LOC101904027, KAT5, RNASEH2C, AP5B1, OVOL1* and *LOC101904071*. Of these *KAT5* encodes a histone acetylase that has a role in DNA repair, apoptosis and signal transduction but none of these genes have established roles in immunity.

Bacteria such as *E. coli* can cause a variety of other important cattle diseases apart from mastitis. In this study two cows with the GG genotype were culled due to respiratory disease and an abscess respectively, and another died with no reason given. Several animals had reported metritis or endometritis and four were culled for infertility. The numbers are too low to draw conclusions, but further work is warranted to assess the linkage between this SNP and immune responses to other infections. In humans, a SNP rs1049728 in the vicinity of *RELA* was significantly associated with the soluble ICAM-1 concentration in the circulation [35]. ICAM-1 is a member of the immunoglobulin superfamily of

adhesion receptors: the soluble form is thought to arise from proteolytic cleavage of the extra-cellular domains and is a recognized genetic marker for inflammation [36].

5. Conclusion

In conclusion, we have reported a significant association between an SNP in *RELA*, longevity and SCC in a population of Holstein-Friesian dairy cows. As NF-κB activation is known to play a central role in the mammary response to bacterial infection, this link seems plausible. The SNP investigated was, however, a synonymous SNP located in *RELA* intron 5. Further work is therefore required to confirm the gene and establish the mutation which influence the traits reported.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Kossaibati, M.A. and Esslemont, R.J. (1997) The Costs of Production Diseases in Dairy Herds in England. *The Veterinary Journal*, **154**, 41-51. https://doi.org/10.1016/S1090-0233(05)80007-3

[2] Bradley, A. (2002) Bovine Mastitis: an Evolving Disease. *The Veterinary Journal*, **164**, 116-128. https://doi.org/10.1053/tvjl.2002.0724

[3] Seegers, H., Fourichon, C. and Beaudeau, F. (2003) Production Effects Related to Mastitis and Mastitis Economics in Dairy Cattle Herds. *Veterinary Research*, **34**, 475-491. https://doi.org/10.1051/vetres:2003027

[4] Smith, K.L. and Hogan, J.S. (1993) Environmental Mastitis. *Veterinary Clinics of North America*: *Food Animal Practice*, **9**, 489-498. https://doi.org/10.1016/S0749-0720(15)30616-2

[5] Vaarst, M. and Enevoldsen, C. (1997) Patterns of Clinical Mastitis Manifestations in Danish Organic Dairy Herds. *Journal of Dairy Research*, **64**, 23-37. https://doi.org/10.1017/S002202999600194X

[6] Schukken, Y.H., Günther, J., Fitzpatrick, J., Fontaine, M.C., Goetze, L., Holst, O., *et al.* (2011) Host-Response Patterns of Intramammary Infections in Dairy Cows. *Veterinary Immunology and Immunopathology*, **144**, 270-289. https://doi.org/10.1016/j.vetimm.2011.08.022

[7] Shuster, D.E., Lee, E.K. and Kehrli Jr., M.E. (1996) Bacterial Growth, Inflammatory Cytokine Production, and Neutrophil Recruitment during Coliform Mastitis in Cows within Ten Days after Calving, Compared with Cows at Mid Lactation. *American Journal of Veterinary Research*, **57**, 1569-1575.

[8] Pyörälä, S. (2008) Mastitis in Post-partum Dairy Cows. *Reproduction in Domestic Animals*, **43**, 252-259. https://doi.org/10.1111/j.1439-0531.2008.01170.x

[9] Günther, J., Esch, K., Poschadel, N., Petzl, W., Zerbe, H., Mitterhuemer, S., Blum, H. and Seyfert, H.M. (2011) Comparative Kinetics of *Escherichia coli*- and Staphylococcus aureus-specific Activation of Key Immune Pathways in Mammary Epithelial Cells Demonstrates That *S. aureus* Elicits a Delayed Response Dominated by Interleukin-6 (IL-6) but Not by IL-1A or Tumor Necrosis Factor Alpha. *Infection and Immunity*, **79**, 695-707. https://doi.org/10.1128/IAI.01071-10

[10] Akira, S. and Takeda, K. (2004) Functions of Toll-Like Receptors: Lessons from KO mice. *Comptes Rendus Biologies*, **327**, 581-589. https://doi.org/10.1016/j.crvi.2004.04.002

[11] Sun, S.C. (2012) The Noncanonical NF-κB Pathway. *Immunological Reviews*, **246**, 125-140. https://doi.org/10.1111/j.1600-065X.2011.01088.x

[12] Senftleben, U. and Karin, M. (2002) The IKK/NF-kappaB Pathway. *Critical Care Medicine*, **30**, S18-S26. https://doi.org/10.1097/00003246-200201001-00003

[13] Ouaaz, F., Li, M. and Beg, A.A. (1999) A Critical Role for the RelA Subunit of Nuclear Factor KappaB in Regulation of Multiple Immune-Response Genes and in Fas-Induced Cell Death. *The Journal of Experimental Medicine*, **189**, 999-1004. https://doi.org/10.1084/jem.189.6.999

[14] Notebaert, S., Carlsen, H., Janssen, D., Vandenabeele, P., Blomhoff, R. and Meyer, E. (2008) *In Vivo* Imaging of NF-kappaB Activity during Escherichia Coli-Induced Mammary Gland Infection. *Cellular Microbiology*, **10**, 1249-1258. https://doi.org/10.1111/j.1462-5822.2008.01123.x

[15] Chen, L., Liu, X., Li, Z., Wang, H., Liu, Y., He, H., *et al.* (2014) Expression Differences of miRNAs and Genes on NF-κB Pathway between the Healthy and the Mastitis Chinese Holstein Cows. *Gene*, **545**, 117-125. https://doi.org/10.1016/j.gene.2014.04.071

[16] Sun, Y., Li, L., Wu, J., Yu, P., Li, C., Tang, J., *et al.* (2015) Bovine Recombinant Lipopolysaccharide Binding Protein (BRLBP) Regulated Apoptosis and Inflammation Response in Lipopolysaccharide-Challenged Bovine Mammary Epithelial Cells (BMEC). *Molecular Immunology*, **65**, 205-214. https://doi.org/10.1016/j.molimm.2015.01.026

[17] Ogorevc, J., Kunej, T., Razpet, A. and Dovc, P. (2009) Database of Cattle Candidate Genes and Genetic Markers for Milk Production and Mastitis. *Animal Genetics*, **40**, 832-851. https://doi.org/10.1111/j.1365-2052.2009.01921.x

[18] Abdel-Shafy, H., Bortfeldt, R.H., Tetens, J. and Brockmann, G.A. (2014) Single Nucleotide Polymorphism and Haplotype Effects Associated with Somatic Cell Score in German Holstein Cattle. *Genetics Selection Evolution*, **46**, 35. https://doi.org/10.1186/1297-9686-46-35

[19] Meredith, B.K., Kearney, F.J., Finlay, E.K., Bradley, D.G., Fahey, A.G., Berry, D.P. and Lynn, D.J. (2012) Genome-Wide Associations for Milk Production and Somatic Cell Score in Holstein-Friesian Cattle in Ireland. *BMC Genetics*, **13**, 21. https://doi.org/10.1186/1471-2156-13-21

[20] Durán Aguila,r M., Román Ponce, S.I., Ruiz López, F.J., González Padilla, E., Vásquez Peláez, C.G., Bagnato, A. and Strillacci, M.G. (2017) Genome-Wide Asso-

ciation Study for Milk Somatic Cell Score in Holstein Cattle using Copy Number Variation as Markers. *Journal of Animal Breeding and Genetics*, **134**, 49-59. https://doi.org/10.1111/jbg.12238

[21] Salojin, K. and Oravecz, T. (2007) Regulation of Innate Immunity by MAPK Dual-Specificity Phosphatases: Knockout Models Reveal New Tricks of Old Genes. *Journal of Leukocyte Biology*, **81**, 860-869. https://doi.org/10.1189/jlb.1006639

[22] Brickell, J.S., Bourne, N., McGowan, M.M. and Wathes, D.C. (2009) Effect of Growth and Development during the Rearing Period on the Subsequent Fertility of Nulliparous Holstein-Friesian Heifers. *Theriogenology*, **72**, 408-416. https://doi.org/10.1016/j.theriogenology.2009.03.015

[23] Brickell, J.S. and Wathes, D.C. (2011) A Descriptive Study of the Survival of Holstein-Friesian Heifers through to Third Calving on English Dairy Farms. *Journal of Dairy Science*, **94**, 1831-1838. https://doi.org/10.3168/jds.2010-3710

[24] Chen, X., Cheng, Z., Werling, D., Pollott, G.E., Salavati, M., Johnson, K.F., *et al.* (2016) Bovine P-Selectin Mediates Leukocyte Adhesion and is Highly Polymorphic in Dairy Breeds. *Research in Veterinary Science*, **108**, 85-92. https://doi.org/10.1016/j.rvsc.2016.08.004

[25] Chen, X., Zhang, S., Cheng, Z., Cooke, J.S., Werling, D., Wathes, D.C. and Pollott, G.E. (2017) Polymorphisms in the Selectin Gene Cluster Are Associated with Fertility and Survival Time in a Population of Holstein Friesian Cows. *PLoS One*, **12**, e0175555. https://doi.org/10.1371/journal.pone.0175555

[26] Chang, C.C., Chow, C.C., Tellier, L.C.A.M., Vattikuti, S., Purcell, S.M. and Lee, J.J. (2015) Second-Generation PLINK: Rising to the Challenge of Larger and Richer Datasets. *GigaScience*, **4**, 7. https://doi.org/10.1186/s13742-015-0047-8

[27] Yoshida, T., Furuta, H., Kondo, Y. and Mukoyama, H. (2012) Association of BoLA-DRB3 Alleles with Mastitis Resistance and Susceptibility in Japanese Holstein Cows. *Animal Science Journal*, **83**, 359-366. https://doi.org/10.1111/j.1740-0929.2011.00972.x

[28] Cole, J.B., Wiggans, G.R., Ma, L., Sonstegard, T.S., Lawlor, T.J., Crooker, B.A., *et al.* (2011) Genome-Wide Association Analysis of Thirty One Production, Health, Reproduction and Body Conformation Traits in Contemporary U.S. Holstein Cows. *BMC Genomics*, **12**, 408. https://doi.org/10.1186/1471-2164-12-408

[29] Cochran, S.D., Cole, J.B., Null D.J. and Hansen, P.J. (2013) Discovery of Single Nucleotide Polymorphisms in Candidate Genes Associated with Fertility and Production Traits in Holstein Cattle. *BMC Genetics*, **14**, 49. https://doi.org/10.1186/1471-2156-14-49

[30] Zhang, F., Gu, W., Hurles, M.E. and Lupski, J.R. (2009) Copy Number Variation in Human Health, Disease, and Evolution. *Annual Review of Genomics and Human Genetics*, **10**, 451-481. https://doi.org/10.1146/annurev.genom.9.081307.164217

[31] Bickhart, D.M., Hou, Y., Schroeder, S.G., Alkan, C., Cardone, M.F., Matukumalli, L.K., *et al.* (2012) Copy Number Variation of Individual Cattle Genomes Using Next-Generation Sequencing. *Genome Research*, **22**, 778-790. https://doi.org/10.1101/gr.133967.111

[32] Liu, Z., Li, A., Schulz, V., Chen, M. and Tuck, D. (2010) MixHMM: Inferring Copy Number Variation and Allelic Imbalance Using SNP Arrays and Tumor Samples Mixed with Stromal Cells. *PLoS One*, **5**, e10909. https://doi.org/10.1371/journal.pone.0010909

[33] Hunt, R.C., Simhadri, V.L., Landoli, M., Sauna, Z.E. and Kimchi-Sarfaty, C. (2014) Exposing Synonymous Mutations. *Trends in Genetics*, **30**, 308-321.

https://doi.org/10.1016/j.tig.2014.04.006

[34] Luo, L., Jiang, S., Huang, D., Lu, N. and Luo, Z. (2015) MLK3 Phosphorylates AMPK Independently of LKB1. *PLoS One*, **10**, e0123927. https://doi.org/10.1371/journal.pone.0123927

[35] Paré, G., Ridker, P.M., Rose, L., Barbalic, M., Dupuis, J., Dehghan, A., *et al.* (2011) Genome-Wide Association Analysis of Soluble ICAM-1 Concentration Reveals Novel Associations at the NFKBIK, PNPLA3, RELA, and SH2B3 Loci. *PLOS Genetics*, **7**, e1001374. https://doi.org/10.1371/journal.pgen.1001374

[36] Raman, K., Chong, M., Akhtar-Danesh, G.G., D'Mello., M, Hasso, R., Ross, S., *et al.* (2013) Genetic Markers of Inflammation and Their Role in Cardiovascular Disease. *Canadian Journal of Cardiology*, **29**, 67-74. https://doi.org/10.1016/j.cjca.2012.06.025