The Journal of Agricultural Science

cambridge.org/ags

Variation in *PLIN2* and its association with milk traits and milk fat composition in dairy cows

Y. H. Li[1], H. Zhou[1], L. Cheng[2], J. Zhao[1] and J. G. H. Hickford[1]

[1]Department of Agricultural Sciences, Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, New Zealand and [2]Faculty of Veterinary and Agricultural Sciences, Dookie College, The University of Melbourne, Victoria 3647, Australia

Animal Research Paper

Cite this article: Li YH, Zhou H, Cheng L, Zhao J, Hickford JGH (2020). Variation in *PLIN2* and its association with milk traits and milk fat composition in dairy cows. *The Journal of Agricultural Science* **158**, 774–780. https://doi.org/10.1017/S0021859621000174

Received: 6 May 2020
Revised: 12 August 2020
Accepted: 8 February 2021
First published online: 30 March 2021

Key words:
Genetic variation; milk fatty acid; *PLIN2*

Author for correspondence:
J. G. H. Hickford,
E-mail: Jonathan.Hickford@lincoln.ac.nz

Abstract

The current study investigated associations between variation in the bovine perilipin-2 gene (*PLIN2*) and milk traits (milk fat content, milk protein content, milk yield and milk fatty acid (FA) component levels) in 409 New Zealand pasture-grazed Holstein-Friesian × Jersey-cross (HF × J-cross or Kiwicross™) cows. Five nucleotide sequence variants were found in three regions of the gene, including c.17C>T in exon 2, c.53A>G in exon 3, c.595+23G>A and c.595+104_595+108del in intron 5, and c.*302T>C in the 3′-untranslated region. The c.*302T>C substitution produces two nucleotide sequence variants (A_5 and B_5), and this variation was associated with variation in milk protein content and milkfat composition for C10:0, C11:0, C12:0, C13:0 and C16:0 FA and medium-chain fatty acid (MCFA) and long-chain fatty acid (LCFA) groups. After correcting for the effect of variation in the diacylglycerol acyl-CoA acyltransferase 1 gene (*DGAT1*) that results in the amino acid substitution p.K232A, variation in the FA binding protein 4 gene (*FABP4*) and variation in the stearoyl-CoA desaturase (Δ-9-desaturase) gene (*SCD*) that results in the amino acids substitution p.A293V, significant differences between A_5A_5 and B_5B_5 cows were found for C10:0, C11:0, C12:0, C13:0, C16:0, and the MCFA, LCFA, total saturated FA and C10:1 index groups. This suggests that nucleotide sequence variation in *PLIN2* may be affecting milk FA component levels.

Introduction

The perilipin-2 gene (*PLIN2* also known as *ADFP*) encodes the protein perilipin-2 (also called the adipose differentiation-related protein (ADRP)) and adipophilin. This protein participates in the regulation of body fat distribution and it is located on the surface of lipid droplets in different tissues (Heid *et al.*, 1996). During lipid droplet formation, upregulation of *PLIN2* expression occurs (Russell *et al.*, 2007), along with an increase in lipid storage (Prats *et al.*, 2006; Listenberger *et al.*, 2007). The *PLIN2* gene is an important candidate gene for fat deposition traits, because muscle tissues will uptake more fatty acids (FAs) for triglyceride formation, when abundant *PLIN2* expression occurs (Imamura *et al.*, 2002; Magra *et al.*, 2006; Imai *et al.*, 2007).

In cattle, *PLIN2* is located on chromosome 8. Nucleotide sequence variation in *PLIN2* has been identified and associated with intramuscular fat content in chicken (Zhao *et al.*, 2009). Cheong *et al.* (2009) reported 25 nucleotide sequence variations in beef cattle and that these variations occur in different gene regions (the promoter region, the coding exons, the untranslated regions and the introns). In the Korean native cattle they studied, the variations in the promoter region were associated with meat-marbling score.

During lactation in dairy cattle, perilipin-2 participates in globule surface membrane formation and it is one of the constituents of the globule surface (Reinhardt and Lippolis, 2006; McManaman *et al.*, 2007). Bionaz and Loor (2008) have described how the expression of *PLIN2* increases during early lactation (with a peak in expression at the 60th day in milk), then subsequently declines and Li *et al.* (2014) have identified seven nucleotide substitutions and six haplotypes of *PLIN2* that are associated with goat milk yield traits.

Although *PLIN2* is a ubiquitously expressed gene (Brasaemle *et al.*, 1997), to date there have been no specific reports of genetic associations between *PLIN2* nucleotide sequence variation and milk traits in dairy cattle. However, Ogorevc *et al.* (2009) have described bovine chromosome 8 (BTA8) QTLs for milking speed, protein content, somatic cell score, somatic cell count and clinical mastitis occurrence, in the region that contains *PLIN2*, and Chong *et al.* (2011) describe how the majority of the lipid produced during lactation is secreted into milk by a novel process of membrane envelopment of cytoplasmic lipid droplets, with PLIN2 hypothesized to play a pivotal role in both the formation and secretion of the milk lipids.

In the current study, variation in *PLIN2* will be searched for in dairy cattle, and if it is identified then associations between that variation and variation in milk traits (milk yield, fat content, protein content and fat composition) will be investigated.

Materials and methods

Animals and milk sample collection

A total of 409 Holstein-Friesian × Jersey (HF × J)-cross dairy cows from two herds (114 cows in herd 1, 295 cows in herd 2) were studied. This is now the most common type of milking cow in New Zealand (NZ), being a cross of the two parent breeds (of no fixed breed proportion at the herd level), and selected based on traits of value to the NZ dairy industry using an index-based selection system (NZAEL, DairyNZ, Hamilton, NZ). All the cows investigated were 3–10 years old and were in their 1st to 7th lactation. They were grazed on pasture (a mixture of perennial ryegrass and white clover) on the Lincoln University Dairy Farm, Canterbury, NZ. All the cows in the current study calved over the months August–September. The cows were milked twice a day throughout lactation (from calving until the end of May) on rotary milking platforms in dairy sheds positioned near the centre of each farm.

The milk samples for trait analysis were collected using Tru-Test Electronic Milk Meters (Tru-test Ltd, Auckland, NZ) with the daily milk yield recorded in litres and samples for the trait analysis being undertaken once a month from September to February (approximately 50 ml stirred samples automatically collected at morning and afternoon milking and pooled for subsequent analysis). The fresh samples were analysed following collection, for fat content and protein content, using Fourier-Transform Infra-Red Spectroscopy on a MilkoScan FT120 milk analyser (Foss, Hillerød, Denmark).

Milk samples for FA analysis were collected from each cow in a single afternoon milking in mid-January (mid-lactation – days in milk (DIM) = 148 ± 19 days). These were frozen at −20°C, and then freeze-dried, prior to being individually ground to a fine powder for component analysis.

Gas chromatography of the fatty acids in the milk sample

The milk FAs were methylated and extracted in n-heptane, before being analysed by gas chromatography (GC) as FA methyl esters (FAMEs). The methylation reactions for ester formation were performed in 10 ml Kimax tubes. Individual powdered milk samples (0.17 g) were dissolved in 900 µl of n-heptane (100%, AR grade), before 100 µl of internal standard (5 mg/ml of C21:0 methyl ester in n-heptane) and 4.0 ml of 0.5 M NaOH (in 100% anhydrous methanol) were added. The tubes were vortexed then incubated in a block heater (Ratek Instruments, Australia) at 50°C for 15 min. After cooling to room temperature, another 2.0 ml of n-heptane and 2.0 ml of deionized water were added to each tube. After vortexing, the tubes were centrifuged for 5 min at 1500 ***g*** (Megafuge 1.0R, Heraeus, Germany). The top layer of n-heptane was transferred with a Pasteur pipette into a second Kimax tube, and another 2.0 ml of n-heptane was added to each of the original tubes. The extraction was repeated and the n-heptane aspirates were then pooled. Finally, anhydrous sodium sulphate (10 mg) was added to the n-heptane extracts, to remove any residual water.

The GC analysis was carried out using a Shimadzu GC-2010 Gas Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and an AOC-20i autosampler. The output was analysed with GC Solution Software (Shimadzu). For analysis, 1 µl of the n-heptane sample extract was injected into a 100 m GC column (250 µm × 0.25 µm capillary column, CP-Select, Varian) with a 1:60 split ratio. The separation was undertaken with a pure helium carrier gas and was run for 92 min. The temperature of both the injector and detector was set at 250°C and the thermal profile of the column consisted of 45°C for 4 min, followed by 27 min at 175°C (ramped at 13°C/min), 35 min at 215°C (ramped at 4°C/min.) and a final 'bake-off' at 250°C for 5 min (ramped at 25°C/min.). The individual FAMEs were identified by the peak retention time compared to commercially obtained external standards (ME61, ME93, BR3, BR2, ME100, GLC411 and GLC463; Laroden AB, Sweden). Quantification of the individual FAMEs was based on peak area assessment and comparison with the internal and external standards. The threshold for peak area determination on the chromatogram was a 500-unit count, with peaks that were under 500-unit count, being ignored. The calculated minimum component of an individual FAME was therefore 0.01 g per 100 g of total FA. The individual FA measurements were recorded and grouped FA levels and various FA indices were calculated.

The groups were, short-chain FAs = C4:0 + C6:0 + C8:0; medium-chain FAs (MCFA) = C10:0 + C12:0 + C14:0; long-chain FAs (LCFA) = C15:0 + C16:0 + C17:0 + C18:0 + C19:0 + C20:0 + C22:0 + C24:0; omega 3 FAs = C18:3 *cis*-9, 12, 15 + C20:5 *cis*-5, 8, 11, 14, 17 + C22:5 *cis*-7, 10, 13, 16, 19; omega 6 FAs = C18:2 *cis*-9, 12 + C18:3 *cis*-6, 9, 12 + C20:3 *cis*-8, 11, 14 + C20:4 *cis*-5, 8, 11, 14; monounsaturated FAs (MUFA) = C10:1 + C12:1 + C14:1 *cis*-9 + C15:1 + C16:1 *cis*-9 + C17:1 + C18:1 *trans*-11 + C18:1 *cis*-9 + C18:1 *cis*-(10 to 15) + C20:1 *cis*-5 + C20:1 *cis*-9 + C20:1 *cis*-11 + C22:1 *trans*-13; polyunsaturated FAs (PUFA) = C18:2 *trans*-9, 12 + C18:2 *cis*-9, trans-13 + C18:2 *cis*-9, *trans*-12 + C18:2 *trans*-9, *cis*-12 + C18:2 *cis*-9, 12 + C18:3 *cis*-6, 9, 12 + C18:3 *cis*-9, 12, 15 + CLA + C20:3 *cis*-8, 11, 14 + C20:4 *cis*-5, 8, 11, 14 + C20:5 *cis*-5, 8, 11, 14, 17 + C22:5 *cis*-7, 10, 13, 16, 19; total branched FA = C13:0 *iso* + C13:0 *anteiso* + C15:0 *iso* + C15:0 *anteiso* + C17:0 *iso*; total UFA = MUFA + PUFA; and total SFA = C4:0 + C6:0 + C8:0 + C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C19:0 + C20:0 + C22:0 + C24:0.

The indices calculated were: C10:1 index = C10:1/(C10:1 + C10:0) × 100; C12:1 index = C12:1/(C12:1 + C12:0) × 100; C14:1 index = C14:1 *cis*-9/(C14:1 *cis*-9 + C14:0) × 100; and C16:1 index = C16:1 *cis*-9/(C16:1 *cis*-9 + C16:0) × 100.

PCR-SSCP analysis and genotyping

A blood sample from each of the cow's coccygeal vein was collected via venepuncture and placed on to FTA™ cards (Whatman™, Maidstone, UK) and air-dried. Genomic DNA was purified from a 1.2 mm punch of the dried blood spot, using a two-step washing procedure described by Zhou *et al.* (2006).

The PCR amplifications were performed in a 15 µl reaction containing the purified genomic DNA (a punch of FTA™ paper), 0.25 µM of each designed primer, 150 µM of each dNTP (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the polymerase enzyme. Five PCR primer sets (Table 1) were designed to amplify five regions of *PLIN2*. The forward primer for the Region 1 amplification was designed based on the cattle *PLIN2* reference sequence ENSBTAT00000007519 (Ensembl). The reversed primer for the Region 1 amplification and the other four sets of primers were designed based on the cattle reference sequence AF239708 (GenBank). These amplified: Region 1, spanning a portion of 5′-untranslated region (5′-UTR), exon 1 and part of intron 1; Region 2, spanning exon

https://doi.org/10.1017/S0021859621000174 Published online by Cambridge University Press

Table 1. PCR-SSCP amplification and analysis conditions

Region	Primers (5′-3′)	Predicted size (bp)	Annealing temperature (°C)	SSCP conditions
1	TGAATTACACGCAGATTC CAAGAAATGAGAACCACGC	589	These primers did not appear to work at 48–62°C	No results due to the absence of a detectable PCR amplicon
2	GAATCTTGTCACAGTGTTCT GATCACTCTCAATGACTATAT	398	58	12% acrylamide gel containing 4% glycerol; 350 V for 19 h at 26°C
3	CCATGTTTCTCACCAGCCAG GAAGAAGTTCCTTGGTGG	446	58	12% acrylamide gel containing 4% glycerol; 390 V for 19 h at 15°C
4	GCTGAATCCACTGCTCATTC TTAGCTGCCTGCCTACTTCAG	483	60	No sequence variation found
5	CCAGATGACAGCTCCTCTTG CCGATCTATTCTGCAGTGAA	393	58	12% acrylamide gel containing 1% glycerol; 300 V for 19 h at 20°C

2, intron 2 and exon 3; Region 3, spanning part of intron 4, exon 5 and part of intron 5; Region 4, spanning part of intron 7 and part of exon 8; and Region 5, spanning part of exon 8 and part of intron 8. The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Amplifications were undertaken using S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA) and the thermal profile included an initial denaturation for 2 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at different annealing temperature (Table 1) and 30 s at 72°C; with a final extension for 5 min at 72°C.

Following amplification, a 0.7 µl aliquot of the PCR products was mixed with 7 µl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95°C for 5 min and rapid cooling on wet ice, the samples were loaded on 16 cm × 18 cm, different percentage acrylamide: bisacrylamide (37.5: 1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad) at different conditions in 0.5× TBE buffer (Table 1), and gels were silver-stained using the method of Byun *et al.* (2009).

The cows were typed for *DGAT1*, *FABP4* and *SCD1* variation using the methods described by Li *et al.* (2020*a*, 2020*b*, 2021) respectively. Only 405 of the original 409 cows could be typed for all three of these genes.

Sequencing of the dairy cattle PLIN2 *Regions 2, 3 and 5 variants and sequence analysis*

Homozygous PCR amplicons identified using PCR-SSCP, or individual bands of interest from heterozygous amplicons that were recovered directly from the SSCP gels as a gel slice using the method of Gong *et al.* (2011), were sequenced at the Lincoln University DNA Sequencing Facility.

The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment and comparisons. The BLAST algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) for the *PLIN2* genotypes was analysed using an online χ^2 calculator (http://www.oege.org/software/hwe-mr-calc.shtml).

All other statistical analyses were carried out using IBM SPSS version 22 (IBM, NY, USA). Associations between variation in *PLIN2* and variation in milk FA traits were tested using General Linear Mixed-effects Models (GLMMs). As some measurements were made in percentages, a γ regression function was adopted in the GLMMs. A GLMM (fixed effect: genotype, DIM, age and herd) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes with a frequency >5% (thus insuring adequate sample size) on milk FA traits.

Interactions between different genes might be expected. The effects of *DGAT1* p.K232A, variation in *FABP4* and variation in *SCD* p.A293V on milk fat composition in these dairy cows have been described in previous studies (Li *et al.*, 2020*a*, 2020*b*, 2021). To correct for the potentially confounding effects of these genes, another GLMM (fixed effect: genotype, DIM, age, herd, *DGAT1* p.K232A genotype, *FABP4* genotype and *SCD1* p.A293V genotype) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes with a frequency >5% (thus insuring adequate sample size) on milk FA traits. As some of the cows were not typed for these genes the number of cattle analysed reduced from 409 to 405 in total.

The effect of sire of cow could not be included in the GLMMs. Some semen straws (sire genetics) used in NZ dairy cattle artificial insemination breeding contain mixed-sire semen purchased from commercial semen producers. In these cases, individual sire identity was impossible to ascertain, but because the straws were mixed-semen straws and because different sires were used for different inseminations in different years, it was unlikely that sire was a strong confounding effect. Cow age and herd might also be confounded with sire, but this cannot be confirmed.

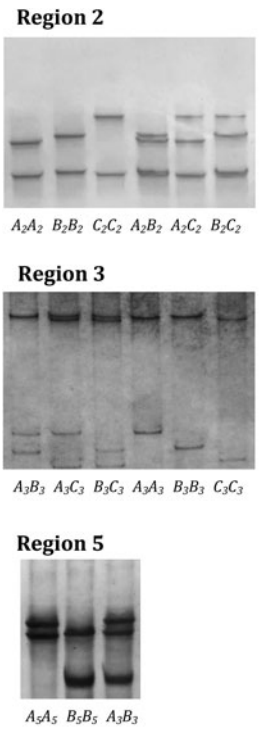
Results

Variation in PLIN2

In the five regions investigated, variations in the PCR-SSCP pattern were observed for Regions 2, 3 and 5. For Region 1, the primers did not appear to amplify the chosen region, and for Region 4, no PCR-SSCP variation was observed.

The PCR-SSCP banding patterns observed for different genotypes of Regions 2, 3 and 5 are illustrated in Fig. 1. Three variants (A_2, B_2 and C_2) of Region 2, three variants (A_3, B_3 and C_3) of Region 3 and two variants (A_5 and B_5) of Region 5 were detected. Five nucleotide sequence variants were found in these three regions, including c.17C>T in exon 2, c.53A>G in exon 3, c.595

https://doi.org/10.1017/S0021859621000174 Published online by Cambridge University Press



Position	Nucleotide substitutions			Reference
	A_2	B_2	C_2	
c.17	T	C	T	rs42211560
c.53	A	A	G	rs110859677

Position	Nucleotide substitutions			Reference
	A_3	B_3	C_3	
c.595+23	A	G	A	rs42211558
c.595+104_c.595+108del	TGGCA	TGGCA	-	rs380629765

Position	Nucleotide substitutions		Reference
	A_5	B_5	
c.*302	C	T	rs134156974

Fig. 1. Variation in bovine *PLIN2*. Unique PCR-SSCP patterns representing different sequence variants of Regions 2, 3 and 5 are shown.

+23G>A and c.595+104_595+108del(TGGCA/−) in intron 5, and c.*302T>C in the 3′-UTR. All these nucleotide sequence variations have been described in Ensembl, with rs numbers allocated (Fig. 1). The sequence variants, c.17C, c.53A, c.595+23G, c.595+104_595+108del(TGGCA) and c.*302T, are found in the reference sequence AF239708.

Six genotypes A_2A_2, B_2B_2, C_2C_2, A_2B_2, A_2C_2 and B_2C_2 were observed for Region 2, with the frequencies of 43.0, 13.4, 0.3, 34.8, 5.7 and 2.7%, respectively. The most common variant was A_2 (63.3%) and the frequency of B_2 and C_2 was 32.2 and 4.5%, respectively. The P value for the χ^2 for deviation from HWE was 0.044, suggesting the population was not at equilibrium.

Six genotypes A_3A_3, B_3B_3, C_3C_3, A_3B_3, A_3C_3 and B_3C_3 were found for Region 3, with the frequencies of 32.3, 32.0, 16.4, 10.3, 6.8 and 2.2%, respectively. The most common variant was A_3 (56.5%) and the frequency of B_3 and C_3 was 29.7 and 13.8%, respectively. The P value for the χ^2 for deviation from HWE was 0.469, suggesting the population was at equilibrium.

Three genotypes A_5A_5, A_5B_5 and B_5B_5 were found in Region 5, with the frequencies of 16.9, 53.3 and 29.8%, respectively. The most common variant was B_5 (56.5%) and the frequency of A_5 was 43.5%. The P value for the χ^2 for deviation from HWE was 0.089, suggesting the population was at equilibrium.

Associations of PLIN2 *variation with milk traits and milk fat composition*

The cows had a phenotypic average milk yield of 20.8 ± 0.41 litres, average milk fat content of 5.1 ± 0.05% and milk protein content of 4.2 ± 0.02 for herd 1 (n = 114) and phenotypic average milk yield of 22.5 ± 0.22 litres, average milk fat content of 5.1 ± 0.03% and milk protein content of 4.1 ± 0.02 for herd 2 (n = 295). Associations between *PLIN2* variation in the amplified regions and gross milk traits (i.e. milk yield, milk fat content and milk protein content) were analysed. No associations were observed between variation in either Region 2 or Region 3 and variation in these traits (results not shown). At the level of milk fat composition level, variation in milk FA profile was also not affected by the variation in Region 2 or 3, or the variation predicted was small (<5%), hence these results are also not shown.

In Region 5, three genotypes (A_5A_5, A_5B_5 and B_5B_5) were identified resulting from the nucleotide substitution c.*302T>C. Associations between these genotypes and milk traits are listed in full in Supplementary Tables S1 and S2, with only the significant associations shown in Tables 2 and 3. The effects of c.*302T>C on the gross milk trait of protein content were significant, and the variation was also associated with variation in milk fat composition for C10:0, C11:0, C12:0, C13:0 and C16:0 FA levels, and the MCFA and LCFA group levels (Table 2). The

Table 2. Associations between milk FA levels and *PLIN2* c.*302T>C (Region 5)

Traits (g/100 g milk FA)	Mean ± s.e.[a]			P value[b]
	A_5A_5 (n = 69)	A_5B_5 (n = 218)	B_5B_5 (n = 122)	
C10:0	3.2 ± 0.05	3.2 ± 0.03	3.3 ± 0.03	**0.013**
C11:0	0.06 ± 0.002	0.06 ± 0.001	0.06 ± 0.002	**0.021**
C12:0	3.9 ± 0.06	3.9 ± 0.04	4.1 ± 0.05	**0.010**
C13:0	0.12 ± 0.003	0.12 ± 0.002	0.13 ± 0.002	**0.027**
C16:0	37.9 ± 0.37	37.0 ± 0.22	36.5 ± 0.29	**0.010**
MCFA	20.7 ± 0.20	20.7 ± 0.12	21.2 ± 0.16	**0.030**
LCFA	49.5 ± 0.33	48.5 ± 0.32	48.1 ± 0.43	**0.014**

[a]Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk' and 'herd' were fitted to the models as fixed effects.
[b]P < 0.05 in bold.

https://doi.org/10.1017/S0021859621000174 Published online by Cambridge University Press

Table 3. Associations between milk fat components and *PLIN2* c.*302T>C (corrected for *DGAT1*, *FABP4* and *SCD* genotype)

	Mean ± s.e.[a] (FA are given in g/100 g milk FA) associated with *LPIN2* genotypes			*P* value[b]			
Traits	A_5A_5 ($n = 68$)	A_5B_5 ($n = 216$)	B_5B_5 ($n = 121$)	*DGAT1*	*FABP4*	*SCD*	*PLIN2*
C10:0	3.2 ± 0.05	3.3 ± 0.03	3.4 ± 0.04	0.320	**0.081**	**0.012**	**0.014**
C11:0	0.06 ± 0.002	0.06 ± 0.002	0.06 ± 0.002	**<0.001**	**0.048**	**0.001**	**0.007**
C12:0	3.9 ± 0.07	3.9 ± 0.05	4.1 ± 0.05	0.393	0.068	0.294	**0.009**
C13:0	0.12 ± 0.003	0.12 ± 0.002	0.13 ± 0.003	**<0.001**	0.063	**<0.001**	**0.017**
C16:0	37.4 ± 0.40	36.6 ± 0.27	36.1 ± 0.33	**<0.001**	**0.017**	0.126	**0.008**
MCFA	20.9 ± 0.22	20.9 ± 0.15	21.4 ± 0.189	**<0.001**	**0.026**	**0.037**	**0.024**
LCFA	48.7 ± 0.43	48.0 ± 0.23	47.6 ± 0.28	**<0.001**	**0.019**	0.120	**0.012**
Total SFA	68.8 ± 0.32	67.9 ± 0.22	67.9 ± 0.26	**<0.001**	**0.040**	0.396	**0.019**
C10:1 index (%)[c]	8.1 ± 0.17	7.9 ± 0.11	7.6 ± 0.14	0.956	0.572	**<0.001**	**0.022**

[a]Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk', 'herd', '*DGAT1* p.K232A', '*FABP4*' and '*SCD* p.A293V' were fitted to the models as fixed effects.
[b]$P < 0.05$ in bold.
[c]C10:1 index = C10:1/(C10:1 + C10:0) × 100.

B_5B_5 cows contained more C10:0, C11:0, C12:0, C13:0 and MCFA, but less C16:0 FA and LCFA.

After correcting for the effects of variation in *DGAT1*, *FABP4* and *SCD1*, associations were observed between c.*302T>C and C10:0, C11:0, C12:0, C13:0, C16:0 FA levels, and the MCFA, LCFA, total SFA and C10:1 index groups (Table 3).

Discussion

Ogorevc *et al.* (2009) summarized the relationship between BTA8 QTLs and milk traits, identifying that the region that contains *PLIN2* (*ADFP*) has QTLs associated with milking speed, protein content, somatic cell score, somatic cell count and clinical mastitis occurrence. They did not find associations with other milk traits. In contrast, Lu *et al.* (2016) found evidence that perilipin-2 levels were associated with variation in milk fat. Their mass spectrometry-based proteomics approach revealed that the concentration of perilipin-2 in bovine milk was higher in large fat globules (7.6 ± 0.9 µm), than in small ones (3.3 ± 1.2 µm). These large fat globules also contained more total SFA, C17:0 and C18:0, but less C10:1, C12:1, C14:1 *cis*-9, C18:1 *cis*-9 FA and conjugated linoleic acid. Whether this was a consequence of sequence variation in the perilipin-2 gene was not tested.

The association reported here between *PLIN2* variation and milk fat composition may be because of variation in gene expression. The c.*302T>C nucleotide substitution was in the 3′-UTR of the gene, and this region of eukaryote genes can contain regulatory elements that influence gene expression. For example, 3′-UTR regions can contain microRNA response elements, AU-rich elements, iron response elements and other 'signatures' that can affect translation and mRNA stability. Sequence variation in these or similar regulatory elements might therefore change their function, and hence the level of gene expression.

For example, a nucleotide substitution c.*382A>G in the 3′-UTR of the high-mobility group box protein 1 gene (*HMGB1*) alters the binding of bta-miR-223, and was found to be associated with somatic cell scores in dairy cows (Li *et al.*, 2012). Similarly, Ju *et al.* (2018) revealed that the 3′-UTR variation c.*301A>G in the neutrophil cytosolic factor 4 gene (*NCF4*) affects the binding of bta-miR-2426, and that cows with the GG genotype had a lower somatic cell score than cows with the AA genotype. Using a quantitative real-time PCR assay, they also revealed that the cows with genotype GG had a higher expression of *NCF4* mRNA, compared to the cows with genotype AA.

Other researchers have also described 3′-UTR variation in genes that affect milk traits. For example, in describing the effect of DGAT1 p.K232A, Grisart *et al.* (2002) described the 3′-UTR variation c.*85T>C, but suggested this was 'more likely to be neutral'. Weikard *et al.* (2005) reported two nucleotide substitutions, c.*967C>A and c.*2922C>T, in the bovine peroxisome proliferator-activated receptor-γ coactivator 1α gene (*PPARGC1A*) 3′-UTR. They reported a trend ($P = 0.076$) that cows with the *PPARGC1A* c.*967C>A genotype AA had a higher milk fat yield (25.04 ± 4.29 kg), than cows with the CC genotype (16.77 ± 3.90 kg). Khatib *et al.* (2006) reported associations between milk fat yield and the 3′-UTR nucleotide substitution c.*223C>A (described as SNP 8232) in the oxidized low-density lipoprotein receptor gene (*OLR1*). They suggested that c.*223C>A might control the translation or stability of *OLR1* mRNA, because expression levels were lower in the AA genotype cows, than in the AC or CC cows. In the context of the above studies, it could be concluded that variant c.*302T>C might affect *PLIN2* expression, but further studies will be needed to ascertain how that may be happening.

Cheong *et al.* (2009) reported that the c.-74A>G (they described it as c.-56-18A>G) in the *PLIN2* promotor region was associated with meat-marbling score in Korean native beef cattle. In NZ pasture-grazed HF × J-cross cows, the Region 1 amplicon might contain the nucleotide sequence variations reported by Cheong *et al.* (2009), such as the variations c.-123G>A, c.-74A>G and c.-57G>C in the promotor region, the variation c.-39G>C in 5′-UTR region and the variations c.−26+128C>G, c.−26+149G>A, c.−26+163T>C and c.−26+175C>T in intron1. However, the primers designed here did not appear to work. Aside from the fact that the primers may have been poorly designed, unaccounted for sequence variation in the primer-binding sites might also be responsible for the amplification failures. All the primers used in the current study were based on the

https://doi.org/10.1017/S0021859621000174 Published online by Cambridge University Press

cattle reference sequence AF239708, except the forward primer for Region 1. The sequence AF239708 reported by Cheong *et al.* (2009) did not contain the 5′ flanking region that was to be targeted, thus the forward primer for Region 1 was instead designed based on the predicted sequence ENSBTAT00000007519. These predicted sequences are generated by the software and thus sequence errors may have been promulgated and led to the failure of the Region 1 amplifications.

In the process of milk fat formation, perilipin-2 regulates the filling of milk lipid droplets with triglyceride. Both Thering *et al.* (2009) and Lu *et al.* (2016) reported that perilipin-2 appeared to affect LCFA transport, lipid sequestration and lipid storage. In the current study, a significant association between *PLIN2* variation and milk C16:0 FA levels was found (Tables 2 and 3). Compared to the C16:0 FA result in the current study, the nucleotide substitution c.*302T>C appeared to have an opposite effect on MCFAs. For example, the B_5B_5 cows had more C10:0, C11:0, C12:0 and C13:0 FA in their milk (Table 2), although the results for the C11:0 and C13:0 levels were difficult to interpret as only the homozygous B_5B_5 and heterozygous A_5B_5 cows differed at $P < 0.05$, but were not significantly different to the A_5A_5 cows. In both cases, the levels of the FAs were very low, and likely close to the detection limits of the GC analysis, hence these enigmatic differences in C11:0 and C13:0 FA levels may simply be a consequence of machine error.

Bionaz and Loor (2008) described gene networks involved in bovine milk fat synthesis and suggested that the C14:0 FA in milk was mainly derived from *de-novo* synthesis in the mammary gland. Previous studies suggest that *DGAT1*, *FABP4* and *SCD* could affect C14:0 FA levels (Li *et al.*, 2020*a*, 2020*b*, 2021), and this was confirmed by the results in Supplementary Table S2, but no effect was observed for C14:0. After correcting for possible interactions with these genes, the effect of variant c.*302T>C on C10:0, C11:0, C12:0, C13:0 and C16:0 FA levels, and the MCFA and LCFA groups appeared to be stronger (i.e. the *P* value for the *PLIN2* associations typically decreased in Table 3). However, there were once again confusing results for C11:0 and C13:0 levels, with A_5B_5 and B_5B_5 genotype cows differing from each other ($P < 0.05$), but not being different to the A_5A_5 cows (Table 3). Given the cows studied were in mid-lactation and thus likely to be synthesizing FA, it may instead be that other *de-novo* synthesis-related genes not investigated here might also be affecting the results, such as the activity of the FA synthase gene (*FASN*), acetyl-coenzyme A carboxylase α gene (*ACACA*) and acyl-CoA synthetase short-chain family member 2 gene (*ACSS2*). More research into the activity of these genes would therefore appear to be needed, and at different stages of lactation.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0021859621000174

Acknowledgements. The authors thank the staffs of the Faculty of Agriculture and Life Science for sampling and care of the animals.

Financial support. The present study was financially supported by the Ministry of Science and Innovation (DRCX 0802; Dairy Systems for Environmental Protection; NZ) and the Lincoln University Gene-Marker Laboratory of New Zealand.

Conflict of interest. None.

Ethical standards. The Lincoln University Animal Ethics Committee (AEC Number 521) approved the current research under the provision of the Animal Welfare Act 1999 (NZ Government).

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