Genome-wide association study and functional analyses for clinical and subclinical ketosis in Holstein cattle

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ABSTRACT

Ketosis is one of the most frequent metabolic diseases in high-yielding dairy cows and is characterized by high concentrations of ketone bodies in blood, urine, and milk, causing high economic losses. The search for polymorphic genes, whose alleles have different effects on resistance to developing the disease, is of extreme importance to help select less susceptible animals. The aims of this study were to identify genomic regions associated with clinical and subclinical ketosis (β -hydroxybutyrate concentration) in North American Holstein dairy cattle and to investigate these regions to identify candidate genes and metabolic pathways associated with these traits. To achieve this, a GWAS was performed for 4 traits: clinical ketosis lactation 1, clinical ketosis lactation 2 to 5, subclinical ketosis lactation 1, and subclinical ketosis lactation 2 to 5. The estimated breeding values from 77,277 cows and 7,704 bulls were deregressed and used as pseudophenotypes in the GWAS. The top-20 genomic regions explaining the largest proportion of the genetic variance were investigated for putative genes associated with the traits through functional analyses. Regions of interest were identified on chromosomes 2, 5, and 6 for clinical ketosis lactation 1; 3, 6, and 7 for clinical ketosis lactation 2 to 5; 1, 2, and 12 for subclinical ketosis lactation 1; and 20, 11, and 25 for subclinical ketosis lactation 2 to 5. The highlighted genes potentially related to clinical and subclinical ketosis included *ACAT2* and *IGF1*. Enrichment analysis of the list of candidate genes for clinical and subclinical ketosis showed molecular functions and biological processes involved in fatty acid metabolism, lipid metabolism, and inflammatory response in dairy cattle. Several genomic regions and SNPs related to susceptibility to ketosis in dairy cattle that were previously described in other studies were confirmed. The

novel genomic regions identified in this study aid to characterize the most important genes and pathways that explain the susceptibility to clinical and subclinical ketosis in dairy cattle.

Key words: β -hydroxybutyrate, clinical ketosis, genome-wide association study

INTRODUCTION

Increased milk production in high-yielding cows is associated with an increased risk of health issues, such as metabolic diseases (Sundrum, 2015). Cows enter a state of negative energy balance (NEB) when they are unable to satisfy the requirements for energy early in lactation. Some cows naturally recover from NEB, but others do not recover and develop metabolic diseases such as ketosis (Andersson, 1988). In addition, previous studies have shown that by contrasting the first lactation with later lactations, cows respond differently to NEB (Jamrozik et al., 2016). Clinical and subclinical ketosis are defined by the presence of clinical symptoms and BHB levels in the blood, respectively (Duffield et al., 2009). The BHB concentration test is the gold standard test for diagnosis of ketosis because ketosis is characterized by high levels of ketone bodies in the blood. Values ≥ 1.2 mM indicate subclinical ketosis, and values ≥ 3.0 mM indicate clinical ketosis (Zhu et al., 2019).

Being one of the most frequent metabolic diseases in dairy cows, ketosis causes high economic losses as the result of decreased productivity and costs of treatment. Due to the dairy industry's focus on the genetic improvement of milk production, energy demands will increase, and cows will continue to present ketosis (Duffield, 2000). In cases of subclinical ketosis, the drop in milk production could reach 1.2 kg/cow per day (McArt et al., 2012). Thus, preventing ketosis is one of the most effective strategies to avoid economic losses associated with milk production because, on average, each case of subclinical and clinical ketosis can result in losses of \$203 and \$289, respectively (McArt et al., 2015; Steeneveld et al., 2020).

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The clinical symptoms of this metabolic disease include weight loss, loss of appetite, and decreased milk yield, along with increased levels of ketone bodies in the blood and milk (Duffield et al., 2009). Many misrepresentations of subclinical ketosis or misdiagnosed clinical ketosis occur because of the difficulty in diagnosing the condition, which makes it difficult to resolve the issue. In addition, ketosis in high-producing dairy cows is a multifactorial trait with low heritability. Heritability estimates for clinical ketosis ranging from 0.02 to 0.16, and for subclinical ketosis ranging from 0.04 to 0.17, have been reported (Lee et al., 2016; Pryce et al., 2016), which shows the need to better understand the genetic origin of this problem (Parker Gaddis et al., 2018). The search for polymorphic genes, whose alleles are associated with different levels of resistance to the development of the disease, is of extreme importance to help selecting for less susceptible animals. In this sense, a GWAS is a powerful strategy for detecting genomic regions involved with metabolic processes associated with this trait (Ku et al., 2010).

Therefore, the goals of this study were as follows: (1) to conduct a GWAS to screen for genetic variants that may influence clinical and subclinical ketosis (BHB concentrations) susceptibility in North American Holstein dairy cattle on first and later lactations, and (2) to pinpoint candidate genes and pathways with biologically relevant associations with clinical and subclinical ketosis susceptibility.

MATERIALS AND METHODS

Animals and Data Set Description

The data set used in this study was provided by the Canadian Dairy Network (CDN), a member of Lactanet (www.lactanet.ca), and contained pedigree, genotypes, and EBV for cows and bulls. Comparing the first lactation with later lactations, previous studies found that cows respond to NEB differently and, for this reason, the first and later (from 2–5) lactations were considered as different traits (Jamrozik et al., 2016). This way, 4 traits were defined as follows: clinical ketosis lactation 1 (**CK1**), clinical ketosis lactation 2 to 5 (**CK2**), subclinical ketosis lactation 1 (**SCK1**), and subclinical ketosis lactation 2 to 5 (**SCK2**). Milk BHB measurements were predicted by mid-infrared spectroscopy in routine DHI milk analyses, and phenotypes for clinical ketosis were recorded either by farmers as part of the Canadian health recording system or by veterinarians via Valacta (a member of Lactanet). A total of 84,981 animals (77,277 cows and 7,704 bulls) with EBV for

CK1, CK2, SCK1, and SCK2 provided by CDN were used for the analyses. The pedigree consisted of 329,958 individuals traced back 7 generations.

Genotypic Data and Quality Control

A total of 9,161 Canadian Holstein animals were genotyped with the Illumina bovine SNP60 (60K; 57,798 SNP) BeadChip (Illumina, Inc.). These SNPs have passed the standard quality control used by CDN. A sample of 652 Canadian Holstein cows were also genotyped with a low-density array containing 998 putative ketosis markers (1K; 998 SNPs). These 998 markers are a combination of novel and previously reported SNP markers that are located within or near candidate genes related to ketosis (Kroezén et al., 2018). None of these 998 markers were located in the sex chromosomes. The 60K and 1K genotypes in the 652 cows were combined to create a new panel (61K SNP). Genotype imputation from the 60K to 61K panel was performed for the remaining animals using the FImpute V2.2 software (Sargolzaei et al., 2014), excluding the sex chromosomes. The algorithm in FImpute is based on the combination of family and population methods to reconstruct haplotypes (Sargolzaei et al., 2014). To estimate the accuracy of imputation, animals were randomly selected from the reference population (61K panel), and their genotypes were masked down to the 60K panel. The squared correlation between imputed genotypes and true genotypes (r^2) was used as a measure of imputation accuracy (Browning and Browning, 2009), and it was $r^2 = 0.98$.

After imputation, a quality control was performed using the BLUPF90 software (Misztal et al., 2019). The genotyping quality control filtered out markers with minor allele frequency lower than 1% and with a heterozygosity that deviated by more than 15% from the expected value under Hardy-Weinberg equilibrium. Only autosomal chromosomes were considered for further analysis. After quality control, 57,957 SNPs remained for further analyses.

Deregressed EBV

Deregressed EBV (**dEBV**) computed following Garrick et al. (2009) were used as response variables for genomic prediction. This method results in dEBV containing only information for each animal along with their descendant's information because it eliminates shrinkage in the EBV and adjusts for ancestral information.

Genome-Wide Association Analyses

The SNP effects were estimated using the weighted single-step method (**wssGBLUP**) proposed by Wang et al. (2012). The wssGBLUP model can be described as follows:

$$\mathbf{y}^* = \mathbf{1}\mu + \mathbf{Z}_a\mathbf{a} + \mathbf{e},$$

where $\mathbf{y}^*$ is the vector of pseudophenotypes (dEBV); $\mathbf{1}$ is a vector of ones, μ is the overall mean; $\mathbf{Z}_a$ is an incidence matrix that relates animals to dEBV; $\mathbf{a}$ is the vector of direct additive genetic effects; and $\mathbf{e}$ is the vector of random residuals. It was assumed that $\mathbf{a} \sim N(0, \mathbf{H}\sigma_a^2)$ and $\mathbf{e} \sim N(0, \mathbf{R}\sigma_e^2)$, where $\mathbf{H}$ is the relationship matrix based on genomic and pedigree information, σ_a^2 is the additive genetic variance, $\mathbf{R}$ is a diagonal matrix, whose elements account for the differences in the reliabilities of the dEBV, and σ_e^2 is the residual variance. The inverse of $\mathbf{H}$ matrix can be defined as (Aguilar et al., 2010):

$$\mathbf{H}^{-1} = \mathbf{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & \mathbf{G}^{-1} - \mathbf{A}_{22}^{-1} \end{bmatrix},$$

where $\mathbf{A}$ is the numerator relationship matrix based on pedigree for all animals; $\mathbf{A}_{22}$ is the numerator relationship matrix based on pedigree for genotyped animals only; and $\mathbf{G}$ is the genomic relationship matrix for genotyped animals.

The solutions of SNP effect estimates ($\hat{\mathbf{u}}$) were obtained using the formula: $\hat{\mathbf{u}} = \mathbf{DZ}'[\mathbf{ZDZ}']^{-1}\hat{\mathbf{a}}$, where $\mathbf{D}$ is a diagonal matrix with weights for SNPs; $\mathbf{Z}$ is an incidence matrix of genotyped for each locus, $\mathbf{Z}'$ is the transpose of $\mathbf{Z}$ matrix, and $\hat{\mathbf{a}}$ is the vector of dEBV of genotyped animals. The $\hat{\mathbf{u}}$ vector and the $\mathbf{D}$ matrix were derived as follows (Wang et al., 2012):

1. In the first iteration, $t = 0$ and $\mathbf{D}_t = \mathbf{I}$, where t is the iteration number, $\mathbf{D}_t$ is matrix $\mathbf{D}$ at iteration t , and $\mathbf{I}$ is an identity matrix.
2. Calculate the SNP effects at iteration t ($\hat{\mathbf{u}}_t$).
3. Recalculate the diagonal elements of $\mathbf{D}$ to obtain $\mathbf{D}_{(t+1)}^*$ as $d_{i(t+1)}^* = \hat{\mathbf{u}}_{i(t)}^2 / 2p_i(1-p_i)$ for all SNPs, where $d_{i(t+1)}^*$ is the diagonal element of the ma-

trix $\mathbf{D}_{(t+1)}^*$ for the i th SNP, and p_i is the allele frequency of the reference allele of the i th SNP.

4. Normalize $\mathbf{D}_{(t+1)} = \left(\text{tr}(\mathbf{D}_{(0)}) / \text{tr}(\mathbf{D}_{(t+1)}^*) \right) \mathbf{D}_{(t+1)}^*$.
5. $t = t + 1$.
6. Exit, or loop to step 2.

The SNP effects and weights were iteratively recomputed over 2 iterations. The iteration process increases the weight of SNPs with large effects and decreases those with small effects, resulting in an increasing proportion of variance explained by the remaining markers. The SNP effects were calculated using POSTGSF90 and sliding windows of 20 adjacent SNPs (Misztal et al., 2019).

Functional Annotation Analyses

The top-20 genomic regions explaining the largest proportion of genetic variance were investigated for putative genes associated with subclinical and clinical ketosis. The ENSEMBL Biomart Martview application (Ensembl Genes 97, <http://www.ensembl.org/biomart/martview>) was used for identification of the genes located within those windows as well as nucleotide and protein sequences of these genes, using the ARS-UCD1.2 assembly as the reference map. All unique sequences were aligned against the sequences in the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) nonredundant database using the BLASTp algorithm from the Blast2GO software (Götz et al., 2008). Sequences with a BLAST match were mapped and annotated. Gene ontology (GO) analysis was performed considering the 3 GO categories: biological process, molecular function, and cellular component. The GO significance levels ($P < 0.05$) were computed following Fisher's exact test for multiple testing in Blast2GO, comparing the list of positional candidate genes versus the functions from the reference (background genes) using the bovine genome assembly ARS-UCD1.2 (release 97). Metabolic pathways associated with the top-20 genomic regions were identified using the Kyoto Encyclopedia of Genes and Genome (KEGG; Kanehisa and Goto, 2000). Additionally, a gene network analysis was performed using STRING database (Szklarczyk et al., 2019) to identify specific protein-protein interactions, and "key nodes" were selected from proteins with degree ≥ 4 , betweenness centrality (BC) ≥ 0.05 and closeness centrality (CC) ≥ 0.2 .

RESULTS

Function Annotation and Gene Network Analyses

Genome-wide Association Analyses

The proportion of variance explained by the top-20 sliding windows associated with CK1 are presented in Table 1, CK2 are presented in Table 2, SCK1 are presented in Table 3, and SCK2 are presented in Table 4. A greater proportion of variance observed using wssGBLUP analyses was found on chromosomes 6 for CK1 and CK2, and on chromosomes 2 and 20 for SCK1 and SCK2, respectively. The window that explained the highest proportion of variance overlapped for both traits (CK1 and CK2). The sum of the proportion of variance explained by the top-20 genomic windows was 16.7, 18.0, 19.2, and 13.4% for CK1, CK2, SCK1, and SCK2, respectively.

The genetic variance explained by the SNPs in the GWAS analyses are shown as Manhattan plots for CK1 (Figure 1), CK2 (Figure 2), SCK1 (Figure 3), and SCK2 (Figure 4). The results indicated important genomic regions on chromosomes 2, 5, 6, 11, 17, and 23 for CK1; 3, 6, 7, 15, and 20 for CK2; and 1, 2, 3, 12, and 23 for SCK1, which each explained more than 1% of the total genetic variance. For SCK2, none of the windows explained more than 1% of the genetic variance.

The annotated genes within the top-20 sliding windows associated with the 4 traits using wssGBLUP analyses are presented in Tables 1 to 4. For CK1, 114 annotated genes were found within 19 genomic windows identified by the GWAS. For CK2, 126 genes were found within the top-20 genomic windows. For SCK1, a total of 145 genes were identified within 18 genomic windows. For SCK2, the highest number of annotated genes was found (i.e., 166 genes within 16 genomic windows).

The GO enrichment analyses were performed for all candidate genes for the 4 traits to identify genes involved in known metabolic pathways. For CK1 (Supplemental Table S1, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a), 29 GO terms were found, whereas 60 GO terms were associated with CK2 (Supplemental Table S2, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a). For SCK1 (Supplemental Table S3, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a) and SCK2 (Supplemental Table S4, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a), 31 and 62 GO terms were found, respectively.

Based on the functional annotation results, significant pathways were found for subclinical and clinical ketosis.

Table 1. Annotated genes within the top 20 sliding windows explaining the highest proportion of genetic variance for clinical ketosis lactation 1

Chr ¹	Position (bp) ²	Var (%) ³	Genes ⁴
6	39470632:40182771	1.70	<i>SLIT2</i> , <i>bta-mir-218-1</i>
5	87558801:88246615	1.48	<i>ETNK1</i> , <i>C2CD5</i> , <i>ST8SIA1</i> , <i>CMAS</i>
2	108277094:109019023	1.27	—
23	50731493:51335705	1.08	<i>SERPINB6</i> , <i>SERPINB9</i> , <i>SERPINB1</i> , <i>RF00003</i> , <i>WRNIP1</i> , <i>MYLK4</i> , <i>GMDS</i>
11	71130818:71946048	1.07	<i>PLB1</i> , <i>FOSL2</i> , <i>BABAM2</i> , <i>RF02271</i> , <i>RBKS</i> , <i>MRPL33</i>
17	42736277:43493467	1.02	<i>CTSO</i> , <i>RF00026</i> , <i>TDO2</i> , <i>ASIC5</i>
4	78071301:79231632	0.97	<i>MRPL32</i> , <i>PSMA2</i> , <i>C4H7orf25</i> , <i>GLI3</i>
12	68852955:69746467	0.88	<i>DCT</i> , <i>RF00001</i> , <i>TGDS</i> , <i>GPR180</i> , <i>RF00004</i> , <i>SOX21</i>
2	125665173:126315385	0.78	<i>IFI6</i> , <i>FGR</i> , <i>AHDC1</i> , <i>WASF2</i> , <i>RF00026</i> , <i>GPR3</i> , <i>CD164L2</i> , <i>MAP3K6</i> , <i>SYTL1</i> , <i>TMEM222</i>
9	96043531:96090673	0.77	<i>ACAT2</i> , <i>TCP1</i> , <i>RF00401</i> , <i>RF00429</i> , <i>MRPL18</i> , <i>PNLDC1</i>
23	48617540:49291906	0.69	<i>F13A1</i> , <i>NRN1</i> , <i>FARS2</i>
3	32800931:32888689	0.68	<i>KCNA2</i>
19	49744592:50605756	0.64	<i>B3GNTL1</i> , <i>TBCD</i> , <i>bta-mir-2344</i> , <i>ZNF750</i> , <i>RF00045</i> , <i>FN3KRP</i> , <i>RAB40B</i> , <i>WDR45B</i> , <i>FOXK2</i> , <i>NARF</i> , <i>CYBC1</i> , <i>HEXD</i> , <i>OGFOD3</i> , <i>SECTM1A</i> , <i>bta-mir-2345</i> , <i>SECTM1</i> , <i>CD7</i> , <i>CSNK1D</i>
27	34061853:35143952	0.62	<i>PLEKHA2</i> , <i>HTRA4</i> , <i>TM2D2</i> , <i>ADAM9</i> , <i>ADAM32</i> , <i>ADAM2</i> , <i>ADAM3A</i> , <i>ADAM18</i> , <i>RF00026</i> , <i>IDO1</i> , <i>IDO2</i>
14	9361706:9949694	0.61	<i>ADCY8</i>
16	4263770:4831223	0.51	<i>IKBKE</i> , <i>RASSF5</i> , <i>EIF2D</i> , <i>DYRK3</i> , <i>MAPKAPK2</i> , <i>IL10</i> , <i>IL19</i> , <i>IL20</i> , <i>IL24</i> , <i>FCMR</i> , <i>PIGR</i> , <i>FCAMR</i>
20	50585802:51392649	0.49	<i>CDH12</i>
3	110042206:110866523	0.48	<i>CLSPN</i> , <i>C3H1orf216</i> , <i>PSMB2</i> , <i>TFAP2E</i> , <i>NCDN</i> , <i>KIAA0319L</i> , <i>ZMYM4</i> , <i>SFPQ</i> , <i>ZMYM1</i> , <i>ZMYM6</i> , <i>TMEM35B</i> , <i>DLGAP3</i> , <i>SMIM12</i>
3	3596795:4313521	0.48	<i>RXRG</i> , <i>LMX1A</i> , <i>PBX1</i>
7	32543025:33469201	0.47	<i>PRR16</i>

¹Chr = chromosome.

²Starting and ending coordinates of the window.

³Var = % genetic variance explained by the SNPs within the window.

⁴Ensembl symbol of annotated genes using the *Bos taurus* ARS-UCD1.2 assembly (<http://www.ensembl.org/biomart/martview>).

Highlighted pathways included synthesis and degradation of ketone bodies, biosynthesis of unsaturated fatty acids (Supplemental Table S5), fatty acid elongation (Supplemental Tables S5 and S8, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a), fatty acid degradation (Supplemental Tables S5, S7, S8, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a), arachidonic acid metabolism (Supplemental Tables S6, S7, S8, <http://doi.org/10.5281/zenodo.4651568>; Soares et al., 2021a), and pyruvate metabolism (Supplemental Tables S5 and S7). The *ACAT2* gene was involved in several pathways for CK1 and SCK1 (Supplemental Tables S5 and S7).

The relationships among the genes in the network were determined by co-expression, gene fusion, protein homology, gene-neighborhood, and gene co-occurrence. A network containing 97 nodes and 61 edges between them [protein-protein interaction (PPI) enrichment $P = 2.2e^{-16}$] was constructed for CK1. Based on degree ≥ 4 , BC ≥ 0.05 , and CC ≥ 0.2 , 6 key nodes were identified, with *IL10* having the highest degree (6), BC (0.72),

and CC (0.69), making it the most interconnected and powerful gene in the network (Supplemental Figure S1, <http://doi.org/10.5281/zenodo.4651578>; Soares et al., 2021b). A network containing 120 nodes and 114 edges between them (PPI enrichment $P = 7.11e^{-15}$) was constructed for CK2. We identified *APOA1* as a key node having the highest degree (6), BC (0.31), and CC (0.24), as shown in Supplemental Figure S2 (<http://doi.org/10.5281/zenodo.4651578>; Soares et al., 2021b). A network containing 135 nodes and 123 edges between them (PPI enrichment $P = 1.1e^{-15}$) was constructed for SCK1. With degree of 8, BC of 0.26, and CC of 0.22, *RPS10* was identified as a key node of this network (Supplemental Figure S3, <http://doi.org/10.5281/zenodo.4651578>; Soares et al., 2021b). A network containing 164 nodes and 186 edges between them (PPI enrichment $P = 1.0e^{-16}$) was constructed for SCK2. We found *SUMF2* was a key node in this network with degree of 10, BC of 0.07, and CC of 0.21 (Supplemental Figure S4, <http://doi.org/10.5281/zenodo.4651578>; Soares et al., 2021b).

Table 2. Annotated genes within the top 20 sliding windows explaining the highest proportion of genetic variance for clinical ketosis lactation 2 to 5

Chr ¹	Position (bp) ²	Var (%) ³	Genes ⁴
6	39825526:40456680	2.35	<i>SLIT2</i> , <i>bta-mir-218-1</i> , <i>PACRGL</i> , <i>KCNIP4</i>
20	8811738:9562025	1.37	<i>TNPO1</i> , <i>ZNF366</i> , <i>PTCD2</i> , <i>MRPS27</i> , <i>MAP1B</i>
7	38298547:38608111	1.32	<i>UNC5A</i> , <i>HK3</i> , <i>UIMC1</i> , <i>ZNF346</i> , <i>RF00026</i> , <i>FGFR4</i>
3	3639255:4343859	1.31	<i>LMX1A</i> , <i>PBX1</i>
15	29240225:30156144	1.23	<i>ARCN1</i> , <i>PHLDB1</i> , <i>bta-mir-12011</i> , <i>TREH</i> , <i>DDX6</i> , <i>RF00001</i> , <i>CXCR5</i> , <i>BCL9L</i> , <i>UPK2</i> , <i>FOXR1</i> , <i>CCDC84</i> , <i>RPS25</i> , <i>TRAPPC4</i> , <i>SLC37A4</i> , <i>HYOU1</i> , <i>VPS11</i> , <i>HMBS</i> , <i>DPAGT1</i> , <i>C2CD2L</i> , <i>HINFP</i> , <i>ABCG4</i> , <i>NLRX1</i> , <i>PDZD3</i> , <i>CCDC153</i> , <i>CBL</i> , <i>MCAM</i> , <i>RNF26</i> , <i>C1QTNF5</i> , <i>MFRP</i> , <i>USP2</i> , <i>THY1</i>
15	26653665:27503740	1.10	<i>RF00285</i> , <i>BUD13</i> , <i>ZPR1</i> , <i>APOA5</i> , <i>APOA4</i> , <i>APOC3</i> , <i>APOA1</i>
6	5063081:6358918	1.04	<i>PDE5A</i> , <i>FABP2</i> , <i>C6H4orf3</i> , <i>USP53</i> , <i>RF00001</i> , <i>MYOZ2</i> , <i>SYNPO2</i>
5	2143145:2940529	0.91	<i>TRHDE</i>
20	65741860:66319602	0.89	<i>ADYC2</i>
9	17170909:19212422	0.77	<i>MEI4</i> , <i>RF00100</i> , <i>IRAK1BP1</i> , <i>PHIP</i> , <i>RF00026</i> , <i>LCA5</i>
20	35757652:36278126	0.74	<i>LIFR</i> , <i>EGFLAM</i>
4	14277959:15633760	0.66	<i>RF01887</i> , <i>RF01888</i> , <i>DLX6</i> , <i>RF01778</i> , <i>DLX5</i> , <i>SDHAF3</i> , <i>TAC1</i> , <i>ASNS</i> , <i>RF00322</i> , <i>C1GALT1</i> , <i>COL28A1</i>
6	38929469:39797151	0.57	<i>SLIT2</i>
19	50691143:51263493	0.55	<i>CCDC57</i> , <i>FASN</i> , <i>DUS1L</i> , <i>GPS1</i> , <i>RFNG</i> , <i>DCXR</i> , <i>RAC3</i> , <i>LRRC45</i> , <i>CENPX</i> , <i>ASPSCR1</i> , <i>bta-mir-2346</i> , <i>NOTUM</i> , <i>MYADML2</i> , <i>PYCR1</i> , <i>MAFG</i> , <i>SIRT7</i> , <i>PCYT2</i> , <i>bta-mir-2347</i> , <i>NPB</i> , <i>ANAPC11</i> , <i>ALYREF</i> , <i>ARHGDI</i> , <i>P4HB</i> , <i>PP1R27</i> , <i>MCRIP1</i> , <i>GCCR</i> , <i>MRPL12</i> , <i>HGS</i> , <i>ARL16</i> , <i>CCDC137</i> , <i>OXLD1</i> , <i>PDE6G</i> , <i>TSPAN10</i> , <i>NPLOC4</i> , <i>FAAP100</i> , <i>FSCN2</i> , <i>ACTG1</i> , <i>bta-mir-3533</i>
18	59412224:60980891	0.54	<i>ZNF677</i> , <i>ZNF331</i> , <i>MGC139164</i> , <i>bta-mir-371</i> , <i>NLRP12</i>
20	59086224:59794504	0.54	<i>RF00026</i> , <i>DNAH5</i>
5	65581391:66435941	0.53	<i>GNPTAB</i> , <i>DRAM1</i> , <i>WASHC3</i> , <i>NUP37</i> , <i>PARPBP</i> , <i>PMCH</i> , <i>IGF1</i>
5	66446463:66777438	0.52	<i>RF00026</i> , <i>PAH</i> , <i>ASCL1</i>
6	7070264:7925817	0.52	<i>NDST3</i>
20	8151139:8771290	0.50	<i>RF00026</i> , <i>TMEM174</i> , <i>TMEM171</i> , <i>FCHO2</i> , <i>TNPO1</i>

¹Chr = chromosome.

²Starting and ending coordinates of the window.

³Var = % genetic variance explained by the SNPs within the window.

⁴Genes: ENSEMBL symbol of annotated genes using the *Bos taurus* ARS-UCD1.2 assembly (<http://www.ensembl.org/biomart/martview>).

Table 3. Annotated genes within the top 20 sliding windows explaining the highest proportion of genetic variance for subclinical ketosis lactation 1

Chr ¹	Position (bp) ²	Var (%) ³	Genes ⁴
2	77688697:78437805	2.43	—
23	36786202:37974716	2.22	<i>CDKAL1, E2F3, RF00001, MBOAT1</i>
3	3043960:3781801	1.52	<i>UCK2, TMC01, ALDH9A1, MGST3, LRRC52, RXRG, LMX1A</i>
1	100122413:101049645	1.15	<i>ZBBX, RF00026</i>
2	78507223:79522994	1.11	<i>RF00612, GYPC, GLS, STAT1</i>
12	59129931:60463147	1.08	<i>RF00026</i>
14	1147948:1315998	0.97	<i>ZC3H3, MAFA, RHPN1, TOP1MT, ZNF696</i>
6	104960055:116222948	0.91	<i>PSAPL1, bta-mir-2452, AFAP1, ABLIM2, bta-mir-95, SH3TC1, HTRA3, ACOX3, TRMT44, CPZ, HMX1, ADRA2C, RF00001, LRPAP1, DOK7, HGFAC, MSANTD1, HTT, bta-mir-2451, bta-mir-2450a, bta-mir-2450b, bta-mir-2450d, GRK4, NOP14, MFSD10, ADD1, RF00026, SH3BP2, TNIP2, FAM193A, RNF4</i>
28	25778236:26552286	0.89	<i>TACR2, TSPAN15, NEUROG3, FAM241B, COL13A1, H2AFY2, AIFM2, TYSND1, SAR1A, PPA1, NPFFR1, LRRC20</i>
23	43155060:43481899	0.75	<i>GFOD1, TBC1D7, PHACTR1</i>
5	13068223:14695474	0.70	<i>RF00026, SLC6A15, LRRIQ1, TSPAN19</i>
22	50423075:51293138	0.67	<i>UBA7, INKA1, CDHR4, IP6K1, GMPPB, RNF123, AMIGO3, MST1, APEH, BSN, DAG1, NICN1, AMT, TCTA, RHOA, GPX1, RF00026, USP4, C22H3orf62, CCDC36, C22H3orf84, KLHDC8B, CCDC71, LAMB2, USP19, QARS, QRICH1, IMPDH2, NDUFAF3, MIR191, bta-mir-425, DALRD3, WDR6, P4HTM, ARIH2, SLC25A20, PRKAR2A, IP6K2, NCKIPSD, CELSR3, SLC26A6, TMEM89, UQCRC1, COL7A1</i>
19	47674203:48522690	0.66	<i>TANC2, CYB561, ACE, KCNH6, DCAF7, TACO1, MAP3K3, LIMD2, STRADA, CCDC47, DDX42, FTSJ3, PSMC5, SMARCD2, bta-mir-10173, TCAM1, GH1, CD79B, SCN4A, ERN1, RF00289, RF00407, RF00598, TEX2</i>
13	6382837:7531220	0.63	<i>SPTLC3, ISM1, TASP1, ESF1, NDUFAF5, SEL1L2</i>
8	67492932:68136948	0.62	—
20	56196377:56292004	0.61	<i>MYO10</i>
10	96631113:97342537	0.60	<i>FLRT2</i>
23	8282432:9205114	0.59	<i>HMGA1, NUDT3, RF00322, RPS10, PACSIN1, SPDEF, ILRUN, RF00001, RF00156, RF00066, SNRPC, UHRF1BP1, TAF11, ANKS1A, TCP11, SCUBE3</i>
14	26094070:26691608	0.57	<i>RAB2A, CHD7</i>
18	6592522:7571766	0.57	<i>DYNLRB2, CDYL2</i>

¹Chr = chromosome.²Starting and ending coordinates of the window.³Var = % genetic variance explained by the SNPs within the window.⁴Ensembl symbol of annotated genes using the *Bos taurus* ARS-UCD1.2 assembly (<http://www.ensembl.org/biomart/martview>).

DISCUSSION

Advances in genomics and systems biology can aid in the understanding the genomic background of clinical and subclinical ketosis. The proportions of genetic variance explained by the top-20 sliding windows identified in the GWAS confirmed the polygenic nature of these traits. The window explaining the largest proportion of the genetic variance for CK1 and CK2 was located in a region on chromosome 6 (1.69% and 2.35%, respectively), with CK2 having 4 windows on the same chromosome. This chromosome was also present in the top-20 genomic windows for SCK1 and SCK2. Parker Gaddis et al. (2018) reported that the largest proportion of the genetic variance for ketosis was associated with a region on chromosome 6 located at 56.1 Mb. In addition, Nayeri et al. (2019) reported SNPs on chromosome 6 (rs109930261, rs109793149, rs110899052) to be associated with milk BHB concentrations in dairy cattle. Regions on chromosome 6 identified in this study were consistent with the ones found by Nayeri et al.

(2019) for CK2 (56Mb and 78.7–98.6Mb) and for SCK2 (87–93Mb). These findings validate the association of chromosome 6 with clinical and subclinical ketosis previously reported in the literature. Thus, chromosome 6 harbors regions of interest for both clinical and subclinical ketosis.

Additional chromosomes with regions explaining high proportions of genetic variance for CK1 include chromosomes 2, 5, 11, and 23. Furthermore, of the top-20 windows explaining the highest genetic variance, 3 windows were present on chromosome 3, which is a region with genes known to affect the concentration of nonesterified fatty acids (**NEFA**). Regions on chromosome 20 were found to be associated with clinical and subclinical ketosis for later lactations. For CK2, 5 windows were associated with regions on chromosome 20, and SCK2 presented 3 windows on this chromosome. These regions were previously related to pathways associated with ketosis, and genes within these regions were associated with inflammatory response (Nayeri et al., 2019). Nayeri et al. (2019) reported a region

Table 4. Annotated genes within the top 20 sliding windows explaining the highest proportion of genetic variance for subclinical ketosis lactation 2 to 5

Chr ¹	Position (bp) ²	Var (%) ³	Genes ⁴
20	65741860:66319602	0.95	<i>SUPT7L</i> , <i>GPN1</i> , <i>ZNF512</i> , <i>C11H2orf16</i> , <i>FNDC4</i> , <i>IFT172</i> , <i>KRTCAP3</i> , <i>NRBP1</i> , <i>PPM1G</i> , <i>ZNF513</i> , <i>SNX17</i> , <i>EIF2B4</i> , <i>GTF3C2</i> , <i>MPV17</i> , <i>TRIM54</i> , <i>DNAJC5G</i> , <i>SLC30A3</i> , <i>CAD</i> , <i>ATRAID</i> , <i>SLC5A6</i> , <i>TCF23</i> , <i>PRR30</i> , <i>PREB</i> , <i>ABHD1</i> , <i>CGREF1</i> , <i>KHK</i> , <i>EMILIN1</i> , <i>AGBL5</i> , <i>TMEM214</i> , <i>MAPRE3</i> , <i>RF00091</i> , <i>DPYSL5</i> , <i>CENPA</i> , <i>SLC35F6</i> , <i>KCNK3</i>
11	71946048:72911805	0.89	—
25	30067534:30543167	0.85	<i>CCDC57</i> , <i>FASN</i> , <i>DUS1L</i> , <i>GPS1</i> , <i>RFNG</i> , <i>DCXR</i> , <i>RAC3</i> , <i>LRRC45</i> , <i>CENPX</i> , <i>ASPSCR1</i> , <i>bta-mir-2346</i> , <i>NOTUM</i> , <i>MYADML2</i> , <i>PYCR1</i> , <i>MAFG</i> , <i>SIRT7</i> , <i>PCYT2</i> , <i>bta-mir-2347</i> , <i>NPB</i> , <i>ANAPC11</i> , <i>ALYREF</i> , <i>ARHGDI</i> , <i>P4HB</i> , <i>PPP1R27</i> , <i>MCRIP1</i> , <i>GCGR</i> , <i>MRPL12</i> , <i>HGS</i> , <i>ARL16</i> , <i>CCDC137</i> , <i>OXLD1</i> , <i>PDE6G</i> , <i>TSPAN10</i> , <i>NPLOC4</i> , <i>FAAP100</i>
19	50660229:51233023	0.82	<i>WASHC3</i> , <i>NUP37</i> , <i>PARBP</i> , <i>PMCH</i> , <i>IGF1</i> , <i>RF00026</i>
5	65743164:66456344	0.77	<i>ST8SIA2</i> , <i>SLCO3A1</i>
21	14564126:14847447	0.74	—
9	5795323:6181422	0.73	<i>FBXW11</i> , <i>STK10</i> , <i>EFCAB9</i> , <i>UBTD2</i> , <i>SH3PXD2B</i>
20	3442790:4360799	0.69	<i>NAA15</i> , <i>NDUFC1</i> , <i>MGARP</i> , <i>ELF2</i> , <i>NOCT</i> , <i>RF00026</i>
17	18266121:18862410	0.69	<i>KCNQ3</i> , <i>HHLA1</i> , <i>EFR3A</i>
14	8996524:9624604	0.63	<i>SNORD42</i> , <i>COX18</i> , <i>ANKRD17</i> , <i>ALB</i> , <i>AFP</i> , <i>AFM</i> , <i>RASSF6</i> , <i>7SK</i> , <i>CXCL8</i>
6	87840175:88893733	0.58	<i>PDE5A</i> , <i>FABP2</i> , <i>C6H4orf3</i> , <i>USP53</i> , <i>RF00001</i> , <i>MYOZ2</i> , <i>SYNP02</i>
6	5063081:6358918	0.58	—
2	77688697:78437805	0.58	<i>ZNF629</i> , <i>BCL7C</i> , <i>CTF1</i> , <i>FBXL19</i> , <i>ORAI3</i> , <i>SETD1A</i> , <i>HSD3B7</i> , <i>STX1B</i> , <i>STX4</i> , <i>ZNF668</i> , <i>ZNF646</i> , <i>PRSS53</i> , <i>VKORC1</i> , <i>BCKDK</i> , <i>KAT8</i> , <i>PRSS8</i> , <i>PRSS36</i> , <i>FUS</i> , <i>PYCARD</i> , <i>TRIM72</i> , <i>ITGAM</i> , <i>ITGAD</i> , <i>COX6A2</i> , <i>ARMC5</i> , <i>TGFB1I1</i> , <i>SLC5A2</i> , <i>C25H16orf58</i> , <i>AHSP</i> , <i>SEPT14</i> , <i>ZNF713</i> , <i>MRPS17</i> , <i>NIPSNAP2</i> , <i>PSPH</i> , <i>CCT6A</i> , <i>RF00414</i> , <i>RF00398</i> , <i>SUMF2</i> , <i>PHKG1</i> , <i>CHCHD2</i> , <i>NUPR2</i> , <i>VKORC1L1</i> , <i>GUSB</i>
25	26903532:27969929	0.56	<i>TTC39B</i> , <i>RF00001</i> , <i>FREM1</i> , <i>CER1</i> , <i>ZDHHC21</i> , <i>NFIB</i>
8	29018683:29865913	0.56	<i>BAHCC1</i> , <i>RF00100</i> , <i>SLC38A10</i> , <i>NDUFAF8</i> , <i>TEPSIN</i> , <i>CEP131</i> , <i>AATK</i> , <i>bta-mir-338</i> , <i>BAIAP2</i> , <i>CHMP6</i> , <i>RPTOR</i> , <i>RF00003</i>
19	51263493:52029384	0.55	<i>OTOF</i> , <i>DRC1</i> , <i>SELENOI</i> , <i>bta-mir-2284y-1</i> , <i>DGRF3</i> , <i>HADHB</i> , <i>HADHA</i>
11	73053087:73269991	0.54	<i>MRPL32</i> , <i>PSMA2</i> , <i>C4H7orf25</i> , <i>GLI3</i>
4	77978159:79139483	0.54	<i>NDUFS4</i> , <i>FST</i> , <i>MOC52</i> , <i>ITGA2</i> , <i>ITGA1</i> , <i>PELO</i>
20	25229625:26352669	0.52	<i>NRCAM</i> , <i>PNPLA8</i> , <i>NME8</i> , <i>SFRP4</i> , <i>EPDR1</i>
4	49216223:49813182	0.519	<i>SUPT7L</i> , <i>GPN1</i> , <i>ZNF512</i> , <i>C11H2orf16</i> , <i>FNDC4</i> , <i>IFT172</i> , <i>KRTCAP3</i> , <i>NRBP1</i> , <i>PPM1G</i> , <i>ZNF513</i> , <i>SNX17</i> , <i>EIF2B4</i> , <i>GTF3C2</i> , <i>MPV17</i> , <i>TRIM54</i> , <i>DNAJC5G</i> , <i>SLC30A3</i> , <i>CAD</i> , <i>ATRAID</i> , <i>SLC5A6</i> , <i>TCF23</i> , <i>PRR30</i> , <i>PREB</i> , <i>ABHD1</i> , <i>CGREF1</i> , <i>KHK</i> , <i>EMILIN1</i> , <i>AGBL5</i> , <i>TMEM214</i> , <i>MAPRE3</i> , <i>RF00091</i> , <i>DPYSL5</i> , <i>CENPA</i> , <i>SLC35F6</i> , <i>KCNK3</i>

¹Chr = chromosome.²Starting and ending coordinates of the window.³Var = % genetic variance explained by the SNPs within the window.⁴Ensembl symbol of annotated genes using the *Bos taurus* ARS-UCD1.2 assembly (<http://www.ensembl.org/biomart/martview>).

on chromosome 20 (55–63 Mb) for SCK2 for the first time. For SCK1, the window that explains the highest genetic variance is located on chromosome 2, which has been previously associated with genes related to insulin resistance (Clempson et al., 2012). Many of the genes located within the regions studied here pertain to at least one common function group, and they will be discussed below. For instance, several genes were involved in insulin metabolism, lipid metabolism, inflammatory response, and immune system.

Clinical Ketosis in First Lactation (CK1)

An important gene associated with CK1 was *ACAT2*, which encodes the enzyme responsible for fatty acid β -oxidation, lipid metabolism, and synthesis and degradation of ketone bodies (Puchalska and Crawford, 2017). The expression of this gene was reported to be lowered in the liver tissues of cows with ketosis, sug-

gesting impaired liver function in utilization of fatty acids (Xu et al., 2008). Thus, reduced concentrations of *ACAT2* may be caused by a negative feedback from elevated concentrations of ketone bodies in ketotic cows. The *ACAT2* was found to be related to *IGF1*, providing evidence that ketone bodies and insulin resistance can be related (Farrés et al., 2010).

The gene *PLB1* is present on chromosome 11 and it is related to lipid digestion and absorption. This gene encodes the membrane-associated phospholipase B1, which cleaves fatty acid residues from the phospholipids (Okada et al., 2014). The gene *ADCY8* mediates the effects of GLP1 and plays an important role in insulin secretion (Delmeire et al., 2003). It is an enzyme required for the activation of glucose-induced signaling pathways in β -cells and for glucose tolerance via regulation of islet insulin secretion. Thus, the reduction of *ADCY8* is related to damaged secretory capacity of β -cells in diabetes (Raoux et al., 2015).

Clinical Ketosis in Later Lactations (CK2)

The main function of apolipoproteins is the transport of lipids throughout the body and in the regulation of energy metabolism, and the genes *APOA1*, *APOA4*, and *APOA5* were found in regions associated with CK2. An association with ketosis and other metabolic diseases has been established to decreased serum concentrations of *APOA1* and other apolipoproteins (İleri-Büyükoğlu et al., 2009). Although *APOA1* is a component of high-density lipoprotein, Kroezen et al. (2018) detected a SNP within the gene *APOA1* that is associated with ketosis and established a relationship between this apolipoprotein and lipid homeostasis. In addition, this gene has been associated with cholesterol metabolism, a pathway that should be further studied when considering the pathogenesis of ketosis.

Along with *APOA1* and *APOA5*, *APOC3* and *FABP2* are also involved in the pathway of regulation of lipid metabolism by PPAR α . Furthermore, *FABP2* is also involved in fatty acid metabolism (Belaguli et al., 2007). The *FABP2* interacts with metabolic enzymes that affect fatty acid utilization (Glatz et al., 1997).

The genes *PYCR1*, *ASNS*, and *PAH* are involved in the biosynthesis of AA. The *PYCR1* gene is a key gene in proline biosynthesis that catalyzes the NADH-dependent conversion of pyrroline-5-carboxylate (P5C) to L-proline (Zeng et al., 2017). This gene is involved in intracellular AA metabolism and it was found to be upregulated during lactation (Dai et al., 2018). The *ASNS* gene catalyzes the transfer of an amino group to aspartic acid to form Asn, which can then be converted

to oxaloacetate, a rate-limiting compound in gluconeogenesis (Richards and Kilberg, 2006). The *PAH* gene is mainly expressed in the liver and kidney and codes for the phenylalanine hydroxylase enzyme (Lichter-Konecki et al., 1999). The concentration of Tyr was found to be lower in the blood plasma of dairy cows with clinical ketosis compared with subclinical ketosis (Wang et al., 2016).

Genes related to the immune system located in the top 20 genomic regions associated with CK2 included *CXCR5* and *ADCY2*. A study evaluating the immune system in neonatal calves showed that *ACTG1* was expressed and categorized as antimicrobial (He et al., 2018). The expression of *CXCR5* was affected during parasitic infection in resistant cattle (Li et al., 2011). The *ADCY2* is essential in accelerating phosphor-acidification and the synthesis and breakdown of glycogen (Rexroad et al., 2001). The *ADCY2* has been associated with immune response and inflammatory diseases (Walker et al., 2012).

Subclinical Ketosis in First Lactation (SCK1)

The gene *SLC26A6*, also known as putative anion transporter-1 (PAT-1), codes for a protein involved in transporting chloride, oxalate, sulfate and bicarbonate. It is associated with diabetic ketoacidosis, which is marked by the triad of the uncontrolled hyperglycemia, acidosis, and ketosis. Also, this gene is related to a phenotype of increased resistance toward insulin (Zhang et al., 2019).

Figure 1. Genetic variance explained by 20-SNP sliding windows for clinical ketosis lactation 1. Chromosome number is shown on the horizontal axis.

Figure 2. Genetic variance explained by 20-SNP sliding windows for clinical ketosis lactation 2 to 5. Chromosome number is shown on the horizontal axis.

Genes involved in fatty acid synthesis and degradation were identified in SCK1, namely *ACOX3* and *SLC25A20*. The *ACOX3* gene is involved in fatty acid oxidation and the degradation of the branched-chain fatty acids. Genes encoding enzymes involved in fatty acid oxidation are relevant because they could reduce the influx of NEFA (Schäff et al., 2013). The *SLC25A20* gene encodes the CACT protein. Defective CACT results in decreased carnitine-acylcarnitine transport and impaired fatty acid β -oxidation. The clinical effects of such changes may include hypoglycemia, hyperammonaemia, cardiomyopathy, hepatic failure, and encephalopathy (Palmieri, 2014). Wu et al. (2020) indicated *SLC25A20* had higher relative expression level when comparing ketotic cows pre and postpartum.

Subclinical Ketosis in Later Lactations (SCK2)

The gene *RASSF6* induces apoptosis and has enhanced ability to kill cells when Ras is activated, and it also controls the degree of inflammatory response (Allen et al., 2007). Nayeri et al. (2019) identified the *RASSF6* gene on chromosome 6 as associated with subclinical ketosis for later lactations in Canadian Holstein dairy cattle, corroborating the results of this study.

The gene *CXCL8* is involved in inflammatory response and mediates neutrophil attraction and activation acting in the site of infection (Harada et al., 1994). In addition, Nayeri et al. (2019) identified a highly significant SNP (rs109793149) on this gene on BTA6 at around 90.5 Mb for SCK1. The result of this

study agrees with previous studies reporting important interactions between metabolic disease and immune response in dairy cow (Wathes et al., 2009).

The genes *HADHA* and *HADHB* are involved in fatty acid synthesis and degradation. The *HADHA* encodes the α subunit, and the gene *HADHB* encodes the β subunit of the mitochondrial trifunctional protein, which catalyzes the last 3 steps of mitochondrial β -oxidation of long-chain fatty acids (Puchalska and Crawford, 2017). The NEFA can be either esterified or oxidized in mitochondria and peroxisomes in the liver to produce acetyl-CoA and the mobilization of fat from body stores builds the transition of NEFA into the liver (Drackley et al., 2005). Thus, genes involved in mitochondrial fatty acid oxidation could affect the influx of NEFA into the liver (Schäff et al., 2013).

Overlapping Regions and Genes Among the Traits

For the first lactation, 2 genes (*SLIT2* and *PBX1*) were identified in both clinical and subclinical ketosis (CK1 and SCK1; Supplemental Figure S5, <http://doi.org/10.5281/zenodo.4651578>; Soares et al., 2021b), and 42 genes were found to be in common between clinical and subclinical ketosis for later lactations (CK2 and SCK2; Supplemental Figure S6, <http://doi.org/10.5281/zenodo.4651578>; Soares et al., 2021b). The overlap of regions and genes is expected due to the existence of genetic correlation between subclinical ketosis (BHB) and clinical ketosis (e.g., $r = 0.48$; Koeck et al., 2014). The genes found in common between these 2

traits stand out for being identified as having a biological significance or being expressed in target tissues for ketosis, as they are related to insulin resistance and lipid metabolism.

Insulin is a key metabolic hormone (Herdt, 2000) and an indicator of energy status (Blum et al., 1983). Insulin acts in a feedback loop where NEFA and ketone bodies stimulate insulin secretion, and insulin then acts to suppress the formation of more ketone bodies (Herdt, 2000). Early lactating dairy cows typically have reduced blood insulin levels to support lactation. Healthy NEB-related diseases such as ketosis have been linked to an insulin-resistant condition in near-parturition dairy cows (Gross et al., 2011).

We found *IGF1* to be associated with clinical and subclinical ketosis for later lactations (CK2 and SCK2). The *IGF1* concentration has been proposed as an early biomarker of ketosis (Piechotta et al., 2015). This gene is expressed in many organs of the body and can have an influence on proliferation, differentiation, and metabolic activities. It has been reported that expression of *IGF1* has a positive effect on ovarian estradiol production (Jorritsma et al., 2003).

The synthesis and secretion of lipids are essential steps in milk-fat formation. This complex biological process is controlled in part by gene expression (Bionaz et al., 2012). The multifunctional protein FASN catalyzes long-chain fatty acid synthesis and plays a central role in mammalian de novo lipogenesis (Matsumoto et al., 2012). Lipogenesis is typically decreased around parturition, in part due to low plasma insulin concen-

trations, and thus there is lower mRNA abundance for *FASN* in the liver (Graber et al., 2010). However, Sun et al. (2019) showed that the amount of mRNA for *FASN* was higher in cows with clinical ketosis than in healthy cows. Zhu et al. (2019) showed that cows with clinical ketosis had increased gene expression of *FASN* than cows with subclinical ketosis; this can be justified by elevated fatty acid β -oxidation in cows with subclinical ketosis, which provides them with a better way of addressing fatty acid overload without the need to promote lipid synthesis. In addition, the bovine *FASN* gene is considered a candidate gene for a milk-fat content QTL (Roy et al., 2006).

For CK2 and SCK2, the *PCYT2* gene is the main regulatory enzyme in biosynthesis of phosphatidylethanolamine (the most abundant lipid on the cytoplasmic layer of cellular membranes) from ethanolamine and diacylglycerol by the CDP-ethanolamine Kennedy pathway (Gibellini and Smith, 2010). The major physiological consequences of deletion of one *PCYT2* allele include development of symptoms of the metabolic syndrome such as elevated lipogenesis and lipoprotein secretion, liver steatosis, and insulin resistance (Pavlovic and Bakovic, 2013).

Functional Annotation

For further functional annotation, enrichment analysis of the list of candidate genes for clinical and subclinical ketosis was performed. The results showed molecular functions and biological processes that are

Figure 3. Genetic variance explained by 20-SNP sliding windows for subclinical ketosis lactation 1. Chromosome number is shown on the horizontal axis.

Figure 4. Genetic variance explained by 20-SNP windows for subclinical ketosis lactation 2 to 5. Chromosome number is shown on the horizontal axis.

associated with fatty acid metabolism, synthesis and degradation of ketone bodies, lipid metabolism, and inflammatory response in dairy cattle.

The GO enrichment analysis performed in this study revealed that different annotated genes are enriched in many GO terms. From these significant GO terms, several terms including GO:0008152 (metabolic process), GO:0044238 (primary metabolic process), GO:0043170 (macromolecule metabolic process), and GO:0009893 (positive regulation of metabolic process) were related to metabolic processes.

The gene-gene network analysis was very effective in the investigation of shared biological processes among the candidate genes. The most informative networks for this study were the networks grouped around the genes *IL10* for CK1, *APOA1* for CK2, *RPS10* for SCK1, and *SUMF2* for SCK2. The gene *IL10* is an anti-inflammatory cytokine that regulates the immune system by preventing inflammatory pathology (Couper et al., 2008). Conditions such as decreased milk yield and lack of appetite in ketotic cows in early lactation might be related to the presence of a systemic inflammation, which is usually associated with greater pro-inflammatory cytokines levels in dairy cows (Zhang et al., 2016). The failure to successfully overcome the transition period is associated with an inflammatory state in cows that might contribute to development of ketosis and insulin resistance, altering normal liver function (Loor et al., 2007). Heiser et al. (2015) showed that *IL10* expression increased during the first week after calving.

Metabolic pathways associated with the genomic regions for clinical and subclinical ketosis were investigated. An important metabolic pathway is pyruvate metabolism, which is associated with the synthesis of formate. Formate production can be altered by formic acid levels, and, as a substitute, pyruvic acid can be transformed into acetyl-CoA. This conversion results in increased levels of ketone bodies in the blood, as seen in a clinically ketotic dairy cows (Wang et al., 2016). The *ACAT2* gene was present in the pyruvate metabolism pathway and several other significant pathways reported in this study for CK1 and SCK1, highlighting its possible association with clinical and subclinical ketosis for first lactation.

CONCLUSIONS

This study reported genomic regions associated with both clinical ketosis and subclinical ketosis and indicated potential candidate genes associated with them. Chromosome 6 and chromosome 20 were of interest for all 4 traits and are known to harbor important genes related to ketosis, and genes within these regions were associated with inflammatory response, respectively. Important biological pathways involved in fatty acid metabolism, lipid metabolism, and inflammatory response in dairy cattle were found. Several genomic regions and SNPs related to susceptibility to ketosis in dairy cattle that were previously described in the literature were confirmed. The findings of this study enhance

the genetic understanding of clinical and subclinical ketosis in dairy cows, which could improve selection decisions in the future. However, further studies are necessary to confirm the role of newly identified SNPs and genes in this study in the susceptibility to ketosis.

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