Association between the *GHR*, *GHRHR*, and *IGF1* gene polymorphisms and milk yield and quality traits in Sarda sheep

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ABSTRACT

The growth hormone receptor (*GHR*), the growth hormone releasing hormone receptor (*GHRHR*), and the insulin-like growth factor 1 (*IGF1*) genes are known to modulate growth, reproduction, and lactation traits in livestock. The aim of the current work was to investigate if the variation of the sheep *GHR*, *GHRHR*, and *IGF1* genes is associated with milk yield and quality traits. Three hundred eighty dairy Sarda sheep were genotyped for 36 single nucleotide polymorphisms (SNP) mapping to these 3 loci, and records for milk yield and daily fat and protein yield, as well as for fat, protein, casein, lactose, and milk urea contents, pH, somatic cell score, logarithmic bacterial count, and milk energy were obtained. The linkage disequilibrium analysis was performed only for *GHR*, as both *GHRHR* and *IGF1* had only 1 polymorphic SNP. Haplotype analysis revealed the existence of 7 haplotype blocks in *GHR*. Two haplotype blocks, including part of the intron 1 and the upstream region, were clearly separated from the remaining 5 blocks by SNP rs412986330, which may be a recombination hotspot. The latter 5 blocks were contiguous, spanning from intron 2 to exon 10. Statistical analysis revealed that the *GHR* polymorphism is significantly associated with milk traits for daily fat and protein yield and fat, milk urea, and lactose content. Moreover, variation in *IGF1* was associated with milk protein and casein content. Data generated in this research provide new insights into the allelic effects of the ovine *GHRHR*, *GHR*, and *IGF1* genes on milk production and quality traits, information that may be useful in gene-assisted selection programs.

Key words: growth hormone receptor, single nucleotide polymorphism, haplotypes, association analysis, sheep milk

INTRODUCTION

In the Mediterranean basin, sheep milk contributes appreciably to the production of many different cheese varieties that differ in texture, color and flavor. With more than 3 million Sarda sheep, Sardinia is one of the main ovine milk providers in southern Europe (Bittante et al., 2017).

The economic importance of sheep milk production has led to investigations of which genomic regions influence ovine growth and dairy traits (Chessa et al., 2017). Three molecules integrated in the somatotrophic axis and linked by a complex regulation (Akers, 2002) could have a major role in milk production: growth hormone receptor (*GHR*), growth hormone-releasing hormone receptor (*GHRHR*), and IGF-1.

The *GHR* is a regulator of postnatal growth and, as the growth hormone, it has important effects on carbohydrate, protein, and lipid metabolism. The *GHR* is a type I cytokine receptor that forms part of the Janus kinase (JAK)/ signal transducers and activators of transcription (STAT) signaling pathways, and it has an extracellular and an intracellular domain. In humans, it is organized as a constitutive dimer, being activated by the binding of the growth hormone monomer (Brooks et al., 2008). The sheep growth hormone receptor is encoded by the *GHR* gene, which maps to Oar16:31.8–32.0 Mb (ovine reference genome Oar_v3.1; https://www.ncbi.nlm.nih.gov/assembly/GCF_000298735.1/) and spans 167.5 kb. The *GHR* gene includes 10 exons, and encodes a transmembrane receptor protein of 651 AA residues. In cattle, mutations in *GHR* have been associated with growth and meat quality traits (Di Stasio et al., 2005). A missense variant (F279Y) located in exon 8 of the bovine *GHR* gene has been associated with milk yield and composition in Ayrshire, Holstein, and Jersey cattle (Blott et al., 2003) as well as with protein percentage and fat percentage (Viitala et al., 2006) and milk yield and BCS (Banos et al., 2008). Pegolo et al. (2016) demonstrated that the *GHR* polymorphism is associated with milk fatty acid composition in dairy cows. In goats, a variable thymine-guanine

Received April 12, 2018.

Accepted July 1, 2018.

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repeat in *GHR* did not display significant associations with dairy traits such as milk yield or composition and SCC (Maj et al., 2007). In addition, the variation of the ovine *GHR* gene was not associated with milk traits in Churra sheep (García-Fernández et al., 2011). By using a genome-wide association study methodology to search for candidate regions targeted by selection, Wang et al. (2015) identified *GHR* as a growth-related gene, potentially affecting meat production and quality, as well as reproduction traits, in the German Mutton Merino sheep breed. Bolormaa et al. (2016) included the ovine *GHR* gene in a group of loci with pleiotropic effects on body composition in meat breeds, but little is known about the effect of *GHR* polymorphisms on sheep dairy traits.

The GHRHR protein is a G-protein-coupled receptor with 7 transmembrane domains and it is expressed specifically in somatotropic cells. The activation of GHRHR in response to growth hormone-releasing hormone binding leads to the release of growth hormone stored in secretory granules and stimulates de novo GH production in somatotropes (Pang and Chan, 2010). The sheep *GHRHR* gene maps to chromosome Oar4: 65.35–65.36 Mb (Oar_v3.1) and includes 14 exons encoding a 426-AA long protein.

The IGF-1 polypeptide hormone is the major mediator of prenatal and postnatal growth (Baker et al., 1993). It is produced in many tissues, but mainly in the liver in response to growth hormone stimulation, and it behaves as an endocrine, paracrine, and autocrine hormone in peripheral tissues (Pang and Chan, 2010). The sheep *IGF1* gene maps to chromosome 3:171,26–171,32 Mb (reverse strand); it spans 59.3 kb and it encompasses 5 exons encoding a 172-AA long protein.

Association studies concerning the association of the polymorphism of these 3 genes with ovine dairy traits are scarce, especially with regard to the Sarda breed, which has a great economic value due to its excellent dairy performance. The main goal of our study was to investigate if the polymorphism of the ovine *GHR*, *GHRHR*, and *IGF1* genes are associated with milk traits in Sarda sheep.

MATERIALS AND METHODS

Animals and Sampling

The resource population included 380 ewes sampled from 19 different farms (20 ewes/farm) located in Sardinia (Italy). All the ewes were officially registered in the Sarda breed flock book and they were included in the selection scheme of the Sarda breed. Farms, animals, and sampling have been reported in Pazzola et al. (2014) and Vacca et al. (2015). Briefly, ewe parity ranged from

1 (primiparous) to 9, and ewes were sampled between 2 and 7 mo after parturition. Each of the 380 ewes was sampled just 1 time (single-day sampling) to obtain an individual milk sample per ewe at each farm. Samples were collected during the afternoon milking in 200-mL sterile plastic containers. Daily milk yield was recorded as the morning plus evening milking. A blood sample was taken from each ewe in K₃EDTA vacuum tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Genomic DNA was isolated with the Gentra Pure-gene Blood Kit (Qiagen, Hilden, Germany) and purity and concentration were measured with an Eppendorf BioPhotometer instrument (Eppendorf, Hamburg, Germany). Once collected, milk samples were kept at 4°C and analyzed within 24 h. Milk fat, protein, casein, lactose, and milk urea (MU) content and pH were measured with a MilkoScan FT6000 device (Foss Electric, Hillerød, Denmark), according to the International Dairy Federation (IDF) standard 141:2013 (ISO-IDF, 2013); daily fat and protein yield (dFPY; measured in g/d) was calculated as the sum of fat and protein content in relation to daily milk yield. Total bacterial count (TBC) was determined with a BactoScan FC150 instrument (Foss Electric) and transformed to log-bacterial count to normalize the distribution of the measurements [$\log\text{-bacterial count} = \log_{10}(\text{total bacterial count}/1,000)$]. Somatic cell count was determined with a Fossomatic 5000 equipment (Foss Electric) according to the IDF 148-2:2006 method (ISO-IDF, 2006) and then transformed into the logarithm score [$\text{SCS} = \log_2(\text{SCC} \times 10^{-5}) + 3$].

Design of a TaqMan Real-Time PCR Assay

A custom TaqMan Real-Time PCR assay (Thermo Fisher Scientific, Waltham, MA) was designed for SNP genotyping. It included a total of 31 SNP mapping to the sheep *GHR* gene. The *GHR* SNP were retrieved from the Ensembl Genome database (http://www.ensembl.org/Ovis_aries/Gene/Variation_Table?db=core;g=ENSOARG00000008837;r=16:31829583-32003795;t=ENSOART00000009622). Moreover, 2 and 3 SNP mapping to the *GHRHR* and *IGF1*, respectively, were retrieved from the dbSNP database (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) and genotyped in the 380 Sarda sheep. Sequences flanking the SNP are given in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-14914>) and the full panel of 36 SNP is reported in Table 1. Each SNP plus 60-bp flanking sequences were submitted to the Custom TaqMan Assay Design Tool web page (<https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/>) to design an assay matching the producer's criteria. Genotyping was carried out in the Servei Veterinari de

Table 1. Description of the markers mapping to the *GHR*, *GHRHR* and *IGF1* genes and associated population parameters in Sarda sheep

Gene and SNP ID ¹	Chr position ²	Gene region	ObsHET ³	PredHET ⁴	HWpval ⁵	%Geno ⁶	MAF ⁷	Alleles ⁷
<i>GHR</i>								
rs161146162	16:31834322	Downstream	0.005	0.005	1.0	97.6	0.003	C:(G)
rs408890407	16:31834412	Exon 10 (synonymous)	0.299	0.334	0.07	94.1	0.212	C:(T)
rs161146164	16:31834495	Exon 10 (missense)	0.003	0.003	1.0	98.1	0.001	T:(G)
rs55631463	16:31834666	Exon 10 (missense)	0.394	0.396	1.0	96.5	0.272	T:(C)
rs413776054	16:31834942	Exon 10 (missense)	0.003	0.003	1.0	98.4	0.001	G:(A)
rs406641730	16:31840531	Intron 9	0.0	0.0	1.0	97.6	0.000	A:(A)
rs405063669	16:31843317	Intron 8	0.414	0.363	0.01	93.8	0.239	G:(A)
rs411154235	16:31851952	Intron 6	0.307	0.328	0.30	96.8	0.206	G:(A)
rs404583153	16:31852174	Intron 6	0.378	0.424	0.05	97.1	0.305	G:(T)
rs162153483	16:31856692	Intron 5	0.52	0.492	0.34	99.5	0.438	T:(G)
rs406893455	16:31856942	Intron 5	0.534	0.5	0.23	98.4	0.485	T:(G)
rs161146229	16:31857882	Intron 5	0.369	0.366	1.0	99.5	0.241	T:(C)
rs161146242	16:31858236	Intron 5	0.515	0.5	0.67	90.1	0.487	A:(G)
rs407871250	16:31868298	Intron 5	0.534	0.492	0.14	93.3	0.437	C:(T)
rs404237321	16:31860904	Exon 5 (missense)	0.014	0.014	1.0	97.9	0.007	C:(T)
rs415419991	16:31861096	Intron 4	0.374	0.374	1.0	99.7	0.249	G:(A)
rs409713530	16:31861705	Intron 4	0.523	0.492	0.29	100	0.438	C:(T)
rs425402906	16:31868809	Intron 4	0.52	0.493	0.36	100	0.442	G:(A)
rs161146298	16:31868958	Exon 4 (synonymous)	0.525	0.49	0.22	96.5	0.429	G:(A)
rs426666828	16:31882869	Intron 3	0.501	0.484	0.57	99.5	0.410	(T):C
rs430067568	16:31898013	Intron 3	0.516	0.497	0.54	99.7	0.462	A:(G)
rs412881843	16:31917480	Intron 3	0.421	0.403	0.49	100	0.280	C:(G)
rs400713333	16:31919259	Intron 2	0.493	0.479	0.66	97.9	0.397	(C):T
rs417896686	16:31996009	Intron 2	0.278	0.299	0.24	97.3	0.183	C:(G)
rs426539270	16:31996860	Intron 1	0.283	0.298	0.42	99.5	0.182	C:(T)
rs412986330	16:31997185	Intron 1	0.184	0.189	0.77	98.9	0.106	C:(T)
rs399882480	16:32001790	Intron 1	0.336	0.34	0.91	98.1	0.217	A:(G)
rs417647459	16:32002309	Upstream	0.31	0.34	0.12	98.7	0.217	T:(C)
rs428862267	16:32002622	Upstream	0.467	0.495	0.32	92.5	0.451	(G):A
rs402337124	16:32004307	Upstream	0.327	0.336	0.69	96.0	0.214	A:(G)
rs425834583	16:32004844	Upstream	0.0	0.0	1.0	100	0.000	A:A
<i>GHRHR</i>								
rs409504706	4:65329776	Intron	0.301	0.314	0.51	96.0	0.195	T:(G)
rs161797246	4:65363733	Intron	0.0	0.0	1.0	94.1	0.000	C:C
<i>IGF1</i>								
rs159876390	3:171055347	Synonymous	0.342	0.476	0.001	83.5	0.391	G:(A)
rs159876394	3:171108350	Missense	0.0	0.0	1.0	99.7	0.000	C:C
rs401028781	3:171113596	Upstream	0.0	0.0	1.0	98.9	0.000	G:G

¹SNP ID = dbSNP reference records (<https://www.ncbi.nlm.nih.gov/projects/SNP/>).²Chr position = chromosome position on Oar_v4.0 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000298735.2/).³ObsHET = observed heterozygosity.⁴PredHET = predicted heterozygosity.⁵HWpval = Hardy-Weinberg test *P*-value.⁶%Geno = percentage of genotyped samples.⁷MAF = minor allele frequency (minor allele in brackets).

Genetica Molecular from the Universitat Autònoma de Barcelona (<http://sct.uab.cat/svgm/en>) by using a 12K Flex QuantStudio instrument (Thermo Fisher Scientific). Genotypes were visualized with the Taqman Genotyper v.1.3 software (Applied Biosystems, Foster City, CA), and SNP with call rates <0.9 were removed from the data set.

Haplotype and Statistical Analyses

The Haploview software package (Barrett et al., 2005) was used to estimate and plot pairwise linkage

disequilibrium (**LD**) measures [normalized coefficient of linkage disequilibrium (**D'**) and squared correlation (r^2)]. The same tool was used to infer haplotype frequencies and to define LD blocks according to the Gabriel criteria (Gabriel et al., 2002). Haplotypes can be defined as specific combinations of alleles in a genomic DNA region. On the other hand, a haplotype block is a DNA region where no recombination has occurred in the ancestors of that population. Gabriel et al. (2002) identified haplotype blocks by examining the recombination patterns across each region on the basis of information provided by markers with minor

allele frequency >1%. This measure is based on the value of the allelic association D' normalized (Daly et al., 2001). As Gabriel et al. (2002) rely on confidence bounds on D' , the one-sided upper 95% confidence bound for strong LD was set to >0.98 (indicating no historical recombination). In contrast, a pair of markers was considered strong evidence of historical recombination when the upper confidence bound on D' was less than 0.9 (Gabriel et al., 2002). Haploview was also used to estimate minor allele frequencies (MAF), observed and expected heterozygosity, and to identify significant departures from the Hardy-Weinberg equilibrium at each polymorphic locus.

Association analysis between *GHR*, *GHRHR*, and *IGF1* genotypes and milk traits was based on the model

$$Y = \mu + G + F + P + \text{DIM} \\ + \text{SIRE}(G) + e_{ijklmn}, \quad [1]$$

where Y is the observed trait; μ is the general mean; G is the fixed effect of the SNP genotype, one at a time (2 to 3 levels: the 2 homozygotes and the heterozygote); F is the fixed effect of the farm, which also includes animal management and feeding (1 to 19 levels; the different farms where animals were reared); P is the fixed effect of parity of the ewes (1 to 4 levels; first to fourth or more parities); DIM is the fixed effect of the days in milking (4 levels; 1 = ≤ 100 d; 2 = 101–140 d; 3 = 141–160 d; 4 = ≥ 161 d); $\text{SIRE}(G)$ is the random effect of the sire (101 different sires) nested within the genotype; and e_{ijklmn} is the random residual effect.

The same model expressed in equation [1] was used to investigate the association between milk traits and each of the 7 LD blocks, one at a time. In the single SNP and LD block analysis we only considered SNP with a MAF >0.050 to make sure that genotypic means were correctly estimated. The MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) was used to carry out the association analysis and correction for multiple testing was implemented with the Bonferroni method (one milk trait for each SNP or LD block at a time). Model effects were significant at $P < 0.05$.

RESULTS

Allele Frequencies of the *GHR*, *GHRHR*, and *IGF1* Polymorphisms in Sarda Sheep

The description of the 36 analyzed SNP and the associated population parameters is provided in Table 1. The ovine *GHR* gene is about 167 kbp long and 98.8% of this gene is made of intronic regions. Many variants

have been reported for this gene, out of which 3,038 are intronic variants, 14 missense and 9 synonymous (Ensembl, <https://www.ensembl.org/index.html>). Among these variants, we chose a panel of 31 SNP spanning the entire gene sequence and genotyped 380 dairy Sarda breed ewes. A total of 6 exonic *GHR* SNP were analyzed and rs161146164 and rs413776054 (both missense variants located in exon 10) and rs404237321 (a missense variant localized in exon 5) markers showed MAF lower than 5%, whereas rs408890407 (exon 10, synonymous), rs55631463 (exon 10, missense), and rs161146298 (exon 4, synonymous) showed MAF higher than 5%. According to the SIFT (<http://sift.jcvi.org/>) prediction algorithm, none of the variants were expected to affect protein function. All intronic SNP were polymorphic in the analyzed population and showed MAF higher than 5%, except rs406641730 (intron 9), which was monomorphic. The only downstream variant analyzed in *GHR* was rare (MAF = 0.003), and 1 of the 4 upstream variants was monomorphic (rs425834583). For *GHRHR*, 1 of the 2 genotyped SNP was monomorphic, and in *IGF1* 2 of the 3 SNP analyzed were monomorphic. Genotype distributions were in Hardy-Weinberg equilibrium, except for rs405063669 (intron 8, *GHR*) and rs159876390 (*IGF1*).

Effect of *GHR*, *GHRHR*, and *IGF1* Genotypes on Dairy Traits

Descriptive statistics of daily milk yield and quality traits recorded in the Sarda sheep are displayed in Table 2. Phenotypes showing the highest variability were daily milk yield and dFPY, with coefficients of variation (CV) above 50%. The CV of MU (35%), LBC (36%), and SCS (46%) were also considerably high. The results of the ANOVA (F -values) of daily milk yield and quality traits versus *GHR*, *GHRHR*, and *IGF1* genotypes are reported in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-14914>). Markers displaying significant effects on phenotype variance are described in detail in Supplemental Table S3 (<https://doi.org/10.3168/jds.2018-14914>).

In *GHR*, the rs55631463 SNP marker was associated with dFPY ($P < 0.05$) and homozygote CC ewes displayed the highest genotypic mean (227.4 g/d, with SE of 11.07). Moreover, the rs404583153 SNP (intron 6) was significantly associated with SCS ($P < 0.05$). In the upstream region of *GHR*, the rs417647459 marker was significantly associated with lactose content ($P < 0.05$), and homozygote CC ewes showed the highest genotypic mean (4.94%, with SE of 0.06). The rs399882480, rs428862267 ($P < 0.05$), and 402337124 ($P < 0.01$) SNP were associated with MU. With regard to *IGF1*,

Table 2. Descriptive statistics for milk daily yield and quality traits in Sarda sheep

Trait	N	Mean	SD	CV (%)	Percentile	
					P1	P99
Daily yield traits, g/d						
Milk yield (dMY)	376	1641.3	899.2	55	200.0	4176.0
Fat and protein yield (dFPY)	376	192.8	102.9	53	23.6	452.0
Milk quality traits						
Fat, %	376	6.50	1.23	19	4.13	10.41
Protein, %	376	5.46	0.72	13	4.29	7.50
Casein, %	376	4.27	0.61	14	3.25	5.96
Lactose, %	376	4.81	0.28	6	4.03	5.36
Milk urea, mg/dL	375	32.9	11.4	35	10.70	59.90
pH	316	6.66	0.09	1	6.50	6.86
SCS ¹	376	4.72	2.18	46	0.75	10.31
LBC ²	355	2.54	0.93	36	0.78	4.23
Milk energy, MJ/kg	376	4.63	0.58	13	3.56	6.34

¹SCS = $\log_2 (\text{SCC} \times 10^{-5}) + 3$.

²Logarithmic bacterial count (LBC) = $\log_{10} (\text{total bacterial count}/1,000)$.

the rs159876390 SNP was associated with milk protein and casein contents ($P < 0.05$), and homozygote AA ewes displayed the highest genotypic means for protein (5.76%, with SE of 0.12) and casein (4.51%, with SE of 0.10).

LD Analysis and Haplotype Effect

The results of LD analysis made with the Haploview software are shown in Figures 1 and 2. Linkage disequilibrium analysis was performed only for *GHR*, as both *GHRHR* and *IGF1* loci had only 1 segregating SNP. To determine the haplotype structure of the sheep *GHR* gene, SNP rs406641730 and rs425834583 were excluded because they were monomorphic. The resulting set of 29 SNP covered about 169.9 kb and the average distance between SNP was 6.07 kb, ranging from 62 to 76.7 kb. Seven regions of LD were identified in *GHR*, the larger (block 4) covered 61 kb, spanning introns 2 to 5, followed by block 1 (the second in size), which covered 8 kb from intron 8 to exon 10. Haplotype block 2 included 2 SNP, both localized on intron 6, only 67 bp away from exon 7. The 3 SNP included in haplotype block 3 were all localized in intron 5, 106 bp upstream of exon 6. Two different haplotype blocks were identified in the upstream region of *GHR*: block 7, including 2 SNP, and block 6, spanning from intron 1 to the upstream region. Haplotype blocks 6 and 7 were adjacent to each other and clearly separated from the remaining 5 blocks by SNP rs412986330. This SNP, which is located at intron 1, physically close to block 6, might be a recombination hotspot. Haplotype block 5 had 2 SNP positioned upstream (rs426539270, intron 1) and downstream (rs417896686, intron 2) of exon 2, forming a haplotype block including exon 2.

Block 4, the largest in size, consisted of 10 SNP. It had 7 possible haplotype combinations, and it could be captured by 6 different haplotype tagging SNP (**htSNP**). Blocks 1 and 3 included 3 SNP and were defined by 4 different haplotype combinations, being tagged by each of the 3 SNP (3 htSNP). Blocks 2, 5, 6, and 7 included 2 SNP and had 2 (block 5) or 3 different haplotype combinations, with 1 htSNP (block 5) or 2 htSNP. Haplotype association analysis (Table 3) revealed a significant effect of block 1 on milk fat content and energy ($P < 0.05$), with the lowest values recorded for haplotype H4 (CCG), and on the casein content, with the highest values featured by haplotype H3 (TTG). Block 5 was significantly associated with milk energy ($P < 0.05$), and haplotype H1 (CC) was showed the highest values.

DISCUSSION

The *GHR* gene plays a fundamental role in the neuroendocrine control of milk synthesis by regulating carbohydrate, protein, and lipid metabolism. Several studies have highlighted *GHR* as a candidate locus for dairy traits in cattle (Blott et al., 2003; Viitala et al., 2006; Pegolo et al., 2016). For this reason, we hypothesized that variations of the ovine *GHR* gene might affect milk quality traits in Sarda sheep. Our investigation highlighted the existence of a significant association between the rs55631463 *GHR* SNP genotype and milk dFPY, with the CC genotype displaying the highest genotypic mean (227.4 g/d). We are also aware that for genotypes with low frequencies, as in the case of CC homozygotes at rs55631463, represented by 28 ewes, the model used for the statistical analysis may lead to large errors, as evidenced by the high values of

Figure 1. Linkage disequilibrium structure of the *GHR* gene in Sarda sheep. Structural organization of the *GHR* gene, retrieved from Ensembl (<https://www.ensembl.org/index.html>), with exons represented by boxes and introns by horizontal lines. The first box on the left corresponds to exon 10 and the first on the right is exon 1, as the *GHR* gene sequence is on the reverse strand of chromosome 16. Haplotype plot of pairwise normalized coefficient of linkage disequilibrium (D'): red (dark gray), $D' = 1.0$ and logarithm of the odds (LOD) ≥ 2.0 ; blue (light gray), $D' = 1.0$ and LOD < 2.0 ; white, $D' < 1.0$ and LOD < 2.0 . Linkage disequilibrium blocks are defined by a black line; SNP in black are within intron regions, whereas SNP in green (gray) are exonic or located in the upstream regions (SNP 28, 29, and 30). Color version available online.

standard error of least squares means (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-14914>).

Crisà et al. (2010) genotyped SNP rs55631463 in 3 Italian sheep breeds, including 34 Sarda breed ewes, and found that, overall, in the 3 breeds, the C allele was associated with decreased milk fat content (−14%). Nonetheless, if we take into account the haplotype analysis, it is evident that the C allele of SNP rs55631463 is associated with a decrease in fat content when included in haplotype CCG (H4) of block 1. In contrast, in the CCA (H2) haplotype the C allele is associated with higher fat, protein, and energy contents. In summary, the association of the C genotype of the rs55631463 SNP depends on the specific haplotype under consideration as well as on its frequency in the resource population. This can be related to the role

of growth hormone-GHR, as well as the GHR effector system linkages within the cell, on lipid metabolism. Specifically, growth hormone-GHR stimulate lipolysis by modulating gene expression and protein activation. The mobilization of lipids from adipose tissue increases the plasma free fatty acids and their utilization by cells of other tissues (Bergan-Roller and Sheridan, 2018). The growth hormone is the only endogenous ligand of GHR (Bergan-Roller and Sheridan, 2018), and association studies with dairy traits showed that polymorphism of the *GH* gene affected milk yield in Serra da Estrela ewes (Marques et al., 2006) and Sarda ewes (Vacca et al., 2013) as well as milk fat content in Sarda ewes (Dettori et al., 2015).

The SNP rs417647459 and rs411154235 were associated with milk lactose content (Supplemental Table S3;

Table 3. Least squares means (and SE between brackets) of milk traits according to the different haplotype blocks in Sarda sheep

Block	Haplotype (H)	H	n	Fat, %	Casein, %	Milk energy, MJ/kg
Block 1	CTG	H1	80	6.51 ^a (0.12)	4.20 ^{ab} (0.06)	4.61 ^a (0.06)
	CCA	H2	11	6.55 ^a (0.29)	4.21 ^{ab} (0.15)	4.62 ^a (0.14)
	TTG	H3	20	6.67 ^a (0.22)	4.40 ^a (0.11)	4.74 ^a (0.10)
	CCG	H4	4	3.36 ^b (0.95)	2.89 ^b (0.50)	3.05 ^b (0.45)
Block 5	CC	H1	246	6.49 (0.08)	4.24 (0.04)	4.61 ^a (0.04)
	GT	H2	15	5.95 (0.27)	3.97 (0.13)	4.33 ^b (0.12)

^{a,b}Means with different superscript lowercase letters in each column differ significantly in genotype comparison at $P < 0.05$.

<https://doi.org/10.3168/jds.2018-14914>). This finding can be related to the role of the growth hormone-GHR axis on carbohydrate metabolism, as lactose is synthesized in the mammary gland from galactose and glucose (Brew and Hill, 1975). The growth hormone-GHR system acts on glucose metabolism as an insulin antagonist; it facilitates the transformation of glucose into glycogen up to the saturation of glycogen cellular deposits. At the same time, it depresses glycolysis, leading to an increase in glycemia (Kim and Park, 2017).

The association of SNP located in the 5' region (upstream and intron 1) of *GHR* on MU content might be linked to the effect of this hormone on protein metabolism. The growth hormone-GHR system exerts an anabolic action, as it promotes AA uptake, RNA synthesis, and protein synthesis (Sheridan, 2011). Moreover, in humans it reduces protein catabolism and decreases deamination of AA and hepatic urea formation (Møller

and Jørgensen, 2009). Urea is synthesized in the liver from ammonia generated by the catabolism of proteins and AA absorption. In ruminants, the excess of ammonia is absorbed through the rumen wall, being transformed into urea and excreted in urine and milk (Roy et al., 2011).

The metabolic action of growth hormone-GHR occurs on multiple tissues and can be directly or indirectly mediated by IGF. Plasma growth hormone stimulates the secretion of IGF-1 from the liver, and IGF-1 stimulates cell growth and differentiation in target tissues by utilizing specific receptors (Bergan-Roller and Sheridan, 2018). The *IGF1* SNP genotyped in Sarda sheep (rs159876390) was associated with protein and casein contents ($P < 0.05$). This result was not described in previous reports, where attention was more focused on growth-related traits (Grochowska et al., 2017) and reproduction traits (He et al., 2012). Crisà et al. (2010)

Figure 2. Haplotype blocks defined by the 31 SNP genotyped in the sheep *GHR* gene. Haplotype frequencies are reported near each haplotype within a block. For each crossing area, a value of multiallelic normalized coefficient of linkage disequilibrium (D') is given for the haplotypes displayed, representing the level of recombination between the 2 blocks. SNP: 2 = rs408890407, 4 = rs55631463, 7 = rs405063669; 8 = rs411154235, 9 = rs404583153; 10 = rs162153483, 11 = rs406893455, 12 = rs161146229; 13 = rs161146242, 14 = rs407871250, 16 = rs415419991, 17 = rs409713530, 18 = rs425402906, 19 = rs161146298, 20 = rs426666828, 21 = rs430067568, 22 = rs412881843, 23 = rs400713333; 24 = rs417896686, 25 = rs426539270; 27 = rs399882480, 28 = rs417647459; 29 = rs428862267, 30 = rs402337124. SNP indicated with a triangle are haplotype tagging SNP (htSNP).

found that *IGF1* was polymorphic in some Italian dairy sheep breeds and showed a significant effect on milk lipid composition.

CONCLUSIONS

Several studies have highlighted an effect of *GHR*, *GHRHR*, and *IGF1* on dairy traits in cattle, but information on a potential effect of the same genes on dairy traits in sheep was scarce. By genotyping 36 SNP distributed in *GHR* (31 SNP), *GHRHR* (2 SNP), and *IGF1* (3 SNP) genes, in 380 Sarda sheep, our investigation has partially filled this gap. The polymorphism of *GHR* was associated with milk daily fat and protein yield (dFPY) and lactose and urea contents, whereas variability in *IGF1* was associated with milk protein and casein contents. These findings may be useful for future breeding strategies based on implementing gene-assisted selection.

ACKNOWLEDGMENTS

This research was funded by the Regione Autonoma della Sardegna (Cagliari, Italy; MIGLIOVIGENSAR - CUP: B82I13000580002).

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