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Association between the *GHR, GHRHR and IGF1* gene polymorphisms and milk coagulation properties in Sarda sheep

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Shortened version of the title suitable as a heading: *GHR, GHRHR, IGF1* genes and sheep milk coagulation

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Summary

The aim of this Research was to investigate if variation of the sheep Growth Hormone Receptor (*GHR*), Growth Hormone Releasing Hormone Receptor (*GHRHR*) and Insulin-Like Growth Factor 1 (*IGF1*) genes was associated with milk coagulation properties (MCP) in sheep. The *GHR*, *GHRHR* and *IGF1* genes are part of the GH system, which is known to modulate metabolism, growth, reproduction, as well as mammogenesis and galactopoiesis in dairy species. A total of 380 dairy Sarda sheep were genotyped for 36 SNPs mapping to these three genes. Traditional MCP were measured as rennet coagulation time (RCT), curd-firming time (k_{20}) and curd firmness at 30 minutes (a_{30}). Modeling of curd firming over time (CF_t) was based on 60 minutes lactodynamographic test, generating a total of 240 records of curd firmness (mm) for each milk sample. The model parameters obtained included: the rennet coagulation time, as a result of modeling all data available (RCT_{eq}, min); the asymptotic potential value of curd firmness (CF_P, mm) at an infinite time; the CF instant rate constant (k_{CF}, % /min); the syneresis instant rate constant (k_{SR}, % /min); the maximum value of CF (CF_{max}, mm), and the time at achievement of CF_{max} (t_{max}, min). Statistical analysis revealed that variation of the *GHR* gene was significantly associated with RCT, k_{SR} and CF_P ($P < 0.05$). These findings may be useful for the dairy industry, as well as for selection programs.

Keywords

Sheep milk, milk coagulation properties, *GHR*, *GHRHR*, *IGF1*

Dairy sheep breeding has growing importance worldwide mainly because of its products. Dairy sheep are reared in several European Countries, especially the southern regions surrounding the Mediterranean Sea (Carta et al. 2009). Italy produces over 4% of the world's ovine milk (FAOSTAT 2016) mainly provided by ewes of Sarda breed, which is considered one of the most important Italian dairy breeds (Dettori et al. 2015). Sarda sheep milk is almost entirely addressed to the production of cheese, and three cheeses produced in Sardinia are recognized by the European Union (EU) as Protected Designation of Origin (PDO) (Cipolat-Gotet et al. 2016). Given the growing economic importance of the sheep cheese production sector, recent investigations have been devoted to better understand sheep milk coagulation properties (MCP), as a valid tool to be made available to the dairy industry.

MCP can be traditionally measured using a lactodynamographic instrument, which detects three single point parameters: rennet coagulation time (RCT, min), curd firming time (k_{20}, min) and curd firmness at 30 minutes of analysis (a_{30}, mm), first described by McMahon & Brown (1982). In dairy cattle MCP have been proved to be independent from milk yield, and mainly influenced by the titratable acidity of milk (Bittante et al. 2012). In addition, RCT and a_{30} are strongly and negatively correlated. In contrast, the correlation between RCT and a_{30} was not evidenced in milk samples from Sarda breed ewes (Pazzola et al. 2014). The same authors extended the MCP analysis up to 60 minutes, obtaining also curd firmness at 45 (a_{45}) and 60 (a_{60}) minutes. The possibility to extend the analysis allowed to observe that sheep milk has a very early gelation time compared with bovine milk (RCT = 8.6 min vs. 10-20 min), a rapid increase in curd firming time (k_{20} = less than 2 min vs. 5-15 min), and a higher curd firmness at a_{30} (a_{30} = 50 mm vs. 35 mm) compared to bovine (Bittante et al. 2012; Pazzola et al. 2014). In addition to MCP, several research papers exploited all available lactodynamograph data to appropriately model curd firming over time (CFt) in milk from different species (Bittante et al. 2014; Stocco et al. 2017; Pazzola et al. 2018). Those modeled parameters have been proved to be more informative than the traditional MCP. A four parameter model was applied to cow milk coagulation and curd firming test prolonged from 30 to 90 min

(Bittante et al. 2013). The four parameter model was modified and applied to test coagulation ability of sheep milk from Sarda sheep by Cipolat-Gotet et al. (2018).

MCP are clearly influenced by genetic factors such as species, breed and individual factor (Bittante et al. 2012), and evidence has been given for the casein genotype (Ceriotti et al., 2005; Noce et al., 2016). The Growth Hormone Releasing Hormone Receptor (*GHRHR*), the Growth Hormone Receptor (*GHR*), and the Insulin-Like Growth Factor 1 (*IGF1*) genes have been considered as potential candidate genes for milk quality traits in cattle (Banos et al. 2008; Viitala et al. 2006). These genes are involved in the GH system. The growth hormone (GH) regulates many physiological functions, such as metabolism, growth, reproduction, feeding, osmoregulation and immune system function (Bergan-Roller & Sheridan 2018), in addition to its effects on mammary development (mammogenesis) and milk production (galactopoiesis) (Akers 2017). The GHRHR protein, expressed in somatotropic cells, mediates the production and release of the growth hormone from the somatotropic cells, upon ligand binding with the hypotalamic factor GH releasing hormone (GHRH) (Pang & Chan 2010). The actions triggered by the GH are mediated by its specific receptors (GHR) distributed among tissues, which in turn are regulated at the expression level by several factors reflecting the metabolic and nutritional status of the organism (Bergan-Roller & Sheridan 2018). GHRs can be associated, within the cell, with different effectors, which in turn, can cause different responses upon GH activation. When GHR is associated with the Janus tyrosine kinase-signal transducer and activator of transcription (JAK/STAT), PI3K-protein kinase B (Akt) and extracellular signal regulated kinase (ERK), it causes the synthesis and secretion of IGF1 polypeptide hormone and therefore the pathway of cell growth (Herington & Lobie 2012). In contrast, when GHRs are associated with intracellular effectors as cAMP/protein kinase C (PKC) pathways, they mediate lipolytic GH signaling by targeting expression and activation of lipases (Chaves et al. 2011). Many of the growth-promoting effects of GH are mediated by the IGF1. The circulating GH stimulates the synthesis and secretion of IGF1 from the liver, and IGF1, in turn,

387 stimulates cell growth and differentiation in a variety of target tissues, through distinct IGF
388 receptors (Laviola et al. 2007).

389 Dettori et al. (2018) investigated association between a panel of 36 SNPs within the *GHR*,
390 *GHRHR* and *IGF1* genes and milk production and quality traits in Sarda sheep, revealing that the
391 *GHR* gene is associated with daily fat and protein yield. They also revealed the *IGF1* gene is
392 associated with milk protein and casein content. Then, the present study aims to explore association
393 between the 36 SNP panel of the *GHRHR*, *GHR* and *IGF1* genes and traditional and modeled MCP
394 in Sarda sheep.

395

396 **Materials and methods**

397 No specific authorization from an animal ethics committee was required, because according to the
398 EC Directive 86/609/EEC and Directive 2010/63/EU, none of the procedures met the criteria to be
399 defined as an experiment or procedure. Blood samples for DNA isolation were collected by
400 experienced veterinarians and milk samples were collected concurrently with official sampling
401 procedures for performance controls of the flock book.

402 A total of 380 lactating ewes, in their first to seventh parity, were sampled from 19 farms
403 (20 ewes per farm) located in Sardinia (Italy). The ewes were included in the selection scheme of
404 the Sarda breed and registered in the flock book. Ewes were between 2 and 7 months after
405 parturition. Detailed description of farms, animals and sampling is given in Pazzola et al. (2014)
406 and Vacca et al. (2015). Ewes from each flock were individually sampled in a single day (one
407 sampling day for each flock). During the afternoon milking 200 mL of milk were collected from
408 each ewe. Milk samples were maintained at 4 °C and were analyzed within 24 hours. Individual
409 blood samples were collected in K3EDTA vacuum tubes (BD Vacutainer, Becton Dickinson,
410 Franklin Lakes, NJ) from each ewe for genomic DNA isolation, performed with the Puregene
411 Blood Kit (Qiagen, Hilden, Germany). The concentration and purity of DNA were determined with
412 an Eppendorf BioPhotometer instrument (Eppendorf, Hamburg, Germany).

MCP were measured with the Formagraph instrument (Foss Italia, Padova, Italy). Individual milk samples (10 mL x 2 replicates) were heated to 35 °C and they were added 200 μL of rennet solution (Hansen Naturen Plus 215, Pacovis Amrein AG, Bern, Switzerland), containing 80 ± 5% chymosin and 20 ± 5% pepsin (215 international milk clotting units per mL, IMCU/mL), which was diluted to 1.2% (wt/ vol) in distilled water to achieve 0.0513 IMCU/mL milk. The traditional single point parameters RCT, k_{20} and a_{30} were recorded, and the analysis was extended to 60 minutes to obtain the values of curd firmness at 45 (a_{45}) and 60 (a_{60}) min. Six milk samples were excluded from the statistical analyses as did not coagulated. In addition to traditional single point parameters, we retrieved from the Formagraph instrument the specific file containing the complete record of curd firming values (expressed as the width of the oscillatory graph, in mm), detected every 15 seconds. This created a total of 240 CF values for each replicate, for a 60 min run. Data obtained was implemented in the four-parameter model described by Bittante et al. (2013):

$$CF_t = CF_P \times [1 - e^{-kCF \times (t - RCTeq)}] \times e^{-kSR \times (t - RCTeq)}$$

where CF_t is curd firmness at time t (mm); CF_P is the asymptotical potential maximum value of CF at an infinite time (mm); k_{CF} is the curd-firming instant rate constant (% × min^{-1}); k_{SR} is the curd syneresis instant rate constant (% × min^{-1}), and RCT_{eq} is the rennet coagulation time estimated by the model, on the basis of all data points (min). The CF_P is conceptually independent from test duration and is not intrinsically dependent on RCT (unlike a_{30}). The parameter k_{CF} describes the shape of the curve from the time of milk gelation to infinity, and is conceptually different from k_{20} as it uses all available information. The parameter k_{CF} is assumed to increase CF toward the asymptotic value of CF_P, whereas k_{SR} is assumed to decrease CF toward a null asymptotic value. In the initial phase of the test, the first rate constant prevails over the second, so that CF_t increases to a point in time (t_{max}) at which the effects of the 2 parameters are equal but opposite in sign; this is

when CF_t attains its maximum level (CF_{max}). Thereafter, CF_t decreases, tending toward a null value due to the effect of curd syneresis and the resulting expulsion of whey.

The 36 SNP panel included 31 SNPs mapping to the sheep *GHR* gene, 2 SNPs of the *GHRHR* gene and 3 SNPs of the *IGF1* gene, genotyped in the 380 Sarda sheep. Genotyping was carried out with a 12K Flex QuantStudio instrument (Thermo Fisher Scientific), based on a custom TaqMan Real-Time PCR assay (Thermo Fisher Scientific, Waltham, MA) as described in Dettori et al. (2018).

The Haploview software package (Barrett et al. 2005) was used to estimate and plot pairwise linkage disequilibrium (LD) measures (D′ and r^2). The same tool was used to infer haplotype frequencies as well as to define LD blocks according to the Gabriel criteria (Gabriel et al. 2002). Haplotype analysis revealed seven LD blocks within the *GHR* gene sequence, described in Dettori et al. (2018).

The 240 CF_t observations available for each sample were fitted with curvilinear regressions using the non-linear procedure (PROC NLIN) of SAS (version 9.4, SAS Institute Inc., Cary, NC). The Marquardt iterative method has been used according to Bittante (2011).

Association analysis between *GHRHR*, *GHR* and *IGF1* genotypes and experimental data regarding CF_t modeling parameters was based on the following model [1]:

$$Y_{ijklmn} = \mu + G_i + F_j + P_k + DIM_l + SIRE(G)_m + e_{ijklmn} \qquad [1]$$

where Y_{ijklmn} is the observed trait (RCT_{eq}, k_{CF}, k_{SR}, C_{FP}, CF_{max}, and t_{max}); μ is the general mean; G_i is the fixed effect of the i^{th} SNP genotype, one at a time (i = 2 to 3 levels: the two homozygotes and the heterozygote); F_j is the fixed effect of the j^{th} farm, which also includes animal management and feeding (j = 1 to 19 levels; the different farms where animals were reared); P_k is the fixed effect of k^{th} parity of the ewes (k = 1 to 4 levels; first to fourth or more parities); DIM_l is the fixed effect of the l^{th} days in milking (l = 4 levels; level 1: ≤100 days; 2: 101–140 days; 3: 141–160 days; level 4:

464 ≥161 days); $SIRE(G)_m$ is the random effect of the m^{th} sire (m = 108 different sires) nested within
465 the genotype, and e_{ijklmn} is the error random residual effect.

466 This model [1] was also used to investigate the association between both traditional and
467 modeled MCP and each of the seven LD blocks, one at a time. In the single SNP and LD block
468 analysis, we only considered SNPs with a MAF > 0.05, to make sure that genotypic means are
469 correctly estimated. The MIXED procedure of SAS (version 9.4, SAS Inst. Inc.) was used to carry
470 out the association analysis and correction for multiple testing was implemented with the
471 Bonferroni method (one milk trait for each SNP or LD block at a time).

472

473 **Results and discussion**

474 Descriptive statistics of traditional MCP and CF_t model parameters of milk samples are displayed in
475 Table 1. All traits exhibited high variability, the coefficient of variation (CV) of traditional MCP
476 traits was between 24.65% (for a_{30}) and 43.45% (for RCT), and k_{SR} had the highest CV value
477 (129.36%). Table 2 displays the *F*-values obtained from the analysis of variance of CF_t model
478 parameters, as a function of genotype of the *GHR*, *GHRHR* and *IGF1* genes. The SNP genotypes
479 exhibiting significant effects on phenotype variance are described in Table 3. Among the three
480 genes analyzed, only the *GHR* polymorphism was significantly associated with the considered
481 traits. The only physiological ligand of GHR is the growth hormone, and in the same breed,
482 polymorphism of the *GH* gene was associated with milk yields (Vacca et al. 2013) and with lipid
483 content, in addition to protein, casein and lactose contents (Dettori et al. 2015).

484 Statistical analysis highlighted a significant association of SNP rs404237321 with k_{SR}. Fig. 1
485 clearly depicted the effect of the SNPs on the pattern of coagulation, in particular, the rs404237321
486 CT genotype showed larger syneresis compared with CC genotype (Fig. 1a). The SNP rs404237321
487 was a missense variant of exon 5, causing the p.Gly147Asp variation in the GHR protein, and
488 according to the SIFT (http://sift.jcvi.org/) prediction algorithm, it was not expected to affect
489 protein function. As regards SNP rs426666828, the CC genotype showed higher k_{SR} compared with

CT and TT genotypes (Fig. 1b). This SNP was located in intron 3 of the *GHR* gene (13.8 kb from exon 4) and it was included in haplotype block 4, which was the largest in size, consisting of ten SNPs (Dettori et al., 2018). The SNP rs412881843 was significantly associated with both traditional RCT (data not shown) and the k_{SR} value ($P<0.05$); its effects on RCT, shorter for GG genotype, and k_{SR}, are shown in Fig. 1c. The SNP rs412881843 is localized in intron 3 (only 427 bp from exon 3) and linkage disequilibrium analysis revealed it was included in haplotype block 4, as was SNP rs426666828. In the resource population of the present paper the GG genotype of SNP rs412881843 was associated with an RCT value of 6.97 min, which is shorter than the average RCT value of 8.6 min found by Pazzola et al. (2014). The heterozygote CG genotype of SNP rs412881843 was associated with a delayed value of RCT (9.09) with a similar mean value of RCT reported for the Brogna breed (Bittante et al. 2014). Finally, the SNP rs402337124, located in the upstream region and included in haplotype block 7, was associated with k_{SR}, lower for AA genotype (Fig. 1d).

Although the literature is poor about this topic, Bittante et al. (2014) showed that an integration of the lipid fraction of the diet with rumen-protected conjugated linoleic acid, doubled the rate of whey expulsion (k_{SR} trait) in Alpine sheep breeds. In a previous investigation on the same resource population (Dettori et al. 2018), the *GHR* gene has been shown to affect variation of the lipid content of milk, possibly indicating that the effect of *GHR* is not direct on coagulation, but mediated by the milk composition, in particular by the lipid content.

Linkage Disequilibrium (LD) analysis was performed from 29 informative SNPs in the *GHR* gene and seven regions of LD were identified (described in Dettori et al. 2018). Haplotype association analysis revealed a significant effect of block 1 on CF_P ($P < 0.05$), with the lowest values recorded for haplotype H4 (CCG) (CF_P of 24.24 mm vs 63.32 of haplotype H1; Table 4). Haplotype H4 of block 1 was also associated with a reduction of lipid and casein contents and of milk energy, in the same animals (Dettori et al. 2018). The molecular bases underlying the observed associations are unknown and need more investigation, especially in sheep. In fact, the *GHR* gene is characterized by high transcriptional complexity, due to the structural organization of the 5′

regulatory region of this gene (Adams 1995). Multiple forms of GHRs are known to exist in vertebrates, with specific tissue expression and differential expression in relation to the distinctive conditions of the organism (Bergan-Roller & Sheridan 2018). In cattle there are multiple forms of *GHR* mRNA variants, with disparate tissue specific expression (Jiang & Lucy 2001), while two specific forms of *GHR* mRNA are currently known in the sheep: the ovine Pl promoter, with liver-specific expression in vivo (Adams et al. 1990) and the P2 promoter, with widespread tissue transcription (Adams 1995).

In conclusion, this is the first research exploring the potential effects of the *GHR*, *GHRHR* and *IGF1* genes on traditional MCP and CF_t parameters, based on prolonged curd firmness recording. In particular, the study demonstrated that polymorphisms of the *GHR* gene are associated with milk rennet coagulation time and syneresis. These findings may be useful for the dairy industry, as well as for selection programs.

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Table 1. Descriptive statistics for traditional (MCP) and curd firming over time (CF_t) coagulation properties of milk from Sarda sheep

					Percentile	
Trait	N	Media	SD	CV,[1]%	P1	P99
Traditional MCP[2]						
RCT	374	8.77	3.81	43.45	4.00	27.15
k_{20}	374	1.93	0.54	28.05	1.30	4.15
a_{30}	376	49.88	12.29	24.65	4.14	67.82
a_{45}	376	45.99	14.72	32.01	8.22	70.44
a_{60}	376	42.19	16.08	38.11	3.98	71.20
CF_t parameters[3]						
CF_P , mm	353	60.58	12.11	20.00	33.23	102.82
k_{CF} , % × min^{-1}	376	0.278	0.132	47.39	0.001	0.734
k_{SR} , % × min^{-1}	309	0.014	0.018	129.36	0.000	0.105
CF_{max} , mm	374	53.92	8.86	16.44	33.53	70.68
t_{max} , min	374	30.00	15.07	50.26	13.00	60.00

[1]CV = coefficient of variation; [2]RCT = measured rennet coagulation time; k_{20} = time interval between coagulation and attainment of curd firmness of 20 mm; a30, a45 and a60 = curd firmness 30, 45 and 60 min after rennet addition; [3]CF_P = asymptotic potential curd firmness; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness achieved within 45 min; t_{max} = time at achievement of CF_{max}; RCT_{eq} = RCT estimated according to curd firm change over time modeling (CF_t).

Table 2. Analysis of variance (ANOVA, *F*-values and significance) of curd firming over time (CF_t) model parameters of milk from Sarda sheep

		CF_t model parameters[1]				
gene	SNP ID	CF_P	k_{CF}	k_{SR}	CF_{max}	t_{max}
GHR	rs161146162	0.01	0.01	0.72	0.79	0.06
	rs408890407	1.79	1.30	0.65	1.62	0.50
	rs161146164	0.04	0.08	0.37	0.78	0.10
	rs55631463	0.81	1.94	2.02	0.03	1.15
	rs413776054	0.04	0.09	0.39	0.77	0.09
	rs405063669	2.52	1.53	2.04	0.09	2.56
	rs411154235	2.42	0.28	0.71	1.75	0.49
	rs404583153	2.08	1.11	1.02	1.05	0.81
	rs162153483	2.51	1.75	3.38*	1.75	1.24
	rs406893455	0.42	0.87	1.79	1.13	1.32
	rs161146229	1.45	0.76	2.28	0.12	1.12
	rs161146242	0.78	0.94	0.94	1.83	0.87
	rs407871250	2.29	1.24	2.04	2.78	1.28
	rs404237321	0.05	0.41	7.90**	1.43	0.34
	rs415419991	1.51	0.68	3.30*	0.06	0.89
	rs409713530	2.53	1.82	3.35*	2.23	1.20
	rs425402906	1.87	1.28	1.94	2.00	0.98
	rs161146298	2.36	1.68	2.91	1.93	0.89
	rs426666828	1.45	1.11	3.48*	0.05	2.79
	rs430067568	0.75	0.36	0.76	0.88	1.59
	rs412881843	0.74	1.64	3.17*	1.07	0.88
	rs400713333	0.13	0.12	0.60	0.76	1.93
	rs417896686	0.28	0.22	0.06	0.26	1.49
	rs426539270	0.23	0.17	0.07	0.57	1.54
	rs412986330	1.42	0.29	0.71	1.06	0.11
	rs399882480	1.21	1.15	2.17	2.15	0.18
	rs417647459	0.59	0.06	0.13	0.34	0.08
	rs428862267	0.05	0.25	1.08	0.91	0.06
	rs402337124	2.42	0.82	3.15*	2.30	0.89
GHRHR	rs409504706	0.17	0.62	0.42	1.00	1.29
IGF1	rs159876390	0.84	0.07	1.50	0.48	0.55

[1]CF_P = asymptotic potential curd firmness; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness achieved within 45 min; t_{max} = time at achievement of CF_{max}. * = P < 0.05; **= P < 0.01.

Table 3. Least square means of traditional (MCP) and curd firming over time (CF_t) coagulation properties of milk from Sarda sheep according to the different genes, SNP and genotypes (n = 380)

				Traditional MCP[1]					CF_t model parameters[2]				
GHR Gene	SNP ID	Genotype	n	RCT	k_{20}, min	a_{30}, mm	a_{45}, mm	a_{60}, mm	CF_P, mm	k_{CF}, %/min	k_{SR}, %/min	CF_{max}, mm	t_{max}, min
	rs404237321	CC	366	8.86	1.99	49.40	45.76	42.27	60.81	27	0.14^{b}	53.74	34.48
		CT	5	7.92	1.66	52.61	45.58	42.37	62.11	24	0.45^{a}	57.77	40.52
	rs426666828	CC	59	8.98	2.08	48.61	44.49	41.58	58.27	26	0.20^{a}	53.27	40.96
		CT	186	8.66	1.98	48.91	44.85	41.11	60.59	28	0.15^{ab}	53.40	33.00
		TT	126	8.87	1.95	49.50	46.62	43.56	61.64	29	0.11^{b}	53.62	33.54
	rs412881843	CC	190	8.84^{ab}	2.00	49.07	44.82	41.05	59.88	27	0.17^{a}	53.23	35.43
		CG	157	9.09^{a}	2.02	48.57	45.51	42.27	61.09	27	0.13^{ab}	53.32	34.22
		GG	26	6.97^{b}	1.75	52.91	49.04	46.66	62.52	32	0.07^{b}	55.52	28.90
	rs402337124	AA	223	8.81	1.96	49.57	46.15	42.50	61.35	29	0.12^{b}	53.45	33.08
		AG	117	8.89	2.05	47.45	43.53	40.41	58.33	27	0.18^{a}	52.87	35.99
		GG	18	8.07	1.82	52.88	48.37	45.26	61.44	27	0.18^{a}	56.98	38.49

[1]RCT = measured rennet coagulation time; k_{20} = time interval between coagulation and attainment of curd firmness of 20 mm; a30, a45 and a60 = curd firmness 30, 45 and 60 min after rennet addition; [2]CF_P = asymptotic potential curd firmness; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness achieved within 45 min; t_{max} = time at achievement of CF_{max}.
Means with different superscript capital or lower-case letters in each column differ significantly in genotype comparison at $P < 0.01$ and $P < 0.05$ respectively.

Table 4. Least square means of milk traits according to the different haplotype blocks in Sarda sheep

Blocks	Haplotype	H	n	CF_P
Block 1	C T G	H1	80	63.32[a]
	C C A	H2	11	57.44[ab]
	T T G	H3	20	60.82[a]
	C C G	H4	4	24.24[b]

SNPs in Block 1 are rs408890407 (C/T; exon 10, synonymous), rs55631463 (C/T; exon 10, missense) and rs405063669 (G/A; intron 8).

Means with different superscript capital or lower-case letters in each column differ significantly in haplotype comparison at $P < 0.01$ and $P < 0.05$ respectively.

646 **Figure 1.**

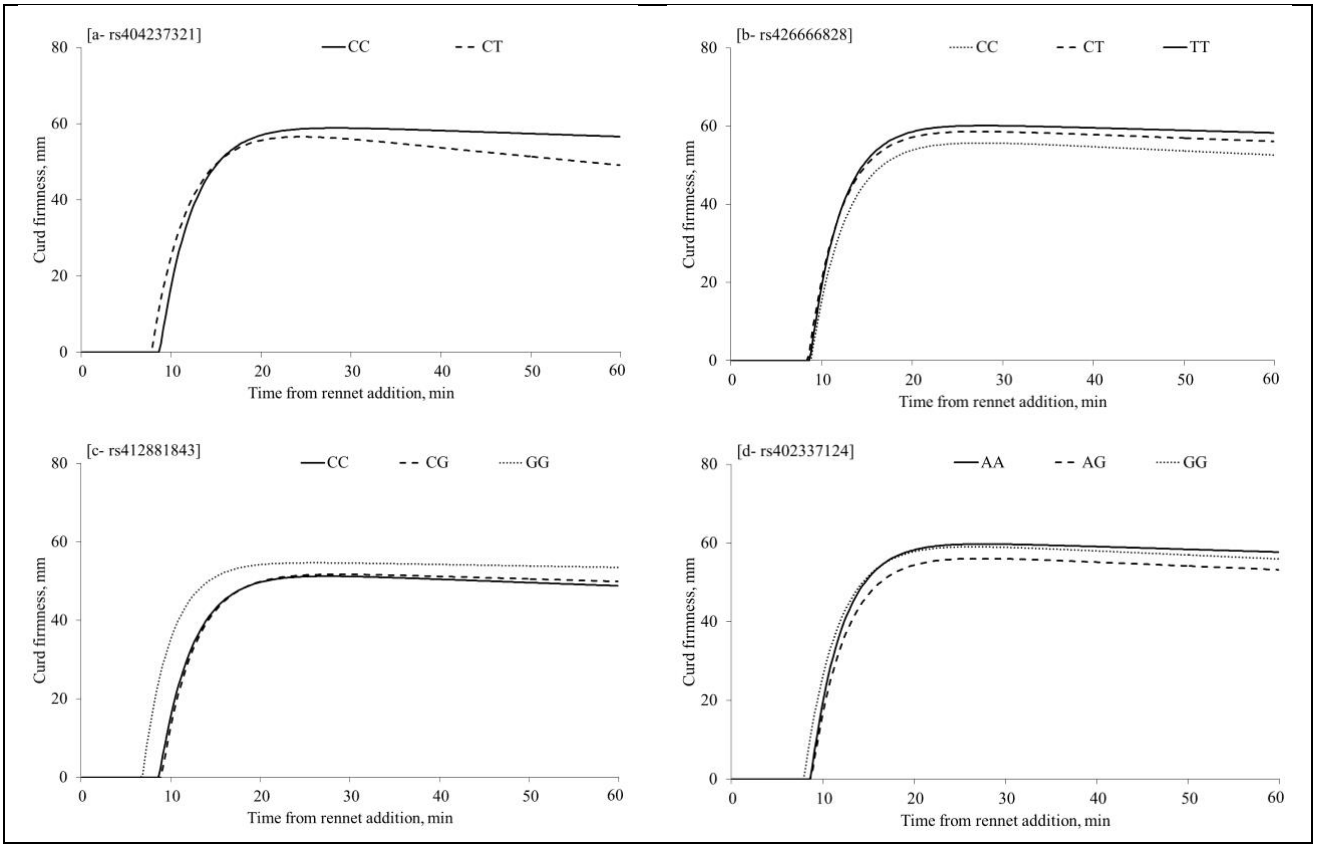

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Figure captions

Fig. 1. Pattern of curd firming over time (CF_t) of milk samples from SNP rs404237321 (a) rs426666828 (b), rs412881843 (c) and rs402337124 (d) according to their genotypes.