

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

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

301

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304

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

307

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310 **Summary**

311 The aim of this Research was to investigate if variation of the sheep Growth Hormone Receptor
312 (*GHR*), Growth Hormone Releasing Hormone Receptor (*GHRHR*) and Insulin-Like Growth Factor
313 1 (*IGF1*) genes was associated with milk coagulation properties (MCP) in sheep. The *GHR*,
314 *GHRHR* and *IGF1* genes are part of the GH system, which is known to modulate metabolism,
315 growth, reproduction, as well as mammogenesis and galactopoiesis in dairy species. A total of 380
316 dairy Sarda sheep were genotyped for 36 SNPs mapping to these three genes. Traditional MCP
317 were measured as rennet coagulation time (RCT), curd-firming time (k_{20}) and curd firmness at 30
318 minutes (a_{30}). Modeling of curd firming over time (CF_t) was based on 60 minutes
319 lactodynamographic test, generating a total of 240 records of curd firmness (mm) for each milk
320 sample. The model parameters obtained included: the rennet coagulation time, as a result of
321 modeling all data available (RCT_{eq} , min); the asymptotic potential value of curd firmness (CF_P ,
322 mm) at an infinite time; the CF instant rate constant (k_{CF} , % /min); the syneresis instant rate
323 constant (k_{SR} , % /min); the maximum value of CF (CF_{max} , mm), and the time at achievement of
324 CF_{max} (t_{max} , min). Statistical analysis revealed that variation of the *GHR* gene was significantly
325 associated with RCT, k_{SR} and CF_P ($P < 0.05$). These findings may be useful for the dairy industry,
326 as well as for selection programs.

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329 **Keywords**

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

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

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

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

364 MCP are clearly influenced by genetic factors such as species, breed and individual factor
365 (Bittante et al. 2012), and evidence has been given for the casein genotype (Ceriotti et al., 2005;
366 Noce et al., 2016). The Growth Hormone Releasing Hormone Receptor (*GHRHR*), the Growth
367 Hormone Receptor (*GHR*), and the Insulin-Like Growth Factor 1 (*IGF1*) genes have been
368 considered as potential candidate genes for milk quality traits in cattle (Banos et al. 2008; Viitala et
369 al. 2006). These genes are involved in the GH system. The growth hormone (GH) regulates many
370 physiological functions, such as metabolism, growth, reproduction, feeding, osmoregulation and
371 immune system function (Bergan-Roller & Sheridan 2018), in addition to its effects on mammary
372 development (mammogenesis) and milk production (galactopoiesis) (Akers 2017). The GHRHR
373 protein, expressed in somatotropic cells, mediates the production and release of the growth hormone
374 from the somatotropic cells, upon ligand binding with the hypothalamic factor GH releasing
375 hormone (GHRH) (Pang & Chan 2010). The actions triggered by the GH are mediated by its
376 specific receptors (GHR) distributed among tissues, which in turn are regulated at the expression
377 level by several factors reflecting the metabolic and nutritional status of the organism (Bergan-
378 Roller & Sheridan 2018). GHRs can be associated, within the cell, with different effectors, which in
379 turn, can cause different responses upon GH activation. When GHR is associated with the Janus
380 tyrosine kinase-signal transducer and activator of transcription (JAK/STAT), PI3K-protein kinase B
381 (Akt) and extracellular signal regulated kinase (ERK), it causes the synthesis and secretion of IGF1
382 polypeptide hormone and therefore the pathway of cell growth (Herington & Lobie 2012). In
383 contrast, when GHRs are associated with intracellular effectors as cAMP/protein kinase C (PKC)
384 pathways, they mediate lipolytic GH signaling by targeting expression and activation of lipases
385 (Chaves et al. 2011). Many of the growth-promoting effects of GH are mediated by the IGF1. The
386 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).

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

$$425$$

$$426 \quad CF_t = CF_P \times [1 - e^{-k_{CF} \times (t - RCT_{eq})}] \times e^{-k_{SR} \times (t - RCT_{eq})}$$

$$427$$

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

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

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

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

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

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

455

$$456 \quad Y_{ijklmn} = \mu + G_i + F_j + P_k + DIM_l + SIRE(G)_m + e_{ijklmn} \quad [1]$$

457

458 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
459 is the fixed effect of the i^{th} SNP genotype, one at a time ($i = 2$ to 3 levels: the two homozygotes and
460 the heterozygote); F_j is the fixed effect of the j^{th} farm, which also includes animal management and
461 feeding ($j = 1$ to 19 levels; the different farms where animals were reared); P_k is the fixed effect of
462 k^{th} parity of the ewes ($k = 1$ to 4 levels; first to fourth or more parities); DIM_l is the fixed effect of
463 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

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

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

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

516 regulatory region of this gene (Adams 1995). Multiple forms of GHRs are known to exist in
517 vertebrates, with specific tissue expression and differential expression in relation to the distinctive
518 conditions of the organism (Bergan-Roller & Sheridan 2018). In cattle there are multiple forms of
519 *GHR* mRNA variants, with disparate tissue specific expression (Jiang & Lucy 2001), while two
520 specific forms of *GHR* mRNA are currently known in the sheep: the ovine P1 promoter, with liver-
521 specific expression in vivo (Adams et al. 1990) and the P2 promoter, with widespread tissue
522 transcription (Adams 1995).

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

528

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531

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617

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

Trait	N	Media	SD	CV, ¹ %	Percentile	
					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

620 ¹CV = coefficient of variation; ²RCT = measured rennet coagulation time; k₂₀ = time interval
 621 between coagulation and attainment of curd firmness of 20 mm; a₃₀, a₄₅ and a₆₀ = curd firmness
 622 30, 45 and 60 min after rennet addition; ³CF_P = asymptotic potential curd firmness; k_{CF} = curd
 623 firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness
 624 achieved within 45 min; t_{max} = time at achievement of CF_{max}; RCT_{eq} = RCT estimated according to
 625 curd firm change over time modeling (CF_t).
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Table 2. Analysis of variance (ANOVA, *F*-values and significance) of curd firming over time (CF_t) model parameters of milk from Sarda sheep

gene	SNP ID	CF_t model parameters ¹				
		CF_P	k_{CF}	k_{SR}	CF_{max}	t_{max}
<i>GHR</i>	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	
<i>GHRHR</i>	rs409504706	0.17	0.62	0.42	1.00	1.29
<i>IGF1</i>	rs159876390	0.84	0.07	1.50	0.48	0.55

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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} . * = $P < 0.05$; ** = $P < 0.01$.

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

<i>GHR</i> Gene	SNP ID	Genotype	n	Traditional MCP ¹				CF _t model parameters ²					
				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

634 ¹RCT = measured rennet coagulation time; k₂₀ = time interval between coagulation and attainment of curd firmness of 20 mm; a₃₀, a₄₅ and a₆₀ =
 635 curd firmness 30, 45 and 60 min after rennet addition; ²CF_P = asymptotic potential curd firmness; k_{CF} = curd firming instant rate constant; k_{SR} =
 636 syneresis instant rate constant; CF_{max} = maximum curd firmness achieved within 45 min; t_{max} = time at achievement of CF_{max}.

637 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
 638 respectively.

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

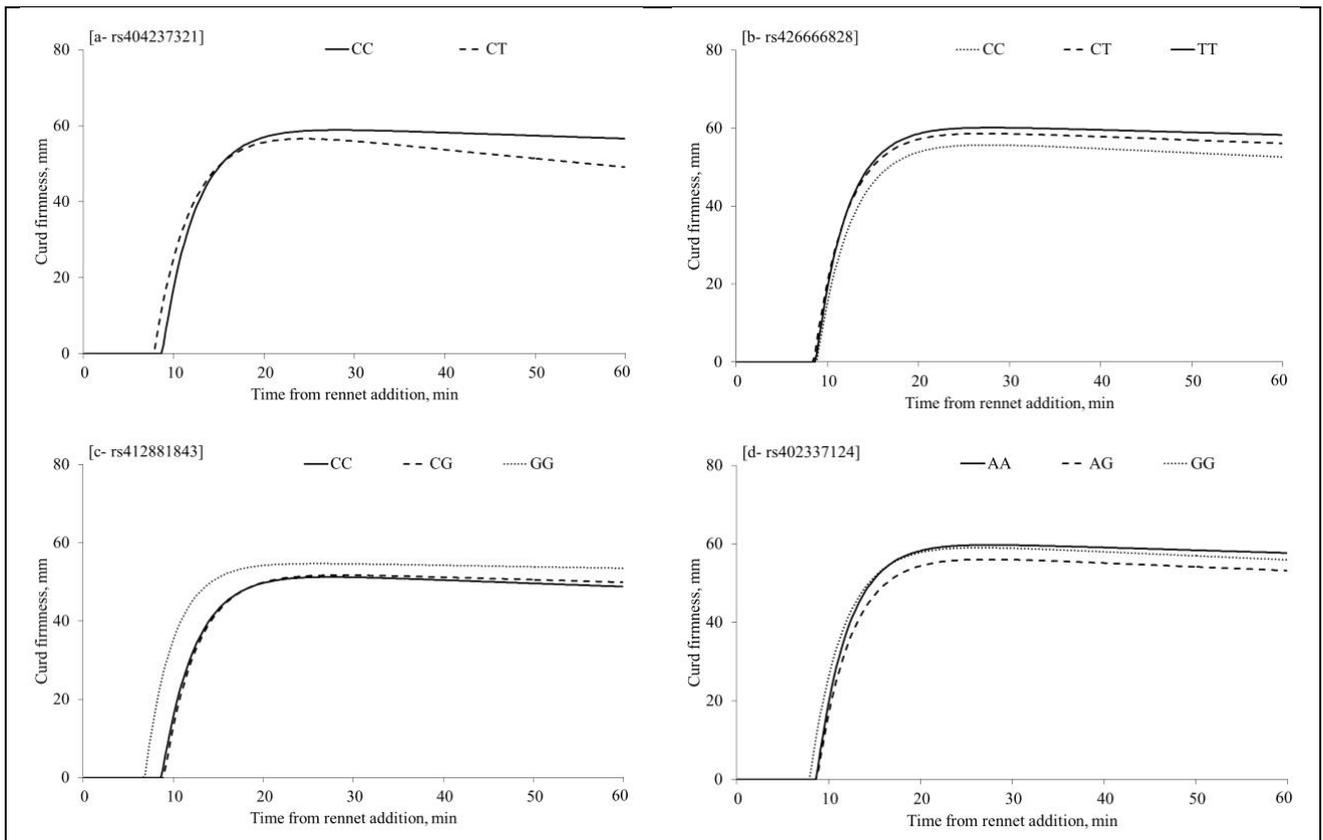
Blocks	Haplotype	H	n	CF _p
Block 1	C T G	H1	80	63.32 ^a
	C C A	H2	11	57.44 ^{ab}
	T T G	H3	20	60.82 ^a
	C C G	H4	4	24.24 ^b

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

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

645

646 **Figure 1.**



647

648 **Figure captions**

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