Exploring polymorphisms and effects of candidate genes on milk fat quality in dairy sheep

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CANDIDATE GENES THAT DETERMINE MILK FAT QUALITY IN DAIRY SHEEP: INTERPRETIVE SUMMARY

Candidate genes that determine milk fat quality in dairy sheep. *By Crisà et al.*

Milk fatty acid profiles play an important role in dairy sheep farming, both as a source of nutraceuticals and as determinants of cheese characteristics. The effect of a large number of SNP, within genes involved in the metabolism of milk fat upon the fatty acid composition, was here evaluated, and some genes that play a role in the desaturation of stearic fatty acid into polyunsaturated fatty acids and in the variability of total fat content were identified. Moreover, a single gene (fatty acid synthetase), that influences medium-chain fatty acids, was identified.

Exploring polymorphisms and effects of candidate genes on milk fat quality in dairy sheep

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ABSTRACT

Aim of the present study was to investigate the genetic control of the fatty acid (FA) composition in milk from three breeds of sheep: Altamurana, Gentile di Puglia and Sarda. SNP within genes, encoding enzymes putatively involved in the synthesis and metabolism of milk fat, were selected for analysis, and the allele substitution effects were determined for 16 genes, that were polymorphic in the three sheep breeds, upon the milk fat composition. Four genes (alpha-1-antichymotrypsin-2; diacylglycerol O-acyltransferase homolog-2; propionyl Coenzyme A carboxylase, beta polypeptide; insulin-like growth factor-I) play a role in the desaturation of stearic FA into polyunsaturated fatty acids (PUFA). Furthermore, two genes (growth hormone receptor and zona pellucida glycoprotein-2) affect the variability of the total fat content, in addition to the butyric and stearic FA profile, and that the fatty acid synthetase gene has an influence on the medium-chain FA. Milk FA profiles play an important role in dairy sheep farming because they have a large effect on cheese characteristics and also because sheep milk may be marketed as a source of nutraceuticals as it contains higher levels of CLA than milk from other ruminants. The current study evaluated the global effects of a large number of SNP and haplotypes on traits that are not commonly investigated in sheep but that are potentially very useful for improving milk quality.

Key words: fatty acids, single nucleotide polymorphisms, dairy sheep, milk composition.

INTRODUCTION

Milk fat triglycerides are synthesized in the mammary epithelial cells but the fatty acids (**FA**) used to synthesize them may arise from either the breakdown of blood lipids, or via de novo synthesis within the mammary epithelial cells. From 40% to 60% of FA come from the blood and are primarily derived from very low density lipoproteins (**VLDL**), which are synthesized in the intestine or liver. Triglycerides in the VLDL are hydrolyzed in the mammary capillaries by lipoprotein lipase (**LPL**) (Fielding and Frayn, 1998).

The FA contained in VLDL are dependent upon dietary lipids and also on mobilized fat from body adipose tissue. The two key enzymes involved in FA synthesis in the mammary gland are acetyl-CoA carboxylase (ACACA), which is the rate limiting step, and fatty acid synthetase (**FASN**), a large complex of enzyme activities responsible for the chain elongation of FA (Bionaz and Loor, 2008). Most of the previous research on milk composition, both in cows and sheep, has focused on the dietary sources of FA variation (Firkins et al., 2006; Tsiplakou et al., 2008) and in particular on those FA that have a range of positive health effects (i.e. polyunsaturated FA (**PUFA**) and CLA) (Tsiplakou et al., 2006; Mele et al., 2007). Other authors have investigated the effects of specific genes, acyl CoA:diacylglycerol acyltransferase (***DGAT1***) and stearoyl CoA desaturase (***SCD***) that are known to directly affect FA desaturation (Mele et al., 2007; Moioli et al., 2006; Schennink et al., 2008). Recently, genome-wide screens for bovine milk-fat composition (Schennink et al., 2009; Stoop et al., 2009) revealed several quantitative trace loci (QTL) for short-, medium- and long chain FA and demonstrated that the QTL for BTA(14) and BTA(26) are associated with polymorphisms in the *DGAT1* and *SCD* genes, respectively. The composition of sheep milk, both between and within breeds, can differ by between 6% and 9% for fat; 4% and 7% for protein; and 17% and 21% for total solids (Haenlein, 2001). Short and medium-chain FA are the most important sources of fat during the processing of cheeses that undergo several weeks of ripening (McSweneey, 2004). The principal flavors and cheesy and lipolysed aromas of raw ewe milk cheeses are derived from short- and medium-chain free fatty acids (FFA), which

originate from the degradation of lipids (Curioni and Bosset, 2002; House and Acree, 2002; Fernandez-Garcia et al., 2006). Moreover, sheep milk contains higher levels of CLA then cow or goat milk (Jahreis et al., 1999), a FA that is considered to have beneficial effects on human health (Bhattacharya et al., 2006).

The main objectives of the present study were to evaluate the association of different genotypes with milk fat composition in an experimental dairy sheep population composed of three breeds: Altamurana, Gentile di Puglia and Sarda.

MATERIALS AND METHODS

Animals, sampling and diet

The study was conducted using 94 sheep of three breeds, Altamurana (36), Gentile di Puglia (24) and Sarda (34). Altamurana is a dairy sheep belonging to the subgroup of South European milk-sheep (Pieragostini and Dario, 1996). Altamurana is a local breed from Apulia (South-eastern Italy) and lives in a rather harsh environment. Gentile di Puglia is an historical triple-purpose Merino-type breed, whose origin may be traced back to the Roman times (Altobella and Muscio, 1996). Until about the mid-1960s, Altamurana and Gentile di Puglia were the most important sheep breeds in southern Italy, numbering about one million head each. During recent decades however, they have undergone a consistent decline in numbers, largely being replaced by the Sarda breed, so that now no more than 5,000 head of each breed remain. The Sarda sheep, a specialized dairy breed native to Sardinia, is rapidly spreading outside of its island of origin, so that its numbers have increased from about 2.5 million head in 1963 to over 5 million in 2000.

The experimental farm in which the present trials were conducted maintains the local breeds with the aim of conservation and sustainable use of animal genetic resources. Sheep were raised using a traditional management system, consisting of lambing in November, suckling for 35–60 days, then regular machine milking of the ewe twice a day. Adult weights of ewes from the three breeds were similar, ranging

between 40 and 45 kg. Milking ewes were allowed to graze on natural pastures and were additionally fed a 250 g pellet concentrate, 150 g oat grains and 1.5 kg oat and vetch hay. The ewes analyzed were all at their second or third lambing. Milk recording was performed three times along the lactation, i.e. the first record at 60–70 days after lambing (at removal of the lamb), the second at 100 days after lambing and the third at 140 days after lambing. Milk samples were collected for analysis of milk quality in accordance with the regulations of the International Committee for Animal Recording (ICAR).

Milk and fatty acid analysis

The following 22 traits were assessed for each animal: daily milk yield, milk fat content, and milk fat composition with regard to the following FA methyl esters (FAME): C4:0; C6:0; C8:0; C10:0; C10:1; C12:0; C14:0; C14:1; C15:0; C15aiso; C15iso; C16:0; C16:1; C17:0; C17:1; C18:0; C18:1; C18:2; C18:3; CLA. FAME were obtained by digesting milk samples (50 ml) with 10 ml of NH_3 (25% v/v) followed by mixing with 40 ml of ethanol (96% v/v). The extraction was then performed with a 100 ml mixture of diethyl ether-pentane (1:1 v/v). The solvent phase was filtered through 25 g of anhydrous Na_2SO_4 and evaporated under a vacuum. This procedure is based on the ISO method (ISO, 2001). The obtained milk fat (100 mg) was diluted in 5 ml of hexane and derivatized as methyl esters by the addition of 0.25 ml KOH 2 N in methanol (ISO, 2002). One milliliter of the upper phase containing the FAME was then diluted with 7 ml of diethyl ether and 2 ml of hexane for on-column injection. Gas chromatography analysis of the FAME was performed using a HP6890 device (Agilent Technologies, Palo Alto, CA) and a DB23 low bleed (J&W, Agilent Technologies, Palo Alto, CA) capillary column (30 m length, 0.32 mm I.D., 0.25 µm film thickness). On-column injections were adopted and hydrogen (1 ml/min) was used as carrier gas. The temperature program was as follows: 40°C for 3 min, 25°C/min up to 120°C for 1 min, 4°C/min up to 162°C for 2 min, 8°C/min up to 220°C for 3 min. An FID detector was used and held at 250°C. The characteristics of the capillary column did not allow the trans fatty acids to be separated. As a consequence the peak indicated as C18:1 includes trans-11, together with cis-9 and cis-7 isomers.

128

129 ***Genes, genotyping, in silico analysis***

130 Sixteen candidate genes were selected for their potential impact upon milk fat quality based on
131 their previous functional descriptions (Marchitelli et al., 2007; Pariset et al., 2006; Williams et al., 2009)
132 and involvement at various level in lipid metabolism, i.e. as regulators, receptors and encoding enzymes
133 involved in fatty acid biosynthesis and desaturation. The first group of genes encoded enzymes of the
134 somatotrophic axis, which consists of growth hormone releasing hormone (**GHRHR**), growth hormone
135 receptor (**GHR**), and insulin-like growth factor I (**IGF1**), all of which play a key role in the metabolism
136 and physiology of mammalian growth (Akers, 2002). *GHR* is considered to be a strong positional and
137 functional candidate gene in cattle (Blott et al., 2003; Viitala et al., 2006) due to its position in a QTL
138 affecting milk production traits that segregate on bovine chromosome 20 (Georges et al., 1995).
139 DGAT1, DGAT2) and FASN are directly involved in triglyceride synthesis. DGAT1 and DGAT2
140 mediate the final and only committed step in the synthesis of triglyceride from acyl CoA and
141 diacylglycerol (Cases et al., 2001). The *DGAT1* gene has been extensively studied in cattle because of its
142 large influence on milk fat percentage and some authors have provided evidence that a variant of this
143 gene, K232A, correlates with an increase in milk yield (Thaller et al., 2003) and CLA content (Schennink
144 et al., 2008). Ovine *DGAT1* has now been fully sequenced by Scatà et al. (2009) who detected two novel
145 SNP associated with milk fat content. The *FASN* gene encodes an enzyme that catalyzes the de novo
146 synthesis of FA in cells and several authors have reported the effects of this gene on fat-related traits in
147 muscle and on milk in cattle (Di Stasio et al. 2009; Morris et al. 2007; Roy et al., 2006).

148 Moreover, enzymes involved in liver metabolism and lipolysis were also considered. This choice
149 was based on the higher energy needs of the liver for gluconeogenesis during lactation, which is further
150 increased by the enhanced lipolysis in adipose tissue and by the increased blood flow to the liver
151 (Vernon, 2005). Propionyl coenzyme A carboxylase, beta polypeptide (**PCCB**), a crucial enzyme in
152 gluconeogenesis in the liver in cows (Murondoti et al., 2004); lecithin-cholesterol acyltransferase
153 (**LCAT**) which is synthesized in the liver and circulates in the blood plasma as a complex with

components of high density lipoprotein (HDL) (Jonas, 2000); LPL, which regulates the hydrolysis of circulating triglycerides and the uptake of fatty acids by most tissues, including the mammary glands and adipose tissue (Eckel, 1989); hormone-sensitive lipase (**LIPE**) which has a vital role in the mobilization of FFA from adipose tissue and regulates energy homeostasis by catalyzing the rate-limiting step in adipose tissue lipolysis (Holm et al., 1988); AMP-activated protein kinase (**PRKAA**) which monitors the intracellular energy status and regulates the uptake and metabolism of glucose and FA as well as their synthesis and oxidation, and also the levels of cholesterol, glycogen, and proteins to meet energy demand (McFadden and Corl, 2009); Alpha-1-antichymotrypsin **(SERPINA3**), a plasma protease inhibitor synthesized in the liver and for which a deficiency is associated with liver disease in humans (Eriksson, 1986).

In addition, the following genes were considered: the integrin ITGB1, a cell adhesion receptor which plays an important role in mammary cell function by inhibiting differentiation (Streuli et al., 1991); the zona pellucida (**ZP**), an extracellular matrix surrounding the oocyte and the early embryo which binds to P47, a protein which shows homology to a family of mammalian secretory proteins (Ensslin et al.,1998), in particular to the mouse milk fat globule protein; tyrosinase related protein 1 (**TYRP1**), a melanogenic enzyme in both cattle and sheep (Deng, 2007) on the basis that selections for milk related traits have often been performed in parallel to selections for coat color. Moreover, *TYRP1* gene maps to Ovis chromosome 2 (Beraldi et al., 2006), a homolog of bovine chromosomes 2 and 8 on which the location of QTL for milk traits has been reported (Ashwell et al., 1997). Finally, myostatin (**GDF8**) which plays a role in cell fate decisions by inducing a differentiation bias toward the adipocyte lineage and away from the muscle lineage (Feldman et al., 2006), and the callipyge gene (***MEG3***), which is associated with muscle hypertrophy and feed intake (Cockett et al., 1994), were also considered.

A total of 41 SNP were chosen for analysis in this study, regardless of their location either in the coding sequence or within the intronic and UTR regions. The locations of the SNP in each gene, their position relative to their GenBank accession number and the amino acid changes, if present, are listed in Table 1. To perform the analysis, genomic DNA was extracted from blood samples using a Genomix kit

(Talent Srl, Trieste, Italy), according to the manufacturer's instructions and all 41 SNP were genotyped at KBioscience Ltd. using their own novel fluorescence-based competitive allele specific PCR (KASPar) assay. Details of the method used can be found on the web at (http://www.kbioscience.co.uk/). For genes that contained more than one SNP, haplotypes were constructed using Arlequin software (Excoffier et al., 2005). Each haplotype was then treated as an allele in a similar manner to the SNP allele and as described previously in Barendse et al. (2008) and Signorelli et al. (2009).

To verify whether any mutations in the 5'UTR of the 16 candidate genes occurred within the putative binding sites for transcription factors, in silico analysis was performed, using MatInspector software (Cartharius et al., 2005, http://www.genomatix.de/). To ascertain if mutations that cause non-synonymous amino acid changes were deleterious to the encoded protein, in silico analyses were performed using different software platforms. These included PolyPhen, a tool which predicts the possible impact of an amino acid substitution on the structure and function of human proteins using straightforward physical and comparative methods (Ramensky et al., 2001, http://genetics.bwh.harvard.edu/pph/); SIFT which predicts whether an amino acid substitution affects protein function, based on sequence homology and on the physical properties of amino acids (Ng and Henikoff, 2003, http://sift.jcvi.org/www/SIFT_seq_submit2.html); I-Mutant 2.0, a predictor of protein stability changes based on single point mutations from the protein sequence and structure (Capriotti et al., 2005, http://gpcr2.biocomp.unibo.it/~emidio/I-Mutant/I-Mutant.htm); and SNAP, a neural-network based method that uses in silico derived protein information to predict the functionality of mutated proteins (Bromberg and Rost, 2007, http://cubic.bioc.columbia.edu/ services/ SNAP/).

Statistical Analysis

For each trait, residuals for each individual were estimated using a Linear Mixed Model, including the breed as a fixed effect and the distance from lambing as a covariate, to take into account the variations resulting from the lactation stage, and the random effects. To estimate the allele substitution effect of each gene on the traits under analysis, the copy number of each allele – either the SNP or the

haplotype – were regressed against the individual residuals of the 22 traits, in a similar manner to the method of Barendse et al. (2008), using a Linear Mixed Model in SAS (SAS Institute Inc. 2007), as described in Sherman et al. (2008). A preliminary single locus association analysis was performed, so to exclude, for each trait, the genes that had no influence ($P<0.05$). Using the same model, a final association analysis was undertaken including, for each trait, all the possible allele combinations of the genes not excluded from the preliminary analysis. The substitution effect of each allele, for each gene, was then obtained by averaging the effects of that allele in all tested combinations, where the number of combinations depended on both the number of genes included in the model for that trait, and the number of alleles of those genes. All genes were considered in the same model because recent reports have suggested that single locus effects cannot explain complex multifactorial traits (Kardia et al., 2008; Manuguerra et al., 2007). This was also done because of the sparseness of data in high dimensions, which often occurs when data are available for a large number of SNP for a relatively small number of samples, as in the present trial, possibly leading to false-positive effects (Mechanic et al., 2008).

RESULTS AND DISCUSSION

Allelic frequencies and analysis of traits

A group of 41 SNP within in 16 candidate genes were analyzed in this study for 94 animals in total. The following SNP were monomorphic in all breed samples: *FASN* g.56G>A; *GHRHR* g.73G>A and g.205T>C; *GDF8* g.4312G>A and g.4319A>C, and were therefore excluded from subsequent haplotype building and association analysis. The allelic frequencies of either the haplotypes (when the gene contained more than one SNP) or single SNP for each breed and overall, are reported in Table 2. In 11 of the 16 genes that had at least one polymorphic allele, the most frequent allele was found to be the same for the three breeds. In the case of the other genes, the most frequent allele was the same in the Sarda and Gentile di Puglia breeds at *GHR* and *GHRHR*, and in the Sarda and Altamurana breeds at *CLLPG*. Different haplotype frequencies between the three breeds were evident at *IGF1* and *LIPE*. The

231 Sarda breed was monomorphic at the *FASN* and *IGTB1* genes and the Gentile di Puglia and Altamurana
232 were so at the *GDF8* and *DGAT2* genes, respectively.
233 Milk yields and the 21 traits denoting milk fat quality were analyzed in the three sheep breeds and
234 mean values for each trait, each breed and overall are reported in Table 3. Daily milk yields from the
235 Altamurana and Gentile di Puglia breeds was found to be about 60% and 30% less than the Sarda yield,
236 whereas fat content is about 2% higher.
237
238 ***Association analysis between alleles and milk traits***
239 The main objective of the present study was to evaluate if and how some SNP located within lipid
240 metabolism genes affect milk fat composition in sheep. The three different breeds of sheep, chosen for
241 analysis in this study, are commonly and regularly milked but differ strongly in terms of milk yield and
242 quality. A core assumption of the approach was that, if one gene has some effects on one particular trait,
243 this should be evident in all breeds. Animals from the same flock were preferentially selected, so to
244 control feeding and management systems, given that the diet can be an important source of variation in
245 milk yield and quality. Based on the assumption that any gene effects should be made evident at any
246 lactation stage, milk composition analyses were performed at different lactation stages for each animal.
247 Table 4 lists the genes that showed a significant ($P<0.05$) effect on the traits selected by
248 preliminary single-locus analysis. Table 5 lists the results of the final association analysis between genes
249 and traits in the form of the average allelic substitution effect of each allele, with the corresponding
250 minimum and maximum values, and the number of allelic combinations in which the allele was tested.
251 The number of combinations depends on the number of genes and alleles considered in the model (Table
252 4). To avoid the likelihood of false-positive associations, the results in Table 5 include only those alleles
253 that showed a significant ($P<0.05$) effect in all tested combinations, and include also the minimum and
254 maximum values of the statistical probability of the effect. However, because of the relatively low
255 sample size, all of these data should be considered to be preliminary results only.
256 ***DGAT2***

Haplotype substitution effects of the *DGAT2* gene were evaluated only in the Sarda breed, being Altamurana monomorphic for haplotype [A;G;T], whereas haplotype [C;A;C] was found only in the Gentile di Puglia breed, but at a frequency (0.03) below the threshold that would allow association analysis. In the Sarda breed, the haplotypes [C;A;T] and [A;G;T] showed significant opposite effects upon the CLA content (Table 5), with sheep carriers of [A;G;T] producing milk that is richer in CLA (0.09%), whilst those carrying [C;A;T] showing significantly decreased levels of CLA (-0.21%), C18:2 (-0.10%), and milk yield (-78.39 g/d). Sørensen et al. (2008) previously performed an in vitro study on *DGAT2* activity and found that variations in triglyceride production in bovine milk were not due to the presence of different gene transcripts, but likely involved events beyond transcription such as post-translational and/or enzyme activity effects. The current results are consistent with this finding as the analyzed haplotype encompasses a DNA region of 135 bp in the 3'UTR of the gene, and could therefore affect post-transcriptional regulation mechanisms.

FASN

Because the first of the two SNP (g.56G>A) was monomorphic in the three analyzed breeds, only SNP g.257C>T was considered for further assessment. This SNP showed a positive substitution effect of allele T on medium chain FA (0.45% on C10:0; 0.01% on C10:1; 0.28% on C12: 0 and 0.35% on C14:0). It was further found that the Sarda breed was monomorphic for allele C. In this regard, given that Signorelli et al. (2008) showed that this breed has a lower medium chain FA content compared with the Altamurana and Gentile di Puglia breeds, it could be hypothesized that the T allele is responsible, at least in part, for the higher content of these FA. The current results are consistent with the function of the enzyme encoded by *FASN* gene, which is to catalyze the de novo synthesis of short and medium-chain FA in cells. However, it is not yet possible to ascribe a direct contribution to the observed effects as although the SNP is located in the coding region of the gene (exon 31), it produces a synonymous mutation.

GHR

The *GHR* SNP (g.121G>A) encodes a non-synonymous mutation causing the amino acid change Asp to Asn. Allele G is associated with a decreased milk fat content (-0.14%) but increased butyric FA levels (0.10%). In contrast, allele A has the opposite effect on these two traits. Allele G also has a negative effect upon the stearic FA levels (-0.24%). The results obtained from the analyses using the Polyphen, Sift, I-mutant and SNAP software indicated that this amino acid change does not affect protein stability. The g.121G>A polymorphism is located in exon 10 which encodes part of the intracellular domain of the GHR protein and may therefore affect the activation of other intracellular signaling components that have yet to be elucidated.

The significant allelic substitution effect of SNP (g.121G>A) upon the butyric and stearic FA content of the sheep milk is consistent with a role for *GHR* in determining the milk fat profile in sheep. The allelic frequency of SNP (g.121G>A) in the three breeds tested also supports this hypothesis as the highest frequency of allele A (>60%) is found in the Gentile di Puglia breed, which is the highest fat-yielding breed among the three under study and also in the Sarda breed which is typically low milk fat yielding breed but the only one, among those of the present trial, that has been selected for the increase of milk and fat yields.

IGF1

The *IGF1* haplotype [C;C] contributes to a decreased CLA (-0.12%) content in sheep milk whereas haplotype [C;T] is associated with an increased level of alfa-linolenic PUFA (0.07% for C18:3). In addition, whereas SNP (g.271C>T) encodes a synonymous mutation in exon 3, the g.855G>C SNP is located within the 5' UTR of the *IGF1* gene. A number of studies have reported that the ovine *IGF1* gene is differentially spliced (Dickson et al., 1991; Ohlsen et al.,1993; Pell et al.,1993) and there is evidence for an additional exon, referred to as exon 1W (Pell et al., 1993), upstream of exon 1. The g.855G>C SNP is located upstream of exon 1W and is therefore in a putative promoter region. In silico analysis of putative transcription factor binding sites revealed that this SNP falls within the core consensus binding sequence (acgaggGGTCatcccagcgccgtct) for the retinoid X receptor heterodimers (RXR), members of a superfamily of nuclear factors that includes VDR (vitamin D receptor). RXR and VDR form a

heterodimeric complex and bind cooperatively to vitamin D responsive elements (VDREs) to activate or repress the transcription of a multitude of genes that regulate a variety of physiological functions (Lemon et al., 1997). In cattle, Maj et al. (2008) have uncovered an SNP in the 5'UTR of the *IGF1* gene and reported a significant association between the *IGF1* genotype and the IGF1 blood levels.

LCAT

The haplotype [T;A;T] of the *LCAT* gene has a negative effect upon the C18:2 levels (-0.06%) whilst haplotype [C;G;C] positively influences milk yield (40.02 g/d). This [C;G;C] haplotype encompasses an 895 bp region from intron 2 to exon 6 and SNP g.806G>A, in exon 6, encodes a non-synonymous mutation (Asp to Asn). The results of the Polyphen, Sift, and I-mutant analysis indicated that this amino acid change produces a protein with reduced stability and may also have damaging effects on the function of the protein; it can then be inferred that the negative effect, on C18:2, of the haplotype [T;A;T], that contains the mutated allele g.806G>A, may be due to the less functional protein encoded by this allele. In contrast, an increase in milk yield was evident for the haplotype containing the wild type allele.

LPL

The haplotype of *LPL* encompasses a 60 bp region in the 3'UTR; haplotype [T;T;A] showed a positive effect upon the CLA content in the milk (0.09%), consistent with the function of the enzyme encoded by *LPL*, which is critical for the uptake and secretion of the long-chain FA in milk and also for the assimilation of a high-fat milk diet by suckling young (Iverson, 1995).

PCCB

Haplotype [T;T;T;A] of *PCCB* gene has a positive effect on milk nutraceutical properties, by contributing to an increase of all PUFAs (0.10% for C18:2; 0.03% for C18:3 and 0.12% for CLA), while decreasing the content of palmitic FA (-0.73%). It is then possible to infer that different genotypes have a different behavior in balancing TAG synthesis with milk secretion.

SERPINA3

Haplotype [T;A] of the *SERPINA3* gene is associated with a lower milk yield (-59.11 g/d), a lower C16:1 content (-0.08%) and lower PUFA levels (-0.05%; -0.03%; -0.13% respectively for C18:2, C18:3 and CLA). On the other hand, haplotype [T;G] correlates with a higher sheep milk yield (43.38 g/d) and lower C4:0 FA level (-0.15%). Because the enzyme encoded by *SERPINA3* plays a role in the complex interactions between liver endocrine changes associated with lactogenesis (Eriksson, 1986), it is possible to speculate that the analyzed haplotype might be responsible for the variability in the unsaturated FA levels in sheep milk.

TYRP1

A negative allelic substitution effect of haplotype [C;C] on short-chain FA (-0.05% on C6:0 and -0.04% on C8:0) and of haplotype [T;T] on the C15:0 (-0.03%) and relevant isomer levels was observed. These haplotypes encompasses exon 1 of the *TYRP1* gene. The first SNP analyzed in this gene (g.102C>T) is a synonymous mutation whereas the second (g.215T>C) causes a valine to alanine change. Results of the Polyphen, Sift, I-mutant, and SNAP in silico analyses indicated that this amino acid change reduces protein stability and possibly damages protein function. The *TYRP* genes encode melanogenic enzymes, but the frequency of *TYRP1* haplotypes is similar in the three white-fleece breeds analyzed herein; this consideration allows the hypothesis that *TYRP1* might be in linkage disequilibrium with alleles of other genes involved in milk fat synthesis in sheep, although such genes have yet to be identified.

ZP2

The *ZP2* gene haplotypes [G;T] and [G;C] seemed to positively influence milk nutraceutical quality, whereas the haplotype [A;C] had the opposite effect. In addition, haplotype [G;T] negatively affects the fat (-0.21%) and C16:0 contents (-0.56%) in sheep milk whilst haplotype [G;C] negatively affects the medium chain saturated FA levels therein (-0.46% for C10:0 and -0.32% for C14:0) while increasing the oleic FA concentration (0.16%). In contrast, an increase in the fat (0.19%) and C16:0 content (0.46%), together with a decrease in the CLA (-0.11%) and C17:1 (-0.005%) levels were found to be associated with haplotype [A;C].

CONCLUSIONS

A global overview of the results of the current study indicates that four genes (*SERPINA3*, *DGAT2*, *PCCB*, *IGF1*) play a role in the desaturation of stearic FA into PUFA. Long chain FA have their origins in the breakdown in blood lipids derived from VLDL, which are synthesized in the intestine or liver. The *SERPINA3* and *PCCB* genes are directly involved in liver metabolism, whilst the activity of the *DGAT2* gene product catalyzes the final step in triglyceride synthesis and *IGF1* has an essential role in mammary function. Some authors (Van den Top et al., 2005) have reported a combined action of the LPL and LCAT enzymes to channel FA away from adipose tissue and into the udder during the post partum period. The present data reveal that the *LPL* and *LCAT* genes influence the CLA and PUFA levels, respectively, in sheep milk.

Both the G and A alleles of the *GHR* gene, in addition to the [G;T] and [A;C] haplotypes of the *ZP2* gene, affect the variability of the milk fat content with opposite effects on this trait. *GHR* also influences the levels of saturated FA of differing chain length (C4:0 and C18:0) whilst *ZP2* influences the medium and long-chain saturated and unsaturated FA content. However, whereas the *GHR* effects are consistent with the essential role this gene plays in mammary function, further investigations are needed to properly elucidate the biological functions of the *ZP2* gene. The current data also confirm the role of the *FASN* gene in affecting the medium-chain FA, i.e. FA that mostly originate from de novo synthesis. Signorelli et al. (2008) have previously analyzed milk fat composition of the same breeds and regarded the effect of the breed as a global genetic effect explaining the variation in the FA levels. These authors found significant differences between breeds in the amount of highly saturated FA, either short, medium or long-chain, but did not find any differences in the CLA and PUFA levels between breeds. It would thus have been expected that the two genes that influence saturated FA (*FASN*, *ZP2*) would have a different frequency in the three sheep breeds analyzed here, but this was found not to be the case. There are thus likely to be further genes that affect these traits. On the other hand, the haplotypes of the *SERPINA3*, *DGAT2*, and *PCCB* genes, that play a role in the desaturation of stearic FA into PUFA, have

a similar frequency in the three breeds of sheep. This may indicate why Signorelli et al. (2008) did not find any difference between breeds for CLA and PUFA, and this would therefore corroborate the findings on the allelic substitution effects of these genes. The haplotypes [T;A] and [C;A;T] of the *SERPINA3* and *DGAT2* genes, respectively, have been associated with lower milk yield, and they are less frequent in the three sheep breeds here assessed. The more frequent [C;G;C] haplotype of the *LCAT* gene positively influences milk yield and it seems therefore that some kind of selection practices were performed in the past by shepherds to increase milk production even in the absence of any official programs.

The findings of the current study present a number of DNA polymorphisms within genes in sheep involved at various levels in lipid metabolism that might contribute to an increased understanding of the genetic basis of milk fat components. These data have the potential to advance future direct selection of specific sheep milk products but, as the analyzed sample size was relatively low, these findings need to be further confirmed in future studies of independent dairy sheep populations.

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591

592 Table 1. Genes and SNPs analyzed in this study

Gene	Gene symbol	Accession	SNP position in the accession	Location	AA change
Diacylglycerol O-acyltransferase homolog 2	*DGAT2*	GQ150553	g.106C>A	3' UTR	
			g.107A>G	3' UTR	
			g.240 C>T	3' UTR	
Fatty acid synthetase	*FASN*	GQ150557	g.56 G>A	intron 31	
			g257 C>T	exon 32	
Myostatin	*GDF8*	AY032689	g.312A>G	intron 3	
			g.319C>A	exon 3	
			g.2156C>T	exon 3	
Growth hormone receptor	*GHR*	AY292283	g.121A>G	exon 10	D523N
Growth hormone releasing hormone receptor	*GHRHR*	AY292289	g.73G>A	intron 10	
			g.205T>C	intron 10	
			g.339G>T	intron 10	
Insulin-like growth factor 1	*IGF1*	X69472	g.855G>C	5' UTR	
		AY737509	g.211C>T	exon 3	
Integrin B1	*ITGB1*	AY787745	g.203C>T	intron 8	
			g.213G>T	intron 8	
Lecithin-cholesterol acyltransferase	*LCAT*	GQ150556	g.181 T>C	intron 2	
			g.806 G>A	exon 6	D101N
			g.1075 T>C	exon 6	
Hormone-sensitive lipase variant B	*LIPE*	DQ647326.1	g.174G>A	exon 1	
			g.291G>C	exon 1	
			g.1891G>A	exon 9	
Lipoprotein lipase	*LPL*	GQ150554	g.74T>C	3' UTR	
			g.130T>C	3' UTR	
			g.133T>A	3' UTR	
Callipyge	*MEG3*	AY017222	g.379G>T	3' UTR	
Propionyl Coenzyme A carboxylase, beta polypeptide	*PCCB*	GQ150555	g.325T>C	intron 11	
			g.368C>T	esone12	
			g.371C>T	esone12	
			g.478T>C	intron 12	
AMP-activated protein kinase alpha2 subunit	*PRKAA2*	EU131097	g 382C>T	exon 4	
Alpha-1-antichymotrypsin 2	*SERPINA3*	DQ383805	g.120C>T	exon 2	
			g.134 A>G	exon 2	
Tyrosinase related protein 1	*TYRP1*	AY737511	g.102C>T	exon 1	
			g.216C>T	exon 1	V68A
Zona pellucida glycoprotein 2	*ZP2*	DQ383806	g.82A>G	intron 8	
			g.105C>T	intron 8	

593

594

595 Table 2. Detected SNP and frequency of the mutated allele in each breed

Gene	Alleles/Haplotype	Frequency			
		Altamurana	Gentile di Puglia	Sarda	overall
DGAT2	[A;G;T]	1	0.97	0.90	0.95
DGAT2	[C;A;T]	0	0	0.10	0.05
DGAT2	[C;A;C]	0	0.03	0	0
FASN	C	0.93	0.93	1	0.95
FASN	T	0.07	0.07	0	0.05
GDF8	C	0.76	1	0.90	0.87
GDF8	T	0.24	0	0.10	0.13
GHR	A	0.33	0.64	0.61	0.51
GHR	G	0.67	0.36	0.39	0.49
GHRHR	T	0.22	0.64	0.56	0.45
GHRHR	G	0.78	0.36	0.44	0.55
IGF1	[C;T]	0.03	0	0.06	0.03
IGF1	[G;T]	0.29	0.71	0.44	0.45
IGF1	[G;C]	0.56	0.12	0.41	0.39
IGF1	[C;C]	0.12	0.17	0.09	0.13
ITGB1	[C;G]	0.94	0.71	1	0.90
ITGB1	[C;T]	0.06	0	0	0.02
ITGB1	[T;T]	0	0.29	0	0.08
LCAT	[C;G;C]	0.99	0.92	0.88	0.93
LCAT	[T;A;T]	0	0.08	0.12	0.07
LCAT	[C;A;T]	0.01	0.01	0	0
LIPE	[A;G;A]	0.13	0.01	0.06	0.07
LIPE	[G;C;A]	0.13	0.14	0	0.09
LIPE	[G;C;G]	0.19	0.47	0.23	0.28
LIPE	[G;G;A]	0.32	0.08	0.12	0.19
LIPE	[G;G;G]	0.22	0.30	0.59	0.37
LPL	[C;C;A]	0.76	0.70	0.92	0.80
LPL	[T;T;A]	0.19	0.27	0.08	0.18
LPL	[T;T;T]	0.03	0.03	0	0.02
LPL	[T;C;A]	0.02	0	0	0
MEG3	G	0.26	0.53	0.25	0.33
MEG3	T	0.74	0.47	0.75	0.67
PCCB	[T;T;TG]	0.47	0.52	0.59	0.53
PCCB	[C;C;C;G]	0.43	0.31	0.38	0.38
PCCB	[T;T;T;A]	0.10	0.17	0.03	0.09
PRKAA2	C	0.70	0.97	0.85	0.82
PRKAA2	T	0.30	0.03	0.15	0.18
SERPINA3	[C;G]	0.98	0.64	0.58	0.74
SERPINA3	[T;A]	0	0.14	0.15	0.09
SERPINA3	[T;G]	0.02	0.22	0.28	0.17
TYRP1	[T;C]	0.56	0.70	0.41	0.54
TYRP1	[C;C]	0.29	0.10	0.36	0.26
TYRP1	[T;T]	0.15	0.20	0.25	0.20
ZP2	[G;T]	0.13	0.14	0.11	0.12
ZP2	[A;C]	0.87	0.72	0.83	0.82
ZP2	[G;C]	0	0.14	0.06	0.06

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597 Table 3. Mean values and standard deviations for each analyzed trait in sheep milk

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Sheep breed	Altamurana		Gentile di Puglia		Sarda		Overall	
Animals	36		24		34		94	
	mean	SD	mean	SD	mean	SD	mean	SD
Milk yield (cl)	392.55	128.70	196.92	93.99	534.95	156.062	396.12	184.41
Fat %	9.842	1.217	9.523	1.581	7.370	0.864	8.870	1.653
C4:0	4.618	0.802	4.181	0.6373	4.993	0.701	4.647	0.786
C6:0	2.863	0.471	2.830	0.531	2.727	0.417	2.806	0.467
C8:0	2.374	0.459	2.523	0.608	2.219	0.502	2.355	0.521
C10:0	6.261	1.535	6.813	1.832	5.696	1.620	6.192	1.680
C10:1	0.230	0.059	0.258	0.0823	0.200	0.045	0.226	0.064
C12:0	3.798	0.919	3.881	0.949	3.562	0.839	3.733	0.898
C14:0	10.43	1.348	9.959	1.087	9.331	0.941	9.918	1.236
C14:1	0.268	0.062	0.287	0.102	0.208	0.038	0.2514	0.074
C15:0	0.885	0.105	0.916	0.214	1.000	0.127	0.934	0.153
C15iso	0.309	0.066	0.351	0.063	0.298	0.057	0.316	0.065
C15aiso	0.492	0.079	0.536	0.128	0.524	0.089	0.515	0.097
C16:0	26.117	2.523	23.841	1.650	23.408	1.433	24.579	2.325
C16:1	1.294	0.221	1.341	0.351	1.130	0.201	1.246	0.266
C17:0	0.542	0.067	0.549	0.146	0.538	0.088	0.542	0.098
C17:1	0.307	0.061	0.327	0.067	0.294	0.066	0.307	0.065
C18:0	9.529	1.917	10.040	2.053	9.714	2.237	9.720	2.058
C18:1	23.185	3.599	24.925	2.894	25.544	2.829	24.464	3.311
C18:2	2.975	0.613	2.808	0.678	4.045	0.930	3.321	0.930
C18:3	0.572	0.134	0.5227	0.191	0.803	0.206	0.643	0.213
CLA(cis9-trans11)	1.336	0.544	1.4776	0.718	1.925	0.662	1.584	0.680

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609 Table 4. Genes that showed a significant (P<0.05) effect on each trait in the preliminary single-locus
610 analysis.

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Trait	Genes	Allele number
Milk yield	*SERPINA 3*	3
	LCAT	2
	DGAT2	2
	LIPE	5
Fat %	*GHR*	2
	ZP2	3
	ITGB1	3
	IGF1	4
C4:0	*GHR*	2
	SERPINA 3	3
	GHRHR	2
C6:0	*TYRP1*	3
C8:0	*TYRP1*	3
	ZP2	3
C10:0	*FASN*	2
	ZP2	3
C10:1	*TYRP1*	3
	FASN	2
C12:0	*FASN*	2
	PRKAA2	2
C14:0	*FASN*	2
	ZP2	3
	PRKAA2	2
C14:1	-	-
C15:0	*FASN*	2
	ZP2	3
	TYRP1	3
	PCCB	3
	GHR	2
C15aiso	*FASN*	2
	ZP2	3
	TYRP1	3
	PCCB	3
C15iso	*TYRP1*	3
	PCCB	3
C16:0	*ZP2*	3
	PCCB	3
C16:1	*SERPINA 3*	3
	LIPE	5
C17:0	*FASN*	2
	ZP2	3
	TYRP1	3
	PCCB	3

C17:1	*FASN*	2
	ZP2	3
	DGAT2	2
	ITGB1	3
C18:0	*GHR*	2
	ITGB1	3
	SERPINA 3	3
C18:1	*ZP2*	3
	TYRP1	3
C18:2	*GHR*	2
	LCAT	2
	DGAT2	2
	PRKAA2	2
	PCCB	3
	SERPINA 3	3
C18:3	*IGF1*	4
	LCAT	2
	PCCB	3
	SERPINA 3	3
CLA	*DGAT2*	2
	ZP2	3
	LPL	2
	PCCB	3
	SERPINA 3	3
	IGF1	3

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627 Table 5. Genes and alleles for which a substitution effect had a significant effect upon the indicated trait

			Effect					*P*<		
Trait	Gene	Allele	No*	mean	Se	min	max	mean	min	max
Milk yield (g/d)	*DGAT2*	[C;A;T]	30	-78.39	9.53	-93.94	-61.61	0.03	0.02	0.07
	LCAT	[C;G;C]	30	40.02	5.82	31.55	52.97	0.02	0.001	0.04
	SERPINA3	[T;A]	20	-59.11	20.23	-74.02	-47.70	0.006	0.0008	0.02
	SERPINA3	[T;G]	20	43.38	15.10	39.99	46.34	0.005	0.003	0.007
Fat %	*GHR*	A	36	0.13	0.05	0.12	0.16	0.009	0.002	0.02
	GHR	G	36	-0.14	0.05	-0.15	-0.12	0.009	0.002	0.02
	ZP	[G;T]	24	-0.21	0.08	-0.25	-0.19	0.008	0.002	0.01
	ZP	[A;C]	24	0.19	0.06	0.16	0.21	0.005	0.001	0.002
C4:0	*GHR*	A	6	-0.09	0.04	-0.12	-0.09	0.03	0.005	0.04
	GHR	G	6	0.10	0.04	0.09	0.12	0.02	0.003	0.03
	SERPINA3	[T;G]	4	-0.15	0.06	-0.18	-0.12	0.02	0.004	0.03
C6:0	*TYRP1*	[C;C]	1	-0.05	0.02			0.02		
C8:0	*TYRP1*	[C;C]	3	-0.04	0.02	-0.04	-0.04	0.05	0.05	0.05
C10:0	*FASN*	T	3	0.45	0.23	0.44	0.46	0.05	0.05	0.06
	ZP	[G;C]	2	-0.46	0.20	-0.46	-0.45	0.03	0.03	0.03
C10:1	*FASN*	T	3	0.01	0.004	0.008	0.01	0.01	0.004	0.02
C12:0	*FASN*	T	2	0.28	0.14	0.28	0.29	0.05	0.04	0.05
C14:0	*FASN*	T	2	0.35	0.16	0.034	0.036	0.03	0.02	0.03
	ZP	[G;C]	3	-0.32	0.14	-0.32	-0.32	0.02	0.02	0.02
C14:1	-									
C15:0	*PCCB*	[T;T;T;A]	36	0.05	0.02	0.04	0.06	0.01	0.002	0.03
	TYRP1	[T;T]	36	-0.03	0.01	-0.04	-0.02	0.02	0.003	0.07
	ZP	[G;T]	36	0.05	0.02	0.04	0.06	0.004	0.0004	0.02
	ZP	[A;C]	36	-0.04	0.01	-0.04	-0.02	0.02	0.001	0.08
C15aiso	*PCCB*	[T;T;T;A]	18	0.04	0.02	0.04	0.04	0.02	0.01	0.03
	TYRP1	[T;T]	18	-0.02	00.01	-0.03	-0.02	0.04	0.01	0.07
	ZP	[G;T]	18	0.04	0.01	0.03	0.04	0.01	0.004	0.04
C15iso	*PCCB*	[T;T;T;A]	3	0.01	0.006	0.01	0.01	0.03	0.02	0.04
	TYRP1	[T;T]	3	-0.01	0.004	-0.01	-0.01	0.05	0.04	0.05
	TYRP1	[C;C]	3	0.01	0.01	0.01	0.03	0.02	0.04	
C16:0	*PCCB*	[T;T;T;A]	3	-0.73	0.33	-0.77	-0.70	0.03	0.02	0.04
	ZP	[A;C]	3	0.46	0.22	0.43	0.50	0.04	0.02	0.05
	ZP	[G;T]	3	-0.56	0.29	-0.59	-0.52	0.05	0.04	0.07
C16:1	*SERPINA3*	[T;A]	5	-0.08	0.03	-0.08	-0.07	0.006	0.004	0.01
C17:0	*PCCB*	[T ;T;T;A]	18	0.02	0.007	0.01	0.02	0.03	0.02	0.05
C17:1	*FASN*	C	18	0.004	0.001	0.003	0.004	0.01	0.004	0.02
	ITGB1	[C;T]	12	0.014	0.004	0.012	0.015	0.004	0.001	0.009
	ZP	[G;T]	12	0.006	0.002	0.005	0.006	0.01	0.006	0.03
	ZP	[A;C]	12	-0.005	0.002	-0.005	-0.004	0.01	0.0009	0.03
C18:0	*GHR*	G	9	-0.24	0.13	-0.29	-0.20	0.07	0.03	0.14
	SERPINA3	[T;A]	6	0.47	0.25	0.42	0.52	0.06	0.04	0.09

C18:1	*ZP*	[G;C]	3	0.16	0.06	0.16	0.17	0.005	0.004	0.005
C18:2	*DGAT2*	[C;A;T]	72	-0.10	0.04	-0.12	-0.07	0.03	0.002	0.08
	GHR	G	72	0.03	0.01	0.02	0.04	0.02	0.002	0.12
	LCAT	[T;A;T]	72	-0.06	0.03	-0.07	-0.05	0.05	0.03	0.12
	PCCB	[T;T;T;A]	48	0.10	0.02	0.09	0.11	0.0001	0.0001	0.0001
	SERPINA3	[T;A]	48	-0.05	0.02	-0.06	-0.04	0.04	0.03	0.10
C18:3	*IGF1*	[C;T]	18	0.07	0.03	0.07	0.08	0.009	0.004	0.02
	PCCB	[T;T;T;A]	24	0.03	0.01	0.03	0.04	0.02	0.005	0.03
	SERPINA3	[T;A]	48	-0.03	0.01	-0.04	-0.03	0.01	0.005	0.04
CLA	*DGAT2*	[C;A;T]	162	-0.21	0.03	-0.20	-0.22	0.01	0.002	0.05
	DGAT2	[A;G;T]	162	0.09	0.01	0.09	0.08	0.03	0.003	0.10
	IGF1	[C;C]	108	-0.12	0.02	-0.13	-0.10	0.03	0.003	0.10
	LPL	[T;T;A]	162	0.09	0.003	0.09	0.09	0.03	0.002	0.13
	PCCB	[T;T;T;A]	108	0.12	0.01	0.12	0.12	0.05	0.005	0.11
	SERPINA3	[T;A]	108	-0.13	0.01	-0.14	-0.12	0.03	0.002	0.13
	ZP	[A;C]	108	-0.11	0.005	-0.11	-0.10	0.007	0.001	0.01

628 [*]Number of combinations that produced the indicated average allelic substitution effect.

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