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

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ABSTRACT

The aim of the present study was to investigate the genetic control of the fatty acid (FA) composition in milk from 3 breeds of sheep: Altamurana, Gentile di Puglia, and Sarda. Single nucleotide polymorphisms 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, which were polymorphic in the 3 sheep breeds, upon the milk fat composition. Four genes (α -1-antichymotrypsin-2; diacylglycerol O-acyltransferase homolog-2; propionyl Coenzyme A carboxylase, β polypeptide; and insulin-like growth factor-I) play a role in the desaturation of stearic FA into polyunsaturated fatty acids. Furthermore, 2 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 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 because it contains higher levels of conjugated linoleic acid than milk from other ruminants. The current study evaluated the global effects of a large number of single nucleotide polymorphisms and haplotypes on traits that are not commonly investigated in sheep but that are potentially very useful for improving milk quality.

Key words: fatty acid, single nucleotide polymorphism, 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 either from the breakdown of blood lipids or via de novo synthesis within the mammary epithelial cells. Between 40 and 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 on dietary lipids and on mobilized fat from body adipose tissue. The 2 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 Looor, 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 conjugated linoleic acid (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; Moiola et al., 2007; Schennink et al., 2008). Recently, genome-wide screens for bovine milk fat composition (Schennink et al., 2009; Stoop et al., 2009) revealed several QTL for short-, medium- and long-chain FA and demonstrated that the QTL for BTA14 and BTA26 are associated with polymorphisms in the *DGAT1* and stearoyl CoA desaturase 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 TS (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 (McSweeney, 2004). The principal flavors and cheesy and lipolyzed aromas of raw ewe milk cheeses

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are derived from short- and medium-chain 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 than cow or goat milk of CLA (Jahreis et al., 1999), an 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 comprising 3 breeds: Altamurana, Gentile di Puglia, and Sarda.

MATERIALS AND METHODS

Animals, Sampling, and Diet

The study was conducted using 94 sheep of 3 breeds: Altamurana ($n = 36$), Gentile di Puglia ($n = 24$), and Sarda ($n = 34$). 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 (southeastern Italy) that lives in a rather harsh environment. Gentile di Puglia is a historical triple-purpose Merino-type breed whose origin may be traced back to the Roman times (Altobella and Muscio, 1995). Until about the mid 1960s, Altamurana and Gentile di Puglia were the most important sheep breeds in southern Italy, numbering about 1 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 more than 5 million in 2000.

The experimental farm on 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 to 60 d, and then regular machine milking of the ewe twice daily. Adult weights of ewes from the 3 breeds were similar, ranging between 40 and 45 kg. Milking ewes were allowed to graze on natural pastures and were additionally fed 250 g of pellet concentrate, 150 g of oat grains, and 1.5 kg of oat and vetch hay. The ewes analyzed were all at their second or third lambing. Milk recording was performed 3 times during the lactation [i.e., the first record at 60–70 d after lambing (at removal of the lamb), the second at 100 d after lambing, and the third at 140 d after lambing]. Milk samples were collected for analysis of milk quality

in accordance with the regulations of the International Committee for Animal Recording (Rome, Italy).

Milk and FA 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, C15 *aiso*, C15 *iso*, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, and CLA. The FAME were obtained by digesting milk samples (50 mL) with 10 mL of NH_3 (25% vol/vol) followed by mixing with 40 mL of ethanol (96% vol/vol). The extraction was then performed with a 100-mL mixture of diethyl ether-pentane (1:1 vol/vol). 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 (2001) method. 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 of 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 an HP6890 device (Agilent Technologies, Palo Alto, CA) and a DB23 low bleed capillary column (30 m length, 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies). 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, and 8°C/min up to 220°C for 3 min. A flame ionization 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.

Genes, Genotyping, and In Silico Analysis

Sixteen candidate genes were selected for their potential effect on milk fat quality based on their previous functional descriptions (Pariset et al., 2006; Marchitelli et al., 2007; Williams et al., 2009) and involvement at various level in lipid metabolism (i.e., as regulators, receptors, and encoding enzymes involved in fatty acid biosynthesis and desaturation). The first group of genes encoded enzymes of the somatotrophic axis, which consists of growth hormone-releasing hormone receptor (**GHRHR**), growth hormone receptor (**GHR**), and IGF1, all of which play a key role in the metabolism and physiology of mammalian growth (Akers, 2002). Growth hormone receptor is considered to be a strong

positional and functional candidate gene in cattle (Blott et al., 2003; Viitala et al., 2006) because of its position in a QTL affecting milk production traits that segregate on bovine chromosome 20 (Georges et al., 1995). Diacylglycerol acyltransferase 1, DGAT2, and FASN are directly involved in triglyceride synthesis; DGAT1 and DGAT2 mediate the final and only committed step in the synthesis of triglyceride from acyl CoA and diacylglycerol (Cases et al., 2001). The *DGAT1* gene has been extensively studied in cattle because of its large influence on milk fat percentage, and some authors have provided evidence that a variant of this gene, K232A, correlates with an increase in milk yield (Thaller et al., 2003) and CLA content (Schennink et al., 2008). Ovine *DGAT1* has now been fully sequenced by Scatà et al. (2009), who detected 2 novel SNP associated with milk fat content. The *FASN* gene encodes an enzyme that catalyzes the de novo synthesis of FA in cells, and several authors have reported the effects of this gene on fat-related traits in muscle and on milk in cattle (Roy et al., 2006; Morris et al., 2007; Di Stasio et al., 2009).

Moreover, enzymes involved in liver metabolism and lipolysis were also considered. This choice was based on the higher energy needs of the liver for gluconeogenesis during lactation, which is further increased by the enhanced lipolysis in adipose tissue and by the increased blood flow to the liver (Vernon, 2005). Enzymes included propionyl coenzyme A carboxylase, β polypeptide (**PCCB**), a crucial enzyme in gluconeogenesis in the liver in cows (Murondoti et al., 2004); lecithin-cholesterol acyltransferase (**LCAT**), which is synthesized in the liver and circulates in the blood plasma as a complex with components of high density lipoprotein (Jonas, 2000); LPL, which regulates the hydrolysis of circulating triglycerides and the uptake of FA 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); and α -1-antichymotrypsin (**SERPINA3**), a plasma protease inhibitor synthesized in the liver and for which a deficiency is associated with liver disease in humans (Eriksson et al., 1986).

In addition, the following genes were considered: integrin B1, a cell adhesion receptor that 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 that binds to P47, a protein that shows homology to a family of mammalian secretory proteins (Ensslin et al., 1998), in particular to the mouse milk fat globule protein; and tyrosinase-related protein 1 (**TYRP1**), a melanogenic enzyme in both cattle and sheep (Deng et al., 2009) 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, also considered were 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).

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 untranslated regions (**UTR**). The locations of the SNP in each gene, their position relative to their GenBank accession number, and the AA 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. (Hoddesdon, UK) using their own novel fluorescence-based competitive allele-specific PCR (KASPar) assay. For genes that contained more than 1 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 whether mutations that cause nonsynonymous AA changes were deleterious to the encoded protein, in silico analyses were performed using different software platforms. These included PolyPhen, a tool that predicts the possible effect of an AA substitution on the structure and function of human proteins using straightforward physical and comparative methods (Ramensky et al., 2002; <http://genetics.bwh.harvard.edu/pph/>); SIFT, which predicts whether an AA substitution affects protein function based on sequence homology and on the physical properties of AA (Ng and Henikoff, 2003; http://sift.jcvi.org/www/SIFT_seq_submit2.html);

Table 1. Genes and SNP analyzed in this study

Gene	Gene symbol	GenBank accession no.	SNP position in the accession	Location ¹	AA change
Diacylglycerol O-acyltransferase homolog 2	<i>DGAT2</i>	GQ150553	g.106C > A g.107A > G g.240C > T	3' UTR 3' UTR 3' UTR	
Fatty acid synthetase	<i>FASN</i>	GQ150557	g.56G > A g.257C > T	Intron 31 Exon 32	
Myostatin	<i>GDF8</i>	AY032689	g.312A > G g.319C > A g.2156C > T	Intron 3 Exon 3 Exon 3	
Growth hormone receptor	<i>GHR</i>	AY292283	g.121A > G	Exon 10	D523N
Growth hormone releasing hormone receptor	<i>GHRHR</i>	AY292289	g.73G > A g.205T > C g.339G > T	Intron 10 Intron 10 Intron 10	
Insulin-like growth factor 1	<i>IGF1</i>	X69472 AY737509	g.855G > C g.211C > T	5' UTR Exon 3	
Integrin B1	<i>ITGB1</i>	AY787745	g.203C > T g.213G > T	Intron 8 Intron 8	
Lecithin-cholesterol acyltransferase	<i>LCAT</i>	GQ150556	g.181 t > C g.806 G > A g.1075 t > C	Intron 2 Exon 6 Exon 6	D101N
Hormone-sensitive lipase variant B	<i>LIPE</i>	DQ647326.1	g.174G > A g.291G > C g.1891G > A	Exon 1 Exon 1 Exon 9	
Lipoprotein lipase	<i>LPL</i>	GQ150554	g.74T > C g.130T > C g.133T > A	3' UTR 3' UTR 3' UTR	
Callipyge	<i>MEG3</i>	AY017222	g.379G > T	3' UTR	
Propionyl Coenzyme A carboxylase, β polypeptide	<i>PCCB</i>	GQ150555	g.325T > C g.368C > T g.371C > T g.478T > C	Intron 11 Esone 12 Esone 12 Intron 12	
AMP-activated protein kinase $\alpha 2$ subunit	<i>PRKAA2</i>	EU131097	g.382C > T	Exon 4	
α -1-antichymotrypsin 2	<i>SERPINA3</i>	DQ383805	g.120C > T g.134A > G	Exon 2 Exon 2	
Tyrosinase related protein 1	<i>TYRP1</i>	AY737511	g.102C > T g.216C > T	Exon 1 Exon 1	V68A
Zona pellucida glycoprotein 2	<i>ZP2</i>	DQ383806	g.82A > G g.105C > T	Intron 8 Intron 8	

¹UTR = untranslated region.

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, 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, 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 (Manuguerra et al., 2007; Kardia et al., 2008). 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

Allelic Frequencies and Analysis of Traits

A group of 41 SNP within 16 candidate genes were analyzed in this study for a total of 94 animals. The following SNP were monomorphic in all breed samples and were therefore excluded from subsequent haplotype building and association analysis: *FASN* g.56G > A, *GHRHR* g.73G > A and g.205T > C, and *GDF8* g.4312G > A and g.4319A > C. The allelic frequencies of either the haplotypes (when the gene contained more than 1 SNP) or single SNP for each breed and overall are reported in Table 2. In 11 of the 16 genes that had at least 1 polymorphic allele, the most frequent allele was found to be the same for the 3 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 the callipyge gene (*MEG3*). Different haplotype frequencies between the 3 breeds were evident at *IGF1* and hormone-sensitive lipase. The Sarda breed was monomorphic at the *FASN* and integrin B1 genes, and the Gentile di Puglia and Altamurana breeds were monomorphic at the *GDF8* and *DGAT2* genes, respectively.

Milk yields and the 21 traits denoting milk fat quality were analyzed in the 3 sheep breeds, and mean values for each trait, each breed, and overall are reported in Table 3. Daily milk yields from the Altamurana and Gentile di Puglia breeds were found to be about 60 and 30% less, respectively, than the milk yield from the Sarda breed, whereas fat content was about 2% higher.

Association Analysis Between Alleles and Milk Traits

The main objective of the present study was to evaluate whether and how some SNP located within lipid metabolism genes affect milk fat composition in sheep. The 3 different breeds of sheep chosen for analysis in this study are commonly and regularly milked but differ strongly in terms of milk yield and quality. A core assumption of the approach was that if one gene has some effects on one particular trait, this should be evident in all breeds. Animals from the same flock were preferentially selected so to control feeding and management systems, given that the diet can be an important source of variation in milk yield and quality.

Based on the assumption that any gene effects should be made evident at any lactation stage, milk composition analyses were performed at different lactation stages for each animal.

Table 4 lists the genes that showed a significant ($P < 0.05$) effect on the traits selected by preliminary single-locus analysis. Table 5 lists the results of the final association analysis between genes and traits in the form of the average allelic substitution effect of each allele, with the corresponding minimum and maximum values and the number of allelic combinations in which the allele was tested. The number of combinations depends on the number of genes and alleles considered in the model (Table 4). To avoid the likelihood of false-positive associations, the results in Table 5 include only those alleles that showed a significant ($P < 0.05$) effect in all tested combinations and also include the minimum and maximum values of the statistical probability of the effect. However, because of the relatively small sample size, all of these data should be considered to be preliminary results only.

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 on the CLA content (Table 5), with sheep carriers of [A;G;T] producing milk that is richer in CLA (0.09%) and with 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 attributable to the presence of different gene transcripts but rather likely involved events beyond transcription such as posttranslational effects, or enzyme activity effects, or both. The current results are consistent with this finding because the analyzed haplotype encompasses a DNA region of 135 bp in the 3' UTR of the gene and could therefore affect posttranscriptional regulation mechanisms.

FASN

Because the first of the 2 SNP (g.56G > A) was monomorphic in the 3 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 the *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 because 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 nonsynonymous mutation causing the AA 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 2 traits. Allele G also has a negative effect on the stearic

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

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

¹See Table 1 for definitions.

Table 3. Mean values and standard deviations for milk yield, fat percentage, and fatty acids (% of total fatty acids) in sheep milk

Item	Altamurana (n = 36)		Gentile di Puglia (n = 24)		Sarda (n = 34)		Overall (n = 94)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Milk yield, g/d	392.55	128.70	196.92	93.99	534.95	156.062	396.12	184.41
Fat percentage	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
C15 <i>iso</i>	0.309	0.066	0.351	0.063	0.298	0.057	0.316	0.065
C15 <i>anteiso</i>	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 (<i>cis</i> -9, <i>trans</i> -11) ¹	1.336	0.544	1.4776	0.718	1.925	0.662	1.584	0.680

¹CLA = conjugated linoleic acid.

FA levels (−0.24%). The results obtained from the analyses using the Polyphen, Sift, I-Mutant, and SNAP softwares indicated that this AA 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 3 breeds tested also supports this hypothesis because the highest frequency of allele A (>60%) is found in the Gentile di Puglia breed, which is the highest fat-yielding breed among the 3 under study, and also in the Sarda breed, a breed that typically yields low milk fat but the only breed 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 α -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. Several 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, members of a superfamily of nuclear factors that includes vitamin D receptor. Retinoid X receptor and vitamin D receptor form a heterodimeric complex and bind cooperatively to vitamin D responsive elements 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 on the C18:2 levels (−0.06%), whereas 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 nonsynonymous mutation (Asp to Asn). The results of the Polyphen, Sift, and I-Mutant analyses indicated that this AA change produces a protein with reduced stability and may also have damaging

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

Trait	Gene ¹	Allele number
Milk yield	<i>SERPINA3</i>	3
	<i>LCAT</i>	2
	<i>DGAT2</i>	2
	<i>LIPE</i>	5
Fat percentage	<i>GHR</i>	2
	<i>ZP2</i>	3
	<i>ITGB1</i>	3
	<i>IGF1</i>	4
C4:0	<i>GHR</i>	2
	<i>SERPINA3</i>	3
	<i>GHRHR</i>	2
C6:0	<i>TYRP1</i>	3
C8:0	<i>TYRP1</i>	3
	<i>ZP2</i>	3
C10:0	<i>FASN</i>	2
	<i>ZP2</i>	3
C10:1	<i>TYRP1</i>	3
	<i>FASN</i>	2
C12:0	<i>FASN</i>	2
	<i>PRKAA2</i>	2
C14:0	<i>FASN</i>	2
	<i>ZP2</i>	3
	<i>PRKAA2</i>	2
C14:1	—	—
C15:0	<i>FASN</i>	2
	<i>ZP2</i>	3
	<i>TYRP1</i>	3
	<i>PCCB</i>	3
	<i>GHR</i>	2
C15 <i>aiso</i>	<i>FASN</i>	2
	<i>ZP2</i>	3
	<i>TYRP1</i>	3
	<i>PCCB</i>	3
C15 <i>iso</i>	<i>TYRP1</i>	3
	<i>PCCB</i>	3
C16:0	<i>ZP2</i>	3
	<i>PCCB</i>	3
C16:1	<i>SERPINA3</i>	3
	<i>LIPE</i>	5
C17:0	<i>FASN</i>	2
	<i>ZP2</i>	3
	<i>TYRP1</i>	3
	<i>PCCB</i>	3
C17:1	<i>FASN</i>	2
	<i>ZP2</i>	3
	<i>DGAT2</i>	2
	<i>ITGB1</i>	3
C18:0	<i>GHR</i>	2
	<i>ITGB1</i>	3
	<i>SERPINA3</i>	3
C18:1	<i>ZP2</i>	3
	<i>TYRP1</i>	3
C18:2	<i>GHR</i>	2
	<i>LCAT</i>	2
	<i>DGAT2</i>	2
	<i>PRKAA2</i>	2
	<i>PCCB</i>	3
	<i>SERPINA3</i>	3
C18:3	<i>IGF1</i>	4
	<i>LCAT</i>	2
	<i>PCCB</i>	3
	<i>SERPINA3</i>	3

Continued

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

Trait	Gene ¹	Allele number
Conjugated linoleic acid	<i>DGAT2</i>	2
	<i>ZP2</i>	3
	<i>LPL</i>	2
	<i>PCCB</i>	3
	<i>SERPINA3</i>	3
	<i>IGF1</i>	3

¹See Table 1 for definitions.

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 attributed 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 on 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 et al., 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 PUFA (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 triacylglycerol 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, and −0.13% for C18:2, C18:3, and CLA, respectively). 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 et al, 1986), it is possible to speculate that the analyzed haplotype is responsible for the variability in the unsaturated FA levels in sheep milk.

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

Trait	Gene ¹	Allele	No. ²	Effect				P <		
				Mean	SE	Minimum	Maximum	Mean	Minimum	Maximum
Milk yield, g/d	<i>DGAT2</i>	[C;A;T]	30	-78.39	9.53	-93.94	-61.61	0.03	0.02	0.07
	<i>LCAT</i>	[C;G;C]	30	40.02	5.82	31.55	52.97	0.02	0.001	0.04
	<i>SERPINA3</i>	[T;A]	20	-59.11	20.23	-74.02	-47.70	0.006	0.0008	0.02
	<i>SERPINA3</i>	[T;G]	20	43.38	15.10	39.99	46.34	0.005	0.003	0.007
Fat %	<i>GHR</i>	A	36	0.13	0.05	0.12	0.16	0.009	0.002	0.02
	<i>GHR</i>	G	36	-0.14	0.05	-0.15	-0.12	0.009	0.002	0.02
	<i>ZP</i>	[G;T]	24	-0.21	0.08	-0.25	-0.19	0.008	0.002	0.01
	<i>ZP</i>	[A;C]	24	0.19	0.06	0.16	0.21	0.005	0.001	0.002
C4:0	<i>GHR</i>	A	6	-0.09	0.04	-0.12	-0.09	0.03	0.005	0.04
	<i>GHR</i>	G	6	0.10	0.04	0.09	0.12	0.02	0.003	0.03
	<i>SERPINA3</i>	[T;G]	4	-0.15	0.06	-0.18	-0.12	0.02	0.004	0.03
	<i>TYRP1</i>	[C;C]	1	-0.05	0.02			0.02		
C6:0	<i>TYRP1</i>	[C;C]	3	-0.04	0.02	-0.04	-0.04	0.05	0.05	0.05
C8:0	<i>FASN</i>	T	3	0.45	0.23	0.44	0.46	0.05	0.05	0.06
C10:0	<i>ZP</i>	[G;C]	2	-0.46	0.20	-0.46	-0.45	0.03	0.03	0.03
C10:1	<i>FASN</i>	T	3	0.01	0.004	0.008	0.01	0.01	0.004	0.02
C12:0	<i>FASN</i>	T	2	0.28	0.14	0.28	0.29	0.05	0.04	0.05
C14:0	<i>FASN</i>	T	2	0.35	0.16	0.034	0.036	0.03	0.02	0.03
	<i>ZP</i>	[G;C]	3	-0.32	0.14	-0.32	-0.32	0.02	0.02	0.02
C14:1	—									
C15:0	<i>PCCB</i>	[T;T;T;A]	36	0.05	0.02	0.04	0.06	0.01	0.002	0.03
	<i>TYRP1</i>	[T;T]	36	-0.03	0.01	-0.04	-0.02	0.02	0.003	0.07
	<i>ZP</i>	[G;T]	36	0.05	0.02	0.04	0.06	0.004	0.0004	0.02
	<i>ZP</i>	[A;C]	36	-0.04	0.01	-0.04	-0.02	0.02	0.001	0.08
C15 <i>aiso</i>	<i>PCCB</i>	[T;T;T;A]	18	0.04	0.02	0.04	0.04	0.02	0.01	0.03
	<i>TYRP1</i>	[T;T]	18	-0.02	0.01	-0.03	-0.02	0.04	0.01	0.07
	<i>ZP</i>	[G;T]	18	0.04	0.01	0.03	0.04	0.01	0.004	0.04
C15 <i>iso</i>	<i>PCCB</i>	[T;T;T;A]	3	0.01	0.006	0.01	0.01	0.03	0.02	0.04
	<i>TYRP1</i>	[T;T]	3	-0.01	0.004	-0.01	-0.01	0.05	0.04	0.05
	<i>TYRP1</i>	[C;C]	3	0.01	0.01	0.01	0.03	0.02	0.04	
C16:0	<i>PCCB</i>	[T;T;T;A]	3	-0.73	0.33	-0.77	-0.70	0.03	0.02	0.04
	<i>ZP</i>	[A;C]	3	0.46	0.22	0.43	0.50	0.04	0.02	0.05
	<i>ZP</i>	[G;T]	3	-0.56	0.29	-0.59	-0.52	0.05	0.04	0.07
C16:1	<i>SERPINA3</i>	[T;A]	5	-0.08	0.03	-0.08	-0.07	0.006	0.004	0.01
C17:0	<i>PCCB</i>	[T;T;T;A]	18	0.02	0.007	0.01	0.02	0.03	0.02	0.05
C17:1	<i>FASN</i>	C	18	0.004	0.001	0.003	0.004	0.01	0.004	0.02
	<i>ITGB1</i>	[C;T]	12	0.014	0.004	0.012	0.015	0.004	0.001	0.009
	<i>ZP</i>	[G;T]	12	0.006	0.002	0.005	0.006	0.01	0.006	0.03
	<i>ZP</i>	[A;C]	12	-0.005	0.002	-0.005	-0.004	0.01	0.0009	0.03
C18:0	<i>GHR</i>	G	9	-0.24	0.13	-0.29	-0.20	0.07	0.03	0.14
	<i>SERPINA3</i>	[T;A]	6	0.47	0.25	0.42	0.52	0.06	0.04	0.09
C18:1	<i>ZP</i>	[G;C]	3	0.16	0.06	0.16	0.17	0.005	0.004	0.005
C18:2	<i>DGAT2</i>	[C;A;T]	72	-0.10	0.04	-0.12	-0.07	0.03	0.002	0.08
	<i>GHR</i>	G	72	0.03	0.01	0.02	0.04	0.02	0.002	0.12
	<i>LCAT</i>	[T;A;T]	72	-0.06	0.03	-0.07	-0.05	0.05	0.03	0.12
	<i>PCCB</i>	[T;T;T;A]	48	0.10	0.02	0.09	0.11	0.0001	0.0001	0.0001
	<i>SERPINA3</i>	[T;A]	48	-0.05	0.02	-0.06	-0.04	0.04	0.03	0.10
C18:3	<i>IGF1</i>	[C;T]	18	0.07	0.03	0.07	0.08	0.009	0.004	0.02
	<i>PCCB</i>	[T;T;T;A]	24	0.03	0.01	0.03	0.04	0.02	0.005	0.03
	<i>SERPINA3</i>	[T;A]	48	-0.03	0.01	-0.04	-0.03	0.01	0.005	0.04
Conjugated linoleic acid	<i>DGAT2</i>	[C;A;T]	162	-0.21	0.03	-0.20	-0.22	0.01	0.002	0.05
	<i>DGAT2</i>	[A;G;T]	162	0.09	0.01	0.09	0.08	0.03	0.003	0.10
	<i>IGF1</i>	[C;C]	108	-0.12	0.02	-0.13	-0.10	0.03	0.003	0.10
	<i>LPL</i>	[T;T;A]	162	0.09	0.003	0.09	0.09	0.03	0.002	0.13
	<i>PCCB</i>	[T;T;T;A]	108	0.12	0.01	0.12	0.12	0.05	0.005	0.11
	<i>SERPINA3</i>	[T;A]	108	-0.13	0.01	-0.14	-0.12	0.03	0.002	0.13
	<i>ZP</i>	[A;C]	108	-0.11	0.005	-0.11	-0.10	0.007	0.001	0.01

¹See Table 1 for definitions.²Number of combinations that produced the indicated average allelic substitution effect.

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 encompass 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 AA 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 3 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 affected the fat (-0.21%) and C16:0 contents (-0.56%) in sheep milk whereas haplotype [G;C] negatively affected 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 (0.46%) contents together with a decrease in the CLA (-0.11%) and C17:1 (-0.005%) levels were found to be associated with haplotype [A;C].

DISCUSSION

A global overview of the results of the current study indicates that 4 genes (*SERPINA3*, *DGAT2*, *PCCB*, and *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, whereas 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 postpartum 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. The *GHR* gene also influences the levels of saturated FA of differing chain length (C4:0 and C18:0), whereas *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. Signorelli et al. (2008) 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 2 genes that influence saturated FA (*FASN*, *ZP2*) would have a different frequency in the 3 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 3 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 3 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.

CONCLUSIONS

The findings of the current study present several 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, because the analyzed sample size was relatively small, these findings need to be further

confirmed in future studies of independent dairy sheep populations.

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