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Association analyses of single nucleotide polymorphisms in the *LEP* and *SCD1* genes on the fatty acid profile of muscle fat in Simmental bulls

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ABSTRACT

The aim was to investigate the effect of the genetic polymorphisms of leptin (*LEP*) and stearoyl-CoA desaturase (*SCD1*) genes on the fatty acid (FA) composition of the muscle of 103 Simmental bulls. Ten single nucleotide polymorphisms (SNP) were detected in exons 2 and 3 of the *LEP* gene, two of them encoding non-synonymous mutations. Allelic substitution effects of all the SNP on 28 single fatty acids, monounsaturated (MUFA) and polyunsaturated (PUFA) and desaturation indexes were estimated. Both the *SCD1* SNP, as well as three SNP of the leptin gene, affected, to different extents, the desaturation of FA into MUFA. Because it was previously proposed that leptin's metabolic action involves down-regulation of *SCD1*, it is possible that, beyond the mere additive effect of *SCD1* gene on FA desaturation, the non-synonymous mutations in the leptin gene also contribute to the variability of FA composition in muscle fat.
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1. Introduction

It is generally accepted that some saturated fatty acids (SFA) commonly found in meat and dairy products, especially myristic and palmitic acids, raise the total and low-density lipoprotein cholesterol, and are thus risk factors in coronary heart disease (Erkkilä, de Mello, Risérius, & Laaksonen, 2008; Webb & O'Neill, 2008). The effect of animal nutrition, during rearing, on the fatty acid (FA) profile of meat and milk has been clearly demonstrated in a number of studies, and the genetic variation in FA synthesis and deposition that influences both marbling and composition is also well established (Dinh et al., 2010), although the molecular mechanisms controlling fatty acid composition are not fully understood (Barton et al., 2010).

Stearoyl-CoA desaturase (SCD) is a rate-limiting enzyme responsible for the conversion of SFA into monounsaturated fatty acids (MUFA). This enzyme, located in the endoplasmic reticulum, inserts a double bond between carbons 9 and 10 of the fatty acyl chain, so affecting the FA composition of membrane phospholipids, triglycerides and cholesterol esters (Ntambi & Miyazaki, 2004). *SCD1* is the coding gene for the SCD isoform that is located on chromosome 26 in cattle, that is considered responsible for the variation of FA profile in beef, because it encodes the non-synonymous mutation Ala239Val, within exon 5, (g.10329C>T; GenBank accession AY241932). Taniguchi et al. (2004) found that the valine residue, encoded by allele T, may change the enzyme's catalytic activity compared to the alanine residue, encoded by allele C; they showed that, in Japanese Black cattle, allele C was more frequently associated with a higher MUFA content in carcasses. Zhang (2005) tested the effect of the Ala239Val variant on the individual FA composition of fat extracted from loin muscle of 172 bulls and found that heterozygous valine/alanine animals had higher C16:1/C16:0 in their intramuscular fat than the homozygous valine ones.

The expression level of lipogenic genes in adipose tissues is regulated by a number of transcription factors; specifically, Hoashi et al. (2007) suggested that sterol regulatory element binding protein (SREBP) is a transcription factor that regulates gene expression levels of SCD, because they could associate a polymorphism within *SREBP1* gene to MUFA content in muscle fat in cattle. However, this association was not confirmed (Barton et al., 2010).

Leptin is an adipocyte-derived hormone that regulates energy balance, metabolism, and the neuroendocrine response to altered nutrition. Nkrumah et al. (2007) demonstrated the existence of a relationship between serum leptin concentration and carcass traits in beef cattle, and suggested that this parameter can be a valuable tool that could be incorporated into appropriate selection programs to improve the carcass quality of cattle, while Liefers et al. (2003) reported that the polymorphisms in the leptin gene (*LEP*) are associated to leptin concentrations.

LEP gene consists of three exons. Several single nucleotide polymorphisms (SNP) have been reported in the coding region of the gene (Konfortov, Licence, & Miller, 1999; Orrù, Napolitano, Catillo, De Matteis, & Moioli, 2006). Liefers, Pas, Veerkamp, and Van der Lende (2002) found that some SNP are associated to production, live weight, energy balance, feed intake and fertility in Holstein heifers, while Madeja et al., 2004 reported on the effect of *LEP* gene polymorphisms

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on milk production traits, and Buchanan et al. (2002) associated a non-synonymous mutation in the bovine *LEP* gene with carcass fat content. In a recent review, Wylie (2010) stated that endogenous leptin remains a potential marker of carcass marbling in cattle, but that no association has been found between leptin and fatty acid composition in intramuscular fat or in any other body fat depots.

Cohen et al. (2002), while examining the mechanism underlying leptin metabolic actions in mice, found that leptin specifically represses RNA levels and enzymatic activity of hepatic *SCD1*. These findings suggested that down-regulation of *SCD1* is an important component of leptin metabolic actions. The purpose of the present work was to estimate the effect of the *LEP* gene polymorphisms, as well as of the *SCD1* Ala239Val variant, on beef fatty acid composition of a group of young bulls.

2. Materials and methods

Animal care and use committee approval was not required because the samples were obtained postslaughter from a beef processing facility.

2.1. Animals and fatty acid analyses

The experiment was carried out on 103 Simmental young bulls belonging to the performance station for beef production of the National Association of Italian Simmental Breeders; they joined the performance station at the age of 5 months, and then were reared under the optimal conditions so as to exclude variations due to management, feeding, and age at slaughter (Piasentier, Valusso, Volpelli, & Failla, 2003). Details of the housing, feeding and fattening can be found in Piasentier et al. (2009). Animals were slaughtered at 18 months and average live weight of 673 kg (±45). Samples of the m. longissimus thoracis (LT) were collected from the left side of the carcasses between the 8th and 11th ribs (approximately 11 cm thick).

The extraction of total lipids was performed according to Folch, Lees, and Stanley (1957). Every meat sample (1.5 g) was minced, and nonadecanoic acid (C19:0) was added, and homogenized in 30 ml of a chloroform–methanol mixture (2:1 v/v) using an Ultra-Turrax T 25 basic (Ika-Werke); the samples were then filtered by vacuum filtration through Whatman filter paper. The extract was washed with 7.5 ml of 0.88% (w/v) KCl, mixed vigorously for 1 min, and then left overnight. The organic phase was separated and the solvents were evaporated under vacuum at 40 °C. Fatty acid methyl esters were prepared using methanolic HCl (Sukhija & Palmquist, 1988). The lipid sample was mixed with 2 ml of hexane and 3 ml of methanolic HCl in 20 ml glass tubes with Teflon-lined caps. The mixture was heated at 70 °C for 2 h in a metal block and cooled to room temperature; the methyl esters were then extracted in 2 ml of hexane after addition of 5 ml K_2CO_3 6% (w/v) and Na_2SO_4, samples were allowed to stand for 30 min and then centrifuged at 540×g for 10 min and the upper hexane layer was removed, concentrated under nitrogen and diluted in hexane.

Methyl ester analysis was performed using a Carlo Erba gas chromatograph (HRGC 5300 mega-series) fitted with an automatic sampler (Model A200S) and FID detector; 1 μl of sample was injected into the gas chromatograph in split mode (split ratio 1:30). The conditions used were: SP-2380-fused silica capillary column (60 m×0.25 mm i.d., film thickness 0.20 μm) (Supelco Inc., Bellafonte, PA), programmed from 50 to 200 °C at 20 °C/min and from 200 to 240 °C at 10 °C/min, and then held for 5 min. The carrier gas was helium at a flow rate of 1.0 ml/min. The individual fatty acid peaks were identified by comparison of retention times with those of known mixtures of standard fatty acids (FAME, Sigma) run under the same operating conditions. Fatty acids were expressed as percentages of total lipids.

2.2. Genotyping

DNA was extracted from 0.3 g muscle, using a Genomix kit (Talent), following the manufacturer's protocols, modified as in Orrù, Napolitano, Catillo, and Moioli (2006). Although several variants are reported in the *SCD1* gene (Medrano, Islas-Trejo, & Johnson, 2003) only the non-synonymous Ala239Val mutation was considered, because it is the mutation that directly affects muscle FA composition (Hoashi et al., 2007; Taniguchi et al., 2004). Genotyping of the Ala239Val mutation was performed through direct sequencing of an amplicon of 212 bp, that encoded a portion of exon 5, spanning 10,232 to 10,443 bp of the GenBank accession AY241932. The primers used were: forward: ACCTGGCTGGTGAATAGTGC; reverse: TGACATATGGAGAGGGGTCA.

SNP of the *LEP* gene were genotyped through direct sequencing of two amplicons, encompassing exons 2 and 3, obtained with the following primers, designed on the Accession U50365: amplicon 1 (395 bp) forward: CCAGGGAGTGCCTTTCATTA; reverse: ATGGCCACGGTTCTACCTC; amplicon 2 (496 bp) forward: CCCTCTCTCCCACTGAGCTC; reverse: TAAAGGATGCCCACATAGGC. Direct sequencing was performed on a Perkin-Elmer ABI Prism 310 DNA sequencer.

2.3. Statistical analysis

For each animal, 33 traits were considered, including the total lipids, the individual fatty acids, the sum of SFA, MUFA, PUFA, together with the Cn index = Cn:1/(Cn:0 + Cn:1), where n = 14, 16 or 18, and the total desaturation index estimated as MUFA/(MUFA + SFA), and the allele substitution effect of each SNP on the traits was estimated, similarly to Sherman et al. (2008) and Barendse, Harrison, Bunch, and Thomas (2008), by regressing the number of copies of each allele against each of the 32 traits, using a Linear Mixed Model in SAS (SAS Institute Inc, 2007) that included the carcass weight of the individual as a covariate.

3. Results and discussion

In Table 1 the allele frequencies of each detected SNP in the analyzed population are reported.

Table 1
Allele frequencies of each detected SNP in the analyzed population, assigned reference identification in the dbSNP, gene location and the resultant aminoacid change.

SNP	Minor allele	MAF %	NCBI dbSNP ID	Gene region	Aminoacid change
SCD1 g.10329C>T	C	49	Not assigned	Exon 5	Ala239Val
LEP g.978C>T	T	30	rs29004484	Intron	
LEP g.1001G>C	C	30	rs29004485	Intron	
LEP g.1127A>T	T	2	rs29004487	Exon 2	Tyr7Phe
LEP g.1180C>T	T	30	rs29004488	Exon 2	Arg25Cys
LEP g.3100C>T	T	30	rs29004508	Exon 3	Ala80Val
LEP g.3157A>G	G	10	not assigned	Exon 3	Asn99Ser
LEP g.3257C>T	T	23	rs29004509	Exon 3	Synonymous
LEP g.3260T>C	C	23	rs29004510	Exon 3	Synonymous
LEP g.3272T>C	C	23	rs29004511	Exon 3	Synonymous
LEP g.3356C>T	T	23	rs29004512	Exon 3	Synonymous

MAF = minor allele frequencies.

While the two alleles of exon 5 of *SCD1* gene have similar frequency, in the *LEP* gene, the major allele of each SNP was always at frequency >70%. From the association analysis *LEP* g.1127A>T was excluded, because of the low frequency of the minor allele (0.02). Moreover, the following were excluded: *LEP* g.1001G>C, *LEP* g.3260T>C and *LEP* g.3356C>T, because their alleles showed a full correspondence with the alleles of the SNP *LEP* g.978C>T, *LEP* g.3257C>T and *LEP* g.3272T>C, respectively.

Mean values and standard deviation for each trait are reported in Table 2, so as to allow direct evaluation of the extent of the allelic substitution effect, presented in Table 3. All these values fall in the range of the average FA's in beef, taking into account deviations reported in other works, due to the use of different breeds and rearing systems.

In Table 3, the results of the association analysis between SNP and traits are shown; both the statistically significant effects and the effects tending to statistical significance are reported ($P<0.10$).

The results appear to only partly agree with previous works that associated the effect of *SCD1* allele coding for the alanine residue (allele C) to higher MUFA contents in carcasses (Taniguchi et al., 2004), but these authors gave results only for total MUFA, and did not specify what MUFA was mostly affected by this allele. The present results also contradict the finding of Zhang (2005) who reported that only the C16 index was significantly higher in carriers of allele C, compared to carriers of allele T. In fact, in the present study, the desaturation effect of *SCD1* seems to be directed only to C18:0 FA, the amount of which is decreased by 0.51% ($P<0.10$) by one copy of allele A, while C18:1n−9 is increased by 0.93 ($P<0.07$). Total desaturation index also increased (+0.007; $P<0.09$) but this is due to the opposite contribution of C18:0 and C18:1n−9. In any case, Taniguchi et al. (2004) reported that the total contribution of *SCD1* to MUFA variation, although significant, was low (4%). More recently, Barton et al. (2010) studied the effect of the same *SCD1* variant in the muscle fat of 370 Simmental bulls, and although they confirmed that total MUFA was positively affected by the variant, this was evident only through the improved C18 and C14 indexes, while they noted no significant effect on the C16 index, contrasting with the findings of Zhang (2005). The use of desaturation indices as an estimate of SCD activity has been questioned by several authors (Archibeque, Lunt, Gilbert, Tume, & Smith, 2005; Chung, Lunt, Kawachi, Yano, & Smith, 2007) as they suggest that desaturation indices do not reflect actual enzyme activity, especially when comparing diets that vary markedly in FA composition (Archibeque et al., 2005). It is thus likely that the different results obtained by various authors reflect the fact that the trials used different animals and management systems, and that the *SCD1* effect is thus mitigated by other sources of variation, either genetic or environmental. Such contrasting results on the effect of *SCD1* stimulated various authors to investigate other factors that might be involved in the regulation of transcription activation of lipogenic genes, such as the sterol regulatory element binding proteins (SREBP); in fact, Hoashi et al. (2007) identified a 84-bp indel polymorphism in bovine *SREBP-1* and demonstrated that different *SREBP-1* genotypes significantly affect MUFA and fat melting points of fat in Japanese Black cattle, suggesting that this gene really affects the expression level of *SCD1*. However, the *SREBP-1* effect, was not confirmed in different populations of Japanese Black cattle (Ohsaki et al., 2009), or in the study of Barton et al. (2010), who assessed the effect of the polymorphisms of both genes, *SCD1* and *SREBP-1*, in the same group of Simmental bulls, and found that only *SCD1* significantly affected the content of MUFA.

In this study, another potential factor for the regulation of *SCD1* was studied, based on the finding of Cohen et al. (2002) that leptin metabolic action involves down-regulation of *SCD1*, and Biddinger, Miyazaki, Boucher, Ntambi, and Khan (2006) who showed that leptin decreased *SCD1* transcript, protein, and activity by >60% in mice, and that the effect of leptin on *SCD1* in liver is independent of insulin and *SREBP-1c*. Therefore, the effect of polymorphisms in both *SCD1* and *LEP* gene polymorphisms in the same animals was studied. It was found that the G allele of *LEP* g.3157A>G contributes to increasing the C18 index (+0.021; $P<0.007$), MUFA (+1.128%; $P<0.09$), C18:1n-9 (+1.21%; $P<0.06$) and to reducing C18:0 (−1.12; $P<0.01$) and all the short–medium chain SFA. Unexpectedly, it was found that this effect is even higher than the effect of the *SCD1* allele A. *SCD1* and *LEP* genes are located on different chromosomes so excluded the fact that the effect could be due to a partial linkage disequilibrium, the hypothesis of a down-regulation of *SCD1*, on behalf of the *LEP* gene, might be taken into account, considering that *LEP* g.3157A>G encodes the non-synonymous mutation Asn>Ser. This SNP (*LEP* g.3157A>G) which is located in exon 3, was first detected by Orrù, Napolitano, Catillo, De Matteis, et al. (2006) who reported that allele G was not found in Holstein cattle and has a frequency of 0.11 in Piedmontese cattle. Thus this allele was not found in the dairy breed, but was present, although with a minor frequency, in two breeds, Piedmontese and Simmental, that have been selected to produce beef of better quality. Thus, either through a direct contribution, or through differential regulation of the *SCD1* gene, *LEP* g.3157A>G may play a role in the desaturation of SFA.

Another interesting variant is allele C of *LEP* g.3100C>T, that encodes a non-synonymous mutation Ala>Val. The effect of allele C is particularly evident on C14:1 (+0.015%; $P<0.03$) and consequently on the C14 index (+0.008; $P<0.003$). Taniguchi et al. (2004), referring to *SCD1* g.10329C>T, reported that the valine residue may change the enzymatic activity compared to alanine; therefore, the hypothesis that different leptin isoforms display different activity levels is possible, although this should be confirmed through independent experiments.

A positive effect on total desaturation index (0.013; $P<0.04$) was noted for allele C of *LEP* g.978C>T, particularly as it produces a decrease in total SFA (−1.37%; $P<0.04$), C18:0 (−0.84%; $P<0.09$) and C14:0 (−0.16%; $P<0.07$). This SNP is located in an intronic region of the leptin gene, therefore the hypothesis that it plays a direct role in the fatty acid composition of beef should be confirmed in studies of independent cattle populations.

Table 2
Mean values and standard deviation for each analyzed trait.

Trait	Mean	STD
Total lipids (mg/g muscle)	47.08	17.54
C10:0	0.035	0.018
C12:0	0.061	0.021
C14:0	2.218	0.450
C14:1	0.101	0.050
C15:0	0.253	0.087
C15:1	0.136	0.060
C16:0	25.971	1.809
C16:1 n−9	2.755	0.703
C17:0	0.647	0.167
C18:0	17.097	2.534
C18:1 n−9	37.704	3.607
C18:1 n−7	2.380	0.442
C18:2 n−6	7.036	3.011
C18:3 n−6	0.019	0.028
C18:3 n−3	0.335	0.127
C20:0	0.090	0.045
C20:1 n−9	0.134	0.074
C20:2 n−6	0.057	0.064
C20:3 n−6	0.373	0.222
C20:4 n−6	1.896	1.063
C20:5 n−3	0.064	0.064
C22:1 n−9	0.011	0.035
C22:4 n−6	0.296	0.150
C22:5 n−3	0.313	0.162
C22:6 n−3	0.019	0.071
SFA	46.370	3.428
MUFA	43.222	3.914
PUFA	10.409	4.613
n−3	0.732	0.320
n−6	9.677	4.342
C14 index	0.043	0.019
C16 index	0.095	0.021
C18 index	0.688	0.044
Total index	0.482	0.032

Table 3
Allelic substitution effects of the SNP in *SCD1* and *LEP* genes, only for those traits where the effect was either significant ($P<0.05$) or tended to significance ($P<0.10$).

Effect of allele	*SCD1* g.10329C>T		*LEP* g.978C>T		*LEP* g.3100C>T		*LEP* g.3157A>G		*LEP* g.3257C>T	
	C	*P*	C	*P*	C	*P*	G	*P*	C	*P*
Total lipids (g/100 g)									−5.490	0.02
C10:0					0.006	0.02	−0.006	0.05		
C12:0			−0.007	0.09			−0.006	0.07		
C14:0			−0.160	0.07						
C14:1					0.015	0.03	−0.031	0.0002	0.018	0.01
C15:0					0.020	0.08	−0.046	0.002	0.016	0.09
C15:1					0.017	0.05	−0.034	0.001		
C17:0					0.051	0.02	−0.083	0.003		
C18:0	−0.510	0.10	−0.840	0.09			−1.120	0.01		
C18:1 n−9	0.930	0.07					1.210	0.06		
C18:3 n−3	0.007	0.06					−0.046	0.04		
C20:0							−0.020	0.01	0.014	0.04
C20:2 n−6							−0.021	0.03		
C20:5n−3					0.014	0.09	−0.019	0.07		
SFA			−1.370	0.04						
MUFA	0.846	0.10					1.128	0.09		
C14 index					0.008	0.003	−0.117	0.0005	0.008	0.006
C18 index	0.011	0.07	0.015	0.09			0.021	0.007		
Total index	0.007	0.09	0.013	0.04			0.011	0.06		

The last SNP (*LEP* g.3257C>T) was the only SNP that seemed to affect total lipid content, which is decreased by 5.49% by one copy of allele C. However, this effect could be fortuitous because the same SNP affected only a few traits that are not correlated. From the present results, however, it is possible to infer that total lipid content and FA composition in muscle are not strictly interdependent.

It is interesting to note that the effect of *SCD1* was evident only on the C18 FA's, which are the most abundant FA's in meat; thus the effect on total MUFA and total index simply reflects the effect on C18. On the contrary, SNP in the leptin gene seemed to influence other FA's, as in the case of *LEP* g.3100C>T. This infers that there is no direct effect of the leptin gene on FA desaturation, but that leptin gene regulates *SCD1* expression. The extent of the effect, of each considered leptin SNP, on the desaturation activity of *SCD1*, could be evaluated in specific gene expression studies. Because the present result suggests that all the examined SNP, to different extents, affect only SFA and MUFA, it confirms the fundamental role of *SCD1* genes in transforming SFA into MUFA, and that this role is mediated by the leptin gene. The existence of such combined actions of the two genes might justify, at least partly, Schenkel et al.'s (2005) claim that the reported associations of *LEP* gene variants with carcass and meat quality traits often diverge.

4. Conclusion

SCD1 has been proposed as a candidate gene for muscle fat quality in cattle because of the Ala239Val mutation in exon 5, that modifies the enzymatic activity, and affects the proportion of SFA and MUFA, so conferring either favourable or un-favourable taste to beef (Taniguchi et al., 2004). The genes that control the *SCD* mRNA expression level have not been fully identified; Hoashi et al. (2007) demonstrated that of one them (*SREBP-1*), altered the SFA/MUFA ratio in beef fat, but this was not confirmed by later studies.

In this study, a number of polymorphisms in the *LEP* gene have been described and their effect on muscle FA composition evaluated, as well as the effect of *SCD1* g.10329C>T. It was found that both SNP g.10329C>T and three SNP of the *LEP* gene (g.978C>T; g.3100C>T; g.3157A>G) affect, to different extents, the desaturation of FA into MUFA. On the contrary, no effect was evident on any of the PUFA. Moreover, the effect of the *SCD1* variant is weaker than the effect of *LEP* SNP. It is then possible that, as well as the additive effect of the *SCD1* gene on FA desaturation, leptin's metabolic action involves down-regulation of *SCD1*, as proposed by Cohen et al. (2002), so that non-synonymous mutations in the *LEP* gene also contribute to the FA composition of muscle fat.

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