SHORT COMMUNICATION: AN ASSOCIATION ANALYSIS BETWEEN ONE MISSENSE POLYMORPHISM AT THE *SREBF1* GENE AND MILK YIELD AND COMPOSITION TRAITS IN GOATS

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Abstract

Sterol regulatory element binding transcription factor 1 (SREBF1) regulates the expression of genes involved in the biosynthesis of fatty acids and cholesterol. Herewith, we have sequenced the near-complete coding region and part of the 3’UTR of the goat *SREBF1* gene. In doing so, we have detected a missense c.353C>T polymorphism causing a proline to leucine substitution at position 118 (P118L). An association analysis with milk composition traits recorded in Murciano-Granadina goats only revealed a statistical tendency linking *SREBF1* genotype and milk omega 3 fatty acid content. The lack of significant associations suggests that the P118L substitution does not involve a functional change.

Keywords: Goat *SREBF1* gene, milk fat content and composition

Résume

Le facteur de transcription dénommé *Sterol regulatory element binding transcription factor 1* (*SREBF1*) régule l'expression des gènes impliqués dans la biosynthèse des acides gras et du cholestérol. Dans cette étude, nous avons séquencé la quasi-totalité de la région codante et une partie du la région no codante 3'UTR de la chèvre *SREBF1* gène. Ce travail, nous a permis d'identifier un polymorphisme non-synonyme c.353C> T causant la substitution d'une Proline en Leucine à la position 118. L'étude d'association avec la composition du lait enregistrée en chèvres Murciano-Granadina, a révélé seulement une tendance statistique reliant *SREBF1* génotype et l'acide gras oméga-3 du lait. L'absence d'associations significatives suggère que la substitution P118L n'implique pas un changement fonctionnel.

Mots clés: gène *SREBF1*de la chèvre, métabolisme des lipides

76 Sterol regulatory element binding transcription factor 1 (SREBF1) plays a key
77 role in energy homeostasis by regulating the expression of lipogenic genes (Brown and
78 Goldstein, 1997). At a cellular level, inactive SREBF1 remains anchored to either the
79 nuclear envelope or the endoplasmic reticulum, while the N- and C-terminal ends
80 localize to the cytoplasm (Párraga et al. 1998). Low levels of cholesterol activate
81 SREBF1 through the proteolytic cleavage of the N-terminal segment by site-1 and site-2
82 proteases (Shimano 2001). Subsequently, SREBF1 is translocated to the nucleus where
83 it forms homodimers and upregulates the expression of as many as 30 genes involved in
84 cholesterol and FA biosynthesis (Párraga et al. 1998; Shimano 2001). In lactating cows,
85 measurement of *SREBF1* mRNA levels has shown that they increase after parturition, a
86 finding consistent with the key role of this molecule in the coordination of the
87 mammary gland lipogenic pathways (Harvatine and Bauman, 2006; Rudolph et al.
88 2007; Viturro et al. 2009). The main goal of the current work was to characterize the
89 genetic variability of the caprine *SREBF1* coding region and find out if it is associated
90 with phenotypic variation of milk fat content and composition in dairy goats.
91 Total RNA was extracted from liver samples obtained from three Murciano-
92 Granadina and two Malagueña goats following protocols reported by Zidi et al. (2008),
93 and cDNA was synthesized with the ThermoScript RT-PCR kit (Invitrogen S.A.,
94 Barcelona, Spain). The amplification of the coding region and part of the 3'UTR of the
95 goat *SREBF1* gene was performed with six primer pairs, following the protocols
96 detailed at Supplementary Table 1. After enzymatic purification (ExoSAP-IT,
97 Amersham Biosciences), PCR products were sequenced by using the BigDye terminator
98 v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing
99 reaction products were purified with the Montage SEQ_{96} cleanup kit (Millipore

100 Corporation, Billerica, MA, USA) and electrophoresed in an ABI Prism 3730 DNA
101 Analyser (Applied Biosystems, Foster City, CA, USA).

102 Molecular analyses yielded the near-complete sequence of the coding region and
103 part of the 3'UTR of the goat *SREBF1* gene, consisting of 3,561 bp and encoding a
104 1,139 amino acid protein (GenBank accession number: HM443643). Partial sequences
105 had been previously submitted to GenBank, but assembled together they just covered
106 half of the coding region of this gene (GenBank accession numbers: DQ487874,
107 DQ455606, DQ480338 and DQ483057). Blast analysis of the goat *SREBF1* cDNA
108 sequence showed that it was very similar to its bovine (96% identity), porcine (88%)
109 and human (86%) orthologs. The goat SREBF1 amino acid sequence obtained in the
110 current work is shown at Supplementary Fig. 1. Functional domains were inferred
111 according to the UniProtKB entry for human SREBF1 (http://www.uniprot.org
112 /uniprot/P36956). The caprine SREBF1 consisted of a N-terminal transcription factor
113 portion, including a basic region/helix–loop–helix/leucine zipper (bHLHZ) DNA-
114 binding motif, a hydrophobic region with two membrane spanning regions, and a C-
115 terminal regulatory segment. The helix-loop-helix (HLH) domain of SREBF1 displayed
116 a 60–100 amino acid residue motif, encoding two amphipathic α helices separated by a
117 variable loop that is essential for forming homodimers (Párraga et al. 1998). This motif
118 is flanked at its N-terminal and C-terminal ends by a highly conserved basic region that
119 binds to DNA, and a leucine zipper motif, respectively (Supplementary Fig. 1). Finally,
120 it is worth to highlight the existence of an acidic transactivation domain, that it is
121 fundamental to activate transcription, adjacent to a proline-rich region.

122 Alignment of goat *SREBF1* cDNA sequences allowed us to detect a c.353C>T
123 missense polymorphism that involved a proline to leucine replacement at position 118
124 (Supplementary Fig. 2). A primer-extension based protocol was implemented to

genotype it. Genomic DNA isolation and amplification reactions (PCR composition and thermal cycling) were performed as reported in Zidi et al. (2010), with the only exception of the annealing temperature (T_{ann} = 64 °C). Primer Express software (Applied Biosystems, Foster city, CA, USA) was employed to design primers to amplify the polymorphic region as well as to make the extension reaction (Supplementary Table 1). The primer-extension reaction was carried out by following manufacturer instructions and using the following thermal cycling profile: 25 cycles of 10 sec at 96 °C, 5 sec at 50 °C and 30 sec at 60 °C. Extension products were run in an ABI PRISM 3730 capillary electrophoresis device (Applied Biosystems, Foster City, CA, USA) and analysed with the ABI PRISM GeneMapper software v.4.0 (Applied Biosystems, Foster city, CA, USA). *In silico* prediction of amino acid substitutions was carried out with the Polyphen software (Ramensky et al. 2002). This software allows to infer the functional consequences of an amino acid substitution by building a multiple alignment of the query protein sequence and related sequences and calculating a profile-matrix. Elements of this matrix (PSIC scores) are logarithmic ratios of the likelihood of given amino acid occurring at a particular position to the likelihood of this amino acid occurring at any position. Subsequently, PolyPhen calculates the absolute value of the difference between PSIC scores of both alleles in the variable site. If this difference is high (*i.e*. above 1.5), it means that the analysed substitution is rarely or never observed in the protein family. According to this criterion, substitutions are classified as benign (do not involve a functional change) or possibly/probably damaging (they may involve a functional change).

Genotypic frequencies of c.353C>T polymorphism in the Murciano-Granadina goat population (N = 427) were 0.39, 0.46 and 0.15, for CC, CT and TT genotypes, respectively (C and T allele frequencies: 0.62 and 0.38 respectively). Hardy-Weinberg

150 equilibrium was assessed with an online Microsoft Excel tool available at
151 http://www.tufts.edu/~mcourt01/Documents (Court Lab-HW calculator file). This
152 approach allowed us to demonstrate that genotype frequencies matched the Hardy
153 Weinberg equilibrium expectation (χ^2 = 0.155, *P*-value: 0.693). The effects of this
154 mutation were predicted *in silico* with the Polyphen software (Ramensky et al. 2002),
155 obtaining a PSIC score difference of 2.55. This result suggested that this polymorphism
156 might be likely damaging. With the aim of gaining additional insights into the
157 biological effects of this substitution, we decided to investigate if the goat *SREBF1*
158 genotype is associated with milk yield and quality traits.

159 In order to achieve this goal, we genotyped and phenotyped two Murciano-
160 Granadina populations consisting of 133 (population 1, 3 herds) and 319 (population 2,
161 1 herd) individuals. The 133 goats of population 1 had records for dairy traits *i.e.* milk
162 yield, milk protein, fat, lactose and dry extract contents and logarithm of the total
163 somatic cell count (logSCC). The management of this population and phenotype
164 recording are fully described in Badaoui et al. (2007). With regard to population 2, 191
165 goats were selected according to the ordinal number of parturition and lactation (from
166 2nd to 5th lactations) and the amount of genealogical information. For each goat, whole
167 milk in the first of the two daily milkings was collected beginning three months after
168 parturition (average 97.7 days) and with a periodicity of every two months (65.5 days
169 average sampling interval). Protein, fat, lactose, dry extract contents and somatic cells
170 were measured with a CombiFoss 600 FC instrument, as reported by Zidi et al. (2010).
171 A small sample of milk from each goat was freeze-dried stored at –20 ºC and afterwards
172 used for the gas chromatography analyses of FA. Samples were weighted before
173 performing all milk FA composition analyses. Separation and quantification of the FA
174 methyl esters were carried out using a gas chromatograph Agilent 6890N Network GS

175 System (Agilent, Santa Clara, CA), as reported by Zidi et al. (2010). At the end of the
176 experiment we obtained valid records for 176 goats (3-4 measurements per individual).

177 The number of goats with registers for dairy traits (milk yield, milk protein, fat,
178 lactose and dry extract contents and logSCC) was 309 (133 goats from population 1 and
179 176 from population 2), while for milk FA composition we only had data from 176
180 goats (population 2). A mixed model for repeated measurements (Littell et al., 1998)
181 implemented with the SAS software (SAS 9.2 Inst. Inc., Cary, NC) was used to detect
182 associations between *SREBF1* genotypes and traits under study. This model included
183 *SREBF1* genotype, ordinal number of lactation, number of kids born, month of
184 lactation, season of sampling and herd as fixed effects; the random animal effect and the
185 residual error term. Milk FA composition phenotypes were obtained in goats belonging
186 to a single herd, so this fixed effect was not considered in the statistical model. In
187 contrast, season of sampling was included as a fixed effect because there is a seasonal
188 effect on lipid composition of plant pastures (Meľuchová et al., 2008). Besides, logSCC
189 was used as a covariate in the statistical model. In this sense Jensen (2002) suggested
190 that udder health might affect milk FA composition.

191 As shown in Tables 1 and 2, the association analysis did not yield significant
192 results. We just found a statistical tendency for omega-3 FA content. This latter result is
193 consistent with the key role of SREBF1 in the synthesis of unsaturated FA. In this way,
194 increased expression of SREBF1 in the mammary gland of rats has been shown to
195 induce an augment in the expression of stearoyl-CoA desaturases (Rodríguez-Cruz et al.
196 2006). Remarkably, when comparing previous studies aimed to associate *SREBF1*
197 genotype with fat content and composition strong inconsistencies were noticed. For
198 instance, Hoashi et al. (2007) identified an association between a 84 bp-indel at intron 5
199 of the *SREBF1* gene and muscle monounsaturated FA content in Japanese Black cattle.

200 Similarly, Bartoň et al. (2010) found an association between this indel and muscle
201 C14:1 cis-9 FA content in Fleckvieh cattle. On the contrary, Ohsaki et al. (2009)
202 analysed the segregation of the 84 bp-indel in two groups of Japanese Black cattle and,
203 overall, they did not find any significant association with an array of seven carcass and
204 fourteen FA composition traits (although significant associations for myristic, palmitic
205 and elongation index were observed in one group of cows). Similarly, Matsuhashi et al.
206 (2011) performed an association study between the aforementioned 84-bp indel and FA
207 composition of the *longissimus thoracis* muscle and carcass and meat quality traits in
208 480 commercial Japanese Black cattle and did not find any significant relationship. Our
209 study is closely aligned with the work carried out by Ohsaki et al. (2009) and
210 Matsuhashi et al. (2011) in the sense that we did not find a significant association
211 between goat *SREBF1* genotype and milk yield and quality traits. Notably, the results of
212 the association analyses oulined in Tables 1 and 2 challenge the *in silico* prediction
213 obtained with PolyPhen that indicated that the P118L might have functional
214 consequences. If so, we would have expected to find a detectable effect on the dairy
215 phenotypes under analysis. Recently, Rincón et al. (2011) identified a non-synonymous
216 SNP at exon 14 of the bovine *SREBF1* gene that according to *in silico* analyses with
217 SIFT (Ng and Henikoff 2003) and PolyPhen was not tolerated. Interestingly, this SNP
218 displayed significant associations with milk fat and polyunsaturated FA content in cows
219 suffering milk fat depression, but not in their healthy counterparts. As a whole, these
220 results illustrate the complexity of assessing the functional effects of SNP on production
221 traits through the performance of association studies, since results can be strongly
222 affected by a plethora of biological (linkage phase between markers and causal
223 mutations, genotype by environment interactions, dominant and epistatic effects etc.)
224 and experimental (sample size, number of records, phenotype recording etc.) factors.

225 This circumstance stresses the need of validating associations between genetic markers
226 and phenotypes in multiple populations and breeding environments in order to reach
227 valid conclusions.
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Table 1. Association between c.353C>T *SREBF1* genotype and dairy traits in Murciano-Granadina goats.

Data available	CC	CT	TT	Total
Number of goats	114	135	41	290
Number of records	361	437	142	940
Traits	**Least square means ± SE**			***P*-value**
Milk yield, kg/day	1.70 ± 0.11	1.72 ± 0.11	1.80 ± 0.13	0.58
Protein, %	3.61 ± 0.05	3.59 ± 0.05	3.65 ± 0.06	0.51
Fat, %	5.74 ± 0.18	5.67 ± 0.18	5.67 ± 0.20	0.78
Lactose, %	4.92 ± 0.04	4.92 ± 0.04	4.97 ± 0.04	0.36
LogSCC	2.71 ± 0.07	2.74 ± 0.07	2.73 ± 0.08	0.75

340 **Table 2. Association between c.353C>T *SREBF1* genotype and and milk fatty acid**

341 **composition traits in Murciano-Granadina goats.**

Data available	**CC**	**CT**	**TT**	**Total**
Number of goats	71	80	23	174
Number of records	194	224	65	483
Trait[z]	**Least square means ± SE**			***P*-value**
SFA	76.15 ± 0.44	76.34 ± 0.42	76.56 ± 0.56	0.79
MUFA	19.86 ± 0.40	19.68 ± 0.38	19.79 ± 0.50	0.82
PUFA	4.15 ± 0.07	4.41 ± 0.06	3.97 ± 0.10	0.26
Ratio PUFA/MUFA	0.20 ± 0.008	0.21 ± 0.007	0.19 ± 0.01	0.40
Total CLA	0.72 ± 0.02	0.73 ± 0.02	0.68 ± 0.04	0.51
cis-9, *trans*-11 CLA	0.39 ± 0.03	0.40 ± 0.03	0.36 ± 0.04	0.61
C18:1 n9c	13.84 ± 0.25	14.03 ± 0.23	14.0 ± 0.38	0.79
Omega 3 FA	0.31 ± 0.007	0.31 ± 0.007	0.29 ± 0.01	0.07
Omega 6 FA	3.03 ± 0.05	3.0 ± 0.05	2.90 ± 0.08	0.42

342 [z]Fatty acid content was expressed as the percentage of total methyl esters, SFA:

343 saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty

344 acids, CLA: conjugated linoleic acid.

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