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1 **Investigation of the *Perilipin 5* gene expression and association study of its**
2 **sequence polymorphism with meat and carcass quality traits in different pig**
3 **breeds**

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10

11

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13

14 Short title: An explorative study on *PLIN5* gene in pig breeds

15

16 **Abstract**

17 The growing request for healthier fatty acid composition of animal products is raising

18 the necessity of a deeper knowledge of the main factors controlling fatty acids

19 storage in muscle and backfat. Perilipin (PLIN) 5, and generally the whole Perilipin

20 family, seems to play a crucial role in the regulation of lipids deposition as codes for

21 proteins coating intracellular lipid droplets surface. Nevertheless, the knowledge of

22 these genes in pig is still incomplete. The present research was aimed at

23 investigating in different pig breeds the *PLIN5* gene, analysing its expression level

24 and the associations of the variability in its downstream gene region with pork meat

25 and carcass quality traits. Moreover, the PLIN5 protein localisation in porcine skeletal

26 muscle was investigated through immunofluorescence, resulting to be widespread in
27 *Semimembranosus* muscle (SM) myofibers. The identified single nucleotide
28 polymorphism (SNP) rs327694326 (NC_010444.4:g.74314701 T>C) located in *PLIN5*
29 downstream region was analysed in different pig populations, represented by 512
30 Italian Large White (ILW) pigs, 300 Italian Duroc (IDU) samples, 100 Italian
31 Landrace, 100 Pietrain and 60 pigs belonging to three Italian native breeds (20
32 samples of Cinta Senese, 20 Calabrese and 20 Casertana pigs). The C allele was
33 found in ILW, IDU and Pietrain pigs. In ILW pigs this SNP showed results indicating a
34 possible association with oleic and cis-vaccenic fatty acid contents in backfat tissue.
35 Furthermore, since PLINs are known to regulate lipases activity, we tested if the
36 rs327694326 SNP was associated with differences in *Hormone-sensitive lipase*
37 (*LIPE*) gene expression levels. In SM of ILW pigs, *PLIN5* C allele was associated
38 with significantly lower *LIPE* transcription levels than T allele ($P = 0.02$ for Student's t
39 test of TT vs. CT samples, $P < 0.0001$ for TT vs CC pigs), while in IDU breed no
40 significant difference was noticed in *LIPE* transcription levels between TT and TC
41 animals. The results may suggest that variations in the *PLIN5* sequence may be
42 linked to *LIPE* expression through a still poorly known regulative molecular process.

43

44 **Keywords:** pig; PLIN5; LIPE; gene expression; immunohistochemistry

45

46 **Implications**

47 This is the first study investigating *Perilipin 5* (*PLIN5*) gene in pigs. The results
48 obtained from the association study suggest that variations in its sequence may be
49 related to yet poorly-known regulative function of *PLIN5* on *Hormone-sensitive lipase*

50 (*LIPE*) gene activity. This study provides new input to the knowledge of one of the
51 regulative pathways linking *PLIN5* and lipases activities.

52

53 **Introduction**

54 Pig selection breeding programs focused mainly on the increase of carcass muscle
55 deposition, causing a consistent reduction in intramuscular fat and backfat deposition
56 and a worsening of porcine meat quality (Wood *et al.*, 2008). During the last decade,
57 the improvement of meat quality has become a main objective for the pork industry.
58 Different studies proved that selecting simultaneously for growth rate and meat
59 quality without reducing excessively intramuscular fat is a possible objective in pig
60 breeding programs (extensively reviewed in Pena *et al.*, 2016). Meat quality traits
61 have been found correlated with intramuscular and backfat fatty acid composition
62 (Wood *et al.*, 2008), suggesting that the selection for meat and carcass traits may
63 also determine changes in pork fatty acid composition. This issue, along with the
64 increasing interest toward healthier pig products, raised the need of a better
65 knowledge on the key molecular regulators of body lipid storage and metabolism
66 (Pena *et al.*, 2016).

67 Perilipin family (including Perilipin 1 to 5) can be considered as interesting candidate
68 involved in the control of intracellular lipid deposition. *Perilipin 5 (PLIN5)* gene, and
69 generally, the whole Perilipin family, has been investigated mainly in human and
70 mouse, and recent studies have found that Plin5 protein may regulate intracellular
71 fatty acid fluxes and oxidation (Laurens *et al.*, 2016). Different Authors (Brasaemle,
72 2007; Laurens *et al.*, 2016) reported that this regulatory function may be mediated by
73 coordinating the access of lipases to lipid droplets surface. In particular, *PLIN5* was
74 indicated as an essential player in the interaction with the Hormone-sensitive lipase

75 (LIPE). The *LIPE* gene maps on a QTL region linked to sensory quality of porcine
76 meat (Pena *et al.*, 2013), and its protein expression was found associated with
77 intramuscular fat content, in porcine *Semimembranosus* muscle (SM) (Zappaterra *et*
78 *al.*, 2016). The results reported by Pena *et al.* (2013) and Zappaterra *et al.* (2016)
79 suggest that LIPE might play an important role in pig meat and carcass traits, and the
80 investigation of physiological and molecular patterns regulating LIPE activity can
81 provide new knowledge to develop more suitable selection tools. However the
82 interaction between PLIN5 and lipases is still unclear, and to our knowledge, this
83 gene has never been analysed in pigs yet. The research aimed at investigating
84 *PLIN5* gene in samples belonging to different pig breeds and at studying PLIN5
85 protein localisation in muscle tissue of Italian Large White (ILW) and Italian Duroc
86 (IDU) pigs. In particular, the study of the gene was addressed to analyse the
87 sequence variability and to investigate the association between single nucleotide
88 polymorphism (SNP) of *PLIN5* and carcass traits, fat deposition and backfat fatty acid
89 composition. For this first investigation on porcine *PLIN5* gene we decided to focus
90 on the identification of sequence variability in the 3'untranslated (UTR) and
91 downstream gene regions, which are known to be highly variable when compared to
92 coding sequences (Tatarinova *et al.*, 2016). Additionally, the PLIN5 protein
93 localisation and the differences in *PLIN5* gene expression levels in SM were also
94 compared between ILW and IDU pigs, the two main pig breeds reared in Italy for
95 heavy pig production. Furthermore, associations between *PLIN5* sequence variation
96 and *LIPE* transcription levels were analysed with the aim of investigating possible
97 interactions between *PLIN5* variants and *LIPE* gene expression.

98

99 **Material and methods**

100 *Sampling*

101 For the present study, we used samples of different pig breeds. For the main breeds,
102 ILW and IDU, we collected 512 and 300 samples respectively. These two breeds
103 have been taken into account because of their importance in selection schemes
104 addressed to obtain high quality seasoned products. Sampled animals were pure
105 breed gilts and barrows included in the Italian sib test genetic evaluation scheme
106 performed by the Italian national pig breeders association (Associazione Nazionale
107 Allevatori Suini, ANAS; www.anas.it), reared in the same environmental conditions at
108 the genetic test station, fed the same diet *quasi ad libitum* (60% of the pigs was able
109 to ingest the entire supplied ration). At about 150 kg of live weight, the animals were
110 transported to a commercial abattoir located at about 25 km from the test station in
111 accordance with Council Rule (EC) No. 1/2005 regarding the protection of animals
112 during transport and related operations. At the slaughterhouse, the pigs were
113 electrically stunned and bled in a supine position in agreement with Council
114 Regulation (EC) No. 1 099/2009 regarding the protection of animals at the time of
115 slaughter. All slaughter procedures were monitored by the veterinary team appointed
116 by the Italian Ministry of Health. After slaughtering, for each individual two samples of
117 backfat and skeletal muscle tissue (obtained from SM) were collected: one aliquot of
118 each tissue was stored at -20 °C and the leftover aliquots were frozen in liquid
119 nitrogen and stored at -80 °C. For the immunohistochemical analysis, 10 samples of
120 SM, five taken from ILW and five from IDU were included in Tissue-Tek[®] O.C.T.
121 Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands). We
122 furthermore investigated on *PLIN5* SNP allele frequencies in samples of different pig
123 breeds available in our lab and that we collected in previous studies. Thereby,
124 besides analysing the ILW and IDU samples, we have also considered 260 additional

125 samples belonging to five pig breeds: 100 Italian Landrace pigs, 100 Pietrain, and 60
126 samples of three local pig breeds (Cinta Senese, Casertana and Calabrese, 20
127 samples per breed).

128

129 *Phenotyping*

130 *Carcass traits.* Carcass trait measures were partly provided by ANAS and partly
131 determined by slaughterhouse operators. Carcass weight was expressed in kg
132 (carcass weight), percentage of lean mass over the total carcass weight was
133 measured with the Fat-O-Meter (lean %) and backfat thickness was measured with
134 Fat-O-Meter at 8 cm off the midline of the carcass and between the third and fourth
135 last ribs. Furthermore, on the SM, the pH was measured one hour after slaughter
136 (pH₁) and at 24 hours post-mortem (pH₂₄), and the objective colour measurements
137 was performed using a Minolta Chroma Meter portable colorimeter applying the
138 L*a*b* colour system, according to the International Commission on Illumination
139 (CIE, 1976).

140

141 *Analysis of backfat fatty acid composition in Italian Large White pigs.* ILW backfat
142 tissue samples (stored at -20 °C) were processed by direct transesterification,
143 following the protocol reported by Murrieta *et al.* (2003) to obtain the fatty acid
144 composition of backfat. From each sample, 50 mg of frozen backfat was used for
145 total lipid extraction then, 0.5 mg of C19:0 methyl ester in hexane was added in each
146 tube, as internal standard. Gas chromatography was performed on GC- 2010 Plus
147 High-end Gas Chromatograph (Shimadzu Corporation, Tokyo, Japan), using SPTM-
148 2560 Capillary GC Column (Sigma- Aldrich, Merck, Darmstadt, Germany). Backfat

149 FA composition was expressed as the ratio between each individual FA or FA family
150 and the total backfat FAs.

151

152 *Immunohistochemical analysis to detect Perilipin 5 protein in muscle tissue*

153 *Immunofluorescence reaction.* SM samples of five ILW and five IDU carcasses were
154 randomly chosen among the 949 ILW and 484 IDU pigs and then analysed (Table 1).
155 Immediately after collection, muscle samples were cut into 1 × 1 cm pieces, parallel
156 to the muscle fiber direction, rapidly frozen in liquid nitrogen-cooled isopentane and
157 embedded in Tissue-Tek®. Serial cross-sections (10 µm thick) were cut on a cryostat
158 microtome at -20 °C and mounted on poly-l-lysine coated glass slides (Sigma-
159 Aldrich, St. Louis, MO, USA). Immunofluorescence was performed using the
160 procedure described previously by Gandolfi *et al.* (2011). The sections were fixed for
161 10 min in 4% paraformaldehyde in phosphate buffer saline (PBS, 0.1 M, pH 7.2),
162 rinsed in PBS and incubated in 5% normal donkey serum (NDS) for 30 min at room
163 temperature (RT) to reduce the non-specific binding of the secondary antibodies. The
164 sections were then incubated at 4 °C in a humid chamber for 24 hours in a mixture of
165 3% NDS and the primary antibodies *rabbit anti-PLIN5* (Cat. NB110-60509, Novus
166 Biologicals, Littleton, Colorado, USA) diluted 1/2 000. The next day, after washing in
167 PBS, the sections were incubated for 1 hour at room temperature in a PBS mixture
168 containing 3% of NDS and the secondary antibody donkey anti-rabbit
169 immunoglobulin G (IgG) Alexa Fluor 488-conjugated antibody (Invitrogen, Thermo
170 Fisher Scientific Inc., Waltham, MA, USA) diluted 1/1 300. After washing in PBS, the
171 sections were mounted with buffered glycerol, pH 8.6. The specificity of the
172 secondary antibodies was tested by performing the staining in the absence of

173 primary antibodies as a control. The specificity of primary antibodies was tested by
174 Western blot.

175

176 *Morphometrical analysis.* The sections were examined using a Zeiss Axioplan
177 microscope equipped with the appropriate filter cubes to discriminate different
178 fluorochromes. The images were recorded with a Polaroid DMC digital camera
179 (Polaroid, Cambridge, Mass., U.S.A.) and the DMCV 2 software images were further
180 processed using Corel Photo Paint and Corel Draw software programs (Corel, Milan,
181 Italy). Morphometrical analyses were carried out considering for each sample the
182 cross-sectional area (CSA, measured in μm^2) of 100 myofibers with a 20x objective
183 lens using KS 300 image analysis software (Kontron Elektronik, Munich, Germany).
184 For each sample, the total CSA defined by PLIN5 negative myofibers was measured
185 by outlining the profiles on the monitor screen using a computer mouse. The relative
186 percentage of PLIN5 immunoreactive (PLIN5-IR) myofibers was calculated
187 considering the total area of the 100 myofibers measured for each pig from which the
188 total area of the PLIN5 negative myofibers was deducted.

189

190 *Statistical analysis.* Data are reported as mean and SD. Differences were tested by
191 Wilcoxon test, using Stats package on R environment version 3.3.2 (R Core Team,
192 2016). $P \leq 0.05$ was considered as the threshold for significance.

193

194 *Immunostaining validation.* Western blot was performed to assure the specificity of
195 the primary antibodies used for PLIN5 immunostaining. About 300 mg of SM sample
196 stored at $-80\text{ }^\circ\text{C}$ was homogenized on ice in 900 μl of T-PER Tissue Protein
197 Extraction (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) following

198 the protocol reported in Zappaterra *et al.* (2016). About 20–60 µg of the total protein
199 was separated in a 12% SDS-polyacrylamide gel and blotted into a nitrocellulose
200 membrane (GE Healthcare/Amersham, Uppsala, Sweden). Beta Actin molecular
201 weight was used as standard to assess PLIN5 molecular weight. After blocking in 5%
202 non-fat milk-Tris-buffered saline (TBS), membranes were incubated overnight with
203 1/4 000 dilutions of *Rabbit anti-PLIN5* and with 1/1 000 dilutions of *Rabbit anti-beta*
204 *actin* antibody (ab8227, abcam, Cambridge, UK). After washing, membranes were
205 incubated for 1 h with 1/1 000 dilutions of horseradish peroxidase-conjugated anti-
206 rabbit IgG (Santa Cruz Biotechnology, Dallas, Texas, USA). The membrane blots
207 were developed with 3,3'-diaminobenzidine (DAB, Santa Cruz Biotechnology, Dallas,
208 Texas, USA), captured using Canon SX500 IS (Canon Inc., Tokyo, Japan), and the
209 images were adapted and contrast-enhanced using Adobe Photoshop CS4 (Adobe
210 Systems Incorporated, San Jose, California, USA). PLIN5 molecular weight was
211 assessed comparing the location on membranes of PLIN5 bands respect to beta-
212 actin bands. The observed molecular weight was then compared using UniProt
213 database (The UniProt Consortium, 2017) with PLIN5 weights in other species.
214
215 *Perilipin 5 single nucleotide polymorphism detection and association study*
216 *Single nucleotide polymorphism detection.* Porcine *PLIN5* gene sequence obtained
217 from Ensembl database (last accessed on September 20th, 2017;
218 <http://www.ensembl.org/index.html>) was compared with sequences in GenBank
219 database (<https://www.ncbi.nlm.nih.gov/genbank/>). The *PLIN5* 3'untranslated region
220 (UTR) and downstream gene regions of fifteen ILW samples randomly chosen were
221 sequenced using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). A primer
222 pair was designed for the sequencing using Primer3web software version 4.0.0

223 (<http://primer3.ut.ee/>; Supplementary Table S1). PCR were performed in a total
224 volume of 20 µl containing 4 µl of 5x standard buffer, 1.5 mM of magnesium chloride
225 solution (MgCl₂), 0.5 µM of each primer, 160 µM of deoxynucleotide solution (dNTP),
226 1 U of GoTaq[®] G2 DNA Polymerase (Promega Corporation, Madison, USA) and 20–
227 50 ng of template DNA. The PCR products of 15 ILW samples were then sequenced
228 with the Big Dye Terminator kit (Applied Biosystems, Thermo Fisher Scientific Inc.,
229 Waltham, MA, USA) and the automatic sequencer ABI PRISM 3100 Genetic
230 Analyzer (Applied Biosystems). The obtained nucleotide sequences and those
231 retrieved from databases were compared by multiple alignments performed with
232 MEGA software version 6.06 (Tamura *et al.*, 2013). Furthermore, the MEME suite
233 (Bailey *et al.*, 2009) was used to test if the SNP was located inside a motif
234 represented in *PLIN5* sequence as well as to predict potential effects of the SNP
235 changing the structure of a DNA motif.

236

237 *Single nucleotide polymorphism genotyping.* 512 ILW samples and 300 IDU pigs
238 were genotyped for the SNP identified through sequencing. Furthermore, the allele
239 frequencies were also investigated in 100 Italian Landrace, 100 Pietrain, and 60 pigs
240 belonging to the Italian autochthonous breeds of Calabrese, Cinta Senese and
241 Casertana (20 samples per breed). Samples were genotyped using High-Resolution
242 Melt (HRM) analysis on Rotor-Gene[®] 6000 (Corbett Life Science, Concorde, New
243 South Wales). Primers were designed using Primer3web software version 4.0.0
244 (Supplementary Table S1), and the amplification procedure was performed in 35
245 cycles with a denaturation step at 95 °C for 20 s, the annealing step at 63 °C for 15 s
246 and the extension at 72 °C for 10 s.

247

248 *Statistical analysis.* Allelic frequencies of the identified SNP were calculated and
249 deviations from Hardy-Weinberg equilibrium were evaluated by a χ^2 test. The
250 association study between the SNP and the phenotypes was performed in ILW
251 samples using two approaches. Firstly, TM, a Bayesian statistics-based software
252 (Legarra *et al.*, 2011), was applied. The used statistical models were: for backfat fatty
253 acid composition the model included backfat thickness, animal's age (days of life)
254 and carcass weight as covariates, slaughter batch as random effect (27 slaughter
255 batches), and the effects of sex (castrated males or female) and animal (using a
256 pedigree with 1 724 individuals) were taken into account. For the other phenotypic
257 measures, the same statistical model was used including among the covariates the
258 interaction between carcass weight and animal's age instead of backfat thickness.
259 For each trait, the estimated means and differences between genotypes were
260 assessed on Rabbit program (Blasco, 2012) using the same models performed in
261 TM. Then, a second statistical approach was considered to test the association found
262 with the Bayesian method. For each trait we chose a group of divergent ILW samples
263 belonging to the two extreme tails and deviating from the population mean value by
264 at least 1.5 SD units. In cases where the considered trait was not normally distributed
265 (Shapiro-Wilk test of normality: P value < 0.05), data were transformed to achieve
266 normal distribution using Box-Cox transformation and then the two extreme groups
267 were identified. Genotype and allele frequencies were compared between the two
268 extreme groups using two tailed Fisher's exact test and Cochran-Armitage trend
269 tests. $P \leq 0.05$ was considered as the threshold for significance. These statistical
270 analyses were carried out in R environment version 3.3.2 (R Core Team, 2016) using
271 *stats* and *car* packages. The associations found with both the Bayesian approach
272 and Cochran-Armitage trend test were analysed and further discussed.

273

274 *Gene expression study*

275 *Sampling.* For the comparison of *PLIN5* gene expression between breeds, 30 ILW

276 and 30 IDU were chosen avoiding as much as possible full and half sibs and to

277 balance the two groups for sex (Table 1). Then, both *PLIN5* and *LIPE* gene

278 expressions were tested on 69 ILW pigs (composed of 30 *TT*, 20 *CT* and 19 *CC*

279 samples for the studied SNP) and on 34 IDU (comprising 13 *TT*, 18 *CT* and 3 *CC*).

280 The sample sizes were established taking into account the higher frequency of *PLIN5*

281 *T* allele in the studied populations.

282

283 *Total RNA extraction from muscle tissue.* *Semimembranosus* muscle samples were

284 collected at the slaughterhouse and immediately frozen at -80°C . Total RNA was

285 then extracted using TRIZOL reagent (Invitrogen Corporation, Carlsbad, California),

286 the quality and integrity of the RNA were both checked and RNA was

287 retrotranscribed to complementary DNA (cDNA) as described in Davoli *et al.* (2011).

288 Primers were designed using Primer3web software version 4.0.0 (Supplementary

289 Table S1). Gene expression was analysed by quantitative Real-Time PCR (qRT-

290 PCR) using the standard curve method. qRT-PCR was performed on Rotor-Gene™

291 6 000 (Corbett Life Science, Concorde, New South Wales) as described in

292 Zappaterra *et al.* (2016), and *beta-2-microglobulin (B2M)*, *tyrosine 3-*

293 *monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ)* and

294 *hypoxanthine phosphoribosyltransferase1 (HPRT1)* have been chosen as

295 normalising genes. The protocols used for the amplification reactions and for the

296 standard curves are the same described in Zappaterra *et al.* (2016).

297

298 *Statistical analysis of gene expression data.* Differences in gene expression levels
299 were tested by two-tailed Student's t-test. Gene expressions were compared: i)
300 between breeds, to test *PLIN5* gene expression variations; ii) between different
301 genotypes within each breed, in order to consider possible associations of the *PLIN5*
302 SNP with *PLIN5* and *LIPE* expression levels. Moreover, Pearson's correlation
303 analysis was performed between the messenger RNA (mRNA) levels of these two
304 genes using `cor.test` of `stats` package in R environment version 3.3.2 (R Core Team,
305 2016). $P \leq 0.05$ was considered as the threshold for significance.

306

307 **Results**

308 *Perilipin 5 protein localisation in Semimembranosus muscle*

309 This is the first study investigating PLIN5 protein localisation in porcine SM. The
310 specificity of the antibody was confirmed by Western-blot, as a unique band was
311 identified (Figure 1A). In Supplementary Figure S1 is reported the full membrane with
312 beta actin band taken as standard to assess the molecular weight of PLIN5. In
313 Western-blot membrane, PLIN5 lied above beta-actin bands, suggesting for PLIN5
314 an approximate molecular weight of 50 kDa, similar to what observed in mouse
315 (Q8BVZ1 in UniProt database) and human (Q00G26 in UniProt database).

316 Furthermore, all porcine SM samples were positive for PLIN5 antigens. Myofibers
317 were PLIN5-IR for the most part, presenting only isolated (Figure 1B) or grouped in
318 pairs (Figure 1C) PLIN5 negative myofibers. PLIN5-IR myofibers showed a broad
319 fluorescence pattern in the whole sarcoplasm, and a strong intensity staining was
320 found localised mainly at sarcolemma and perimysial collagen levels (Figure 1B and
321 C). Supplementary Table S2 reports the results of the morphometrical analysis for
322 both ILW and IDU samples. No significant difference for the morphometrical

323 measures between the two breeds was detected: all the samples showed PLIN5
324 staining CSA percentages above 87%, with peaks of the 100% of immunoreactivity in
325 some sections.

326

327 *Single nucleotide polymorphism genotyping and allele frequencies*

328 The results of the sequencing performed on ILW DNA samples for *PLIN5* 3'UTR and
329 downstream region showed a unique SNP lying in *PLIN5* gene downstream region.
330 Comparing this polymorphism with those present in Ensembl database we noticed
331 that the same SNP was already reported among the nucleotide variants associated
332 with *PLIN5* and *PLIN4* genes (variant ID: rs327694326, HGVS name
333 NC_010444.4:g.74314701 T>C). The polymorphism was not located in a genomic
334 sequence with binding sites for microRNAs nor in a repeated motif. The SNP
335 rs327694326 deviated from Hardy-Weinberg equilibrium in ILW samples ($P < 0.0001$)
336 with the *C* allele showing a lower frequency than the *T* allele in ILW samples (0.14 for
337 the *C* allele against 0.86 observed for the *T* allele; Table 2). In IDU samples the *C*
338 allele was rare, displaying an allele frequency of 0.04 (Table 2) and an expected
339 number of *CC* animals that was too low and it was not possible to test the Hardy-
340 Weinberg equilibrium in this population (expected number of *CC* animals < 5). Due to
341 the very low frequency of the *C* allele in IDU samples, the association study between
342 SNP and phenotypes was performed only in ILW samples. The *C* allele was very rare
343 in Pietrain pigs with the frequency of 0.03 (three *CC* individuals out of the 100 tested
344 Pietrain), while Italian Landrace pigs and the three Italian native breeds showed only
345 the *T* allele. Considering the limited size of the sample available for the Italian local
346 breeds and the excessively low frequency of the *C* allele in Pietrain (found to be

347 absent in the samples of the native breeds), the association study between the SNP
348 and the phenotypic traits was carried out in ILW pigs.

349

350 *Associations between single nucleotide polymorphism and backfat fatty acid*
351 *composition and carcass traits*

352 In ILW individuals, both the statistical approaches suggested that rs327694326 SNP
353 could be associated with backfat contents of oleic, cis-vaccenic and
354 monounsaturated fatty acids and with *CIE a** colour parameter in SM. The Bayesian
355 statistics analysis identified a dominant effect of the *C* allele for backfat
356 monounsaturated fatty acids, and an overdominant effect of the heterozygote *TC* for
357 both oleic and cis-vaccenic fatty acids (Table 3). The Cochran Armitage test trend
358 produced quite concordant and convergent results because the comparison of the
359 SNP allele frequencies in the two divergent groups showed that the *C* allele was
360 significantly more frequent in the samples with higher contents of oleic and cis-
361 vaccenic fatty acids ($P = 0.026$; Table 4). Moreover, rs327694326 *T* allele seemed to
362 be linked to higher *CIE a** colour values, passing from the frequency of 0.760 in pigs
363 with low values for *CIE a** colour parameter to 0.934 in high *CIE a** individuals (Table
364 4). The overall results of the Bayesian analysis and the statistic trend test used to
365 study associations between the SNP and traits are reported in Supplementary Table
366 S3, Supplementary Table S4 and Supplementary Table S5.

367

368 *Gene expression study*

369 The results highlighted a higher transcription level of this Perilipin gene in ILW
370 samples than in IDU ($P = 0.005$; Figure 2A). Furthermore, rs327694326 was also
371 tested to evaluate the association between *PLIN5* and *LIPE* gene expression levels.

372 The transcription of both genes was quantified and compared between samples with
373 different *PLIN5* genotypes . ILW individuals showing *TT* genotype presented higher
374 *LIPE* mRNA levels than *CT* ($P = 0.02$) and *CC* pigs ($P < 0.0001$) (Figure 2B). IDU
375 samples presented the highest *PLIN5* transcriptional levels in *CT* pigs ($P = 0.01$ for
376 *CT* vs. *TT*; Figure 2C). The low frequency of *C* allele in IDU did not permit
377 comparisons of the mRNA levels between the *CC* samples and *CT* or *TT* individuals.
378 The results of the correlation analysis between *PLIN5* and *LIPE* expression levels
379 can be seen in Table 5. The two breeds presented significant correlations between
380 *PLIN5* and *LIPE* expressions, and in particular IDU samples showed a higher
381 correlation compared to ILW ($r = 0.597$ in IDU and $r = 0.371$ in ILW pigs).

382

383 **Discussion**

384 The widespread expression of *PLIN5* protein detected in SM myofibers of both ILW
385 and IDU pigs is consistent with the literature, which reports a higher presence of
386 *PLIN5* in oxidative tissues, such as type I skeletal muscle fibers, cardiac muscle and
387 liver (Dalen *et al.*, 2007; MacPherson *et al.*, 2012). Ruusunen and Puolanne (2004)
388 and Lefaucheur *et al.* (2010) observed that, unless it is classified as white skeletal
389 muscle, porcine SM is mainly composed of type IIA myofibers, characterized by a
390 higher oxidative capacity and a red appearance if compared to glycolytic (type IIB)
391 muscle fibers. Hence, the prevalence of *PLIN5*-IR myofibers detected in the present
392 research is in accordance with the SM oxidative capacity reported by Ruusunen and
393 Puolanne (2004) and Lefaucheur *et al.* (2010). Furthermore, the broad fluorescence
394 pattern in sarcoplasm confirms the evidences observed in other species, where
395 *PLIN5* protein localised to intracellular lipid droplets surface (Yamaguchi *et al.*, 2006),
396 to mitochondria (Bosma *et al.*, 2012), as well as a free form in cytosol (Wolins *et al.*,

397 2006). No difference was observed between the two considered breeds in the
398 number and area of PLIN5-IR myofibers, while distinct *PLIN5* gene expression levels,
399 with a higher transcription level in ILW samples, were displayed. These data need to
400 be validated in other pig populations before any conclusive assumption can occur.
401 Anyway, it is worth noting that the observed differences in *PLIN5* expression levels
402 between ILW and IDU and the molecular processes influencing gene expression
403 could be related to the different genetic background and different adipogenic
404 potential of these breeds. In particular Large White pigs, compared to Duroc, show a
405 lower capacity to store fat in muscle tissue (Jung *et al.*, 2015).
406 The relation between PLIN5 and lipases was evidenced by the positive correlation
407 value linking *PLIN5* and *LIPE* transcription levels, identified in both breeds,
408 particularly strong in IDU pigs. Current literature lacks studies describing the link
409 between *PLIN5* and *LIPE* transcription patterns in livestock species. However, the
410 *PLIN5* and *LIPE* co-expression found in the present research may be consistent with
411 the observations of Wang *et al.* (2009), who noticed that PLIN5 and LIPE proteins co-
412 expressed on lipid droplets surface in cultured cells. Furthermore, it is worth noting
413 that both *PLIN5* and *LIPE* genes have been reported to be up-regulated by
414 Peroxisome Proliferator-Activated Receptor alpha (PPAR α) (Deng *et al.*, 2006;
415 Bindsbøll *et al.*, 2013), thus implying that the identified co-expression may represent
416 the effect of a common transcriptional regulative process linking the two investigated
417 genes. Additionally, another possible explanation could be that *PLIN5* expression
418 directly affects *LIPE* gene activity. This last hypothesis seems to be supported by the
419 associations found between the SNP located in the *PLIN5* gene downstream region
420 and both *PLIN5* and *LIPE* gene expression levels. Anyway, as the SNP is not located
421 in a binding site for microRNAs nor in a repeated motif, we believe that the

422 polymorphism could be in linkage disequilibrium with other causal mutations affecting
423 *PLIN5* gene expression, and consequently also *LIPE* gene expression. Although the
424 positive co-expression between *PLIN5* and *LIPE* identified in the present work seems
425 to contradict the generally-accepted view that *PLIN5* limits lipolysis (MacPherson and
426 Peters, 2015; Wang *et al.*, 2015), it is worth noting that the information about the
427 specific function of *PLIN5* on fat metabolism is still contradictory. In fact, while some
428 studies identified this protein as a player limiting lipid droplets hydrolysis (Laurens *et*
429 *al.*, 2016), others reported that its overexpression increases fat oxidation (Bosma *et*
430 *al.*, 2012). The controversy around the role played by *PLIN5* in skeletal muscle could
431 be explained considering the complexity of biological actions and the number of
432 factors involved in lipases activities. Bosma *et al.* (2012) and Pollak *et al.* (2015)
433 reported that both the phosphorylation of *PLIN5* protein in particular sites (i.e. the
434 PAT-1 domain) and the phosphorylation of *LIPE*, are among the regulative
435 mechanisms hypothesised in lipase activity control. The results observed for the SNP
436 rs327694326 seemed to suggest that the interaction of *PLIN5* with lipases could also
437 be regulated at the gene level. Anyway, due to the location of this variant in the
438 downstream region of *PLIN5*, we suppose that the SNP rather than a direct causative
439 variant could be an indirect marker in linkage disequilibrium with the functional
440 mutation involved in changes in *LIPE* expression. Furthermore, the SNP
441 rs327694326 seemed also to be associated in ILW samples with oleic and cis-
442 vaccenic backfat contents and with CIE a^* value in SM. Oleic acid is the most
443 abundant monounsaturated fatty acid in pork and its amounts have been linked to
444 consumers' acceptability of high quality cured products (Pena *et al.*, 2016). Thus, the
445 identification of genetic markers associated with this trait may represent a valuable
446 contribution in improving meat and seasoned product quality. In the tested ILW

447 samples, the *CT* heterozygotes for rs327694326 showed an overdominant effect,
448 with higher estimated means for the traits compared to both the homozygotes.
449 Underdominant and overdominant effects were also observed by Gol *et al.* (2016) for
450 SNPs in the sequence of two other Perilipin family members, suggesting that there
451 may be epistatic effects linking this gene family.
452 The complete understanding of the effects associated to rs327694326 remains to be
453 elucidated and further studies are needed to deepen the role of *PLIN5* on fat traits in
454 pig. On the whole, the results suggest that this gene could have a potential role in pig
455 fat traits mainly concerning the backfat content of oleic fatty acid. Further
456 investigations may offer new opportunities to understand the underlying genetic
457 factors affecting porcine fat deposition and composition.

458

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466

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563

564 **Table 1** *The considered Italian Large White and Italian Duroc samples used for the*
 565 *detection of PLIN5 antigens through immunohistochemical analysis, for the*
 566 *genotyping of rs327694326 SNP and for the comparison between breeds of PLIN5*
 567 *gene expressions.*

	Immunohistochemical analysis		Genotyping		Gene expression study	
	ILW ¹	IDU ²	ILW ¹	IDU ²	ILW ¹	IDU ²
Total	5	5	512	300	30	30
Sows	3	3	339	203	20	10
Barrows	2	2	173	97	18	12
Days of slaughter	2	2	27	19	16	10
Litters	5	5	270	148	28	25

568 ¹ Italian Large White pigs.

569 ² Italian Duroc pigs.

570 **Table 2** Genotyping results and rs327694326 allele frequencies in Italian Large

571 White and Italian Duroc samples.

	Allele									
	N	TT		CT		CC		frequencies		HWE ¹
		N	%	N	%	N	%	T	C	
ILW ²	512	395	0.771	92	0.180	25	0.049	0.861	0.139	P<0.0001
IDU ³	300	278	0.927	19	0.063	3	0.010	0.958	0.042	-

572 For Italian Duroc pigs the C allele frequency was too low to calculate the deviance from Hardy-

573 Weinberg equilibrium

574 ¹ Hardy-Weinberg equilibrium.

575 ² Italian Large White pigs.

576 ³ Italian Duroc pigs.

577 **Table 3** Additive and dominant effects of rs327694326 SNP on backfat contents of oleic, cis-vaccenic, monounsaturated fatty acids
 578 and CIE a* parameter measured on Semimembranosus muscle, with the means and differences of the estimated marginal posterior
 579 distribution for the genotypes in Italian Large White pigs.

Backfat fatty acids ¹	Additive (a) and dominant (d) effects				Estimated means			Estimated differences between genotypes					
								<i>TT-CT</i>		<i>CT-CC</i>		<i>TT-CC</i>	
	a	P(>0)	d	P(>0)	<i>TT</i>	<i>CT</i>	<i>CC</i>	Mean	P(>0)	Mean	P(>0)	Mean	P(>0)
C18:1, cis-9 ²	0.001	0.64	0.002	0.85	38.41	38.66	38.39	-0.25	0.07	0.27	0.79	0.02	0.52
C18:1, cis-11 ³	-0.00002	0.48	0.0003	0.83	2.18	2.22	2.20	-0.03	0.10	0.03	0.69	-0.008	0.44
MUFA ⁴	0.0005	0.61	0.003	0.90	43.61	43.94	43.61	-0.33	0.05	0.33	0.81	-0.001	0.50
CIE a*	0.003	0.91	0.003	0.86	9.17	9.22	8.69	-0.05	0.42	0.53	0.88	0.48	0.88

580 ¹ All the fatty acids or fatty acid categories are expressed as percentage on the total fatty acids amount.

581 ² Oleic acid; ³ Cis-vaccenic acid; ⁴ Monounsaturated fatty acids.

582 CIE stands for International Commission on Illumination.

583 P (>0): Posterior probability of a value being positive. In bold, probabilities above (or equal to) 0.90 or below 0.10. Oleic and cis-vaccenic levels were reported
 584 in Table 3 as the observed P(>0) were close to the considered thresholds of 0.90 and 0.10.

585 **Table 4** Number of samples per each extreme group, genotypic and allelic frequencies inside the two tails of extreme and divergent
586 samples, and the results of the two tailed Fisher's exact test and Cochran-Armitage trend tests performed on the genotypic
587 frequencies of rs327694326 SNP in Italian Large White pigs.

Traits	Groups		<i>TT</i>		<i>CT</i>		<i>CC</i>		Allele frequencies (%)		Fisher's exact	X ²	Cochran-Armitage
	Type	N	N	%	N	%	N	%	<i>T</i>	<i>C</i>	<i>P</i> -value		<i>P</i> -value
C18:1, cis-9 ¹	low	29	28	0.965	0	0.000	1	0.035	0.965	0.035	0.007	4.984	0.026
	high	35	24	0.686	7	0.200	4	0.114	0.786	0.214			
C18:1, cis-11 ²	low	29	28	0.965	0	0.000	1	0.035	0.965	0.035	0.007	4.984	0.026
	high	35	24	0.686	7	0.200	4	0.114	0.786	0.214			
MUFA ³	low	31	29	0.935	1	0.032	1	0.032	0.952	0.048	0.108	3.494	0.062
	high	38	28	0.737	7	0.184	3	0.079	0.829	0.171			
CIE a*	low	25	17	0.680	4	0.160	4	0.160	0.760	0.240	0.036	5.650	0.017
	high	38	33	0.868	5	0.132	0	0.000	0.934	0.066			

588 ¹Oleic acid; ²Cis-vaccenic acid; ³Monounsaturated fatty acids. All the fatty acids or fatty acid categories are expressed as percentage on the total fatty acids
589 amount.

590 CIE stands for International Commission on Illumination.

591

592 **Table 5** *The Pearson's correlation results between PLIN5 and LIPE expressions in*
 593 *Italian Large White and Italian Duroc samples.*

	<i>PLIN5</i> gene expression		
	n ¹	r ²	<i>P</i> value
<i>LIPE</i> gene expression			
ILW ³	69	0.371	0.003
IDU ⁴	34	0.597	0.0002

594 ¹ The number of the considered samples

595 ² the Pearson's correlation coefficient.

596 ³ Italian Large White pigs.

597 ⁴ Italian Duroc pigs.

598

599 **Figure captions**

600

601 **Figure 1** Western blot results confirming the specificity of primary antibody rabbit
602 anti-PLIN5 (A). Cross-sections from *Semimembranosus* muscle in Italian Duroc (B)
603 and Italian Large White (C) pigs stained by PLIN5. The images show that the majority
604 of muscle fibers are PLIN5 immunoreactive, while some isolated or coupled fibers
605 appear unlabeled (asterisks).

606 **Figure 2** Gene expression results and Student's t test *P* values of the comparisons
607 between *PLIN5* levels in the two breeds (A) and between *PLIN5* and *LIPE*
608 expressions for the different genotypes of rs327694326 SNP in Italian Large White
609 (B) and Italian Duroc pigs (C).