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

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

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

Abstract

The growing request for healthier fatty acid composition of animal products is raising the necessity of a deeper knowledge of the main factors controlling fatty acids storage in muscle and backfat. Perilipin (PLIN) 5, and generally the whole Perilipin family, seems to play a crucial role in the regulation of lipids deposition as codes for proteins coating intracellular lipid droplets surface. Nevertheless, the knowledge of these genes in pig is still incomplete. The present research was aimed at investigating in different pig breeds the *PLIN5* gene, analysing its expression level and the associations of the variability in its downstream gene region with pork meat and carcass quality traits. Moreover, the PLIN5 protein localisation in porcine skeletal

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

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

Implications

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

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

Introduction

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

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

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

Material and methods

Sampling

For the present study, we used samples of different pig breeds. For the main breeds, ILW and IDU, we collected 512 and 300 samples respectively. These two breeds have been taken into account because of their importance in selection schemes addressed to obtain high quality seasoned products. Sampled animals were pure breed gilts and barrows included in the Italian sib test genetic evaluation scheme performed by the Italian national pig breeders association (Associazione Nazionale Allevatori Suini, ANAS; www.anas.it), reared in the same environmental conditions at the genetic test station, fed the same diet *quasi ad libitum* (60% of the pigs was able to ingest the entire supplied ration). At about 150 kg of live weight, the animals were transported to a commercial abattoir located at about 25 km from the test station in accordance with Council Rule (EC) No. 1/2005 regarding the protection of animals during transport and related operations. At the slaughterhouse, the pigs were electrically stunned and bled in a supine position in agreement with Council Regulation (EC) No. 1 099/2009 regarding the protection of animals at the time of slaughter. All slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health. After slaughtering, for each individual two samples of backfat and skeletal muscle tissue (obtained from SM) were collected: one aliquot of each tissue was stored at -20 °C and the leftover aliquots were frozen in liquid nitrogen and stored at -80 °C. For the immunohistochemical analysis, 10 samples of SM, five taken from ILW and five from IDU were included in Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands). We furthermore investigated on *PLIN5* SNP allele frequencies in samples of different pig breeds available in our lab and that we collected in previous studies. Thereby, 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 (pH_1) and at 24 hours post-mortem (pH_{24}), 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

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

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

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

Immunostaining validation. Western blot was performed to assure the specificity of the primary antibodies used for PLIN5 immunostaining. About 300 mg of SM sample stored at −80 °C was homogenized on ice in 900 µl of T-PER Tissue Protein 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

(http://primer3.ut.ee/; Supplementary Table S1). PCR were performed in a total volume of 20 µl containing 4 µl of 5x standard buffer, 1.5 mM of magnesium chloride solution ($MgCl_2$), 0.5 µM of each primer, 160 µM of deoxynucleotide solution (dNTP), 1 U of GoTaq® G2 DNA Polymerase (Promega Corporation, Madison, USA) and 20–50 ng of template DNA. The PCR products of 15 ILW samples were then sequenced with the Big Dye Terminator kit (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the automatic sequencer ABI PRISM 3 100 Genetic Analyzer (Applied Biosystems). The obtained nucleotide sequences and those retrieved from databases were compared by multiple alignments performed with MEGA software version 6.06 (Tamura *et al*., 2013). Furthermore, the MEME suite (Bailey *et al*., 2009) was used to test if the SNP was located inside a motif represented in *PLIN5* sequence as well as to predict potential effects of the SNP changing the structure of a DNA motif.

Single nucleotide polymorphism genotyping. 512 ILW samples and 300 IDU pigs were genotyped for the SNP identified through sequencing. Furthermore, the allele frequencies were also investigated in 100 Italian Landrace, 100 Pietrain, and 60 pigs belonging to the Italian autochthonous breeds of Calabrese, Cinta Senese and Casertana (20 samples per breed). Samples were genotyped using High-Resolution Melt (HRM) analysis on Rotor-Gene® 6 000 (Corbett Life Science, Concorde, New South Wales). Primers were designed using Primer3web software version 4.0.0 (Supplementary Table S1), and the amplification procedure was performed in 35 cycles with a denaturation step at 95 °C for 20 s, the annealing step at 63 °C for 15 s and the extension at 72 °C for 10 s.

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

Gene expression study

Sampling. For the comparison of *PLIN5* gene expression between breeds, 30 ILW and 30 IDU were chosen avoiding as much as possible full and half sibs and to balance the two groups for sex (Table 1). Then, both *PLIN5* and *LIPE* gene expressions were tested on 69 ILW pigs (composed of 30 *TT*, 20 *CT* and 19 *CC* samples for the studied SNP) and on 34 IDU (comprising 13 *TT*, 18 *CT* and 3 *CC*). The sample sizes were established taking into account the higher frequency of *PLIN5* *T* allele in the studied populations.

Total RNA extraction from muscle tissue. Semimembranosus muscle samples were collected at the slaughterhouse and immediately frozen at −80 °C. Total RNA was then extracted using TRIZOL reagent (Invitrogen Corporation, Carlsbad, California), the quality and integrity of the RNA were both checked and RNA was retrotranscribed to complementary DNA (cDNA) as described in Davoli *et al*. (2011). Primers were designed using Primer3web software version 4.0.0 (Supplementary Table S1). Gene expression was analysed by quantitative Real-Time PCR (qRT-PCR) using the standard curve method. qRT-PCR was performed on Rotor-Gene™ 6 000 (Corbett Life Science, Concorde, New South Wales) as described in Zappaterra *et al*. (2016), and *beta-2-microglobulin* (*B2M*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta* (*YWHAZ*) and *hypoxanthine phosphoribosyltransferase1* (*HPRT1*) have been chosen as normalising genes. The protocols used for the amplification reactions and for the standard curves are the same described in Zappaterra *et al*. (2016).

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

Results

Perilipin 5 protein localisation in Semimembranosus *muscle*

This is the first study investigating PLIN5 protein localisation in porcine SM. The specificity of the antibody was confirmed by Western-blot, as a unique band was identified (Figure 1A). In Supplementary Figure S1 is reported the full membrane with beta actin band taken as standard to assess the molecular weight of PLIN5. In Western-blot membrane, PLIN5 lied above beta-actin bands, suggesting for PLIN5 an approximate molecular weight of 50 kDa, similar to what observed in mouse (Q8BVZ1 in UniProt database) and human (Q00G26 in UniProt database). Furthermore, all porcine SM samples were positive for PLIN5 antigens. Myofibers were PLIN5-IR for the most part, presenting only isolated (Figure 1B) or grouped in pairs (Figure 1C) PLIN5 negative myofibers. PLIN5-IR myofibers showed a broad fluorescence pattern in the whole sarcoplasm, and a strong intensity staining was found localised mainly at sarcolemma and perimysial collagen levels (Figure 1B and C). Supplementary Table S2 reports the results of the morphometrical analysis for both ILW and IDU samples. No significant difference for the morphometrical

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

Single nucleotide polymorphism genotyping and allele frequencies

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

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

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

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

Gene expression study

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

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

Discussion

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

2006). No difference was observed between the two considered breeds in the number and area of PLIN5-IR myofibers, while distinct *PLIN5* gene expression levels, with a higher transcription level in ILW samples, were displayed. These data need to be validated in other pig populations before any conclusive assumption can occur. Anyway, it is worth noting that the observed differences in *PLIN5* expression levels between ILW and IDU and the molecular processes influencing gene expression could be related to the different genetic background and different adipogenic potential of these breeds. In particular Large White pigs, compared to Duroc, show a lower capacity to store fat in muscle tissue (Jung *et al*., 2015).

The relation between PLIN5 and lipases was evidenced by the positive correlation value linking *PLIN5* and *LIPE* transcription levels, identified in both breeds, particularly strong in IDU pigs. Current literature lacks studies describing the link between *PLIN5* and *LIPE* transcription patterns in livestock species. However, the *PLIN5* and *LIPE* co-expression found in the present research may be consistent with the observations of Wang *et al*. (2009), who noticed that PLIN5 and LIPE proteins co-expressed on lipid droplets surface in cultured cells. Furthermore, it is worth noting that both *PLIN5* and *LIPE* genes have been reported to be up-regulated by Peroxisome Proliferator-Activated Receptor alpha (PPARα) (Deng *et al*., 2006; Bindesbøll *et al*., 2013), thus implying that the identified co-expression may represent the effect of a common transcriptional regulative process linking the two investigated genes. Additionally, another possible explanation could be that *PLIN5* expression directly affects *LIPE* gene activity. This last hypothesis seems to be supported by the associations found between the SNP located in the *PLIN5* gene downstream region and both *PLIN5* and *LIPE* gene expression levels. Anyway, as the SNP is not located in a binding site for microRNAs nor in a repeated motif, we believe that the

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

		TT		*CT*		*CC*		Allele frequencies		
	N	N	%	N	%	N	%	*T*	*C*	HWE[1]
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					
								TT-CT		*CT-CC*		*TT-CC*	
	a	P(>0)	d	P(>0)	*TT*	*CT*	*CC*	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.

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

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

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

CIE stands for International Commission on Illumination.

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

	PLIN5 gene expression		
	n[1]	r[2]	*P* value
LIPE gene expression			
ILW[3]	69	0.371	0.003
IDU[4]	34	0.597	0.0002

[1] The number of the considered samples

[2] the Pearson's correlation coefficient.

[3] Italian Large White pigs.

[4] Italian Duroc pigs.

Figure captions

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

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