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Identification of differentially expressed genes in early-postmortem Semimembranosus muscle of Italian Large White heavy pigs divergent for glycolytic potential

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1 **Identification of differentially expressed genes in early-*postmortem***
2 ***Semimembranosus* muscle of Italian Large White heavy pigs divergent for**
3 **glycolytic potential**

4
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17 board. The other co-authors declare that they have no competing interests.

18

19 **Abstract**

20 Glycolytic potential (GP) is one of the *postmortem* traits utilized to predict the quality of the final
21 meat products. Despite that, the knowledge of the molecular and metabolic pathways controlling
22 this trait is still not complete. To add some information on this field we used two pools of Italian
23 Large White heavy pigs divergent for GP to investigate through a microarray the differences of
24 gene expressions between the two pools. On the whole, 32 genes were differentially expressed, and
25 among them 31 were overexpressed in low GP pool. These genes were involved in mitochondrial
26 functions and ATP biosynthetic processes, in calcium homeostasis, and in lipid metabolism, with
27 PPAR signaling being a possible master regulator of the molecular differences observed between
28 the two pools. The different GP levels between the two pools could have determined in low GP
29 muscles a more rapid occurrence of the molecular cascade related to the events triggering cell death.

30

31 **Keywords**

32 Swine; skeletal muscle; gene expression; glycogen; pHu; meat quality.

33

34 **1. Introduction**

35 Glycolytic potential (GP) is a biochemical parameter that defines in the skeletal muscle the amount
36 of carbohydrates susceptible to conversion into lactate during the *postmortem* phase (Monin &
37 Sellier, 1985). The major component of GP and the prevalent storage form of carbohydrate in
38 skeletal muscle is glycogen, whose content and breakdown play a major role in the biochemical
39 changes occurring *postmortem* in the muscle tissue. Changes in the GP of a muscle affect the pH
40 decline: after slaughtering, the oxygen level decreases and, to satisfy the energy demand of the
41 cells, muscle glycogen is mobilized towards the formation of glucose and lactate via glycogenolysis
42 and anaerobic glycolysis pathway (Nanni Costa et al., 2009). As anaerobic glycolysis is undeniably
43 the dominant pathway *postmortem*, muscles with high GP levels usually show a lower ultimate pH

44 that, in turn, affects other quality traits such as meat color, water holding capacity (WHC), drip loss,
45 tenderness, and processing yield (Enfält, Lundström, Hansson, Johansen, & Nyström, 1997; Nanni
46 Costa et al., 2009). Hence, GP at 24 hours (h) after slaughtering may be considered, together with
47 glycogen and lactate contents separately, among the muscle parameters capable of predicting the
48 final meat quality (Monin, Mejenes-Quijano, & Talmant, 1987; Monin, 1988; Henckel, Karlsson,
49 Mogens, Oksbjerg & Petersen, 2002; Boler et al., 2010; Chauhan & England, 2018). Even though
50 this parameter is often used to explain or predict ultimate pH (pH_u), Scheffler, Scheffler, Kasten,
51 Sosnicki & Gerrard (2013) reported that complex metabolic properties of muscle likely play a more
52 critical role than glycogen content and *postmortem* glycolysis in determining the course and
53 culmination of the pH decline. Certainly, lactate production is not the only metabolic process
54 influencing *postmortem* pH decline because some reports show that mitochondria may contribute to
55 muscle *postmortem* metabolism influencing pH drop. Several studies have indicated that
56 mitochondria, to sustain an increase in ATP demand early after slaughter, may enhance the muscle
57 glycogen degradation with aerobic glucose oxidation by restoring ATP levels (England et al., 2018;
58 Matarneh, England, Scheffler, Yen, & Gerrard, 2017; Matarneh, Beline, de Luz e Silva, Shi, &
59 Gerrard, 2018). Afterward, once oxygen is extremely depleted after slaughtering, ATP hydrolysis
60 contributes the most to the H^+ production, lowering pH, and influencing meat quality. However, the
61 same Authors showed that the exact underlying mechanism of how mitochondria contribute to
62 muscle metabolism and biochemical changes in the *postmortem* period is not yet clear (England,
63 Scheffler, Kasten, Matarneh, & Gerrard, 2013; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard,
64 2021).

65 The amount of glycogen stored in muscle tissue depends on several factors, such as the animal
66 species, the breed within a given species, the genetics of the animal within a breed, the muscle fiber
67 type composition (Shen et al., 2015), the finishing diet, and the occurrence of stressful events before
68 and during slaughter (reviewed in Xing et al., 2019). Concerning the animals' genetics, several

69 studies have focused on the discovery of genes and Quantitative Trait Loci (QTL) related to
70 glycogen and GP. Even though many QTLs have been detected and markers identified in some
71 candidate genes associated with GP (Kaminski et al., 2010), the knowledge of genes affecting GP is
72 still incomplete. To date, the gene *Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 3*
73 (*PRKAG3*) has been indicated as one of the major molecular players affecting GP levels in pig
74 muscles (Milan et al., 2000; Ciobanu et al., 2001) but other genes have been investigated as
75 candidates influencing GP, meat color and WHC in different pig breeds (Ma et al., 2014;
76 Zappaterra, Sami, & Davoli, 2019). However, the knowledge of the gene expression framework
77 associated with porcine muscle *postmortem* metabolism and glycogen degradation is still scant. In
78 this scenario, the present study utilizes two pools of Italian Large White (ILW) pigs divergent for
79 pHu, glycogen and GP measured at 24 h *postmortem* and aims to investigate early *postmortem*
80 differences in the gene expression profile between *Semimembranosus* muscle (SM) samples
81 belonging to the two pools. The identification of differentially expressed genes (DEGs) may
82 provide insight into molecular events occurring in early *postmortem* muscle and likely influencing
83 the metabolic and biochemical processes during the conversion of muscle to meat in samples
84 characterized by the presence of divergent amounts of muscle residual glycogen. To our knowledge,
85 this is the first study investigating the DEGs related to GP content 24 h *postmortem* in ILW heavy
86 pigs intended for the production of high-quality seasoned pork products.

87

88 **2. Material and methods**

89 **2.1 Animals and phenotypes**

90 Eight purebred ILW pigs (four females and four barrows) were selected from a larger group of 277
91 sib-tested pigs for their GP value measured in SM. The 277 pigs were reared at the Sib-Test genetic
92 station managed by the Italian Pig Breeders National Association (Associazione Nazionale
93 Allevatori Suini; ANAS; <http://www.anas.it>). Italian Sib-test relies on the use of triplets (two gilts

94 and one barrow) of full sibs of candidate boars tested to become breeders. The 277 pigs (183 gilts
95 and 94 barrows) were therefore subjects belonging to triplets coming from 154 litters, originated
96 from 154 dams and 80 sires. The 277 pigs were reared in a unique testing station, and thus in a
97 standard environment, in order to avoid any environmental effects on the phenotypes of the sib-
98 tested population. The testing period lasted from 30-45 days of age to about 9 months of age, up to
99 a final live weight of 155 kg, in line with the specifications reported on the document describing the
100 rules and the procedures to obtain Protected Designation of Origin Parma dry-cured hams. During
101 the testing period, siblings were kept separated and all animals were fed the same growing and
102 finishing diets in order to avoid any possible effects related to diet composition. The pigs were fed
103 with a *quasi ad libitum* nutrition level, which means that about 60% of pigs were able to ingest the
104 entire supplied ration. All animals used in this study were reared according to Italian and European
105 law for pig production and all slaughter procedures complied with national and European Union
106 regulations for animal care and slaughtering. At the end of the testing period, pigs were fasted for
107 about 12 h before being transported and immediately slaughtered at a commercial abattoir located
108 near the genetic station. Pigs were transported and slaughtered in compliance with European rules
109 on the protection of animals during transport and at slaughtering (Council Regulation (EC) No.
110 1/2005 and Council Regulation (EC) No. 1099/2009). At the slaughterhouse, the pigs were stunned
111 by CO₂ at a concentration of 87% in the air (Butina, Holbaek, Denmark) and bled in a supine
112 position. Slaughter procedures were monitored by the veterinary team appointed by the Italian
113 Ministry of Health. Pigs were slaughtered during six slaughtering days. The date of slaughter and
114 sex were recorded for each animal. SM muscle samples were gathered from the whole population of
115 277 pigs. One aliquot of SM samples was collected from the 277 pigs approximately 1 h after
116 slaughtering, immediately frozen in liquid nitrogen, then stored in a deep freezer at -80°C until
117 DNA or RNA extraction. From the same thigh used to collect SM samples for DNA and RNA
118 extraction, a portion of SM muscle was also gathered at 24 h *postmortem* from the 277 pigs, frozen

119 in liquid nitrogen and stored in a deep freezer at -80°C until performing the analyses for the
120 determination of glycogen, lactic acid and GP content.

121 Meat pH₁ (measured about 1 h *postmortem*) and pH_u (measured 24 h *postmortem*) were measured
122 directly on the SM of the left thigh of each animal. Glycogen, lactic acid content, and GP were
123 measured in a portion of SM collected from the same thigh at 24 h *postmortem*. All samples were
124 processed within 4 months after sampling. Two separate analyses were carried out: one for the
125 lactate and the other for the sum of glycogen (the main component of GP degraded to glucose with
126 amyloglucosidase), glucose, and glucose-6-phosphate using the L-Lactic acid and D-Glucose
127 Enzymatic Bio-Analysis kits (R-Biopharma, Milan, Italy), respectively. GP was calculated
128 according to Monin & Sellier (1985) by summing: 2[glycogen + glucose + glucose-6-phosphate] +
129 [lactate] and expressed as micro-moles (μmoles) of lactic acid equivalent per gram of fresh muscle.

130 The animals used to perform the microarray analysis were chosen avoiding full-sib pigs and
131 selecting eight animals with extreme and divergent GP values: Pool L, four pigs with a GP value
132 less than the mean - 2 standard deviations (S.D.); Pool H with four pigs with a GP value greater
133 than the mean + 2 S.D. The pigs comprised in each group (Pool H and Pool L) were selected by
134 balancing for the animals' sex, thus selecting two gilts and two barrows for their inclusion in each
135 group. Kruskal-Wallis test, performed in the R environment (R Core Team, 2020), was then applied
136 to test whether the samples used for the two pools had statistically different values for the measured
137 phenotypes. Data of the two pools were considered significantly different for *P*-values < 0.05.

138

139 **2.2 Genotyping for the mutations *PRKAG3* p R200Q and *RYRI* c.1843C>T**

140 The stored samples of SM muscle gathered from the whole population of 277 pigs were submitted
141 to DNA extraction using a standard Phenol:Chloroform protocol (Sambrook and Russell, 2006).
142 After the extraction, the quality of the DNA was tested with an ND-1000 spectrophotometer
143 (NanoDrop Technologies, Willmington, DE, USA). The 277 samples were then genotyped for the

144 major mutations affecting pH decline and meat quality. In particular, the samples were genotyped
145 for i) the p.R200Q mutation (rs1109104772) of the *PRKAG3* gene (Milan et al., 2000), causative for
146 the acid meat defect; ii) for the c.1843C>T mutation (rs344435545) of the *Ryanodine receptor 1*
147 (*RYR1*) gene (Fujii et al., 1991), responsible of the meat quality defect Pale, Soft, Exudative (PSE).
148 *PRKAG3* genotyping was carried out with Polymerase Chain Reaction- Restriction Fragment
149 Length Polymorphism (PCR-RFLP) as described by Fontanesi, Davoli, Nanni Costa, Scotti, &
150 Russo (2003) and Fontanesi et al. (2008); *RYR1* genotyping was performed as reported by Russo,
151 Fontanesi, Davoli, Chiofalo, Liotta, & Zumbo (2004) with PCR-RFLP.

152

153 **2.3 Total RNA extraction**

154 From the total population of 277 pigs, eight pigs (four gilts and four barrows) were chosen for their
155 extreme and divergent values of GP, assessed on the SM muscles gathered 24 h after slaughter. The
156 SM samples of the eight pigs, which were stored at -80°C, were submitted to total RNA extraction
157 using the Ribo pure kit (Ambion, Thermo Fisher Scientific Inc., Waltham, MA, USA), following
158 the manufacturer's instructions. The RNA quality and integrity were checked with the ND-1000
159 Spectrophotometer (NanoDrop Technologies, Willmington, DE, USA), and by visualization on 1%
160 agarose gel. The RNA samples were treated with DNaseI (Invitrogen, Thermo Fisher Scientific
161 Inc., Waltham, MA, USA) and for each sample, an equal amount of total RNA (2,500 ng) was
162 mixed to obtain two pools of 10,000 ng of RNA each utilized for the next steps of analysis.

163

164 **2.4 Oligonucleotides microarray analysis**

165 The DIPROVAL-OPERON *Sus scrofa* AROS V1.0 oligonucleotide microarray is described in the
166 GEO database with the accession number GPL5468. The microarray is composed of 10,665 70-mer
167 oligonucleotides (probes). Each oligonucleotide was spotted twice on each slide. The hybridization
168 and post-hybridization protocols are detailed on the same GEO entry indicated above. For the

169 present research, six slides were used: Pool H was labeled with Cy3 (green) on slides 1, 2, and 5,
170 while Pool L was labeled with Cy5 (red) on the same three slides. Opposite labeling of the two
171 pools was used for the hybridization of slides 3, 4, 6. For the cDNA synthesis and the labeling
172 procedure, the SuperScript Plus Indirect cDNA Labelling System (Invitrogen, Thermo Fisher
173 Scientific Inc. Waltham, MA, USA) was used according to the manufacturer's recommendations.
174 The obtained cDNA samples were labeled with the fluorochromes Cy3 and Cy5 overnight and in
175 the dark. After hybridization and washing, the slides were scanned with ScanArray Gx scanner
176 (Perkin Elmer, Waltham, MA, USA) and the images were processed using ScanArray Express
177 software.

178

179 **2.5 Oligonucleotides re-annotation**

180 The original annotation of the oligonucleotides was updated for the present work using the
181 annotation available for *Sus scrofa*, *Sscrofa* 11.1 genome assembly in Ensembl (Howe et al., 2021)
182 and the most updated genome annotation available for *Sus scrofa*, *Homo sapiens* and other species
183 using Nucleotide BLAST (Zhang, Schwartz, Wagner, & Miller, 2000) were used to search for
184 sequences similar to the probes. The parameters to select sequences resulting from the local
185 alignments were percent of similarity of at least 80% and length of the alignment between 64 and
186 72 nucleotides. This type of analysis was carried out first in the *Sus scrofa* species, using both
187 “Nucleotide collection (nr/nt)” and “Expressed sequence tags (est)” databases, subsequently, the
188 oligonucleotides that were not identified were aligned against *Homo sapiens* sequences. After those
189 two steps, the oligonucleotides that were still lacking an identification were used to carry out
190 another alignment step against the Nucleotide collection (nr/nt) of all other animal species. The
191 GenBank codes identified for the oligonucleotides were then entered in the DAVID Bioinformatic
192 Resources v. 6.8 Gene ID Conversion Tool (Huang, Sherman, & Lempicki, 2009) and in Ensembl
193 BioMart (Howe et al., 2021) to assign a gene to each oligonucleotide.

194

195 **2.6 Identification of differentially expressed genes and functional analysis**

196 Microarray data were analyzed using the *limma* R package (v. 3.46.0) (Ritchie et al., 2015). Both
197 mean and median data were submitted to background correction using two different methods:
198 Subtract and Edwards (Edwards, 2003). The first is the traditional background correction method
199 where the background fluorescence intensity is subtracted from the foreground one for each spot.
200 The latter method uses a log-linear interpolation method to adjust lower intensities as described in
201 Edwards (2003): the background is corrected only if the difference between foreground and
202 background is larger than a small threshold value. If this difference is lower than the threshold, a
203 smooth monotonic function which is linear with respect to background intensity on the log scale is
204 used instead of subtraction (Edwards, 2003). The main difference between these two methods is that
205 Edwards avoids negative intensities which lead to loss of information and bias (since it is most
206 likely to occur when the expression levels are low) in the subsequent logarithmic transformation.
207 The obtained four datasets (Mean subtract- MNS; mean Edwards- MNE; median subtract- MDS;
208 median Edwards- MDE) were submitted to the within and the between arrays normalization by
209 using the “loess” (Yang et al., 2002) and the “scale” methods (Bolstad, Irizarry, Astrand, & Speed,
210 2003), respectively.

211 The resulting data were analyzed with the *lmFit* and *eBayes* functions included in *limma* package.
212 Both models were performed using the “robust” method that allows reducing the changes to
213 consider a hypervariable gene as a DEG and increase statistical power for the other genes (Phipson,
214 Lee, Majewski, Alexander, & Smyth, 2016). Raw *P*-values were adjusted applying the False
215 Discovery Rate (FDR) correction method (Benjamini & Hochberg, 1995). The cut-off criteria for
216 defining a gene as differentially expressed (DE) were the adjusted *P*-value < 0.05 and a fold change
217 (FC), expressed as the ratio between the normalized intensities of pool H and pool L, > 1 . At the
218 end of the procedure, four different sets of DEGs were obtained (two for the mean intensity and two

219 for the median intensity). The online web tool Bioinformatics and Evolutionary Genomics
220 (Bioinformatics and Evolutionary Genomics, 2021) was then used to graphically represent with a
221 Venn diagram the DEGs found, and the intersection obtained with each statistical approach.
222 The functional annotation was obtained using Cytoscape 3.8.2 (Shannon et al., 2003) with the
223 ClueGO app (Bindea et al., 2009). Functional characterization was performed selecting human
224 database as a reference and the DEGs were significantly enriched in KEGG (Kyoto Encyclopedia of
225 Genes and Genomes) Pathway, biological process (BP), cellular component (CC), and molecular
226 function (MF). The P -values of the obtained functional categories were adjusted with the
227 Bonferroni step-down method. The adjusted P -values were considered significant for $P < 0.10$,
228 following the significance threshold used in other studies (such as Lorenz et al., 2019), and the
229 significant functional categories were graphically displayed with Cytoscape tools.

230

231 **2.7 Validation with quantitative Real-time PCR**

232 The expression levels of some of the DEGs were also analyzed using the relative quantitative Real-
233 Time polymerase chain reaction (qRT-PCR) standard curve method (Pfaffl, Tichopad, Prgomet, &
234 Neuvians, 2004). Six out of the found DEGs were chosen for the validation, and their expression
235 levels were normalized against the geometric mean for the transcription level of the normalizing
236 genes *Beta-2-microglobulin (B2M)* and *RNA polymerase II subunit A (POLR2A)*. Those two
237 normalizing genes were used because they were found to be the most stable in previous studies
238 (Zappaterra, Braglia, Bigi, Zambonelli, & Davoli, 2015) and were chosen following GENORM
239 software based on the method proposed by Vandesompele et al. (2002). The total RNA of the eight
240 samples used also to create the two pools was individually reverse-transcribed. For each sample, 1
241 μg of total RNA was retrotranscribed to cDNA, according to the manufacturer's instructions, using
242 the Improm-IITM Reverse Transcription System and Oligo-dT primers (Promega Corporation,
243 Madison, WI, USA). Then, the obtained cDNA was diluted 1:10 and used to perform the qRT-PCR

244 on Light Cycler 1.0 System (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex
245 TaqTM (TAKARA Bio INC, Olsu, Shiga, Japan), 10 pmol of each primer, and 2 µl of cDNA
246 template diluted 1:10, for a total volume of 10 µl. The Light Cycler protocol was optimized using
247 specific annealing temperatures for each primer pair as shown in Supplementary Table S1. The
248 same protocol reported in Zappaterra, Braglia, Bigi, Zambonelli, & Davoli (2015) for standard
249 curve creation and calculation of qRT-PCR efficiency was applied. The samples were quantified in
250 triplicate and the quantitation for each sample was retained if the coefficient of variation was less
251 than or equal to 0.20.

252 The obtained normalized quantitation for the samples was then used to calculate the mean for each
253 pool and to obtain for each gene the log₂ of the FC value (log₂FC), which was obtained by dividing
254 the average expression level of the Pool H for the average expression identified in Pool L. The
255 log₂FC values of the gene expression quantified with qRT-PCR and with microarray analysis were
256 used to perform Spearman correlations and test whether the trend of the gene expression levels was
257 maintained between the qRT-PCR and the four statistical procedures applied to microarray data to
258 find DEGs (MNE, MNS, MDE, MDS). Spearman correlations were estimated with *Hmisc* R
259 package (v 4.5.0; Harrell Jr, 2021). The correlations were considered significant for $P < 0.05$.

260

261 **3. Results**

262 **3.1 Description of the two pools**

263 The samples were grouped in two pools selected according to the divergence of GP level measured
264 in SM. The mean value of GP in the whole sample of 277 ILW pigs was 103.50 µmoles/g while the
265 two pools were characterized by an extreme and divergent mean value of this parameter with
266 significant differences between the two pools, as reported in Table 1.

267

268 **Table 1.** Descriptive statistics (Mean and Standard Deviation, S.D.) for the phenotypic parameters
 269 measured on the pigs of Pool H (high glycolytic potential), of Pool L (low glycolytic potential) and
 270 on the whole sample of 277 Italian Large White heavy pigs. For the Pool H vs. Pool L comparison,
 271 Kruskal-Wallis test statistics (χ^2 and P -value) were also reported.

Phenotypes	Total sample (N = 277)	Pool H (n = 4)	Pool L (n = 4)	Kruskal-Wallis (Pool H vs. Pool L)	
				χ^2	P -value
pH _i ¹	5.94 ± 0.24	6.14 ± 0.14	6.21 ± 0.15	0.33	0.564
pH _u ²	5.67 ± 0.21	5.48 ± 0.07	6.08 ± 0.16	5.33	0.021
Glycogen (μmoles/g) ³	47.33 ± 22.79	114.39 ± 12.98	13.15 ± 6.38	5.33	0.021
Lactate (μmoles/g) ³	56.30 ± 15.16	47.65 ± 7.35	42.88 ± 5.72	0.33	0.564
Glycolytic Potential (GP) (μmoles/g) ³	103.68 ± 23.15	162.03 ± 12.62	56.03 ± 5.73	5.33	0.021

272 ¹ pH_i was measured about 1 h *postmortem* directly on the *Semimembranosus* muscle of the left thigh.

273 ² pH_u was measured about 24 h *postmortem* directly on the *Semimembranosus* muscle of the left thigh.

274 ³ These parameters were measured 24 h *postmortem* on *Semimembranosus* muscle samples and were expressed as
 275 micro-moles (μmoles) of lactic acid equivalent per gram of fresh muscle.

276

277 The characteristics of the eight selected samples are reported in Supplementary Table S2. The
 278 average levels of glycogen and GP found at 24 h after slaughter in the Pool L were significantly
 279 lower (13.15 ± 6.38 μmoles/g and 56.03 ± 5.73 μmoles/g, respectively) than the average of the
 280 whole group of 277 pigs, whereas the mean values of the glycogen and GP levels in the Pool H
 281 were 114.39 ± 12.98 μmoles/g and 162.03 ± 12.62 μmoles/g, respectively.

282 The average pH_i values of the two groups did not differ significantly, while the pH_u values showed
 283 a significant difference ($P = 0.021$) between the samples of the two pools. This result for pH_u agrees

284 with the result of the comparison of the GP values between the two pools: the pool with the lowest
285 pH_u mean values also showed the highest values of GP (Table 1). Similarly, the glycogen level
286 differed too between the two pools ($P = 0.021$) with divergent GP. In Table 1 the lactate level of
287 both pools is also shown. We did not observe different lactate content between the two groups of
288 samples.

289 The pigs of the two pools did not display the alleles of the two major genes influencing negatively
290 porcine meat quality traits so far detected: p.200Q of the *PRKAG3* gene (Milan et al., 2000) and
291 c.1843T of the *RYR1* gene (Fujii et al., 1991).

292

293 **3.2 Microarray results and functional analysis**

294 Supplementary Table S3 reports the oligonucleotides identified as DE with each statistical method
295 and the complete output of each analytical procedure used to identify sequences differentially
296 expressed between pools. The complete information concerning oligonucleotides sequence and their
297 relative gene identification is reported in Supplementary Table S4. Table 2 shows the DE
298 oligonucleotides identified by each method and Figure 1 graphically displays the number of DE
299 oligonucleotides retained in the different considered methods.

300

301 **Figure 1.** VENN diagram showing the number of differentially expressed oligonucleotides found
302 with the four statistical methods (MNE = Mean Edwards; MNS = Mean Subtract; MDE = Median
303 Edwards; MDS = Median Subtract) and the number of oligonucleotides obtained by the
304 intersections of the four methods. On the central part of the diagram are indicated the 17
305 differentially expressed oligonucleotides identified by all four statistical methods.

306

307 **Table 2.** The 35 oligonucleotides (oligo ID) found differentially expressed with the four statistical methods (MNE = Mean Edwards; MNS = Mean
308 Subtract; MDE = Median Edwards; MDS = Median Subtract) and their gene identification. Data are reported with the log₂ of the fold change
309 (log₂FC) and the adjusted *P*-value.

Oligo ID	Gene symbol	MNE		MNS		MDE		MDS	
		Log ₂ FC	Adjusted <i>P</i> - value	Log ₂ FC	Adjusted <i>P</i> - value	Log ₂ FC	Adjusted <i>P</i> - value	Log ₂ FC	Adjusted <i>P</i> - value
SS00000291	<i>MYH3</i>	ns	ns	1.8410	1.63E-03	1.7273	2.37E-02	2.2504	1.43E-03
SS00000459	<i>NTRK3</i>	ns	ns	ns	ns	-1.1864	3.96E-04	-1.1706	1.01E-04
SS00000539	<i>HBB</i>	-1.0303	9.71E-04	-1.0394	1.15E-03	ns	ns	ns	ns
SS00000679	<i>SCD</i>	-2.5490	1.53E-02	-2.5348	1.48E-02	-2.9639	1.30E-02	-2.9573	1.01E-02
SS00000695	<i>PLN</i>	-2.1164	5.01E-03	-2.0850	4.76E-03	-2.0953	6.58E-03	-2.0634	5.76E-03
SS00000696	<i>GOT1</i>	ns	ns	ns	ns	-1.0043	9.03E-04	-1.0245	2.48E-03
SS00000767	<i>UGP2</i>	-1.0962	9.58E-03	-1.0726	8.32E-03	-1.1868	4.31E-03	-1.1674	3.09E-03
SS00001044	<i>FABP3</i>	-1.3071	9.71E-04	-1.3264	1.63E-03	-1.3513	4.46E-04	-1.3737	7.45E-04
SS00001123	<i>PPARA</i>	-1.0346	1.72E-02	-1.0460	3.00E-02	-1.0382	5.95E-03	-1.0405	7.36E-03
SS00001972	<i>FABP5</i>	-1.2372	2.15E-03	-1.2114	1.20E-03	-1.2275	7.55E-04	-1.1894	2.39E-04

SS00002255*		ns	ns	ns	ns	-1.4485	3.22E-02	ns	ns
SS00002454	<i>DVL3</i>	-1.5981	2.14E-02	-1.3220	1.41E-02	-1.4296	1.19E-02	-1.3706	8.89E-03
SS00002786	<i>ATP5F1A</i>	-1.0630	4.21E-02	ns	ns	ns	ns	ns	ns
SS00002870	<i>MYOZ2</i>	-1.9427	3.08E-02	-1.9236	2.81E-02	-2.0071	2.12E-02	-1.9818	1.85E-02
SS00003271	<i>HSPB6</i>	-1.1909	7.50E-03	-1.1810	6.37E-03	ns	ns	ns	ns
SS00003290	<i>EIF2B2</i>	ns	ns	ns	ns	-1.1900	8.35E-03	-1.0254	1.20E-02
SS00003487*		ns	ns	ns	ns	-1.1355	4.98E-02	ns	ns
SS00004032	<i>CIQBP</i>	ns	ns	ns	ns	-1.0172	5.06E-03	ns	ns
SS00004323	<i>ATP5MC3</i>	-1.0196	1.02E-02	-1.0071	8.30E-03	-1.0124	1.83E-03	-1.0072	9.42E-04
SS00004770	<i>HSPB7</i>	-1.3000	1.18E-02	-1.2853	1.01E-02	-1.2715	1.35E-02	-1.2675	1.32E-02
SS00005142	<i>FCHSD1</i>	ns	ns	ns	ns	-1.0535	4.43E-03	-1.0215	3.43E-03
SS00005172	<i>POLA2</i>	-1.7040	1.88E-02	-1.6282	2.04E-02	ns	ns	-1.4361	4.55E-02
SS00005914	<i>HOMER2</i>	-1.1198	3.76E-03	-1.1020	3.00E-03	-1.1156	6.46E-03	-1.0967	5.76E-03
SS00005997	<i>APIG2</i>	-1.2248	4.29E-02	ns	ns	-1.3253	1.35E-02	-1.0503	1.83E-02
SS00006189	<i>CSRP3</i>	-1.6951	1.42E-02	-1.6674	1.10E-02	-1.6566	7.10E-03	-1.6071	4.76E-03
SS00006369	<i>TSSK6</i>	ns	ns	ns	ns	-1.7502	4.05E-02	ns	3.40E-04

SS00006853	<i>SCD</i>	-2.0247	3.02E-03	-2.0062	2.85E-03	-1.9751	5.02E-04	-1.9667	ns
SS00006888	<i>LPL</i>	-1.5160	1.40E-03	-1.4980	1.06E-03	-1.4952	2.80E-04	-1.4807	1.74E-04
SS00006966	<i>RNF220</i>	ns	ns	ns	ns	-2.7826	4.08E-03	ns	ns
SS00007208	<i>COQ9</i>	-1.8260	3.92E-02	ns	ns	-3.2519	2.69E-04	-3.7321	4.59E-02
SS00007292	<i>MCU</i>	ns	ns	ns	ns	-1.1740	1.30E-02	ns	ns
SS00008224	<i>DNAJB1</i>	-1.1845	3.73E-02	-1.1640	3.56E-02	-1.1004	3.84E-02	-1.0803	3.66E-02
SS00008616	<i>ACSL1</i>	-1.0801	5.40E-03	-1.0681	5.55E-03	-1.1553	7.64E-03	-1.1440	5.76E-03
SS00009114	<i>HSPB2</i>	Ns	ns	ns	ns	-1.2000	2.39E-02	ns	ns
SS00009459	<i>SPARCL1</i>	-1.0378	2.89E-02	-1.1434	1.10E-02	-1.0704	9.18E-03	-1.1538	6.33E-03

310 ns= not significant

311 *not further considered

312

313 The four statistical approaches allowed the identification of a total of 35 oligonucleotides
314 differentially expressed between the two pools. Among them, two oligonucleotides (SS00002255
315 and SS00003487) correspond to Expressed Sequence Tags (ESTs). The sequence of the EST
316 corresponding to the oligonucleotide SS00002255 was found in *Sus scrofa* ovary
317 (OVRM10048C10) and the oligonucleotide SS00003487 partially recognizes the cDNA of the *Sus*
318 *scrofa* gene *Enhancer of mRNA decapping (EDC4)*. Both these oligonucleotides were not further
319 considered. The thirty-three remaining oligonucleotides corresponded to 32 unique genes as the
320 sequence of two oligonucleotides (SS00000679 and SS00006853) targeted the gene *Stearoyl-CoA*
321 *Desaturase delta 9 (SCD)* (Table 2 and Supplementary Table S4).

322 All but one of the DEGs were over-expressed in the Pool L. The oligo SS00000291 coding for a
323 putative *Myosin heavy chain isoform (MYH3)* was the only one overexpressed in Pool H. However,
324 to utilize microarray data more comprehensively, all 32 unique genes identified with the four
325 methods were submitted to functional analysis and the results are reported in Table 3 and Figure 2.

326

327 **Table 3.** Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly enriched for the genes
 328 differentially expressed between Pool L and Pool H. Functional categories are sorted by decreasing Bonferroni adjusted *P*-values.

Associated Genes Found	GO/KEGG ID	GO Term/KEGG pathway	N. Genes	<i>P</i> -value	Bonferroni adjusted <i>P</i> - value
<i>ACSL1, FABP3, FABP5, LPL, PPARA, SCD</i>	KEGG:03320	PPAR signaling pathway	6	3.41E-09	1.64E-07
<i>CSRP3, FBXO32, HSPB6, MYH3, MYOZ2, PLN, PPARA</i>	GO:0003012	muscle system process	7	1.12E-05	5.27E-04
<i>CSRP3, EIF2B2, FABP3, GOT1, LPL, PLN, PPARA</i>	GO:0043434	response to peptide hormone	7	1.60E-05	7.37E-04
<i>ACSL1, ATP5F1A, ATP5MC3, PPARA, SCD</i>	GO:0006164	purine nucleotide biosynthetic process	5	3.26E-05	1.47E-03
<i>ACSL1, ATP5F1A, ATP5MC3, PPARA, SCD</i>	GO:0009260	ribonucleotide biosynthetic process	5	3.64E-05	1.60E-03
<i>CSRP3, MYH3, MYOZ2</i>	GO:0045214	sarcomere organization	3	3.88E-05	1.67E-03
<i>ACSL1, FABP3, FABP5, LPL</i>	GO:0006641	triglyceride metabolic process	4	5.40E-05	2.27E-03
<i>CSRP3, FABP5, FBXO32, HSPB6, HSPB7, PLN, PPARA</i>	GO:0044057	regulation of system process	7	7.69E-05	3.15E-03
<i>ACSL1, FABP3, FABP5, LPL, PPARA, SCD</i>	GO:0006631	fatty acid metabolic process	6	7.94E-05	3.18E-03

<i>ACSL1, ATP5F1A, ATP5MC3, FABP3, FABP5, PPARA, SCD</i>	GO:0090407	organophosphate biosynthetic process	7	1.18E-04	4.61E-03
<i>ACSL1, FABP3, PPARA</i>	GO:2000191	regulation of fatty acid transport	3	1.25E-04	4.75E-03
<i>ACSL1, FABP3, FABP5, PPARA</i>	GO:0015908	fatty acid transport	4	1.35E-04	4.87E-03
<i>ATP5F1A, ATP5MC3, PPARA</i>	GO:0006754	ATP biosynthetic process	3	1.32E-04	4.87E-03
<i>CSRP3, MYH3, MYOZ2, PPARA</i>	GO:0055001	muscle cell development	4	2.37E-04	8.31E-03
<i>ATP5F1A, ATP5MC3, COQ9, MYH3, PPARA</i>	GO:0046034	ATP metabolic process	5	2.49E-04	8.45E-03
<i>FABP5, GOT1, LPL, SCD, UGP2</i>	GO:0046394	carboxylic acid biosynthetic process	5	2.78E-04	9.16E-03
<i>ACSL1, FABP3, LPL, PPARA</i>	GO:1905952	regulation of lipid localization	4	3.51E-04	1.12E-02
<i>CSRP3, HSPB7, PLN</i>	GO:0060047	heart contraction	3	1.28E-02	1.28E-02
<i>ATP5F1A, ATP5MC3, PPARA</i>	GO:0009144	purine nucleoside triphosphate metabolic process	3	5.25E-04	1.52E-02
<i>ATP5F1A, ATP5MC3, PPARA</i>	GO:0009142	nucleoside triphosphate biosynthetic process	3	5.09E-04	1.53E-02
<i>ATP5F1A, ATP5MC3, PPARA</i>	GO:0009199	ribonucleoside triphosphate metabolic process	3	5.96E-04	1.67E-02

<i>HOMER2, MCU, MYOZ2, PLN</i>	GO:0019722	calcium-mediated signaling	4	7.90E-04	1.98E-02
<i>ACSL1, FABP5, SCD</i>	GO:0120162	positive regulation of cold-induced thermogenesis	3	8.42E-04	2.02E-02
<i>FBXO32, HSPB6, PLN, PPARA</i>	GO:0090257	regulation of muscle system process	4	7.78E-04	2.02E-02
<i>FABP3, FABP5, PPARA</i>	GO:0019217	regulation of fatty acid metabolic process	3	7.54E-04	2.04E-02
<i>ATP5F1A, ATP5MC3, MCU</i>	GO:1990542	mitochondrial transmembrane transport	3	1.01E-03	2.13E-02
<i>FABP3, FABP5, LPL, PPARA</i>	GO:0044242	cellular lipid catabolic process	4	9.32E-04	2.14E-02
<i>FBXO32, MYOZ2, PPARA</i>	GO:0043500	muscle adaptation	3	9.87E-04	2.17E-02
<i>ACSL1, ATP5F1A, ATP5MC3, PPARA, SCD</i>	GO:0009150	purine ribonucleotide metabolic process	5	1.16E-03	2.33E-02
<i>CSRP3, FABP5, MCU</i>	GO:0042593	glucose homeostasis	3	1.19E-02	2.39E-02
<i>ACSL1, FABP5, SCD</i>	GO:0033559	unsaturated fatty acid metabolic process	3	1.41E-03	2.54E-02
<i>CSRP3, MYH3, MYOZ2, PPARA</i>	GO:0051146	striated muscle cell differentiation	4	1.39E-03	2.65E-02

<i>ATP5F1A, ATP5MC3, COQ9</i>	GO:0006119	oxidative phosphorylation	3	2.31E-03	3.93E-02
<i>C1QBP, MCU, NTRK3</i>	GO:0050921	positive regulation of chemotaxis	3	2.31E-03	3.93E-02
<i>ACSL1, FABP5, SCD</i>	GO:0106106	cold-induced thermogenesis	3	2.49E-03	3.98E-02
<i>CSRP3, MYOZ2, PLN, PPARA</i>	GO:0014706	striated muscle tissue development	4	4.33E-03	4.33E-02
<i>CSRP3, GOT1, LPL</i>	GO:0032869	cellular response to insulin stimulus	3	9.03E-03	4.51E-02
<i>FBXO32, GOT1, NTRK3</i>	GO:0051384	response to glucocorticoid	3	3.01E-03	4.52E-02
<i>DNAJB1, HSPB2, HSPB7</i>	GO:0006986	response to unfolded protein	3	4.20E-03	4.62E-02
<i>CSRP3, HSPB6, MYH3, PLN</i>	GO:0006936	muscle contraction	4	3.66E-03	4.75E-02
<i>FABP5, LPL, SCD</i>	GO:0006633	fatty acid biosynthetic process	3	3.61E-03	5.05E-02
<i>CSRP3, HSPB7, PLN</i>	GO:0008016	regulation of heart contraction	3	8.83E-03	5.30E-02
<i>FABP5, GOT1, PPARA</i>	GO:0006006	glucose metabolic process	3	8.64E-03	6.05E-02
<i>MCU, NTRK3, PLN</i>	KEGG:04020	calcium signaling pathway	3	8.26E-03	6.61E-02
<i>ACSL1, ATP5F1A, ATP5MC3</i>	KEGG:04714	Thermogenesis	3	7.53E-03	6.77E-02

329

330

331 Among the DEGs upregulated in Pool L we have found the genes *ATP Synthase Membrane Subunit*
332 *C Locus 3 (ATP5MC3)*, *ATP Synthase F1 Subunit Alpha (ATP5F1A)*, *Coenzyme Q9 (COQ9)*,
333 *Mitochondrial Calcium Uniporter (MCU)*, *F-box protein 32 (FXBO32)*, *Glutamic-Oxaloacetic*
334 *Transaminase 1 (GOT1)*, and *Complement C1q Binding Protein (C1QBP)*, which are expressed in
335 mitochondria and are involved in important functions mainly aimed to support the energy needs of
336 the cells. In particular, the genes *ATP5F1A*, *ATP5MC3*, and *COQ9*, together with *Myosin Heavy*
337 *Chain 3 (MYH3)*, and *Peroxisome Proliferator Activated Receptor Alpha (PPARA)* were
338 significantly enriched in “ATP metabolic process” (adjusted $P = 8.45E-03$), and *ATP5F1A*,
339 *ATP5MC3*, and *MCU* significantly entered in “mitochondrial transmembrane transport” category
340 (adjusted $P = 2.13E-02$). This would suggest these genes are involved in determining mitochondrial
341 respiratory function and play pivotal roles to maintain the cellular energy state. The DEGs *Acyl-*
342 *CoA Synthetase Long Chain Family Member 1 (ACSL1)*, *Fatty Acid Binding Protein 3 (FABP3)*,
343 *Fatty Acid Binding Protein 5 (FABP5)*, *Lipoprotein Lipase (LPL)*, *PPARA*, and *Stearoyl-CoA*
344 *Desaturase (SCD)* were significantly enriched in “fatty acid metabolic process” (adjusted $P =$
345 $3.18E-03$); *ACSL1*, *FABP3*, *FABP5*, and *PPARA* significantly entered in “fatty acid transport”
346 (adjusted $P = 4.87E-03$). A group of DEGs (*Heat Shock Protein Family B (Small) Member 2-*
347 *HSBP2*, *DnaJ Heat Shock Protein Family (Hsp40) Member B1- DNAJB1*, *Heat Shock Protein*
348 *Family B (Small) Member 7- HSPB7*) belonging to the Heat Shock Protein family were significantly
349 clustered in the functional category “Response to unfolded proteins” (adjusted $P = 4.62E-02$). The
350 functional classification of the DEGs *Cysteine And Glycine Rich Protein 3 (CSRP3)*, *Myozenin 2*
351 (*MYOZ2*), *Phospholamban (PLN)*, and *PPARA* showed to be significantly involved in “Striated
352 muscle tissue development” (adjusted $P = 4.33E-02$), *Homer Scaffold Protein 2 (HOMER2)*, *MCU*,
353 *MYOZ2*, *PLN* were included in “Calcium-mediated signaling” (adjusted $P = 1.98E-02$), and *MCU*,
354 *Neurotrophic Receptor Tyrosine Kinase 3 (NTRK3)*, *PLN* were significantly clustered in “Calcium
355 signaling pathway” (adjusted $P = 6.61E-02$). The gene *UDP-Glucose Pyrophosphorylase 2*

356 (*UGP2*), which is a precursor of the glycogen synthesis, was found included in the functional
357 category “Carboxylic acid biosynthetic process”, together with the DEGs *FABP5*, *GOT1*, *LPL*, and
358 *SCD* (adjusted $P = 9.16E-03$). Besides the functional classification of the DEGs, the main
359 connections among them were also evaluated (Table 3; Figure 2). The functional class “PPAR
360 signaling pathway” showed to be related with most of the functional categories containing the DEG
361 genes identified (Table 3; Figure 2), thus suggesting that *PPARA*, the main gene of the class, can be
362 considered the master regulator of the identified pathways.

363

364 **Figure 2.** Cytoscape functional analysis of the genes differentially expressed between Pool H and
365 Pool L.

366 Legend: hexagons = KEGG pathways; circles = biological processes (GO BP); shape size =
367 according to the P -value of the term in its own group; different colors are used to identify functional
368 categories pertaining to the same group of terms; interaction line thickness = according to Kappa
369 Score value, represents the strength of the interactions, lighter color corresponds to a lower strength
370 while darker color to a higher strength. The differentially expressed genes labelled with a frame are
371 those that will be further discussed.

372

373 **3.3 Validation of microarray results**

374 To validate the results obtained with the microarray we decided to analyze the expression level by
375 qRT-PCR of six of the DEG found (*ATP5MC3*, *LPL*, *MYOZ2*, *PLN*, *SCD*, *UGP2*) after microarray
376 hybridization; these six genes were selected for their relevant role in muscle metabolism. A high
377 correlation value was found between the \log_2FC found with qRT-PCR and the \log_2FC values
378 identified for the same genes with the four statistical methods ($r = 0.94$, $P < 0.005$ for all the
379 correlations qRT-PCR vs. MNE, qRT-PCR vs. MNS, qRT-PCR vs. MDE, and qRT-PCR vs. MDS).

380 The obtained correlations support the robustness of the results found with the four statistical
381 methods used in this study.

382 **4. Discussion**

383 To our knowledge, this is the first study investigating by microarray the molecular frame of early
384 *postmortem* muscle tissue in Italian Large White heavy pigs divergent for GP measured 24 h after
385 slaughter. The results can help outline the molecular players participating in the metabolic processes
386 involved in pH decline after slaughtering and influencing GP at 24 h *postmortem*.

387 In *postmortem* muscle, the energy demand of the tissue is supported by glycogen, glucose, and
388 lactate that are the main metabolites used to generate glycolytic substrates. It is widely known that
389 the metabolic and molecular processes related to muscle glycogen storage and glycogen degradation
390 to glucose can influence *postmortem* biochemical processes in the conversion of muscle to meat,
391 with particular reference to the rate of pH drop. Different Authors indicated that muscles with lower
392 glycogen and higher lactate show, early *postmortem*, a faster glycolytic rate than muscles with
393 higher glycogen and lower lactate level (Chauahn & England, 2018; England et al., 2016; Moreno,
394 Lipová, Ladero, García, & Cava, 2020; Henckel, Karlsson, Jensen, Oksbjerg & Petersen, 2002;
395 Matarneh, England, Scheffler, & Gerrard, 2017; Pösö & Puolanne, 2005).

396 Henckel, Karlsson, Jensen, Oksbjerg, & Petersen (2002) reported that the minimum amount of
397 glycogen required for a normal *postmortem* pH decline from 7.2 to 5.6, is 53 $\mu\text{moles/g}$ tissue. In the
398 present research, the amount of glycogen ($13.15 \pm 6.38 \mu\text{moles/g}$) and the level of GP (56.03 ± 5.73
399 $\mu\text{moles/g}$) found at 24 h *postmortem* in Pool L are much lower than the threshold indicated in
400 Henckel, Karlsson, Jensen, Oksbjerg, & Petersen (2002). This result could be consistent with the
401 limited pH decline at 24 h after slaughter observed in Pool L, which showed a quite elevated pH_u
402 value of 6.08 ± 0.16 compared to the glycogen and pH_u values of Pool H ($\text{pH}_u = 5.48 \pm 0.07$;
403 glycogen = $114.39 \pm 12.98 \mu\text{moles/g}$; GP = $162.03 \pm 12.62 \mu\text{moles/g}$). The conversion of muscle to

404 meat is traditionally viewed as an anaerobic process that is largely governed by the degradation of
405 glycogen to lactate and H^+ . However, different Authors reported the evidence that glycolytic
406 capacity of muscle tissue is only partially associated with pH decline *postmortem* and that glycogen
407 content and *postmortem* glycolysis are not the unique factors responsible for pH drop (Scheffler &
408 Gerrard, 2007; Pösö & Puolanne, 2005; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013).
409 These Authors showed that other elements and aspects related to the complex metabolic properties
410 of muscle likely play a more critical role in determining the course and outcome of pH drop after
411 slaughter (Scheffler & Gerrard, 2007; Pösö & Puolanne, 2005; Scheffler, Scheffler, Kasten,
412 Sosnicki, & Gerrard, 2013).

413 The amount of lactate is considered by different Authors as an indicator of *postmortem* pH decline.
414 There is however lack of agreement in the scientific community with respect to the relationship
415 linking lactate and *postmortem* pH decline. Other Authors have indeed reported that even though
416 there is a significant connection between muscle glycolysis, GP, lactate accumulation, and
417 *postmortem* pH, the relationship between glycogen content and pH_u is not complete and not linear,
418 as samples showing similar lactate values may display different pH_u (England et al., 2016;
419 Scheffler, Park, & Gerrard, 2011; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013; Pösö &
420 Puolanne, 2005; Chauhan & England, 2018; Huff-Lonergan, Baas, Malek, Dekkers, Prusa, &
421 Rothschild, 2002; Monin & Sellier, 1985). In agreement with results found in other studies (Van
422 Laack & Kauffman, 1999; Van Laack, Liu, Smith, & Loveday, 2000; Choe et al., 2008), in the
423 present research, we noticed different pH_u values between the samples of the two pools but similar
424 values of lactate at 24h *postmortem* were present between the two pools. This result seems to
425 indicate that the difference noticed between the pH_u values of the two pools may not be ascribed to
426 glycogen breakdown leading to lactate formation. The similar values of pH_u and lactate between the
427 two pools may indicate that the differences noticed in GP at 24 h *postmortem* should not be
428 attributed to different preslaughter handling conditions or different *premortem* environmental

429 factors. Previous research indicated higher levels of lactate at 24 h *postmortem* in muscles
430 belonging to animals that had higher exsanguination blood glucose and lactate and thus
431 experiencing more muscle activity and stress before slaughter (Choe et al., 2015). In the present
432 study, we hypothesize that other aspects (not related to pre-slaughter handling) may have caused the
433 observed differences between the two pools in the levels of glycogen, glycolytic potential, and pH_u.
434 Preslaughter stressing conditions or particular feeding protocols are known to reduce the content of
435 glycogen stored in muscles, producing lower pH₁ and pH_u values during the muscle to meat
436 transformation (Rosenvold & Andersen, 2003a; Pösö & Puolanne, 2005; England, Matarneh,
437 Oliver, Apaoblaza, Scheffler, Shi, & Gerrard, 2016; Rosenvold & Andersen, 2003b; Chauhan &
438 England, 2018). The pigs used in the present study were, however, reared in the same
439 environmental conditions, fed the same diet, and slaughtered in the same abattoir. Accordingly, we
440 can infer that the observed phenotypic differences between the pigs of the two pools for the
441 indicated parameters (glycogen and GP), together with the similar lactate level and pH₁, could
442 likely depend on the effects of other factors, such as physiological conditions before slaughtering
443 related to muscle metabolism. It could be plausible also to hypothesize that in Pool L a limited
444 muscle glycogen reserve may have been already present in *antemortem* muscle and/or that
445 dysfunctions of enzymes related to glycogen metabolism may have caused in Pool L a rapid
446 glycogen breakdown. The hypothesized rapid glycogen degradation could have been caused by
447 enzymatic altered activity represented by an up-regulation of proteins involved in glycogenolysis or
448 by the inhibition of the enzyme glycogen synthetase. However, neither glycogen phosphorylase nor
449 glycogen synthetase genes were found to be DE between the two pools. The only gene coding for
450 an enzyme related to muscle glycogen synthesis found overexpressed in low GP samples is *UGP2*,
451 which gives origin to UDP-glucose, a direct precursor of glycogen. Nevertheless, the
452 overexpression of a single enzyme may change the level of a metabolite but may not be enough to
453 control the whole pathway of glycogen metabolism. Accordingly, Reynolds et al. (2005) reported

454 that the overexpression of *UGP2* and an associated increase in the levels of UDP-glucose alone are
455 not able to affect glycogen synthesis in mouse skeletal muscles. The regulation of glycogen content
456 is indeed a highly complex process and appears to be associated with several enzymatic proteins
457 and metabolic conditions (Mookerjee, Nicholls, & Brand, 2016; Roach, Depaoli-Roach, Hurley, &
458 Tagliabracci, 2012; Daran-Lapujade et al., 2007; Tanner et al., 2018). Several Authors showed that
459 altered functionality and activity of enzymes involved in glycogen synthesis and catabolism could
460 be hardly evidenced at the mRNA level (Mookerjee, Nicholls, & Brand, 2016; Roach, Depaoli-
461 Roach, Hurley, & Tagliabracci, 2012; Daran-Lapujade et al., 2007; Tanner et al., 2018). The
462 glycogen metabolism was reported to be mainly regulated by a complex pattern of posttranslational,
463 conformational changes or phosphorylation and dephosphorylation of the main enzymes regulating
464 glycogen metabolism (Daran-Lapujade et al., 2007; Roach, 2002; Roach, Depaoli-Roach, Hurley, &
465 Tagliabracci, 2012). Bouskila et al. (2010) showed that a reduced capacity to synthesize muscle
466 glycogen in glycogen synthase knockout mice may have led to metabolic adaptations and flexibility
467 resulting in the muscles of these animals being more efficient at utilizing extracellular glucose
468 and/or fatty acids as substrates for energy production.

469 Glucose is the major source of energy for cells. This carbohydrate represents the preferred energy
470 substrate used in muscle for ATP production. In normoxic cells, glucose is metabolized via
471 anaerobic glycolysis to pyruvate, which is then oxidatively metabolized to CO₂ in the tricarboxylic
472 acid (TCA) cycle to generate large amounts of ATP through the mitochondrial oxidative
473 phosphorylation (Vamecq, Colet, Vanden Eynde, Briand, Porchet, & Rocchi, 2012; Lunt & Vander
474 Heiden, 2011; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021).

475 In early *postmortem* muscle, when the level of oxygen starts to drop, the conversion of pyruvate to
476 lactate is important to support ATP production by enhancing glucose catabolism via the
477 mitochondrial TCA cycle in presence of an impaired or restrained oxidative phosphorylation due to
478 the limited supply of oxygen. The Lactate Dehydrogenase (LDH) reaction converting pyruvate to

479 lactate contributes to a continuous supply of NAD⁺ required for the glycolysis progression and the
480 transfer to lactate of glycolysis-generated NADH reducing equivalents (Arago, Formentini, &
481 Cueva, 2013; Gladden, 2004; Lunt, & Vander Heiden, 2011; England, Scheffler, Kasten, Matarneh,
482 & Gerrard, 2013). While in normoxic conditions, most pyruvate is oxidized to CO₂, when hypoxic
483 conditions are established, mitochondria respiration is arrested and the mitochondrial contribution
484 to glucose oxidation can be limited/blocked. In these conditions, cells develop only the glycolytic
485 contribution to glucose oxidation, and pyruvate, which is obtained from glycolysis, is reduced by
486 LDH to lactate. The latter tends to be accumulated as a byproduct of glycolysis under anaerobic
487 conditions and mitochondrial dysfunction (Glancy, Kane, Kavazis, Goodwin, Willis, & Gladden,
488 2021; Ferguson, Rogatzki, Goodwin, Kane, Zachary, & Gladden, 2018; England et al., 2018;
489 Martinez-Reyes & Chandel, 2017; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021). In this
490 situation, the oxidation of one molecule of glucose would produce only two molecules of ATP
491 instead of the 36 (2 cytosolic and 34 mitochondrial) obtained through the glycolysis and subsequent
492 complete oxidation of one molecule of glucose in mitochondria. Anaerobic glucose catabolism does
493 not require oxygen, but it is much less efficient in generating ATP when compared with the TCA
494 cycle coupled to oxidative phosphorylation. Moreover, it is worth noting that in the reaction of
495 reduction of pyruvate to lactate, the latter can function as a regulator of cellular redox state
496 consuming H⁺ without contributing to the pH drop (Scheffler, Scheffler, Kasten, Sosnicki, &
497 Gerrard, 2013; England et al., 2018; Glancy, Kane, Kavazis, Goodwin, Willis, & Gladden, 2021).
498 In the samples of the present research, it might be possible to assume that the glycogen degradation
499 and lactate could not be responsible for the different *postmortem* pH decrease between the two
500 pools considering that 24 h after slaughtering lactate is accumulated in muscle *postmortem* of all
501 samples in a similar way.
502 Different mechanisms and processes related to the early *postmortem* muscle metabolism could be
503 considered to explain the different pH declines between the two pools later after slaughtering. A

504 graphical representation of the hypothesized events occurring in the early *postmortem* phases in
505 Pool L samples is reported in Figure 3.

506

507 **Figure 3.** Graphical representation of the hypothesized events occurring in the early *postmortem*
508 phases in Pool L samples.

509 Legend: Genes overexpressed in Pool L samples are highlighted in red; those genes are included in
510 rectangular or oval shapes depending on whether they express respectively for enzymatic proteins
511 or other proteins; asterisks indicate genes whose expression is directly regulated by *Peroxisome*
512 *Proliferator Activated Receptor Alpha (PPARA)*.

513

514 In the present research, the condition of low glycogen level in Pool L pig muscles may have already
515 been present before slaughter for some imbalance or dysfunction regarding metabolic pathways of
516 lipids and/or carbohydrates. After slaughter, this altered metabolic state may have been worsened
517 by the development of *postmortem* crucial changes due to the cessation of blood flow (Chauhan &
518 England, 2018; England et al., 2018). In that condition, when the level of oxygen of the cells starts
519 to lower in the early *postmortem* period, substrates other than glucose can contribute to ATP
520 production. In particular, under glycogen-limiting conditions, the switch towards fatty acid
521 oxidation might have occurred as a compensatory mechanism in muscle energy metabolism (Muioio
522 et al., 2002; Burri, Thoresen, & Berge, 2010; Lunt & Vander Heiden, 2011; Domenis, Bisetto,
523 Rossi, Comelli, & Mavelli, 2012; Phua, Wong, Liao, & Tan, 2018; Rakhshandehroo, Knoch,
524 Müller, & Kersten, 2010; Arago, Formentini, & Cueva, 2013). However, the conditions reported in
525 the scientific literature are not always comparable with those found *postmortem* in muscles during
526 the biochemical processes leading to meat production. Therefore, despite being supported by the
527 literature, the hypothesized switch towards fatty acid oxidation in low GP muscles would need to be

528 validated with ad hoc studies specifically addressed to better understand biochemical processes
529 taking place *postmortem* in muscles coming from animals reared for meat purposes.

530 The hypothesized metabolic switch towards fatty acid oxidation may also be supported by the
531 upregulation in Pool L samples of some genes that have a direct impact on cellular metabolism and
532 energy production, such as *PPARA*. *PPARA* is a master regulator that controls the expression of
533 numerous target genes involved in many pathways related to lipid metabolism, including fatty acid
534 uptake. The upregulation of *PPARA* gene can activate mitochondrial fatty acid oxidation in muscle
535 tissue (Muio et al., 2002; Burri, Thoresen, & Berge, 2010; Goto et al., 2011; Fan & Evans, 2015;
536 Bougarne et al., 2018; Phua, Wong, Liao, & Tan, 2018). Furthermore, several genes found DE in
537 the present study have been previously identified to be regulated by *PPARA*. For example, a large
538 body of literature reports that *PPARA* regulates genes involved in fatty acid uptake (such as the DE
539 gene *LPL*), fatty acid intracellular transport (such as *FABP3*), and fatty acid phosphorylative
540 oxidation (such as mitochondrial ATPase complex V) (Hue & Taegtmeyer, 2009; Rakhshandehroo,
541 Knoch, Müller, & Kersten, 2010; Fan & Evans, 2015; Phua, Wong, Liao, & Tan, 2018; Bougarne
542 et al., 2018). Moreover, *PPARA* can also activate *SCD* gene, which codes for a key lipogenic
543 enzyme. The *SCD* overexpression in turn promotes the release of monounsaturated fatty acids
544 (oleate and palmitoleate). These fatty acids can induce an increase in *PPARA* expression and
545 mitochondrial fatty acids oxidative phosphorylation, in order to avoid excessive triglyceride
546 accumulation (Goto et al., 2011). Accordingly, in the present research, the upregulation of *PPARA*
547 gene may have promoted the transcription of genes overexpressed in Pool L that are related to fatty
548 acid synthesis, transport, and oxidative metabolism (*FABP3*, *FABP5*, *ACSL1*, *LPL*, *SCD*), or that
549 drive ATP production through fatty acid β -oxidation and oxidative phosphorylation (Wilson, 2017;
550 Teodoro et al., 2017; Young et al., 2018). The upregulation of the target genes activated by *PPARA*
551 could have triggered, in Pool L samples, the oxidation of fatty acids instead of glucose for cellular
552 energy production. This result is consistent with the early *postmortem* compensatory events that

553 may take place in response to hypoxia and low glycogen conditions (Smolkova et al., 2010; Wilson,
554 2017). The observed up-regulation of the *PPARA* gene may have, therefore, regulated the
555 expression of several other genes involved in energy metabolism, promoted mitochondrial
556 functions, and may have created conditions favorable to establish a pro-apoptotic *postmortem*
557 condition, as will be further discussed later.

558 Among the genes found DE in the present study, several mitochondrial genes were up-regulated in
559 Pool L samples (such as *ATP5F1A*, *ATP5MC3*, *CIQBP*, *GOT1*, *FBXO32*, and *MCU*), likely
560 suggesting a *postmortem* functionality of mitochondria in these samples. The genes *CIQBP*, *GOT1*,
561 *FBXO32*, and *MCU* are involved in mitochondrial activity and oxidative phosphorylation of fatty
562 acids, which are activities that need oxygen supply since oxygen is the final electron acceptor in the
563 electron transport chain (ETC) (Matarneh, Beline, de Luz e Silva, Shia, & Gerrard, 2018). This
564 result may appear to be surprising at first glance, as in *postmortem* muscle, oxygen is continuously
565 decreasing due to hypoxic/anoxic conditions. However, different Authors reported that the muscle
566 oxygen concentration after slaughter declines gradually within the first 2 h *postmortem*, and thus
567 oxygen is not immediately depleted after exsanguination. In this condition, if energy substrates and
568 oxygen are available, mitochondrial functionality still exists for some time after slaughter and
569 contributes to ATP production through oxidative phosphorylation (England et al., 2018; Matarneh,
570 Yen, Bodmer, El-Kadi, & Gerrard, 2021). As a consequence, in Pool L samples, mitochondria may
571 directly influence *postmortem* muscle metabolism sustaining ATP production in the first hours after
572 slaughter (Scheffler, Matarneh, England, & Gerrard, 2015; Matarneh, Yen, Bodmer, El-Kadi, &
573 Gerrard, 2021). Matarneh, Yen, Bodmer, El-Kadi, & Gerrard (2021) carried out a study aimed to
574 analyze mitochondrial functionality relying on *in vitro* conditions simulating *postmortem*
575 metabolism in porcine *Longissimus lumborum* and *masseter* muscles. These Authors reported that,
576 under those conditions, mitochondria are capable of mobilizing pyruvate originated from glycolysis
577 (Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021). These data, together with the gradual

578 reduction in oxygen reported in their previous study (England et al., 2018), strongly support the
579 evidence that mitochondrial activity is maintained *postmortem*, albeit at reduced levels. It could be
580 argued that in our samples, in presence of low glycogen and low GP, the mitochondria functionality
581 and fatty acid oxidation could have played a noticeable role.

582 Domenis, Bisetto, Rossi, Comelli, & Mavelli (2012) and Arago, Formentini, & Cueva (2013)
583 reported that the expression of the enzymatic complex V of mitochondrial ATP synthase (F1F0
584 ATP synthase) is directly related to the activity of oxidative phosphorylation, whereas it inversely
585 correlates with the rate of glucose utilization by aerobic glycolysis. In Pool L samples we have
586 found a similar situation, with the overexpression of the *ATP5F1A* and *ATP5MC3* and the
587 upregulation of genes coding for proteins involved in fatty acids uptake and activation of
588 mitochondrial oxidative phosphorylation. Different Authors reported that the mitochondrial
589 membrane ATP synthase is a major determinant of muscle energy metabolism, has a pivotal role in
590 cell physiology, and can influence the *postmortem* metabolism of the muscle tissue (Senior,
591 Nadanaciva, & Weber, 2002; Matarneh, England, & Gerrard, 2015; England et al., 2018; Matarneh,
592 England, Scheffler, Yen, & Gerrard, 2017; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021;
593 Aaon, Bhatt, & Cortassa, 2014; Junge & Nelson, 2015). In the present research, the overexpression
594 of genes coding for subunits of the mitochondrial ATPase complex V observed in the samples with
595 low glycogen, may allow us to suppose that the overexpression of these genes in Pool L may also
596 have influenced the *postmortem* skeletal muscle metabolism by regulating the expression of other
597 genes related to energy metabolism. Mitochondrial membrane ATP synthase (F1F0 ATP synthase
598 or Complex V) can synthesize or hydrolyze ATP upon changes in cellular conditions in a reversible
599 reaction and in particular, it produces ATP from ADP and inorganic phosphate in the presence of an
600 electrochemical gradient of protons across the inner membrane generated by electron transport
601 complexes of the respiratory chain during oxidative phosphorylation (Walker, Lutter, Dupuis, &
602 Runswick, 1991; Boyer, 1997; Walker, 2013; Rühle & Leister, 2015). The flow of protons through

603 F1F0 ATP synthase (complex V) back to the mitochondrial matrix enhances ATP synthesis. The
604 description and characterization of the functions of the F1F0 ATPase complex V have been the
605 objectives of a large body of literature. This enzymatic complex consists of two structural domains,
606 the extra-membrane hydrophilic portion F1- which hosts the catalytic sites responsible for ATP
607 synthesis (and hydrolysis)-, and the membrane-embedded F0 domain- which contains the proton
608 channel (Walker, Lutter, Dupuis, & Runswick, 1991; Boyer, 1997; Walker, 2013; Rühle & Leister,
609 2015; Devenish, Prescott, & Rodgers, 2008; Zhou et al., 2015). *ATP5MC3* is one of the three genes
610 that encode for the C-subunit of the proton channel of ATP synthase (Bonora et al., 2013). The C-
611 subunit is produced from three nuclear genes, *ATP5G1*, *ATP5G2*, and *ATP5G3* (*ATP5MC3*)
612 encoding identical copies of the mature protein (Yan, Lerner, Haines, & Gusella, 1994; He, Forda,
613 Carrolla, Dinga, Fearnleya, & Walkera, 2017). *ATP5F1A* codes for a subunit of the ATP synthase
614 complex V domain F1 that protrudes in the mitochondrial matrix and is involved in the catalytic
615 activity of ATP synthesis and hydrolysis (He, Forda, Carrolla, Dinga, Fearnleya, & Walkera, 2017).
616 Despite their known importance in cell function and metabolism, the role of mitochondria and ATP
617 synthase in *postmortem* muscle remains mostly unknown.

618 The activation of mitochondrial oxidative phosphorylation in samples with low glycogen and low
619 GP can be also supported by the overexpression of the gene *CIQBP* that plays an important role in
620 mitochondrial metabolism (Fogal, Richardson, Karmali, Scheffler, Smith, & Ruoslahti, 2010; Chen
621 et al., 2016; Gotoh et al., 2018; Noh et al. 2020). In particular, this gene is described as an important
622 promoter of fatty acid oxidation and oxidative phosphorylation and may be required for efficient
623 ATP production through oxidative phosphorylation (Fogal, Richardson, Karmali, Scheffler, Smith,
624 & Ruoslahti, 2010; Aaon, Bhatt, & Cortassa, 2014). Gotoh et al. (2018) showed that loss of *CIQBP*
625 function in mice dendritic cells induced a metabolic reprogramming characterized by increased
626 glycolysis and impaired oxidative phosphorylation. In Pool L samples, with an extremely low level

627 of glycogen, the upregulation of the gene *CIQBP* could have supported the utilization of fatty acids
628 as an alternative energy substrate to produce ATP.

629 The hypothesis of an activation of oxidative phosphorylation to produce ATP in Pool L samples
630 may also be supported by the overexpression of the genes *MCU* and *PLN* found in that pool. *MCU*
631 codes for the inner mitochondrial membrane Ca^{2+} channel and promotes the increase of Ca^{2+} ions
632 levels in mitochondria (Shaikh, Sahoo & Periasamy, 2016). Mitochondria can receive Ca^{2+} ions from
633 the sarcoplasmic reticulum, the main calcium depot of the cell (Baughman et al., 2012; Giorgi,
634 Marchi, & Pinton, 2018). In Pool L samples, it may be suggested that the overexpression of the
635 gene *PLN* may inhibit the activity of the sarcoplasmic reticulum calcium pumps Ca^{2+} -ATPases
636 (SERCAs), and the flux of calcium ions into the sarcoplasmic reticulum. This condition, together
637 with the upregulation of the mitochondrial gene *MCU*, may, in turn, have promoted the increase of
638 calcium uptake into mitochondria (Fajardo et al., 2013; De Stefani, Patron, & Rizzuto, 2015). As a
639 consequence, it is possible to hypothesize that the MCU-mediated Ca^{2+} uptake within mitochondria
640 may have produced regulatory effects on the functions of these organelles and may also have
641 contributed to stimulating ATP production by oxidative phosphorylation.

642 Several studies described that when sarcoplasmic reticulum Ca^{2+} pumps are blocked and Ca^{2+} ions
643 are sequestered by the mitochondria, the first effect is a stimulation of the aerobic metabolism with
644 the activation of the F1F0 ATP synthase complex V to produce ATP, followed by parallel
645 activation of ATP-consuming processes in the cytosol, which are aimed to prevent significant
646 alterations in the energy balance of the cell (Giorgi, Marchi, & Pinton, 2018; Martínez, Marmisolle,
647 Tarallo, & Quijano, 2020; Nath, 2020). Ca^{2+} ions overload and accumulation in mitochondria
648 trigger mitochondrial necrosis and can lead to the opening of the mitochondrial permeability
649 transition pore (mPTP), with a consequent dissipation of mitochondrial membrane potential and
650 activation of the events leading to cell death (Rasola & Bernardi, 2007; Nesci, Trombetti, Ventrella,
651 & Pagliarani, 2018; Tait & Green, 2010; Arago, Formentini, & Cueva, 2013; England et al., 2018).

652 In consideration of the significant differences between pools for the glycogen and GP levels, the
653 high glycogen content in Pool H may have allowed muscle cells to utilize carbohydrates as a rapidly
654 available source of energy, contributing to a delay in the biological cascade leading to cell death.

655

656 **5. Conclusions**

657 The molecular patterns highlighted in the present research allow drawing some hypotheses bringing
658 out biological events that characterize the different *postmortem* cellular metabolism in the samples
659 belonging to the two pools. In particular, the results obtained in the Pool L in animal tissues
660 collected *postmortem* evidenced the upregulation of groups of genes related to mitochondrial
661 activity, lipid metabolism, ATP synthase complex V function and mitochondrial calcium uptake.

662 The overexpression of these genes in Pool L samples seems to be implicated in metabolic processes
663 taking place in *postmortem* muscle cells. The different GP levels between the two pools could have
664 determined in Pool L a more rapid occurrence of the molecular cascade related to events triggering
665 cell death. In conditions of reduced glycogen and GP, the overexpression of genes related to
666 mitochondrial activity may have influenced the pH drop after slaughtering.

667 To date, the knowledge of the muscle cell *postmortem* metabolism is still scant, and the exact role
668 played by mitochondria in the muscle-to-meat transformation remains largely unknown. The results
669 obtained in the present research could be helpful to add pieces of information to these complex
670 metabolic processes.

671

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676 with a huge passion for research.

677

678 **Supplementary material**

679 **Supplementary Table S1.** Primer sequences and amplification conditions used for the quantitative
680 real-time PCR analysis of the porcine genes found differentially expressed between the two pools.

681 **Supplementary Table S2.** The description of the pigs selected for their extreme and divergent
682 glycolytic potential (GP) and used to obtain the two pools (Pool L and Pool H).

683 **Supplementary Table S3.** Lists of oligonucleotides identified as differentially expressed between
684 pools with the four statistical methods.

685 **Supplementary Table S4.** Complete information of oligonucleotide sequences and their relative
686 gene identification.

687

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