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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

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

2. Material and methods

2.1 Animals and phenotypes

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

and one barrow) of full sibs of candidate boars tested to become breeders. The 277 pigs (183 gilts and 94 barrows) were therefore subjects belonging to triplets coming from 154 litters, originated from 154 dams and 80 sires. The 277 pigs were reared in a unique testing station, and thus in a standard environment, in order to avoid any environmental effects on the phenotypes of the sib-tested population. The testing period lasted from 30-45 days of age to about 9 months of age, up to a final live weight of 155 kg, in line with the specifications reported on the document describing the rules and the procedures to obtain Protected Designation of Origin Parma dry-cured hams. During the testing period, siblings were kept separated and all animals were fed the same growing and finishing diets in order to avoid any possible effects related to diet composition. The pigs were fed with a *quasi ad libitum* nutrition level, which means that about 60% of pigs were able to ingest the entire supplied ration. All animals used in this study were reared according to Italian and European law for pig production and all slaughter procedures complied with national and European Union regulations for animal care and slaughtering. At the end of the testing period, pigs were fasted for about 12 h before being transported and immediately slaughtered at a commercial abattoir located near the genetic station. Pigs were transported and slaughtered in compliance with European rules on the protection of animals during transport and at slaughtering (Council Regulation (EC) No. 1/2005 and Council Regulation (EC) No. 1099/2009). At the slaughterhouse, the pigs were stunned by CO_2 at a concentration of 87% in the air (Butina, Holbaek, Denmark) and bled in a supine position. Slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health. Pigs were slaughtered during six slaughtering days. The date of slaughter and sex were recorded for each animal. SM muscle samples were gathered from the whole population of 277 pigs. One aliquot of SM samples was collected from the 277 pigs approximately 1 h after slaughtering, immediately frozen in liquid nitrogen, then stored in a deep freezer at -80°C until DNA or RNA extraction. From the same thigh used to collect SM samples for DNA and RNA extraction, a portion of SM muscle was also gathered at 24 h *postmortem* from the 277 pigs, frozen

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

Meat pH_1 (measured about 1 h *postmortem*) and pH_u (measured 24 h *postmortem*) were measured directly on the SM of the left thigh of each animal. Glycogen, lactic acid content, and GP were measured in a portion of SM collected from the same thigh at 24 h *postmortem*. All samples were processed within 4 months after sampling. Two separate analyses were carried out: one for the lactate and the other for the sum of glycogen (the main component of GP degraded to glucose with amyloglucosidase), glucose, and glucose-6-phosphate using the L-Lactic acid and D-Glucose Enzymatic Bio-Analysis kits (R-Biopharma, Milan, Italy), respectively. GP was calculated according to Monin & Sellier (1985) by summing: 2[glycogen + glucose + glucose-6-phoshate] + [lactate] and expressed as micro-moles (µmoles) of lactic acid equivalent per gram of fresh muscle. The animals used to perform the microarray analysis were chosen avoiding full-sib pigs and selecting eight animals with extreme and divergent GP values: Pool L, four pigs with a GP value less than the mean - 2 standard deviations (S.D.); Pool H with four pigs with a GP value greater than the mean + 2 S.D. The pigs comprised in each group (Pool H and Pool L) were selected by balancing for the animals' sex, thus selecting two gilts and two barrows for their inclusion in each group. Kruskal-Wallis test, performed in the R environment (R Core Team, 2020), was then applied to test whether the samples used for the two pools had statistically different values for the measured phenotypes. Data of the two pools were considered significantly different for *P*-values < 0.05.

2.2 Genotyping for the mutations *PRKAG3* p R200Q and *RYR1* c.1843C>T

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

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

2.3 Total RNA extraction

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

2.4 Oligonucleotides microarray analysis

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

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

2.5 Oligonucleotides re-annotation

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

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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

for the median intensity). The online web tool Bioinformatics and Evolutionary Genomics (Bioinformatics and Evolutionary Genomics, 2021) was then used to graphically represent with a Venn diagram the DEGs found, and the intersection obtained with each statistical approach.

The functional annotation was obtained using Cytoscape 3.8.2 (Shannon et al., 2003) with the ClueGO app (Bindea et al., 2009). Functional characterization was performed selecting human database as a reference and the DEGs were significantly enriched in KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway, biological process (BP), cellular component (CC), and molecular function (MF). The *P*-values of the obtained functional categories were adjusted with the Bonferroni step-down method. The adjusted *P*-values were considered significant for $P < 0.10$, following the significance threshold used in other studies (such as Lorenz et al., 2019), and the significant functional categories were graphically displayed with Cytoscape tools.

2.7 Validation with quantitative Real-time PCR

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

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

The obtained normalized quantitation for the samples was then used to calculate the mean for each pool and to obtain for each gene the $\log_2$ of the FC value ($\log_2$FC), which was obtained by dividing the average expression level of the Pool H for the average expression identified in Pool L. The $\log_2$FC values of the gene expression quantified with qRT-PCR and with microarray analysis were used to perform Spearman correlations and test whether the trend of the gene expression levels was maintained between the qRT-PCR and the four statistical procedures applied to microarray data to find DEGs (MNE, MNS, MDE, MDS). Spearman correlations were estimated with *Hmisc* R package (v 4.5.0; Harrell Jr, 2021). The correlations were considered significant for $P < 0.05$.

3. Results

3.1 Description of the two pools

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

Table 1. Descriptive statistics (Mean and Standard Deviation, S.D.) for the phenotypic parameters measured on the pigs of Pool H (high glycolytic potential), of Pool L (low glycolytic potential) and on the whole sample of 277 Italian Large White heavy pigs. For the Pool H *vs.* Pool L comparison, Kruskal-Wallis test statistics ($\chi 2$ 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_1[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
Glycolitic Potential (GP) (μmoles/g)[3]	103.68 ± 23.15	162.03 ± 12.62	56.03 ± 5.73	5.33	0.021

[1] pH_1 was measured about 1 h *postmortem* directly on the *Semimembranosus* muscle of the left thigh.

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

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

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

The average pH_1 values of the two groups did not differ significantly, while the pH_u values showed a significant difference ($P = 0.021$) between the samples of the two pools. This result for pH_u agrees

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

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

3.2 Microarray results and functional analysis

Supplementary Table S3 reports the oligonucleotides identified as DE with each statistical method and the complete output of each analytical procedure used to identify sequences differentially expressed between pools. The complete information concerning oligonucleotides sequence and their relative gene identification is reported in Supplementary Table S4. Table 2 shows the DE oligonucleotides identified by each method and Figure1 graphically displays the number of DE oligonucleotides retained in the different considered methods.

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

Table 2. The 35 oligonucleotides (oligo ID) found differentially expressed with the four statistical methods (MNE = Mean Edwards; MNS = Mean Subtract; MDE = Median Edwards; MDS = Median Subtract) and their gene identification. Data are reported with the log_2 of the fold change (log_2FC) and the adjusted *P*-value.

Oligo ID	Gene symbol	MNE		MNS		MDE		MDS	
		Log_2FC	**Adjusted *P*-value**	**Log_2FC**	**Adjusted *P*-value**	**Log_2FC**	**Adjusted *P*-value**	**Log_2FC**	**Adjusted *P*-value**
SS00000291	*MYH3*	ns	ns	1.8410	1.63E-03	1.7273	2.37E-02	2.2504	1.43E-03
SS00000459	*NTRK3*	ns	ns	ns	ns	-1.1864	3.96E-04	-1.1706	1.01E-04
SS00000539	*HBB*	-1.0303	9.71E-04	-1.0394	1.15E-03	ns	ns	ns	ns
SS00000679	*SCD*	-2.5490	1.53E-02	-2.5348	1.48E-02	-2.9639	1.30E-02	-2.9573	1.01E-02
SS00000695	*PLN*	-2.1164	5.01E-03	-2.0850	4.76E-03	-2.0953	6.58E-03	-2.0634	5.76E-03
SS00000696	*GOT1*	ns	ns	ns	ns	-1.0043	9.03E-04	-1.0245	2.48E-03
SS00000767	*UGP2*	-1.0962	9.58E-03	-1.0726	8.32E-03	-1.1868	4.31E-03	-1.1674	3.09E-03
SS00001044	*FABP3*	-1.3071	9.71E-04	-1.3264	1.63E-03	-1.3513	4.46E-04	-1.3737	7.45E-04
SS00001123	*PPARA*	-1.0346	1.72E-02	-1.0460	3.00E-02	-1.0382	5.95E-03	-1.0405	7.36E-03
SS00001972	*FABP5*	-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	*DVL3*	-1.5981	2.14E-02	-1.3220	1.41E-02	-1.4296	1.19E-02	-1.3706	8.89E-03
SS00002786	*ATP5F1A*	-1.0630	4.21E-02	ns	ns	ns	ns	ns	ns
SS00002870	*MYOZ2*	-1.9427	3.08E-02	-1.9236	2.81E-02	-2.0071	2.12E-02	-1.9818	1.85E-02
SS00003271	*HSPB6*	-1.1909	7.50E-03	-1.1810	6.37E-03	ns	ns	ns	ns
SS00003290	*EIF2B2*	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	*C1QBP*	ns	ns	ns	ns	-1.0172	5.06E-03	ns	ns
SS00004323	*ATP5MC3*	-1.0196	1.02E-02	-1.0071	8.30E-03	-1.0124	1.83E-03	-1.0072	9.42E-04
SS00004770	*HSPB7*	-1.3000	1.18E-02	-1.2853	1.01E-02	-1.2715	1.35E-02	-1.2675	1.32E-02
SS00005142	*FCHSD1*	ns	ns	ns	ns	-1.0535	4.43E-03	-1.0215	3.43E-03
SS00005172	*POLA2*	-1.7040	1.88E-02	-1.6282	2.04E-02	ns	ns	-1.4361	4.55E-02
SS00005914	*HOMER2*	-1.1198	3.76E-03	-1.1020	3.00E-03	-1.1156	6.46E-03	-1.0967	5.76E-03
SS00005997	*AP1G2*	-1.2248	4.29E-02	ns	ns	-1.3253	1.35E-02	-1.0503	1.83E-02
SS00006189	*CSRP3*	-1.6951	1.42E-02	-1.6674	1.10E-02	-1.6566	7.10E-03	-1.6071	4.76E-03
SS00006369	*TSSK6*	ns	ns	ns	ns	-1.7502	4.05E-02	ns	3.40E-04

SS00006853	*SCD*	-2.0247	3.02E-03	-2.0062	2.85E-03	-1.9751	5.02E-04	-1.9667	ns
SS00006888	*LPL*	-1.5160	1.40E-03	-1.4980	1.06E-03	-1.4952	2.80E-04	-1.4807	1.74E-04
SS00006966	*RNF220*	ns	ns	ns	ns	-2.7826	4.08E-03	ns	ns
SS00007208	*COQ9*	-1.8260	3.92E-02	ns	ns	-3.2519	2.69E-04	-3.7321	4.59E-02
SS00007292	*MCU*	ns	ns	ns	ns	-1.1740	1.30E-02	ns	ns
SS00008224	*DNAJB1*	-1.1845	3.73E-02	-1.1640	3.56E-02	-1.1004	3.84E-02	-1.0803	3.66E-02
SS00008616	*ACSL1*	-1.0801	5.40E-03	-1.0681	5.55E-03	-1.1553	7.64E-03	-1.1440	5.76E-03
SS00009114	*HSPB2*	Ns	ns	ns	ns	-1.2000	2.39E-02	ns	ns
SS00009459	*SPARCL1*	-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	*P*-value	Bonferroni adjusted *P*-value
ACSL1, FABP3, FABP5, LPL, PPARA, SCD	KEGG:03320	PPAR signaling pathway	6	3.41E-09	1.64E-07
CSRP3, FBXO32, HSPB6, MYH3, MYOZ2, PLN, PPARA	GO:0003012	muscle system process	7	1.12E-05	5.27E-04
CSRP3, EIF2B2, FABP3, GOT1, LPL, PLN, PPARA	GO:0043434	response to peptide hormone	7	1.60E-05	7.37E-04
ACSL1, ATP5F1A, ATP5MC3, PPARA, SCD	GO:0006164	purine nucleotide biosynthetic process	5	3.26E-05	1.47E-03
ACSL1, ATP5F1A, ATP5MC3, PPARA, SCD	GO:0009260	ribonucleotide biosynthetic process	5	3.64E-05	1.60E-03
CSRP3, MYH3, MYOZ2	GO:0045214	sarcomere organization	3	3.88E-05	1.67E-03
ACSL1, FABP3, FABP5, LPL	GO:0006641	triglyceride metabolic process	4	5.40E-05	2.27E-03
CSRP3, FABP5, FBXO32, HSPB6, HSPB7, PLN, PPARA	GO:0044057	regulation of system process	7	7.69E-05	3.15E-03
ACSL1, FABP3, FABP5, LPL, PPARA, SCD	GO:0006631	fatty acid metabolic process	6	7.94E-05	3.18E-03

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

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

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

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330

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

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

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

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

3.3 Validation of microarray results

To validate the results obtained with the microarray we decided to analyze the expression level by qRT-PCR of six of the DEG found (*ATP5MC3*, *LPL*, *MYOZ2*, *PLN*, *SCD*, *UGP2*) after microarray hybridization; these six genes were selected for their relevant role in muscle metabolism. A high correlation value was found between the $\log_2$FC found with qRT-PCR and the $\log_2$FC values identified for the same genes with the four statistical methods ($r = 0.94$, $P < 0.005$ for all the correlations qRT-PCR *vs*. MNE, qRT-PCR *vs*. MNS, qRT-PCR *vs*. MDE, and qRT-PCR *vs*. MDS).

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

4. Discussion

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

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

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

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

The amount of lactate is considered by different Authors as an indicator of *postmortem* pH decline. There is however lack of agreement in the scientific community with respect to the relationship linking lactate and *postmortem* pH decline. Other Authors have indeed reported that even though there is a significant connection between muscle glycolysis, GP, lactate accumulation, and *postmortem* pH, the relationship between glycogen content and pH_u is not complete and not linear, as samples showing similar lactate values may display different pH_u (England et al., 2016; Scheffler, Park, & Gerrard, 2011; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013; Pösö & Puolanne, 2005; Chauhan & England, 2018; Huff-Lonergan, Baas, Malek, Dekkers, Prusa, & Rothschild, 2002; Monin & Sellier, 1985). In agreement with results found in other studies (Van Laack & Kauffman, 1999; Van Laack, Liu, Smith, & Loveday, 2000; Choe et al., 2008), in the present research, we noticed different pH_u values between the samples of the two pools but similar values of lactate at 24h *postmortem* were present between the two pools. This result seems to indicate that the difference noticed between the pH_u values of the two pools may not be ascribed to glycogen breakdown leading to lactate formation. The similar values of pH_u and lactate between the two pools may indicate that the differences noticed in GP at 24 h *postmortem* should not be 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 pH_1 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 pH_1, 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 CO_2 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

lactate contributes to a continuous supply of NAD^+ required for the glycolysis progression and the transfer to lactate of glycolysis-generated NADH reducing equivalents (Arago, Formentini, & Cueva, 2013; Gladden, 2004; Lunt, & Vander Heiden, 2011; England, Scheffler, Kasten, Matarneh, & Gerrard, 2013). While in normoxic conditions, most pyruvate is oxidized to CO_2, when hypoxic conditions are established, mitochondria respiration is arrested and the mitochondrial contribution to glucose oxidation can be limited/blocked. In these conditions, cells develop only the glycolytic contribution to glucose oxidation, and pyruvate, which is obtained from glycolysis, is reduced by LDH to lactate. The latter tends to be accumulated as a byproduct of glycolysis under anaerobic conditions and mitochondrial disfunction (Glancy, Kane, Kavazis, Goodwin, Willis, & Gladden, 2021; Ferguson, Rogatzki, Goodwin, Kane, Zachary, & Gladden, 2018; England et al., 2018; Martinez-Reyes & Chandel, 2017; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021). In this situation, the oxidation of one molecule of glucose would produce only two molecules of ATP instead of the 36 (2 cytosolic and 34 mitochondrial) obtained through the glycolysis and subsequent complete oxidation of one molecule of glucose in mitochondria. Anaerobic glucose catabolism does not require oxygen, but it is much less efficient in generating ATP when compared with the TCA cycle coupled to oxidative phosphorylation. Moreover, it is worth noting that in the reaction of reduction of pyruvate to lactate, the latter can function as a regulator of cellular redox state consuming H^+ without contributing to the pH drop (Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013; England et al., 2018; Glancy, Kane, Kavazis, Goodwin, Willis, & Gladden, 2021). In the samples of the present research, it might be possible to assume that the glycogen degradation and lactate could not be responsible for the different *postmortem* pH decrease between the two pools considering that 24 h after slaughtering lactate is accumulated in muscle *postmortem* of all samples in a similar way.

Different mechanisms and processes related to the early *postmortem* muscle metabolism could be considered to explain the different pH declines between the two pools later after slaughtering. A

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

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

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

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

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

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

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

Among the genes found DE in the present study, several mitochondrial genes were up-regulated in Pool L samples (such as *ATP5F1A, ATP5MC3, C1QBP, GOT1, FBXO32,* and *MCU*), likely suggesting a *postmortem* functionality of mitochondria in these samples. The genes *C1QBP, GOT1, FBXO32,* and *MCU* are involved in mitochondrial activity and oxidative phosphorylation of fatty acids, which are activities that need oxygen supply since oxygen is the final electron acceptor in the electron transport chain (ETC) (Matarneh, Beline, de Luz e Silva, Shia, & Gerrard, 2018). This result may appear to be surprising at first glance, as in *postmortem* muscle, oxygen is continuously decreasing due to hypoxic/anoxic conditions. However, different Authors reported that the muscle oxygen concentration after slaughter declines gradually within the first 2 h *postmortem,* and thus oxygen is not immediately depleted after exsanguination. In this condition, if energy substrates and oxygen are available, mitochondrial functionality still exists for some time after slaughter and contributes to ATP production through oxidative phosphorylation (England et al., 2018; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021). As a consequence, in Pool L samples, mitochondria may directly influence *postmortem* muscle metabolism sustaining ATP production in the first hours after slaughter (Scheffler, Matarneh, England, & Gerrard, 2015; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021). Matarneh, Yen, Bodmer, El-Kadi, & Gerrard (2021) carried out a study aimed to analyze mitochondrial functionality relying on *in vitro* conditions simulating *postmortem* metabolism in porcine *Longissimus lumborum* and *masseter* muscles. These Authors reported that, under those conditions, mitochondria are capable of mobilizing pyruvate originated from glycolysis (Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021). These data, together with the gradual

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

Domenis, Bisetto, Rossi, Comelli, & Mavelli (2012) and Arago, Formentini, & Cueva (2013) reported that the expression of the enzymatic complex V of mitochondrial ATP synthase (F1F0 ATP synthase) is directly related to the activity of oxidative phosphorylation, whereas it inversely correlates with the rate of glucose utilization by aerobic glycolysis. In Pool L samples we have found a similar situation, with the overexpression of the *ATP5F1A* and *ATP5MC3* and the upregulation of genes coding for proteins involved in fatty acids uptake and activation of mitochondrial oxidative phosphorylation. Different Authors reported that the mitochondrial membrane ATP synthase is a major determinant of muscle energy metabolism, has a pivotal role in cell physiology, and can influence the *postmortem* metabolism of the muscle tissue (Senior, Nadanaciva, & Weber, 2002; Matarneh, England, & Gerrard, 2015; England et al., 2018; Matarneh, England, Scheffler, Yen, & Gerrard, 2017; Matarneh, Yen, Bodmer, El-Kadi, & Gerrard, 2021; Aaon, Bhatt, & Cortassa, 2014; Junge & Nelson, 2015). In the present research, the overexpression of genes coding for subunits of the mitochondrial ATPase complex V observed in the samples with low glycogen, may allow us to suppose that the overexpression of these genes in Pool L may also have influenced the *postmortem* skeletal muscle metabolism by regulating the expression of other genes related to energy metabolism. Mitochondrial membrane ATP synthase (F1F0 ATP synthase or Complex V) can synthesize or hydrolyze ATP upon changes in cellular conditions in a reversible reaction and in particular, it produces ATP from ADP and inorganic phosphate in the presence of an electrochemical gradient of protons across the inner membrane generated by electron transport complexes of the respiratory chain during oxidative phosphorylation (Walker, Lutter, Dupuis, & 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 *C1QBP* 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 *C1QBP*
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

of glycogen, the upregulation of the gene *C1QBP* could have supported the utilization of fatty acids as an alternative energy substrate to produce ATP.

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

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

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

5. Conclusions

The molecular patterns highlighted in the present research allow drawing some hypotheses bringing out biological events that characterize the different *postmortem* cellular metabolism in the samples belonging to the two pools. In particular, the results obtained in the Pool L in animal tissues collected *postmortem* evidenced the upregulation of groups of genes related to mitochondrial activity, lipid metabolism, ATP synthase complex V function and mitochondrial calcium uptake. The overexpression of these genes in Pool L samples seems to be implicated in metabolic processes taking place in *postmortem* muscle cells. The different GP levels between the two pools could have determined in Pool L a more rapid occurrence of the molecular cascade related to events triggering cell death. In conditions of reduced glycogen and GP, the overexpression of genes related to mitochondrial activity may have influenced the pH drop after slaughtering.

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

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Supplementary material

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

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

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

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

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