Gene co-expression network analysis for porcine intramuscular fatty acid composition

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ABSTRACT

In pigs, meat quality depends markedly on the fatty acid (**FA**) content and composition of the intramuscular fat, which is partly determined by the gene expression in this tissue. The aim of this work was to identify the link between muscle gene expression and its FA composition. In an (Iberian × Duroc) × Duroc backcrossed pig population, we identified modules of co-expressed genes, and correlation analyses were performed for each of them versus the phenotypes, finding four relevant modules. Two of the modules were positively correlated with saturated FAs (**SFAs**) and monounsaturated FAs (**MUFAs**), while negatively correlated with polyunsaturated FAs (**PUFAs**) and the omega-6/omega-3 ratio. The gene-enrichment analysis showed that these modules had over-representation of pathways related with the biosynthesis of unsaturated FAs, the Peroxisome proliferator-activated receptor signalling pathway and FA elongation. The two other relevant modules were positively correlated with PUFA and the n-6/n-3 ratio, but negatively correlated with SFA and MUFA. In this case, they had an over-representation of pathways related with fatty and amino acid degradation, and with oxidative phosphorylation. Using a graphical Gaussian model, we inferred a network of connections between the genes within each module. The first module had 52 genes with 87 connections, and the most connected genes were *ADIPOQ,* which is related with FA oxidation, and *ELOVL6* and *FABP4,* both involved in FA metabolism. The second module showed 196 genes connected by 263 edges, being *FN1* and *MAP3K11* the most connected genes. On the other hand, the third module had 161 genes connected by 251 edges and *ATG13* was the top neighbouring gene, while the fourth module had 224 genes and 655 connections, and its most connected genes were related with mitochondrial pathways. Overall, this work successfully identified relevant muscle gene networks and modules linked with FA composition, providing further insights on how the physiology of the pigs influences FA composition.

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Implications

Pig meat quality is influenced by the fatty acid composition in intramuscular fat, which, in turn, is determined by different factors such as the diet, the animal age or the breed. Aiming to deepen the knowledge of the links between muscle gene expression and fatty acid composition, our study identified groups of genes that play a role in controlling fatty acid metabolism. Groups with positive relationships with saturated and monounsaturated fatty acids had genes involved in the synthesis of fatty acids and groups with positive relationships with polyunsaturated fatty acids had genes involved in the degradation of fatty acids.

Introduction

Pig is a main livestock species, being pork one of the most produced meats worldwide only surpassed by poultry, with 1.35 billion swine heads produced in 2019 and 110 million tonnes of meat (FAOSTAT, 2022). This high productivity is sustained by the efficiency of the commercial pig breeds, which are strongly

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selected based on their prolificity, growth capacity, and muscle and fat deposition, among other traits (Latorre et al., 2008). Nowadays, other traits are also added to the selective breeding schemes, as consumers demand healthier meats, but also keeping their good organoleptic qualities such as flavour or juiciness (Aaslyng et al., 2007), being determinant of the level of intramuscular fat (Fernandez et al., 1999; van Laack et al., 2001). Lipid metabolism plays an important role in energy production and storage among other processes (Bergen and Mersmann, 2005), and it is a key factor in pork production, as it affects the composition of the fat and, therefore, consumer acceptance of the meat (Fernandez et al., 1999). In addition, pig is used in biomedicine as a model species for obesity and cardiovascular diseases, therefore, increasing the importance of deepening the knowledge on its lipid metabolism (Spurlock and Gabler, 2008).

Iberian pig is a Spanish rustic breed, less efficient than the highly efficient lean breeds, but also endowed with a high−fat deposition, both intramuscular and subcutaneous (Nieto et al., 2019). Iberian pork fatty acid (**FA**) profile, when compared with leaner breeds, has higher content in monounsaturated FAs (**MUFAs**), but also in saturated FAs (**SFAs**) (Serra et al., 1998). Monounsaturated FAs are involved in meat quality and taste, but SFAs have been associated with obesity and cardiovascular diseases in humans (Briggs et al., 2017; Livingstone et al., 2022). Iberian sows can be crossed with Duroc boars to enhance the growth performance while keeping good organoleptic properties of the meat (Ayuso et al., 2016). This crossbreed and the backcross (Duroc × Iberian) × Iberian are also used in the dry-cured products industry, producing more affordable pork while keeping an Iberian background (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2014).

Recent studies have been performed by our research group to assess relationships between the genes and the FA composition in muscle (Valdés-Hernández et al., 2023) using different approaches. This analysis found candidate genes for intramuscular FA profile. However, these results can be further explored using co-expression networks that highlight the relationships between the genes.

The analysis of gene expression data is a challenging task, and extensive efforts have been made to provide a large range of methods to extract relevant interaction networks from transcriptome data. Among these approaches, the Weighted Gene Co-expression Network Analysis (**WGCNA**) detects co-expressed genes associated with complex target traits (Horvath and Dong, 2008; Langfelder and Horvath, 2008; Zhang and Horvath, 2005). Weighted Gene Co-expression Network Analysis has been used to investigate co-expression modules associated with obesity (Kogelman et al., 2014) or feed efficiency in pigs (Ramayo-Caldas et al., 2018). However, WGCNA and other network inference approaches lack the ability to distinguish between direct and indirect relations among the expressed genes in a statistically sound way. A feasible alternative that can overcome this limitation is the combination of the WGCNA approach with graphical Gaussian models (**GGMs**) (Schäfer and Strimmer, 2005). Under this statistical framework, gene regulatory networks are modelled as sparse graphs where genes are nodes, i.e. Gaussian random variables, and edges between nodes are the relationships between genes, i.e. conditional dependencies between random variables. Graphical Gaussian models distinguish direct dependencies (true biological genes regulation network) from spurious correlations (undirected relationships between genes). Furthermore, this approach has been already used in plants (Ma et al., 2007), on other omics (Benedetti et al., 2017) or in cancer research (Svoboda et al., 2018).

Overall, WGCNA and GGM are complementary approaches to unravel the complexity of biological mechanisms (Le Novère, 2015) and in this article, we used a two-step approach similar to

L. Zhang et al. (2018) to apply them to an RNA-Seq dataset that targets the pig muscle transcriptome in animals with intramuscular FA composition.

The aim of this work was to study the relationships between the muscle gene expression and the FA profile, in order to identify genes that may be influencing the FA composition in muscle and therefore, the meat quality. For that purpose, we first used the WGCNA method to identify muscle co-expressed gene modules associated with FA composition. Subsequently, in each module, we used the statistical framework of GGMs to infer regulatory networks.

Material and methods

Animal material

The 129 pigs used in this study belonged to an experimental backcrossed population (BC1_DU), which was obtained by mating five Iberian × Duroc F1 boars with 22 Duroc sows. The population is fully described in Crespo-Piazuelo et al. (2020). In brief, the backcrossed animals (59 females and 70 males) were raised in intensive conditions and fed a cereal-based diet. Pigs were slaughtered at an age of 190 ± 15 days in a commercial abattoir, where samples from the *Longissimus dorsi* muscle were collected and snap-frozen in liquid nitrogen. Muscle samples were later stored at −80 °C until further use.

The FA profile was determined by a gas chromatography protocol for methyl esters, as explained in Crespo-Piazuelo et al. (2020). Then, the results of the FA composition were represented as relative abundances in percentages (Table 1). In addition, other indices such as the sum of SFAs, MUFAs and polyunsaturated FAs (**PUFAs**), and the ratio between the omega-6 and omega-3 (n-6/n-3) PUFA were also calculated.

Transcriptome sequencing and estimation of gene expression

The RiboPure[TM] Isolation kit for High Quality Total RNA (Ambion; Austin, TX, USA) was used, following the manufacturer's recommendations, to isolate the RNA from the *Longissimus dorsi* muscle of the 129 animals. A NanoDrop ND-1000 spectrophotometer (NanoDrop products; Wilmington, DE, USA) was used to quantify the RNA, and its integrity was checked with an Agilent Bioanalyzer-2100 equipment (Agilent Technologies, Inc.; Santa Clara, CA, USA). The CNAG institute (Centre Nacional d'Anàlisi Genòmica; Barcelona, Spain) performed the library preparation and sequencing. The analysis was run in an Illumina HiSeq 3000/4000 machine (Illumina, Inc.; San Diego, CA, USA) using the paired-end library prepared with Tru-Seq Stranded mRNA kit also from Illumina. Full details are available in Crespo-Piazuelo et al. (2020).

Bioinformatic analyses for RNA alignment and quantification can be found in Valdés-Hernández et al. (2023). In brief, initial quality control was performed with FastQC v0.11.9 (Andrews, 2010). STAR v2.7.9a (Dobin et al., 2013) was used to map the reads against the *Sscrofa11.1* pig reference genome and gene expression was assessed with RSEM v1.2.28 (B. Li and Dewey, 2011). In summary, a mean of 45.09 million of 2×75 bp paired-end reads per sample was obtained, resulting in an average of 90.06% of uniquely mapped reads. The raw count matrix consisted of 19 263 genes.

Weighted gene co-expression network analysis and gene enrichment analysis

The FA composition and ratios indicated in Table 1 were used in the WGCNA analysis. The list of expressed genes obtained from the RNA-Seq expression data was filtered, keeping 3 538 genes related to energy metabolism. The filter was created using a list of gene functional annotations based on gene ontology and pathway terms,

Table 1
Mean relative abundance (%) and SD values of fatty acid (FA) composition in the *Longissimus dorsi* muscle (LD) of the 129 BC1_DU pigs.

Phenotypes	Name	Mean	SD
Saturated FA			
SFA_LD	Total SFA[1]	39.98	3.107
C140_LD	Myristic acid	1.27	0.231
C160_LD	Palmitic acid	23.90	1.663
C180_LD	Stearic acid	14.35	1.727
Monounsaturated FA			
MUFA_LD	Total MUFA[2]	43.37	6.233
C161n7_LD	Palmitoleic acid	2.79	0.524
C181n9_LD	Oleic acid	35.83	5.800
C181n7_LD	Vaccenic acid	3.83	0.303
C201n9_LD	Gondoic acid	0.73	0.166
Polyunsaturated FA			
PUFA_LD	Total PUFA[3]	16.16	8.306
C182n6_LD	Linoleic acid	12.25	5.962
C183n3_LD	α-linolenic acid	0.40	0.131
C202n6_LD	Eicosadienoic acid	0.43	0.122
C203n6_LD	Dihomo-γ-linolenic acid	0.46	0.294
C203n3_LD	Eicosatrienoic acid	0.18	0.098
C204n6_LD	Arachidonic acid	2.62	1.983
W6W3_LD	Ratio n-6/n-3	26.60	8.159

[1] SFA: saturated fatty acid.
[2] MUFA: monounsaturated fatty acid.
[3] PUFA: polyunsaturated fatty acid.

which included the following gene ontology databases: Kyoto Encyclopedia of Genes and Genomes (**KEGG**), Reactome, WikiPathways, STRING-db, AmiGO 2, MGI, and BioSystems from NCBI. This list was manually curated by our research group, as further explained in Valdés-Hernández et al. (2023). The list with the pathways and the source can be found in Supplementary Table S1. The counts were corrected by sex and batch, normalised using the

counts per million calculation and log-transformed with the edgeR package v3.38.4 (Robinson et al., 2010). A complete list of the lipid-related genes can be found in Supplementary Table S2. To identify co-expressed and highly interconnected genes associated with the intramuscular profile of 17 FAs (Table 1), the WGCNA package v.1.72–1 (Langfelder and Horvath, 2008) was used in R as follows:

(1) The network construction as well as the identification of the modules of co-expressed genes was done on gene expression data. The soft-thresholding power as a function of the scale-free topology index was defined at seven, which represent a power-law model of $R^2 = 0.88$.

(2) Pearson's correlation between the module eigengene and the FA phenotype information was estimated. The eigengene is defined as the first principal component of a given module and can be considered a representative of the gene expression profiles in a module (Langfelder and Horvath, 2008). A module was chosen for downstream analysis if it presented module-trait relationship $\geq |0.1|$ and *P*-value ≤ 0.05 in at least six of the analysed traits.

Gene function classification and pathway enrichment analyses were performed using the ClueGO Cytoscape plug-in (Bindea et al., 2009). The cut-off for considering a significant over-representation was established with a *P*-value ≤ 0.05 after Benjamini and Hochberg multiple-test correction (Benjamini and Hochberg, 1995).

Network inference using graphical Gaussian model

Within each module of genes, we inferred a network of "direct" regulation between genes (i.e., conditional dependencies) using the
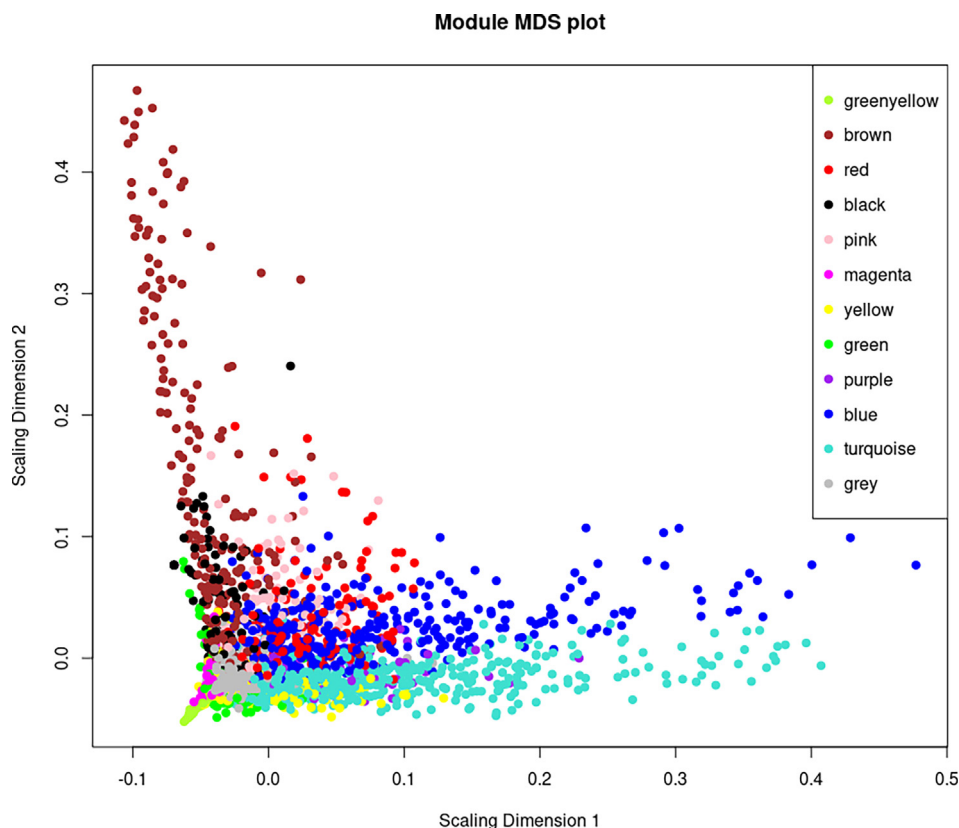

Fig. 1. MDS (Multidimensional Scaling) plot of the distribution of the expressed genes in the *Longissimus dorsi* muscle of the 129 BC1_DU pigs. The colours represent the modules assigned for each gene.

graphical Gaussian model in high dimension (Friedman et al., 2008). Analyses were implemented in R by using the glasso (Friedman et al., 2008) and huge (Zhao et al., 2012) packages.

For consistent estimation of the network, it was inferred 30 times on 30 subsets from the original dataset (sampling with replacement). Based on the Bolasso variable selection algorithm (Bach, 2008), only edges appearing all the times in the inferred networks were kept in the final model. Inferred networks were subsequently uploaded to Cytoscape program for visualisation.

Results

Co-expressed gene modules and correlation with fatty acid traits

The co-variation between gene-expression and FA phenotypic information was estimated by using the WGCNA approach. First, genes were clustered according to their connectivity, resulting in 12 modules. Supplementary Table S3 provides the complete list of genes with their module membership. The WGCNA procedure assigned a specific colour to each gene-module that will henceforth be used to refer to each module, as seen in the summary plot of Fig. 1, which also shows the colour assigned to each significant module. Fig. 2 pictures the correlation coefficient between the eigengene values of these modules and the 17 FA-related phenotypes. From the 12 modules, we only kept for further analyses the ones that had six or more significant correlations (P-value $\leq$ 0.05) of their eigengene values with the phenotypes, which resulted in four relevant modules (Magenta, Yellow, Green and Brown), as shown in Fig. 2. SFA and MUFA followed similar relationships with the module eigengene values, while PUFA showed opposite correlation patterns (Fig. 2). This is an expected result taking into consideration the negative phenotypic correlations between the different FA. As Fig. 3 shows, SFA and MUFA have positive correlations between them and negative with PUFA.

The eigengene values of Magenta and Yellow modules showed a similar correlation trend, which was opposite to the patterns observed in the Green and Brown modules. Magenta and Yellow modules included 57 and 221 genes, respectively, which resulted to be positively correlated with palmitoleic (C16:1n-7), oleic (C18:1n-9) and the sum of the MUFA in Magenta module, and with stearic acid (C18:0) in Yellow module, but both modules were negatively correlated with linoleic acid (C18:2n-6), arachidonic (C20:4n-6), the ratio n-6/n-3 and the sum of the PUFA. The Magenta module was also negatively correlated with other PUFA such as α-linolenic acid (C18:3n-3), eicosadienoic acid (C20:2n-6), eicosatrienoic acid (C20:3n-3) and dihomo-γ-linolenic acid (C20:3n-6). Meanwhile, the Green and Brown modules included 174 and 234 co-expressed genes, respectively; both of them following an opposite relationship than Magenta and Yellow modules, being positively correlated with C18:2n-6, C20:4n-6, C20:3n-6, C20:2n-6, ratio n-6/n-3 or PUFA, but negatively correlated with C14:0, C18:1n-9 and MUFA.

Biological processes and pathways enriched within gene modules

The gene-enrichment analysis indicated that the genes gathered in the four modules strongly associated with FA are involved in a wide variety of physiological and biological events (Supplementary Table S4). Among significantly enriched pathways, it is worth highlighting the pathways listed in Table 2.

To be noted, module gene-enrichment analysis agreed with the observed module and FA correlation patterns. As previously commented, modules Magenta and Yellow were positively correlated with MUFA and SFA, showing an over-representation of pathways related to Biosynthesis of unsaturated fatty acids (KEGG:01040),

Peroxisome Proliferator-Activated Receptor (**PPAR**) signalling pathway (KEGG:03320) and Fatty acid elongation (KEGG:00062). Meanwhile, genes belonging to Green and the Brown modules

Module-trait relationships

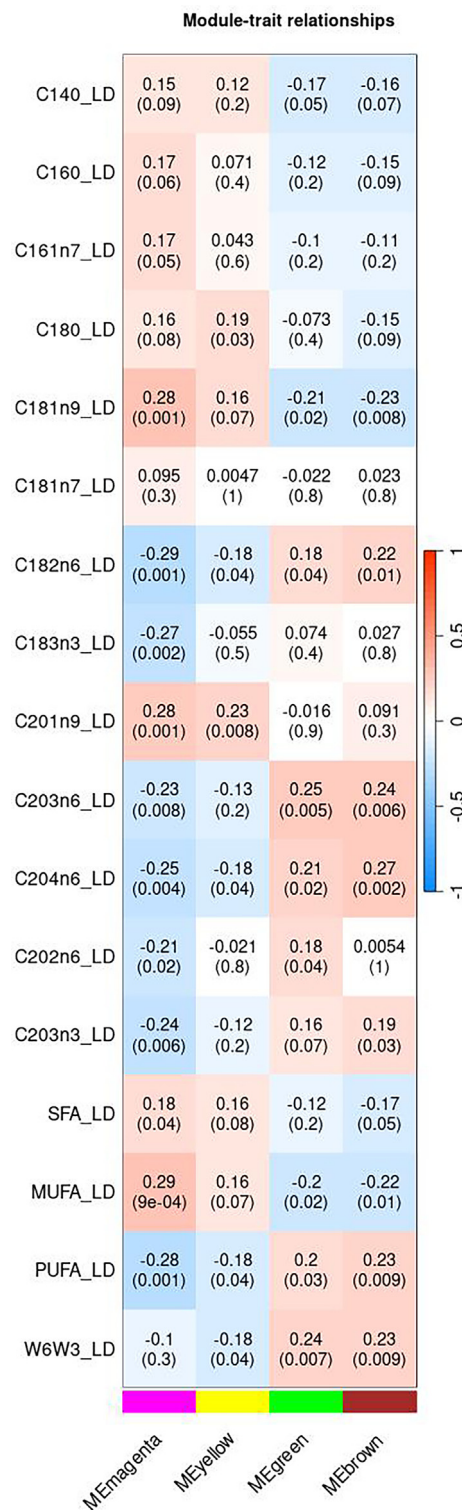|	MEmagenta	MEyellow	MEgreen	MEbrown
C140_LD	0.15 (0.09)	0.12 (0.2)	-0.17 (0.05)	-0.16 (0.07)
C160_LD	0.17 (0.06)	0.071 (0.4)	-0.12 (0.2)	-0.15 (0.09)
C161n7_LD	0.17 (0.05)	0.043 (0.6)	-0.1 (0.2)	-0.11 (0.2)
C180_LD	0.16 (0.08)	0.19 (0.03)	-0.073 (0.4)	-0.15 (0.09)
C181n9_LD	0.28 (0.001)	0.16 (0.07)	-0.21 (0.02)	-0.23 (0.008)
C181n7_LD	0.095 (0.3)	0.0047 (1)	-0.022 (0.8)	0.023 (0.8)
C182n6_LD	-0.29 (0.001)	-0.18 (0.04)	0.18 (0.04)	0.22 (0.01)
C183n3_LD	-0.27 (0.002)	-0.055 (0.5)	0.074 (0.4)	0.027 (0.8)
C201n9_LD	0.28 (0.001)	0.23 (0.008)	-0.016 (0.9)	0.091 (0.3)
C203n6_LD	-0.23 (0.008)	-0.13 (0.2)	0.25 (0.005)	0.24 (0.006)
C204n6_LD	-0.25 (0.004)	-0.18 (0.04)	0.21 (0.02)	0.27 (0.002)
C202n6_LD	-0.21 (0.02)	-0.021 (0.8)	0.18 (0.04)	0.0054 (1)
C203n3_LD	-0.24 (0.006)	-0.12 (0.2)	0.16 (0.07)	0.19 (0.03)
SFA_LD	0.18 (0.04)	0.16 (0.08)	-0.12 (0.2)	-0.17 (0.05)
MUFA_LD	0.29 (9e-04)	0.16 (0.07)	-0.2 (0.02)	-0.22 (0.01)
PUFA_LD	-0.28 (0.001)	-0.18 (0.04)	0.2 (0.03)	0.23 (0.009)
W6W3_LD	-0.1 (0.3)	-0.18 (0.04)	0.24 (0.007)	0.23 (0.009)

Fig. 2. Relationships between the four significant modules identified in WGCNA and the FA phenotypes in the *Longissimus dorsi* muscle of the BC1_DU pigs. Red colour points out the positive correlations, while blue represents the negative ones. The *P*-value of the correlation is shown between parentheses. LD=*Longissimus dorsi*; FA=fatty acid; SFA=saturated fatty acid; MUFA=monounsaturated fatty acid; PUFA=polyunsaturated fatty acid; W6W3 = omega-6/omega-3 ratio; WGCNA=Weighted Gene Co-expression Network Analysis; ME=Module of Expression.
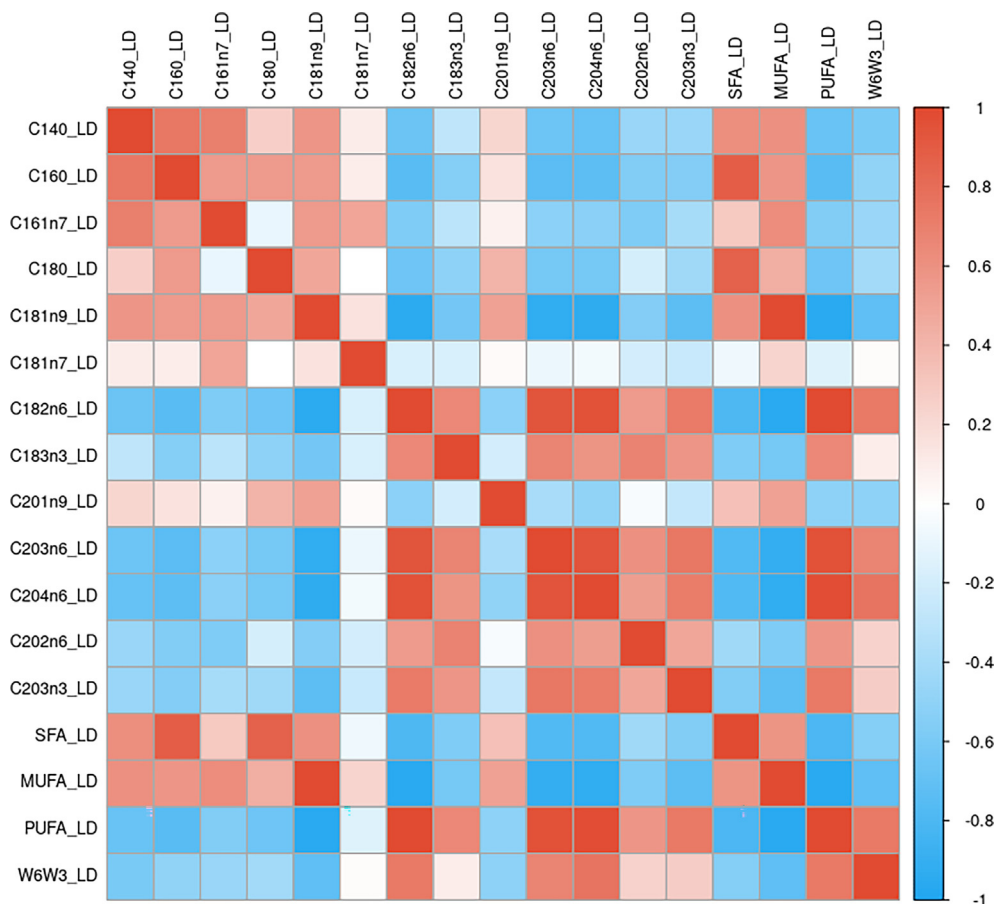
Fig. 3. Phenotypic correlations between the analysed FAs in *Longissimus dorsi* muscle of the 129 BC1_DU pigs. LD=*Longissimus dorsi*; FA=fatty acid; SFA=saturated fatty acid; MUFA=monounsaturated fatty acid; PUFA=polyunsaturated fatty acid; W6W3 = omega-6/omega-3 ratio.

were negatively correlated with those FAs, but positively linked to PUFA, showing over-representation of pathways associated with Fatty acid (KEGG:00071) and amino acid degradation (KEGG:00280; KEGG:00380; KEGG:00620; KEGG:00310) or Oxidative phosphorylation (KEGG:00190).

Network inference in each module

For each of the four WGCNA modules associated with FA composition, we performed network inference by using the graphical Gaussian model. Fig. 4 shows the connections between the most connected genes and their neighbours from the Magenta module; the remaining networks of the significant modules (Yellow, Green and Brown) are available in Supplementary Figure S1. The Magenta network had 52 nodes with 87 edges. The most connected gene was *ADIPOQ* with 10 neighbours; *TRARG1* and *ELOVL6* with eight neighbours; *FABP4* with seven neighbours, and *GYS2, PLIN1, CIDEC,* and *AK4* with six. The Yellow network inferred using the graphical Gaussian model had 196 nodes with 263 edges. The most connected genes were *FN1* and *MAP3K11* with eight neighbours, and *PLAU, CSF1R* and *EHD2* with seven neighbours. The Green network had 161 nodes with 351 edges. The most connected gene was *ATG13* with 11 neighbours. *MEF2C, GOT1, IRS2, NCEH1* and *MAP2K3* had nine neighbours, and *PBX1*, ENSSSCG00000002433 (*PSMC1*), *ILVBL, GPAT4* and *EIF4A1* had eight neighbours. The Brown network had 224 and 655 edges. The most connected gene was ENSSSCG00000034019 (*ATP5MF*) with 16 neighbours, whereas *COX6A2, ATP5F1C, ATP5PB, COX7B* and ENSSSCG00000002907 (*COX6B*) had 13 neighbours.

Discussion

Co-expression networks enable the clustering of different genes that have similar behaviours. This is especially useful when working with a high number of data such as tables of expressed genes analysed in a whole-transcriptome approach, but it is still often needed to define subsets of genes of particular interest. In our study, these groups were defined as modules of co-expressed genes in muscle that showed correlations with FA composition. Among the four significant modules identified, Magenta and Yellow modules showed positive correlations with SFA and MUFA, while Green and Brown modules had positive correlations with PUFA and its omega-6/omega-3 ratio, which is in agreement with previous results of our research group (Valdés-Hernández et al., 2023). Out of the four modules, the Magenta module clustered the least number of co-expressed genes, while the Brown module had the highest number of co-expressed genes. The most connected genes in the Magenta module were related to the insulin and FA biosynthesis pathways and also aligned with the significant phenotypic correlations found in this module, which showed positive results with three of the MUFAs and their sum. On the other hand, the most connected genes of the Brown module were related to the mitochondrial FA oxidation. In this case, the phenotypic correlations showed positive results with four of the PUFAs, their sum, and the omega-6/omega-3 ratio.

In the following sections, we provide an in-depth analysis of the interaction network defining the gene-gene connections within each of the four modules.

Table 2

Over-represented pathways for each significant module of co-expressed genes in the *Longissimus dorsi* muscle of the 129 BC1_DU pigs. Only the pathways with a corrected (by Benjamini and Hochberg) *P*-value of ≤ 0.05 are considered.

Over-represented pathways[1]	Corrected *P*-value
Magenta module	
Biosynthesis of unsaturated fatty acids (KEGG[2]:01040)	0.0013
PPAR[3] signalling pathway (KEGG:03320)	0.0013
Fatty acid elongation (KEGG:00062)	0.0076
Cholesterol metabolism (KEGG:04979)	0.0294
Yellow module	
Chemokine signalling pathway (KEGG:04062)	0.0000
Platelet activation (KEGG:04611)	0.0000
Glutamatergic synapse (KEGG:04724)	0.0009
Calcium signalling pathway (KEGG:04020)	0.0013
Phospholipase D signalling pathway (KEGG:04072)	0.0037
PI3K-Akt signalling pathway (KEGG:04151)	0.0041
Regulation of lipolysis in adipocytes (KEGG:04923)	0.0298
Green module	
Adipocytokine signalling pathway (KEGG:04920)	0.0225
Brown module	
Oxidative phosphorylation (KEGG:00190)	0.0000
Citrate cycle (TCA cycle) (KEGG:00020)	0.0000
Fatty acid degradation (KEGG:00071)	0.0001
Amino acid degradation	
Valine, leucine and isoleucine degradation (KEGG:00280)	0.0004
Tryptophan metabolism (KEGG:00380)	0.0004
Pyruvate metabolism (KEGG:00620)	0.0044
Lysine degradation (KEGG:00310)	0.0058

[1] Numbers between parentheses are the KEGG entry for the pathway.
[2] KEGG: Kyoto Encyclopedia of Genes and Genomes.
[3] PPAR: Peroxisome Proliferator-Activated Receptor.

Insulin pathway and fatty acid biosynthesis (Magenta module)

In the Magenta module, we found *ADIPOQ* (adiponectin) as the most connected gene. *ADIPOQ* is an adipokine involved in meat quality, and this module was found positively correlated with the palmitoleic acid (C16:1n-7). Besides, the palmitoleic acid has been described as a lipokine and has been related with metabolism homeostasis (Frigolet and Gutiérrez-Aguilar, 2017). In addition to being mainly synthesised by the adipocytes, *ADIPOQ* is involved in FA oxidation and inhibits lipogenesis, but it also regulates insulin sensitivity (Kadowaki and Yamauchi, 2005) and has been associated with average daily weight gain in pigs (Dall'Olio et al., 2009). The insulin sensitivity regulation is thought to be performed also by TRARG1 (trafficking regulator of *GLUT4-1*) (Duan et al., 2018), which was found in this module with a positive correlation with *ADIPOQ*. *ADIPOQ* is associated with one of the enriched pathways of this module, the PPAR (peroxisome proliferator-activated receptor) signalling pathway, which has associated with other highly connected genes such as *GYS2* (glycogen synthase 2) and *PPARG* (peroxisome proliferator−activated receptor gamma). *GYS2* plays a role in obesity through abnormal carbohydrate and lipid metabolism (Morton et al., 2011). PPARs are regulators of the lipid metabolism gene expression (Varga et al., 2011), and *PPARG* can be found, in addition to adipose cells, in other tissues such as breast and colon (Walczak and Tontonoz, 2002). The *PPARG* gene is a key regulator of adipogenesis (Tontonoz et al., 1995), but also plays a role in insulin sensitivity and glucose homeostasis (Walczak and Tontonoz, 2002). It interacts with other of the highly neighbouring genes of this module, such as *FABP4* (fatty acid binding protein 4), whose encoded protein promotes insulin resistance (Garin-Shkolnik et al., 2014), and *CIDEC* (cell death inducing DFFA−like effector c), which has been found directly activated by *PPARG* in liver (Matsusue et al., 2008) and up-regulated by *PPARG2* in bovine adipose tissue (Zhou et al., 2018).

PLIN1 (perilipin 1), which belongs to the perilipin family, is found in the lipid droplets of the adipocytes and is involved in intramuscular fat deposition (Gol et al., 2016; Kimmel et al., 2010). Our research group found in a previous work (Valdés-Hernández et al., 2023) that this gene is positively correlated to palmitoleic acid (C16:1n-9), oleic acid (C18:1n-9) and total MUFA content, and negatively correlated to the PUFAs eicosadienoic acid (C20:2n-6) and α-linolenic acid (C18:3n-3). In addition, other studies found that *PLIN1* interacts with *CIDEC* to promote the formation of larger lipid droplets (Li et al., 2018; Moreno-Navarrete et al., 2014), which is in agreement with the connections found in our analysis (Fig. 4).

Among the other highly connected genes in this module, *ELOVL6* (elongation of long-chain fatty acids family number 6) has also an important role in insulin sensitivity, and it acts in the elongation of saturated and monounsaturated long-chain FAs (Matsuzaka and Shimano, 2009). This enzyme is involved in the elongation of FAs, e.g., from myristic (C14:0) to palmitic acid (C16:0) or from palmitic to stearic acid (C18:0). Although another gene found in this module, *SCD* (stearoyl-CoA desaturase), did not have a direct connection in the network analysis with *ELOVL6*, both participate in FA metabolism. *SCD* converts palmitic to palmitoleic acid (C16:1n-7) and stearic acid to oleic acid (C18:1n-9), being both of them MUFA, which were positively correlated with this module. Genetic polymorphisms in *ELOVL6* have been previously reported by our research group (Corominas et al., 2013, 2015; Crespo-Piazuelo et al., 2020) as associated with the abundance of myristic, palmitic and palmitoleic acids (C14:0, C16:0 and C16:1n-7). In a previous study, we also found this elongase positively associated with MUFA content (Valdés-Hernández et al., 2023). In addition, the analysis suggested (corrected *P*-value < 0.1) a positive correlation between the Magenta module and the saturated FA: myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids (Fig. 2). *ELOVL6* is also related to *PPARA*, which, interestingly, has been found in the Green module. A deficiency in *ELOVL6* implies a reduction of *PPARA* and a suppression of FA synthesis and degradation, and increases insulin sensitivity (Matsuzaka and Shimano, 2009).

Fatty acid synthesis genes (Yellow module)

Even though the Yellow module showed many enriched pathways related with cancer and other diseases, there were two significant pathways related with lipid metabolism: PI3K-Akt signalling pathway and the regulation of lipolysis in adipocytes. The PI3K-Akt axis plays a central role in the insulin pathway and acts in liver increasing gluconeogenesis and glycogen synthesis, but also lipid synthesis (Khorami et al., 2015; Petersen and Shulman, 2018), which agrees with the positive correlations found with the SFA and MUFA. Interestingly, one of the most connected genes of this module, *FN1* (fibronectin 1), an activator of the PI3K-Akt signalling pathway (Ji et al., 2020), was found enriched in this pathway. This pathway also had *PIK3CG* as an associated gene, which is one of the four genes that constitute the catalytic subunit of the class I PI3K and works in a wide variety of processes (Engelman et al., 2006). Other enriched genes found, such as PDGFRA and PDGFRB, were positive regulators of the PI3K-Akt signalling pathway (Cunningham et al., 2022).

The other enriched pathway related with lipid metabolism is the one that regulates lipolysis in adipocytes, which breaks the stored triacylglycerol into glycerol and FAs (Duncan et al., 2007). Three genes of the same family of adenylate cyclases (*ADCY3*, *ADCY4* and *ADCY5*), which regulate lipolysis in adipocytes (Kuo et al., 2018), were enriched in this pathway. *MGLL*, another gene enriched in this pathway, is involved in the final step of lipolysis, as it breaks monoglycerides into glycerol and FAs (Fredrikson et al., 1986).
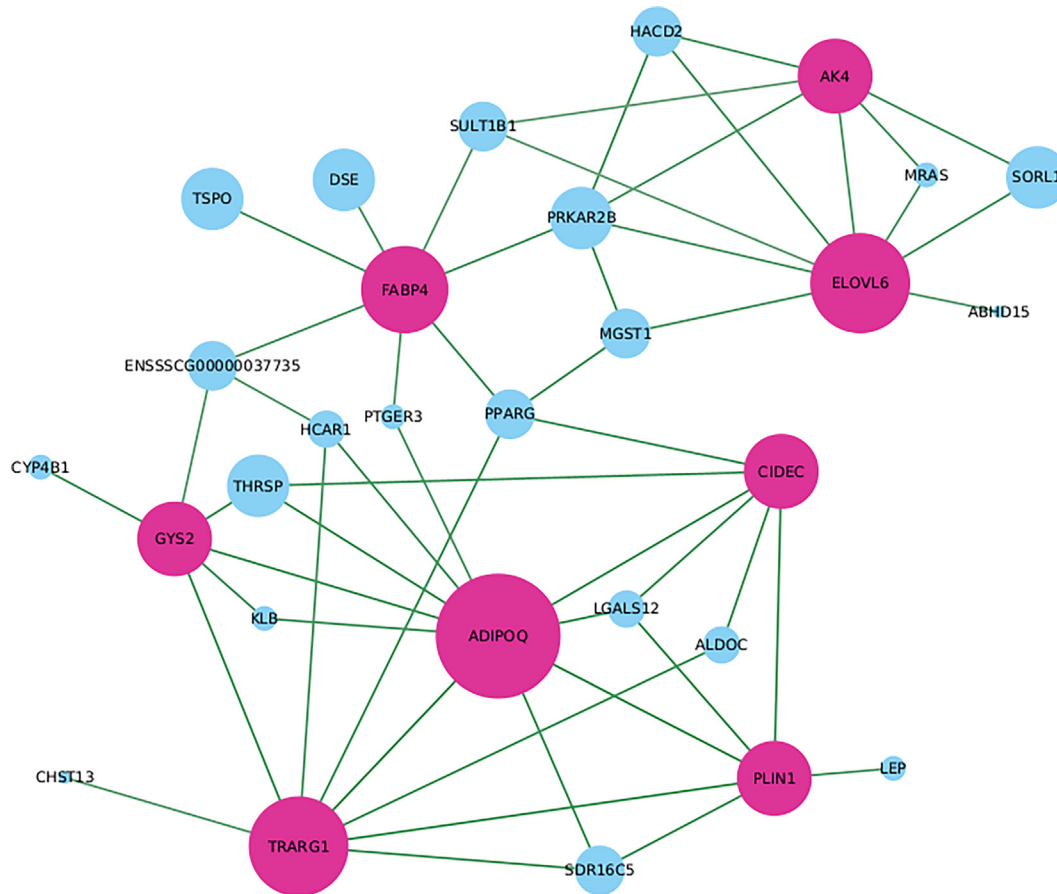
Fig. 4. Magenta module gene network of the most connected genes expressed in the *Longissimus dorsi* muscle of the 129 BC1_DU pigs (genes with six or more neighbours are highlighted in magenta colour and, for visualisation purposes, genes without direct connection with these most connected items are hidden). The size of the node is based on the number of direct neighbours of the gene. The edges are coloured based on the correlation between the genes, green for positive and red for negative. In this network, all the correlations are positive.

Another of the most connected genes, *EHD2* (EH domain-containing 2), can be linked to obesity, as it controls the pathway related with caveolate-dependent FA uptake (Matthaeus et al., 2020). Its over-expression suppresses the activity of *PPARG*, a gene that was enriched in the previous module, and its silencing affects insulin sensitivity, lipid storage capacity and lipolysis (Matthaeus et al., 2020; Morén et al., 2019). *EHD2* and *caveoline-1* are up-regulated during adipocyte differentiation, while *caveoline-1* also regulates the adenylate cyclase pathway (Kuo et al., 2018; Morén et al., 2019).

Adipocytokine signalling (Green module)

The Green module only had one significantly enriched pathway, the adipocytokine signalling pathway, which plays a central role in metabolic homeostasis and is an important component in obesity (Cao, 2014). The gene with the most neighbouring genes that was associated with the adipocytokine signalling pathway was *IRS2* (insulin receptor substrate 2). *IRS2* is involved in insulin signalling and may be related with human life span and nutrient homeostasis (Taguchi et al., 2007). *PPARA* and *PPARGC1A* (peroxisome proliferator-activated receptor gamma-coactivator 1A) genes were also associated with this pathway. As previously explained for the Magenta module results, *PPARA* interacts with *ELOVL6*. PPARA is a nuclear receptor related to FA oxidation, and its decrease can promote obesity (Matsuzaka and Shimano, 2009). *PPARGC1A* is involved in lipid and energy metabolism, and it has been found as a candidate gene for pig meat quality traits (Gandolfi et al., 2011).

Regarding the highly neighbouring genes, some were also found in previous works of our research group, such as *GOT1*, *ILVBL* or *ACSL1*. *GOT1* (glutamate oxaloacetate transaminase 1) is involved in different catalytic processes and also in FA homeostasis (Cunningham et al., 2022), while *ILVBL* (IlvB acetotlactate synthase like) is involved in the α-oxidation of FAs (Kitamura et al., 2017). *ACSL1* (acyl-CoA synthetase long–chain family number 1) plays a key role in lipid metabolism, as seen for humans and cattle (Brasaemle et al., 2004; Zhao et al., 2020), but also for different pig breeds (Li et al., 2012) and it may promote lipogenic-related genes such as *FABP4*, *APOE* and *FASN* (Shan et al., 2010). These three genes were found in the Magenta and Yellow modules, which have enriched pathways related with FA biosynthesis.

Mitochondrial fatty acid oxidation (Brown module)

The FA degradation pathway was found enriched in the Brown module. One of the highly neighbouring genes found was related with this pathway, *ACADVL* (acyl-CoA dehydrogenase very long chain). *ACADVL* is a target gene of *PPARA* and is involved in mitochondrial FA β-oxidation, and has already been found differentially expressed in the muscle of pigs with extreme FA composition (Puig-Oliveras et al., 2014). Another gene related to this pathway is *HADH* (hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex), whose subunits alpha and beta, are also involved in FA β-oxidation.

The FA degradation pathway produces metabolites that can be used in other enriched pathways of this module related with the mitochondria, such as the citrate pathway (Raimundo et al., 2011;

Scheffler, 2007). The most neighbouring genes of this module were composed of three different ATP synthase subunits (*ATP5MF*, *ATP5F1C* and *ATP5PB*) and three cytochrome c oxidases (*COX6A2*, *COX6B* and *COX7B*). ATP synthase and the COX proteins are related to mitochondrial energy metabolism. The ATP synthase is part of an enzyme complex that stimulates the production of ATP more efficiently (Jonckheere et al., 2012). *COX6A2*, which can be found in striated muscle (Taanaman et al., 1993), is a regulator of respiratory uncoupling in muscle, and it is involved in obesity and insulin resistance (Quintens et al., 2013). *COX6B* is involved in mitochondrial respiration (Lazarou et al., 2009), whereas *COX7B* plays a role in apoptosis (Indrieri et al., 2012). The genes of other ATP synthase subunits were highly neighbouring genes, and they were also found in previous studies of our research group (Valdés-Hernández et al., 2023). However, other highly neighbouring genes, such as some genes from the NADH:ubiquinone reductases family (*NDUFAF7*, *NDUFS1*, and *NDUFS2*), were newly reported.

Another member of this family, NDUFB8 (NADH:ubiquinone reductase subunit B8), is also another of the top neighbouring genes. *NDUFB8* is a subunit of the mammalian mitochondrial complex I, and it is involved in the stability and activity of the mitochondrial complex (Arumugam and Manickam, 2022). *GOT1* and *SCD* were located close to the *NDUFB8* gene. *GOT1* was one of the top neighbouring genes in the Green module, while *SCD* was found in the Magenta module. Furthermore, *ELOVL3* (elongation of long-chain fatty acids family number 3) and *HIF1AN* (hypoxia−inducible factor 1 subunit alpha inhibitor) were also found within this SSC14 region. *ELOVL3* is involved in SFA elongation, whereas *HIF1AN* is related to the PI3K signalling pathway, which was enriched in the Yellow module (Puig-Oliveras et al., 2016).

Conclusions

Here, we present an analysis of related gene regulatory networks in the porcine muscle that are potentially related to FA composition in the pig meat. We found four modules that contain relevant muscle gene networks linked with FA composition of pig meat, providing further insights on how the physiology of the pigs influences this relevant phenotype for both meat quality and human health.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101259.

Ethics approval

Animal handling and sample collection were performed based on the regulations from the Spanish Policy for Animal Protection RD1201/05, in accordance with the European Union Directive 86/609 about the protection of animals used in experimentation. In addition, this study was carried out meeting the relevant guidelines and regulations of the animal care and use committee of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA). Therefore, the protocol was approved by the Ethical Committee of the IRTA. These regulations adopt the European Code of Conduct for Research Integrity and Good Experimental Practices. In addition, the study is also reported in full compliance with ARRIVE guidelines (https://arriveguidelines.org/).

Data and model availability statement

All data used in this study are present in the paper or its Supplementary Material. The RNA sequencing data used for these analyses are available in Sequence Read Archive (SRA) from the NCBI BioProject, under the accession number PRJNA882638 (https://www.ncbi.nlm.nih.gov/sra/).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

The authors declare no competing interests.

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