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Identification of genomic regions, genetic variants and gene networks regulating candidate genes for lipid metabolism in pig muscle

M. Passols [a,*], F. Llobet-Cabau [a,b], C. Sebastià [a,b], A. Castelló [a,b], J. Valdés-Hernández [a,b], L. Criado-Mesas [a], A. Sánchez [a,b], J.M. Folch [a,b]

[a] Plant and Animal Genomics, Centre for Research in Agrigenomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, Bellaterra, España
[b] Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona (UAB), Bellaterra, España

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ABSTRACT

The intramuscular fat content and fatty acid composition of porcine meat have a significant impact on its quality and nutritional value. This research aimed to investigate the expression of 45 genes involved in lipid metabolism in the *longissimus dorsi* muscle of three experimental pig backcrosses, with a 25% of Iberian background. To achieve this objective, we conducted an expression Genome-Wide Association Study (**eGWAS**) using gene expression levels in muscle measured by high-throughput real-time qPCR for 45 target genes and genotypes from the PorcineSNP60 *BeadChip* or Axiom Porcine Genotyping Array and 65 single nucleotide polymorphisms (**SNPs**) located in 20 genes genotyped by a custom-designed *Taqman* OpenArray in a cohort of 354 animals. The eGWAS analysis identified 301 eSNPs associated with 18 candidate genes (*ANK2, APOE, ARNT, CIITA, CPT1A, EGF, ELOVL6, ELOVL7, FADS3, FASN, GPAT3, NR1D2, NR1H2, PLIN1, PPAP2A, RORA, RXRA* and *UCP3*). Three *cis*-eQTL (expression quantitative trait *loci*) were identified for *GPAT3*, *RXRA*, and *UCP3* genes, which indicates that a genetic polymorphism proximal to the same gene is affecting its expression. Furthermore, 24 *trans*-eQTLs were detected, and eight candidate regulatory genes were located in these genomic regions. Additionally, two *trans*-regulatory hotspots in *Sus scrofa* chromosomes 13 and 15 were identified. Moreover, a co-expression analysis performed on 89 candidate genes and the fatty acid composition revealed the regulatory role of four genes (*FABP5*, *PPARG*, *SCD*, and *SREBF1*). These genes modulate the levels of α-linolenic, arachidonic, and oleic acids, as well as regulating the expression of other candidate genes associated with lipid metabolism. The findings of this study offer novel insights into the functional regulatory mechanism of genes involved in lipid metabolism, thereby enhancing our understanding of this complex biological process.

Implications

The relationships between the genes that regulate lipid metabolism and the fatty acids are complex. Through an expression genome wide-association study and co-expression analysis, we identified 27 expression quantitative trait loci affecting the gene expression of the 18 candidate genes for lipid metabolism and their relationship with fatty acid composition in *longissimus dorsi* muscle. This research delved into the genetic factors regulating the genes associated with lipid metabolism in pig skeletal muscle. Deepening the knowledge in this field is key to have a better understanding of how fatty acids are produced and, therefore, how can they be modulated.

Introduction

The percentage of intramuscular fat (**IMF**) and its fatty acid (**FA**) composition are recognised as key factors that influence meat quality and play a significant role in determining the nutritional value of meat (Wood et al., 2008). Moreover, IMF is directly related to meat flavour, juiciness, tenderness and firmness, which are relevant traits for consumers. Hence, in accordance with consumers, a high quantity of backfat has less acceptance, although meat with high IMF is considered a desirable trait (Schwab et al., 2006; Wood et al., 2008). Some FAs are essential for humans, such as omega-3 (**ω-3**) and omega-6 (**ω-6**) polyunsaturated FAs (**PUFAs**), as they are not produced by *de novo* biosynthesis in the organism and must be provided through the diet. Therefore, the FA profile of pork is a key factor determining its quality and overall healthiness (Simopoulos, 2002) can also affect meat nutritional values and its sensory quality parameters (Chernukha et al., 2023). How-

* Corresponding author.
E-mail address: magi.passols@cragenomica.es (M. Passols).

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ever, excessive fat can result in a less desirable texture and taste. To ensure a high-quality product, pork producers carefully balance genetics, nutrition, and management practices to optimise the level of IMF in the meat. With proper techniques, consumers can appreciate pork that is both flavourful and healthy, as the right amount of IMF can provide essential nutrients such as ω-3 and ω-6 FAs (Chernukha et al., 2023). Commercial pig breeds, such as Pietrain and Landrace, exhibit superior efficiency in growth and leaner carcasses compared to Duroc or Iberian pigs. However, these carcasses have less IMF, which affects its meat quality. Conversely, meat from Iberian breed is characterised by high IMF deposition and higher mono-unsaturated FA (**MUFA**) percentage, which provide more oxidative stability and improve meat taste and colour. Furthermore, this breed is widely used for dry-cured products, such as loin and ham (Lopez-Bote, 1998). It is a common practice to cross the Iberian pig with Duroc breed to enhance growth and feed efficiency.

Three different backcrosses, between Iberian × Duroc (**BC1_DU**), Iberian × Landrace (**BC1_LD**), and Iberian × Pietrain (**BC1_PI**) pigs were generated (Martínez-Montes et al., 2018) producing animals with large phenotypic differences in growth, carcass and meat quality traits, such as the IMF content and FA composition. Different studies based on these animals used several analytical techniques such as quantitative trait loci (**QTL**) mapping and genome-wide association studies (**GWASs**) to identify genes associated with growth, IMF content, and FA composition (Crespo-Piazuelo et al., 2020; Puig-Oliveras et al., 2014a and 2014b; Ramayo-Caldas et al., 2012).

In previous studies of our group, the expression of candidate genes for lipid metabolism was analysed in *longissimus dorsi* muscle, adipose tissue, and liver of an Iberian × Landrace backcross (Puig-Oliveras et al., 2016; Revilla et al., 2018; Ballester et al., 2017). Moreover, Criado-Mesas et al. (2020) conducted an expression genome wide-association study (**eGWAS**) with the same list of candidate genes of Puig-Oliveras et al. (2016) but including *longissimus dorsi* muscle expression data of 355 animals of the three backcrosses. These studies were specifically centred on the expression of candidate genes implicated in FA metabolism, aiming to identify expression quantitative trait *loci* (**eQTLs**) regulating gene expression. Detecting eQTLs is a valuable strategy to study complex trait genetics, revealing genetic variants linked to gene transcription levels that may contribute to phenotypic variation.

The main goal of this research is to study the expression and regulation of a selected set of 45 candidate genes for lipid metabolism in the porcine *longissimus dorsi* muscle in a total of 354 animals belonging to three different backgrounds to identify potential variants that may explain the expression differences in the analysed genes. Additionally, we aimed to investigate the interrelationship between the 45 candidate genes for lipid metabolism utilised in this study and the 44 lipid metabolism candidate genes employed in the study conducted by Criado-Mesas et al. (2020) and the FA composition derived from the same tissue and population.

Material and methods

Pig population

The IBMAP population was obtained by crossing Iberian boars with Duroc, Landrace and Pietrain sows, and then, F1 boars were crossed again with the respective Duroc, Landrace and Pietrain sows. In the present study, 354 animals were used, of which 122 belong to the BC1_DU (25% Iberian and 75% Duroc), 114 to the BC1_LD (25% Iberian and 75% Landrace) and 118 to the BC1_PI (25% Iberian and 75% Pietrain). All animals were maintained under the same intensive conditions and fed *ad libitum* with cereal-based commercial diet on NOVA GENÈTICA S.A. experimental farm (Lleida, Spain). Detailed information on generation schemes, diet, growth, and housing condition of the three backcrosses is described in (Martínez-Montes et al., 2018). Slaughtering procedures were conducted in a certified abattoir according to the institutional and national guidelines for the Good Experimental Practices and approved by the Ethical Committee of the Institution (IRTA – Institut de Recerca i Tecnologia Agroalimentàries). The *longissimus dorsi* muscle samples were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until further RNA isolation. Diaphragm samples were collected for DNA extraction.

Selection of lipid-related metabolism candidate genes in muscle

In prior researches conducted by our group, we successfully identified strong candidate genes that influence IMF content and FA composition in the *longissimus dorsi* muscle of the IBMAP backcrosses (BC1_DU, BC1_LD and BC1_PI), through the utilisation of GWAS, RNA-Seq and co-association network methodologies (Crespo-Piazuelo et al., 2020; Puig-Oliveras et al., 2014a and 2014b; Ramayo-Caldas et al., 2012). In this study, a total of 45 genes functionally related to lipid metabolism and FA composition pathways were selected. These 45 genes have been described in the literature to play different roles, including involvement in fatty acid synthesis, transport, storage, oxidation, as well as acting as transcriptional and nuclear factors that modulate gene expression. Primers used for the analyses were designed using PrimerExpress 2.0 software (Applied Biosystems) and are detailed in Supplementary Table S1.

Genotyping

Genomic DNA was isolated from diaphragm tissue by the standard method of phenol–chloroform extraction and was quantified with a NanoDrop-2000 spectrophotometer (Thermo Scientific). Animals from BC1_LD and BC1_PI were genotyped using the PorcineSNP60 Beadchip (*Illumina* Inc.; San Diego, USA) (Ramos et al., 2009), and BC1_DU animals were genotyped using Axiom Porcine Genotyping Array (*Affymetrix*). PLINK v1.90b4.3 software (Chang et al., 2015) was used to remove markers that showed a minor allele frequency of less than 5% and single nucleotide polymorphisms (**SNPs**) with more than 5% of missing genotypes. For eGWAS analysis, we used 38,423 SNPs that were common between the two SNP-genotyping arrays and were mapped in the *Sscrofa11.1* assembly. Moreover, 65 SNPs located in positional candidate genes were genotyped in the 354 pigs using custom-designed *Taqman* OpenArray genotyping plates in a QuantStudio™ 12 K flex Real-Time PCR System (ThermoFisher Scientific) and were also included in the eGWAS analysis. Of these 65 SNPs, 11 SNPs were located within the fatty acid elongase 6 (*ELOVL6*) gene. Eight, nine and three were SNPs located on the fatty acid desaturase 1, 2 and 3 (*FADS1*, *FADS2* and *FADS3*) genes, respectively. Five of them were located in fatty acid synthase (*FASN*) gene, four in fatty acid elongase 1 (*ELOVL1*) gene, three in fatty acid elongase 7 (*ELOVL7*) gene, three in fatty acid binding protein 4 (*FABP4*) gene and three in thrombospondin 1 (*THBS1*) gene. The remaining 18 were located with one or two SNPs in the *ACACA*, *ACSL4*, *ANK2*, *CPT1A*, *GPAT3*, *LPL*, *NR1D2*, *PLIN1*, *SLC27A1*, *SREBF2* and *USF1* genes. The SNPs and their positions are detailed in Supplementary Table S2. A total of 38,488 SNPs were used for further analysis.

RNA isolation and gene expression

Total RNA extraction from *longissimus dorsi* tissue was performed with the Ribopure kit (Ambion), following the manufac-

turer's protocol. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and purity and quantification using a NanoDrop-2000 spectrophotometer (Thermo Scientific). Only the RNAs with integrity above seven were used for the analysis. One μg of RNA was converted to cDNA using *High-Capacity cDNA Reverse Transcription Kit* (Applied Biosystems) in 20 μl total volume, following the manufacturer's instructions. The cDNA samples were loaded into a *Dynamic Array* 48.48 chip in a BioMark system (Fluidigm: San Francisco, CA, USA) through an integrated fluidic circuit controller following the manufacturer's instructions.

The expression of 45 target genes was analysed. After checking their stability, *ACTB* and *TBP* were used as reference genes. Data were collected using the Fluidigm Real-Time PCR analysis software 3.0.2 (Fluidigm) and analysed using the DAG expression software 1.0.4.11 (Ballester et al., 2013) applying the relative standard curve method. Samples targeted in this study were analysed in duplicate. The normalised quantity (**NQ**) values of each sample and assay were used to compare the gene expression among animals. Data normality was checked by applying the Shapiro-Wilk test in R, and $\log_2$ transformation of the NQ value was applied when required. The sex and breed effects were also tested by using a linear model with the *lm* function in R.

Furthermore, the gene expression data of 44 candidate genes generated by Criado-Mesas et al. (2020) in the same 3BCs population were used to analyse the gene co-expression patterns and its association with FA composition.

Expression genome-wide association study

Genomic association studies between each gene expression measure and SNP genotypes (eGWAS) were performed through a linear model using GEMMA software (Zhou & Stephens, 2012).

$$\mathbf{y} = \mathbf{W}\alpha + \mathbf{x}\beta + \mathbf{u} + \varepsilon;\ \mathbf{u} \sim \mathbf{MVNn}\,(0, \lambda\tau - 1\ \mathrm{K}),\ \varepsilon \sim \mathbf{MVNn}\,(0, \tau - 1\mathrm{In}),$$

in which: **y** is the vector of phenotypes for **n** individuals; **W** is a matrix nxc of covariables (fixed effects) that includes sex (2 levels), backcross (3 levels) and slaughtering batch (9 levels); **α** is a c vector with corresponding coefficients, including the intercept; **x** is an n vector with the marker genotypes; **β** is the size of the marker effect, **u** is an n vector of random effects (additive genetic effects), **ε** is an n vector of errors. The random effects vector is assumed to follow a normal multivariate n-dimensional distribution (MVNn) where $\tau - 1$ is the variance of residual errors; λ is the quotient between the two components of variance; **K** is an nxn Kinship matrix calculated from the SNPs. The vector of errors is assumed to follow a distribution MVNn, where **In** is an nxn identity matrix.

GEMMA software calculates the *P*-value from the Wald statistical test for each SNP comparing the null hypothesis that the SNP has no effect versus the alternative hypothesis that the SNP effect is different from zero.

An eGWAS using GEMMA software was conducted between 38,488 SNPs distributed along the genome of the animals and 45 lipid-related genes in *longissimus dorsi* muscle. The False Discovery Rate (FDR) multiple testing correction method of Benjamini and Hochberg (Benjamini & Hochberg, 1995) was using the *p.adjust* function of R. For each gene, SNPs were considered significant at a threshold of FDR $\leq$ 0.05.

Identification of cis and trans expression quantitative trait loci

The *cis*-eQTL mapping window was defined from 1 Mb upstream of the start of the gene to 1 Mb downstream of the gene end, and all other regions were considered as *trans*-eQTLs. Significant SNPs separated less than 10 Mb apart were considered as belonging to the same genomic interval or eQTL. This long distance was used to ensure that SNPs located in different eQTLs were not in linkage disequilibrium, being r^2 = 0.05 the mean linkage disequilibrium between SNPs separated by 10 Mb. In this study, only eQTL intervals containing two or more SNPs were considered for further analysis.

Gene annotation

The annotation of the genes contained in the eQTLs was performed with Biomart (Smedley et al., 2015) tool from the Ensembl project (https://www.ensembl.org; release 108) using the *Sscrofa 11.1* reference assembly. Additionally, a 1 Mb extension was included at both ends of the genomic region. Functional predictions of the significant SNPs comprised in the eQTL regions were carried out using Variant Effect Predictor (VEP) (McLaren et al., 2010) and the Ensembl Genes 108 Database. With these tools, the location of eSNPs regarding a gene can be classified as outside of the gene, in untranslated regions, in intron regions or in the coding sequence.

Co-expression and functional analysis

For this analysis, we used the gene expression data of the 45 candidate genes mentioned earlier, besides to the 44 candidate genes employed by Criado-Mesas et al. (2020) measured in the 345 animals from the 3BCs. Furthermore, this analysis incorporated the relative quantification data of 14 different FAs in the *longissimus dorsi* muscle, as previously obtained by Crespo-Piazuelo et al. (2020). The FA composition values used in this analysis are shown in Supplementary Table S3.

Weighted gene expression networks were calculated using the PCIT algorithm (Watson-Haigh et al., 2009), which employ first-order partial correlation coefficients and an information theory approach to detect primary gene interactions. Only significant interactions between genes were considered for further analysis. Networks were represented with the Cytoscape v3.9.1 (Shannon et al., 2003) program.

Gene functional classification

The ShinyGO v0.77 (Ge et al., 2020) program was used to identify the main biological functions and the gene ontology association from the most important pathways of the genes mapped within the eQTLs. Moreover, STRING v11.5 (Jensen et al., 2009) was used to perform the functional enrichment analysis of genes found significantly associated in the eGWAS analysis, and also to integrate and cluster the genes regarding their Gene Ontology.

Results

Sex and genetic background effect on gene expression

In this study, including the three backcrosses (**3BCs**), 24 out of the 45 genes presented significant sex effect (*P*-value $\leq$ 0.05) on pig muscle gene expression: *ADIPOQ, ADIPOR1, ADIPOR2, AGPAT2, ANK2, APOE, ARNT, CD36, CYP2U1, EGF, ELOVL5, ELOVL6, ESRRA, FADS2, FASN, GPAT3, HADH, LPL, ME1, NR1D2, NR1H2, PDK4, PLIN1,* and *USF1* (Fig. 1). We have identified more genes over-expressed in females (20), than in males (4).

Furthermore, a significant backcross effect (*P*-value < 0.05) on gene expression levels was detected in 35 out of 45 analysed genes: *ACACA, ACSL4, ADIPOQ, ADIPOR1, ADIPOR2, AGPAT2, ANK2, APOE, ARNT, CD36, CPT1A, EGF, ELOVL1, ELOVL5, ELOVL6, ELOVL7, ESRRA, ETFDH, FADS1, FADS2, FADS3, FASN, GPAT3, HADH, HADHA, ME1, NR1H2, NRF1, PDK4, PLIN1, PNPLA2, RORA, RXRA, SLC27A1, SREBF2* (Fig. 2). Overall, 24, 7 and 4 genes were over-expressed in BC1_DU, BC1_LD and BC1_PI backcrosses, respectively.

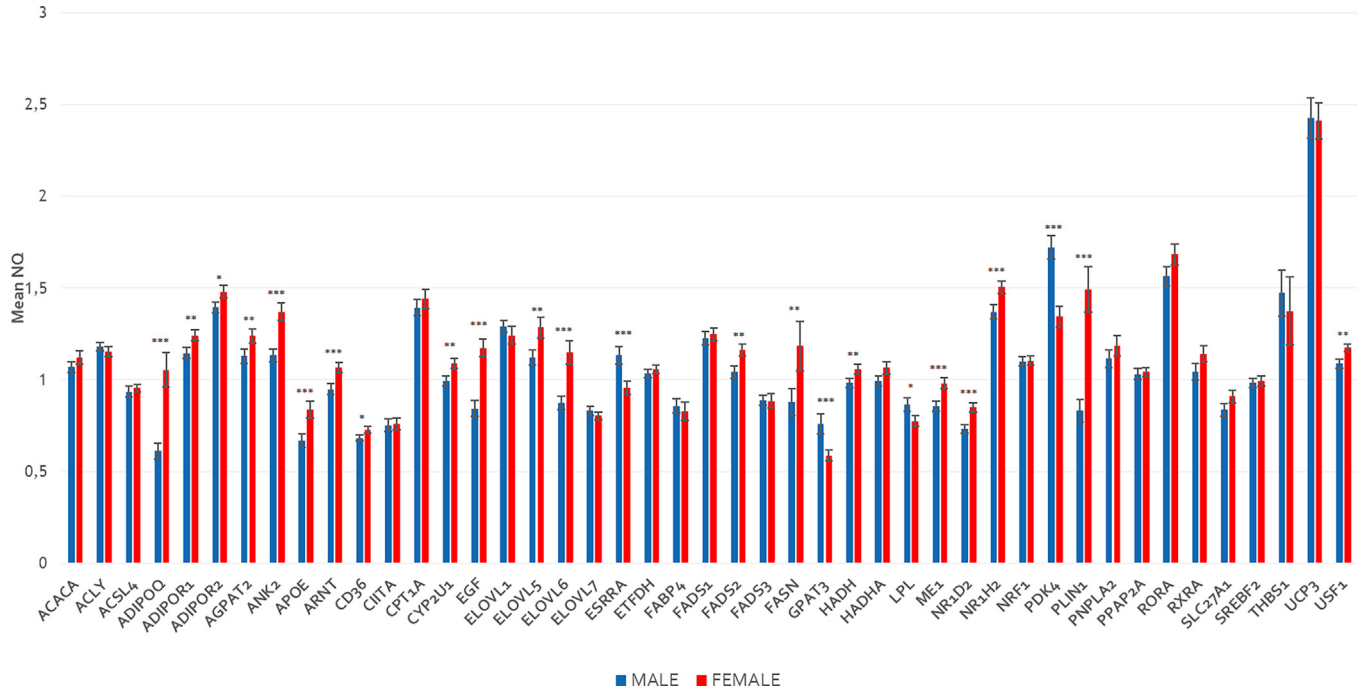

Fig. 1. Comparison between females (red bars) and males (blue bars) of the mRNA levels (Mean Normalised Quantity (NQ)) of 45 lipid-related genes in animals from the three backcrossed pig animals. Data are represented as mean ± SEM. Significant differences are labelled as: **P*-value ≤0.05, ***P*-value ≤ 0.01, ****P*-value ≤ 0.001.

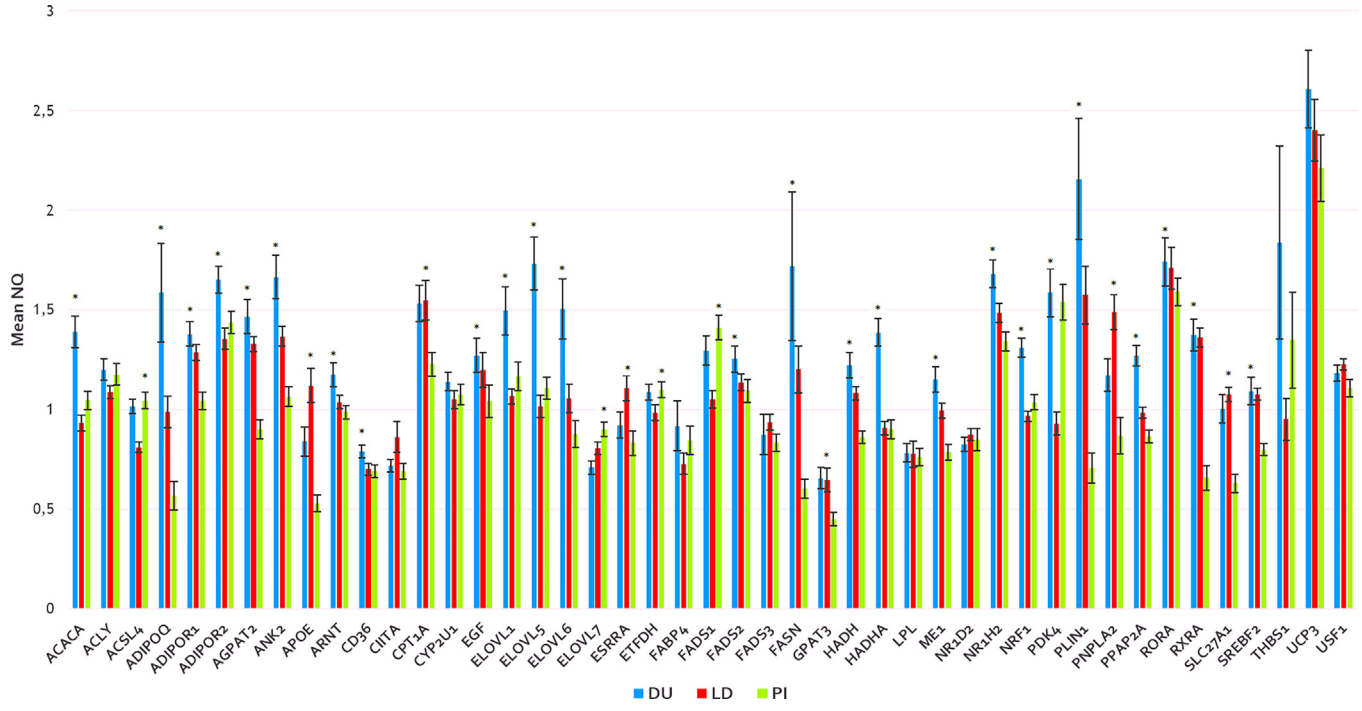

Fig. 2. Comparison between the three backcrossed pig animals in the mRNA levels of 45 lipid-related genes. Data present mean ± SEM. Significant differences are labelled as: **P*-value ≤ 0.05. Abbreviations: DU = Duroc; LD = Landrace; PI = Pietrain; BC1_DU = Backcross between Iberian × Duroc; BC1_LD = Backcross between Iberian × Landrace; BC1_PI = Backcross between Iberian × Pietrain.

Genome-wide association studies for gene expression and expression quantitative trait loci identification

An eGWAS was performed between the muscle gene expression values and the genotypes of 38,488 SNPs distributed along the *Sus scrofa* chromosomes (**SSCs**) in 354 animals. The eGWAS identified 301 eSNPs located in 27 genomic *Sus scrofa* chromosome regions associated with the expression of *ANK2, APOE, ARNT, CIITA, CPT1A, EGF, ELOVL6, ELOVL7, FADS3, FASN, GPAT3, NR1D2, NR1H2, PLIN1, PPAP2A, RORA, RXRA* and *UCP3* (FDR < 0.05) genes. Unfortunately, none of the 65 SNPs located in candidate genes and genotyped by *Taqman* OpenArray were found significant in the eGWAS analysis.

A total of 24 eQTLs showed *trans*-regulatory effects on gene expression of 12 genes (Table 1). In addition, three were classified

Table 1
Significant eQTLs for the 45-muscle gene expression study in the three backcrossed pig animals. Start and end positions refer to the eQTL interval and are based on *Sus scrofa* 11.1 assembly. Lengths are given in base pairs. Gene annotation was performed considering one additional Mb at the start and the end of the eQTL interval. The SNPs column indicates the number of SNPs within the eQTL.

Inter-val	Gene	Chr eQTL	Start position (bp)	End position (bp)	Size (Mbp)	SNPs	Top SNP	*q*-value	MAF	eQTL	Candidate genes
1	*ANK2*	6	129477727	132846185	3.36	3	rs81391604	0.0431	0.2	Trans	
2	*ANK2*	7	90911258	92399480	1.4	3	rs340169919	0.0096	0.22	Trans	
3	*ANK2*	7	105856850	105892246	0.04	2	rs81223355	0.0065	0.24	Trans	
4	*ANK2*	14	55291076	82159674	26.9	118	rs80792689	0.0065	0.18	Trans	*NRBF2*
5	*APOE*	13	77105634	82197802	5.1	14	rs80831731	0.0035	0.11	Trans	
6	*APOE*	15	93185092	94923471	1.74	6	rs333806503	0.0135	0.11	Trans	*STAT1*, *STAT4*
7	*ARNT*	14	56474441	58410680	1.99	5	rs80792689	0.0033	0.18	Trans	
8	*EGF*	8	131373563	131452301	0.08	2	rs81211121	0.0032	0.38	Trans	
9	*ELOVL6*	13	24228663	24253641	0.02	2	rs80853212	0.0429	0.05	Trans	
10	*ELOVL6*	13	77105634	82197802	5.1	14	rs80831731	0.0002	0.11	Trans	
11	*FASN*	5	27631915	28327581	0.7	2	rs81334652	0.0269	0.06	Trans	
12	*FASN*	8	79839602	79853747	0.02	2	rs81401770	0.0426	0.08	Trans	*NR3C2*
13	*FASN*	13	52057695	61140536	9.1	3	rs80909668	0.0374	0.41	Trans	
14	*FASN*	13	77105634	82197802	5.1	14	rs80831731	0.0065	0.11	Trans	
15	*FASN*	15	74207778	74235458	0.03	3	rs80850172	0.0040	0.11	Trans	
16	*FASN*	15	93185092	94923471	1.74	6	rs81301298	0.0183	0.09	Trans	*STAT1*, *STAT4*
17	*GPAT3*	8	134733478	135607348	0.88	8	rs81336088	0.0003	0.25	Cis	
18	*NR1D2*	6	99307882	99580947	0.27	3	rs81347503	0.0246	0.29	Trans	
19	*PLIN1*	13	77105634	82197802	5.1	14	rs81447187	0.0078	0.11	Trans	
20	*PPAP2A*	6	45156998	46387903	1.23	8	rs81395741	0.0089	0.11	Trans	*USF2*
21	*RORA*	14	2722953	2758196	0.36	2	rs80785221	0.0291	0.21	Trans	
22	*RXRA*	1	29427957	31162256	1.74	2	rs80801544	0.0086	0.12	Trans	
23	*RXRA*	1	270278445	274019182	0.37	11	rs81352834	3.79E-08	0.13	Cis	
24	*RXRA*	4	111702541	119798655	8.09	2	rs80985433	0.0014	0.09	Trans	
25	*RXRA*	12	20659791	20767619	0.1	5	rs81214864	0.0383	0.07	Trans	*MLX, STAT3, STAT5*
26	*UCP3*	9	8362141	8406364	0.44	2	rs81413811	0.0228	0.3	Cis	*UCP2*
27	*UCP3*	X	96608820	96624858	0.02	2	rs81473579	0.0228	0.09	Trans	

Abbreviations: eQTL = expression Quantitative Trait *Loci*; Chr = Chromosome; bp = base pairs; Mbp = Megabase pairs; SNP = Single Nucleotide Polymorphism; MAF = Minor Allele Frequency.

as *cis*-acting eQTLs for *GPAT3, RXRA* and *UCP3* genes, which suggests that there is a mutation in the same gene or in a proximal genomic region affecting its expression (Table 1). Both *cis* and *trans*-eQTLs were represented in Fig. 3.

In addition, in SSC13, we found an eQTL that affects the expression of four genes (*APOE, ELOVL6, FASN* and *PLIN1*). Moreover, in SSC15, there is another eQTL that regulates two other genes (*APOE* and *FASN*).

From the 258 eQTL-associated SNPs, 230 were successfully annotated with VEP of Ensembl (*Sscrofa* 11.1 annotation release 108) of which 34.8% (80 SNPs) were located in intergenic regions. The remaining 65.2% (150) of SNPs were mapped within 93 genes: 121 (52.6%) in intronic regions, 10 in upstream regions, five in downstream regions, four in non-coding transcript regions, three in 3′UTR regions and six in the coding regions of genes, five determining synonymous mutations and one being a missense mutation (Supplementary Table S4).

Cis-expression quantitative trait loci

Concerning the *GPAT3* gene in the eGWAS results, located on *SSC*8, one of the annotated *cis*-SNPs (*GPAT3* g.134933342 T > C) was mapped within the *GPAT3* gene. However, this SNP was not the most significant associated SNP (*P*-value = 9.00×10^{-8}). The most significant *cis*-SNP for *GPAT3* (g.135550523A > C; *P*-value = 1.70×10^{-8}) was located in the *SCD5* gene, also located on *SSC*8 (Fig. 4).

Regarding the *RXRA cis*-eQTL, the variant *NCS1* g.270313674C > T (rs81352834), located on *SSC*1, was the most significantly associated polymorphism (*P*-value = 9.84×10^{-13}), and the second most significantly associated variant, also located on *SSC*1, was *RXRA* g.273242436A > G SNP (rs80827620) (*P*-value = 9.73×10^{-11}) (Fig. 5).

In relation to the Uncoupling Protein 3 (*UCP3*) gene, which is located on *SSC*9, the SNP g.8362141G > A (rs81413811) located within the *UCP2* gene, also located on *SSC9*, was the most significantly associated SNP with the *UCP3* gene expression in muscle (*P*-value = 6.54×10^{-7}) (Fig. 6).

Trans-expression quantitative trait loci

A total of 786 genes located within 24 *trans*-eQTL genomic regions were identified in our study. Among them, we detected potential lipid metabolism regulatory genes in six genomic regions (Table 1).

Gene expression correlations

In order to identify co-expression patterns in the candidate genes, a co-expression correlation using PCIT algorithm (Watson-Haigh et al., 2009) was performed. The analysis included the muscle expression data of the 45 candidate genes used in this study, the 44 candidate genes utilised in the study of Criado-Mesas et al. (2020), and the composition data of 14 different FAs in the 3BCs pigs. Hence, a total of 89 candidate genes and 14 FAs were analysed, and a network graph was generated by Cytoscape software (Shannon et al., 2003) (Fig. 7). Notably, the genes *PDHX*, *HIF1AN*, *ACAA2*, and *NCOA6* exhibited the highest number of correlated connections with other genes. Regarding the 14 analysed FAs, only oleic acid (C18:1n-9), α-linolenic acid (C18:3n-3) and arachidonic acid (C20:4n-6) displayed significant correlations with genes. Specifically, C18:1n-9 exhibited positive correlations with the expression of the genes *SCD* (r = 0.32) and *PPARG* (r = 0.32). On the other hand, C18:3n-3 exhibited a negative correlation with the *SREBF1* gene expression (r = –0.3) and a positive correlation with the *FABP5* gene (r = 0.3). Conversely,

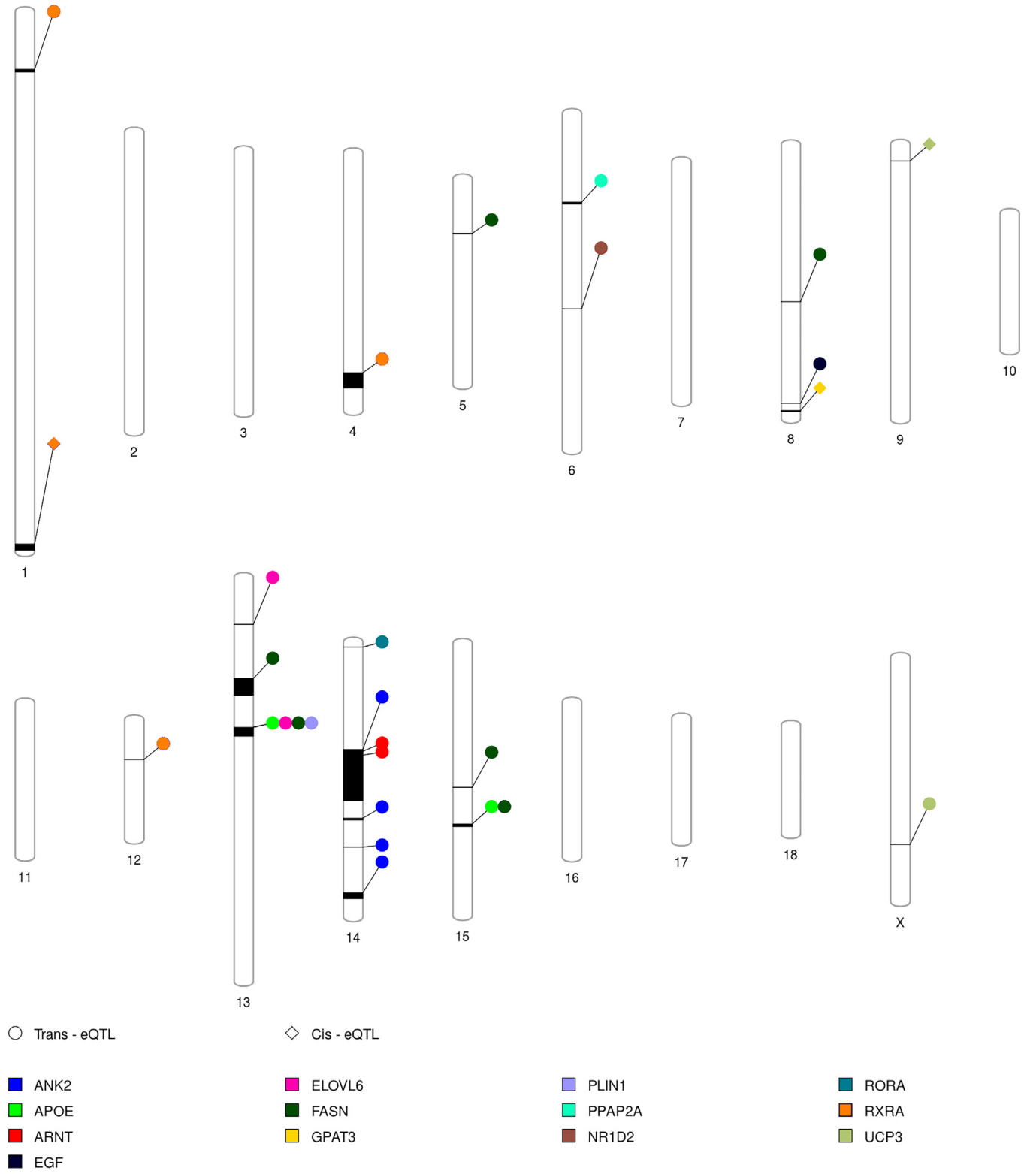

Fig. 3. PhenoGram plot representing regions associated with gene expression of 45 lipid-related genes along pig chromosomes in the three backcrossed pig animals. The shape indicates the type of expression quantitative trait *loci* (eQTL) and the colour indicates the gene name as indicated in the legend.

C20:4n-6 displayed a negative correlation with the *PPARG* gene expression. Finally, the strongest correlations within the entire network were observed between the genes *ADIPOQ1* and *PLIN1*, with a correlation value of 0.96, between the genes *SCD* and *PPARG*, with a correlation value of 0.84, between the linoleic acid (C18:2n-6) and C20:4n-6, with a correlation value of 0.9 and between C18:2n-6 and C18:1n-9, with a correlation value of –0.89.

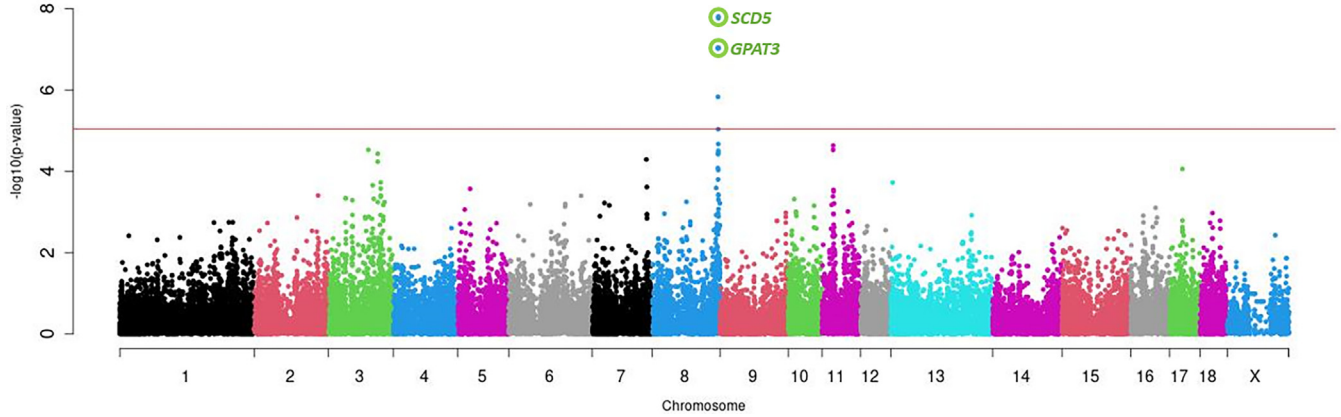

Fig. 4. Genome wide-association study (GWAS) plot of *GPAT3* gene expression in muscle. Chromosome positions in Mb based on *Sscrofa 11.1* assembly of the pig genome are represented in the X-axis and the $-\log_{10}$ (*P*-value) is on the Y-axis. The red horizontal line represents the threshold for an False Discovery Rate (FDR) < 0.05. The *SCD5* g.135550523A > C (rs81344869) and *GPAT3* g.134933342 T > C (rs81269758) single nucleotide polymorphisms (SNPs) are circled and labelled in colour green.

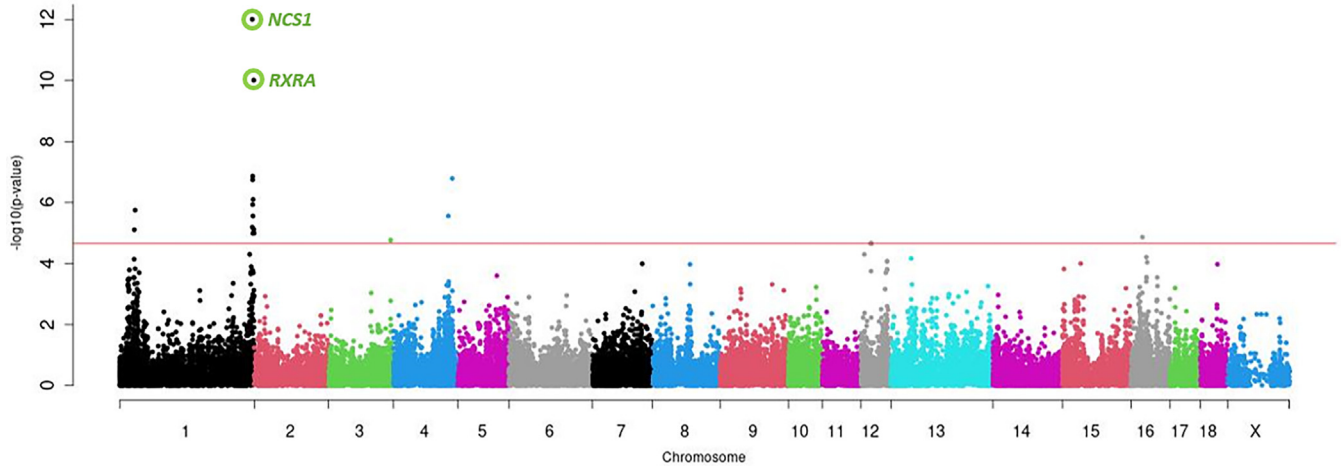

Fig. 5. Genome wide-association study (GWAS) plot of *RXRA* gene expression in muscle. Chromosome positions in Mb based on *Sscrofa 11.1* assembly of the pig genome are represented in the X-axis and the $-\log_{10}$ (*P*-value) is on the Y-axis. The red horizontal line represents the threshold for an False Discovery Rate (FDR) < 0.05. The *NCS1* g.270313674C > T (rs81352834) and *RXRA* g.273242436A > G (rs80827620) single nucleotide polymorphisms (SNPs) are circled and labelled in colour green.

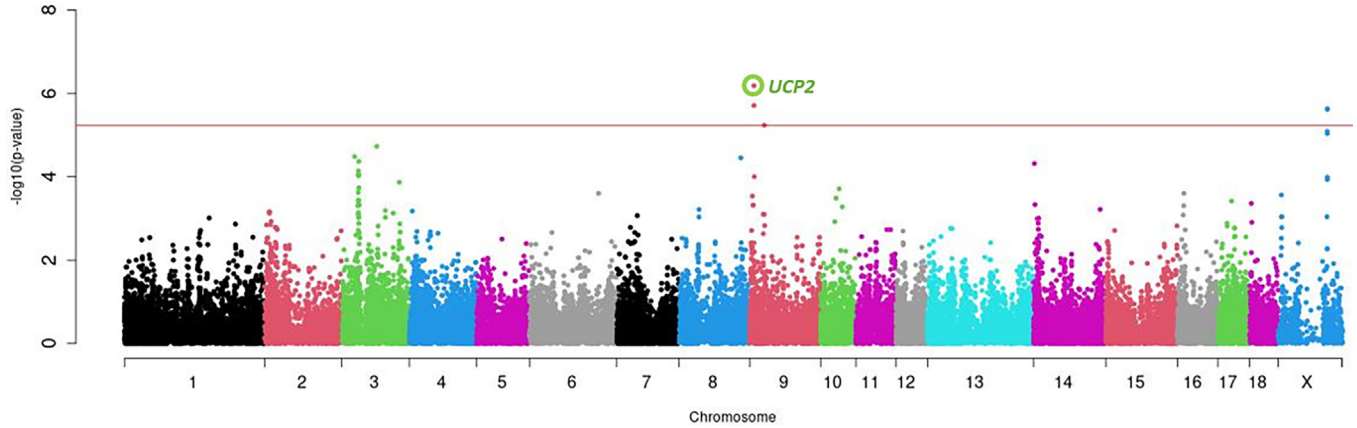

Fig. 6. Genome wide-association study (GWAS) plot of *UCP3* gene expression in muscle. Chromosome positions in Mb based on *Sscrofa 11.1* assembly of the pig genome are represented in the X-axis and the $-\log_{10}$ (*P*-value) is on the Y-axis. The red horizontal line represents the threshold for an False Discovery Rate (FDR) < 0.05. The *UCP2 g.8362141G > A* single nucleotide polymorphism (SNP) is circled and labelled in colour green.

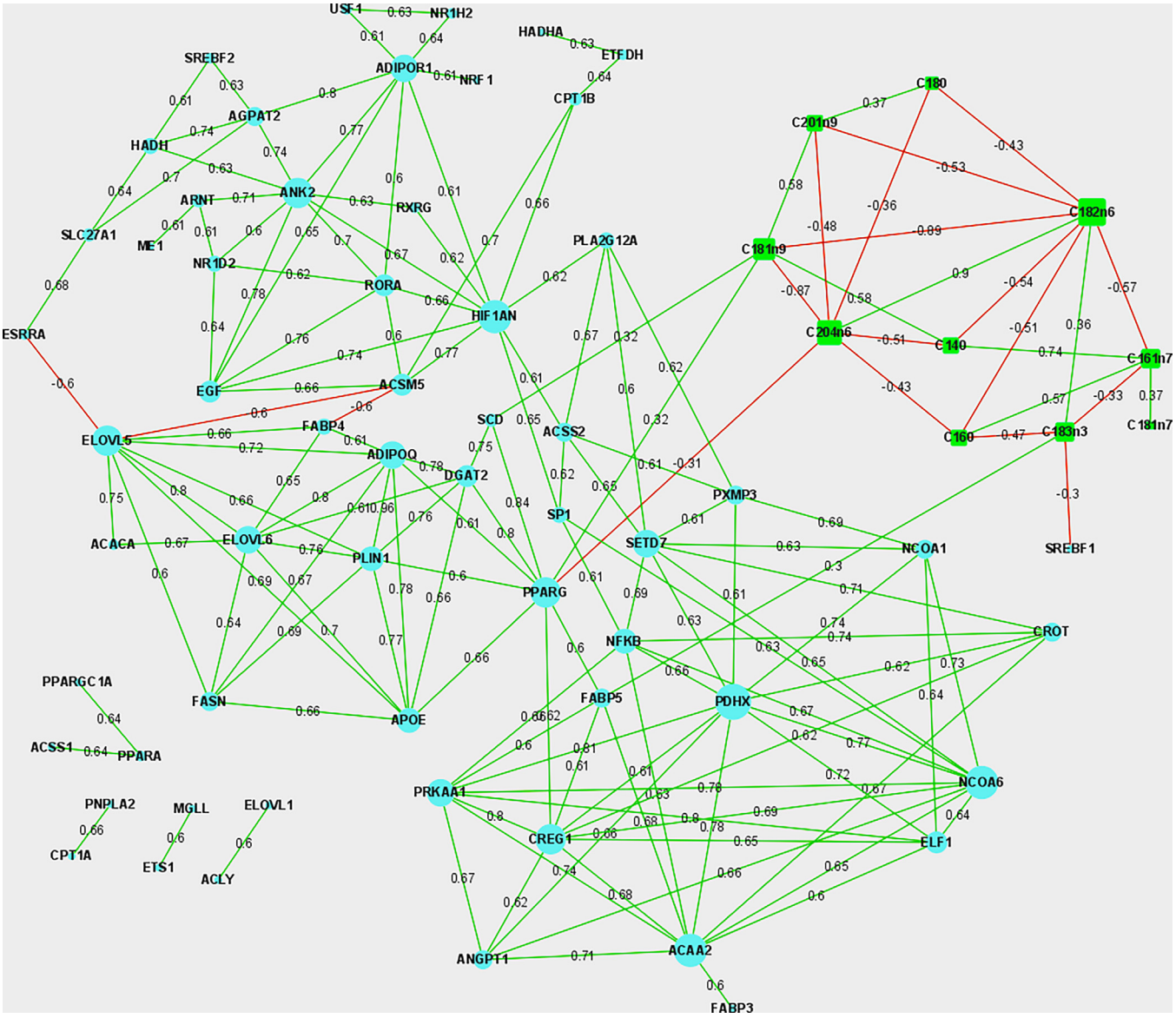

Fig. 7. Gene co-expression network in three backcrossed pig animals using the PCIT algorithm (Watson-Haigh et al., 2009). After filtering by significance and r ≥ |0.6| for genes and r ≥ |0.3| for fatty acids (FAs), 58 of the 90 initial genes and 10 different FAs are shown in this network. Node size represents the degree of a node. Green and red lines indicate the positive and negative correlations, respectively.

Discussion

Sex and genetic background effects

In general terms, our results show that there are differences in muscle gene expression between males and females, particularly in genes associated with fat accumulation and storage. In females, the expression of genes related to lipid metabolism is generally higher, which may contribute to differences in fat distribution between the sexes (Varlamov et al., 2014). Hormonal differences between males and females are thought to play a role in the regulation of gene expression. For instance, oestrogen and progesterone are more abundant in females and can stimulate the expression of genes associated with fat storage (Varlamov et al., 2014). Previously, a sexual dimorphism in the transcriptional regulation of genes related to lipid metabolism has been reported in various tissues, such as muscle, liver, and backfat (Ballester et al., 2017; Puig-Oliveras et al., 2016; Revilla et al., 2018). Therefore, it is relevant to comprehend the mechanisms underlying sexual dimorphism in gene expression.

The genes over-expressed in females are involved in FA metabolism (*APOE, CYP2U1, EGF, ELOVL5, ELOVL6, FADS2, FASN, HADH, PLIN1*), transcriptional regulation and control (*ANK2, ARNT, NR1D2, NR1H2, USF1*), energy metabolism (*ADIPOQ, ADIPOR1, ADIPOR2*) and lipid metabolic process (*AGPAT2, CD36, ME1*). Conversely, some of the four genes showing higher expression in males (*ESRRA, GPAT3, LPL* and *PDK4*) are relevant regulators of lipolytic pathways (Cunningham et al., 2022). In humans, men exhibit higher activity in lipolytic pathways, while women tend to have higher rates of lipogenesis and triglyceride accumulation, putting them at a greater risk for weight gain and obesity development (Varlamov et al., 2014). In a similar way, female pigs seem to develop obesity more readily than male pigs (Zhang & Lerman, 2016). On the other hand, a comparison between Iberian and Duroc pigs reported a breed effect on the expression of genes involved in energy balance and lipogenesis (Bahelka et al., 2007; Benítez et al.,

2018; Font-I-Furnols et al., 2019). In summary, genes more related to lipogenic pathways and biosynthesis pathways were more expressed in BC1_DU whereas genes related to lipolytic pathways were higher expressed in BC1_LD. Finally, 4 out of 45 genes were over-expressed in BC1_PI and were mainly related to lipogenic pathways and biosynthesis of unsaturated FAs.

Altogether, these results indicated a strong effect of sex and breed on gene expression levels. Therefore, they were considered in association studies and included as co-factors in our model.

Cis-expression quantitative trait loci

The Glycerol-3-Phosphate Acyltransferase 3 (*GPAT3*) gene is involved in pathways such as the triglyceride biosynthetic process through the conversion of glycerol-3-phosphate to lysophosphatidic acid in the synthesis of triacylglycerol, and pathways related with gluconeogenesis (Cao et al., 2006). In the *GPTA3* eGWAS results, the most significant polymorphism was located on an intronic region of Stearoyl-CoA Desaturase 5 (*SCD5*) gene, at 0.57 Mb upstream from the *GPAT3* gene. The associated pathways of *SCD5* gene include lipid metabolism through stearoyl-CoA 9-desaturase and acyl-CoA desaturase activity, as well as biosynthesis of FAs and unsaturated FAs (Stelzer et al., 2016). On the other hand, the second most significant SNP was located on an intronic region of *GPTA3* gene. These results suggest the presence of another polymorphism within or near of this gene as a causative mutation affecting *GPTA3* gene expression levels.

Regarding Retinoid X Receptor Alpha (*RXRA*) in the eGWAS findings, this gene is involved in pathways such as hormone-mediated signalling pathway and regulation of RNA transcription (Stelzer et al., 2016). Furthermore, the *RXRA* gene forms the complex *PPARA-RXRA*, which increases lipid catabolism and FA β-oxidation (Tontonoz et al., 1994; Vitali et al., 2018). In the *RXRA* eGWAS results, the most significant SNP was located on an intronic region of *NCS1* gene, at 3.4 Mb downstream from the *RXRA* gene, and the second most significantly associated variant was located within an intronic region of *RXRA* gene. These findings indicate the presence of an additional polymorphism located within or in close proximity to this gene, which may act as a causal mutation influencing *RXRA* gene expression levels.

The Uncoupling Protein 3 (*UCP3*) gene, a target of another *cis*-eQTL region identified, is implicated in pathways including β-oxidation of FAs, and it has been involved in chemical reactions and pathways such as FA metabolism process and adaptative thermogenesis (Han et al., 2012; Lin et al., 2017). This *cis*-eQTL, located in SSC9 contains a mutation located in an intronic position of *UCP2* gene, at 0.15 Mb downstream from the *UCP3* gene. *UCP2* gene, a paralog of *UCP3* gene, plays a role in non-shivering thermogenesis, obesity and diabetes *mellitus*, while its pathways include respiratory electron transport, ATP synthesis and heat production (Lin et al., 2017).

In this study, the associations between gene expression and genetic markers may be influenced by linkage disequilibrium with the causal mutation. Nevertheless, additional investigations are necessary to validate the findings derived from these analyses.

Trans-expression quantitative trait loci

The Ankyrin 2 (*ANK2*) eGWAS results unravelled a total of four *trans*-eQTLs, but only one located at 55.2 Mb – 82.1 Mb in SSC14 (spanning 26.9 Mb and with 118 significant SNPs) contained a candidate gene (*NRBF2*) that could regulate the expression of *ANK2*. The *ANK2* gene belongs to the ankyrin family and has been suggested as a susceptibility gene for obesity, based on studies in mice with a human variant linked to type 2 diabetes (Lorenzo et al., 2015). Furthermore, *AnkB*-deficient adipocytes displayed increased levels of *SLC2A4* (also known as *GLUT4*), plasma membrane, glucose uptake, and lipid accumulation (Lorenzo & Bennett, 2017). Nuclear Receptor Binding Factor 2 (*NRBF2*) was located within the *ANK2* *trans*-eQTL region and was associated with a lipid metabolism regulatory gene. Polymorphisms in *NRBF2* gene have been associated with specific PUFA levels in plasma in humans (Hu et al., 2015). Furthermore, *NRBF2* has also demonstrated its ability to interact with several other receptors, including peroxisome proliferator-activated receptor alpha (*PPARA*), thyroid hormone receptor beta (*THRB*), retinoic acid receptor alpha (*RARA*), and retinoid X receptor alpha (*RXRA*), which is also related to different candidate genes for lipid metabolism, such as *FABP4, FASN, LPL* and *PLIN1* (Ouyang et al., 2020).

The Fatty Acid Synthase (*FASN*) eGWAS results identified *trans*-eQTL covering the genomic region of 79.8–79.85 Mb, wherein a transcription factor associated with lipid metabolism, the Nuclear Receptor Subfamily 3 Group C Member 2 (*NR3C2*), was identified. Polymorphisms in the *NR3C2* gene have been associated with neuroendocrine parameters, carcass composition and meat quality traits in pigs, suggesting its important role in the regulation of lipidic genes (Terenina et al., 2013). Unfortunately, no evidence has been found linking the gene *NR3C2* to the regulation of *FASN*. However, further analysis would be necessary to determine if there is any relationship between these genes.

The further *trans*-eQTL region identified in the eGWAS results of the candidate gene *PPAP2A* was located in SSC6 at 45.1–46.3 Mb, where Upstream Transcription Factor 2 (*USF2*) was found. The *USF2* gene has been identified as a transcriptional regulator of the human *APOC3* gene which is recognised as an inhibitor of lipoprotein lipase, and as such, its overexpression in mice has been observed to result in a substantial increase in plasma triglyceride concentrations, consistent with its inhibitory function (Lai et al., 2005). With this information, it suggests that the *USF2* gene could play a regulatory role in genes related to lipid metabolism.

The Retinoid X Receptor Alpha (*RXRA*) eGWAS unravelled a *trans*-eQTL, positioned at 20.6–20.7 Mb in SSC12. Through a comprehensive analysis of this genomic *locus*, which spans 0.1 Mb, a total of five SNPs have been identified that show significant associations with *RXRA* gene expression levels, with three candidate regulatory genes being mapped (*MLX*, *STAT3, STAT5*).

The MAX Dimerisation Protein (*MLX*) gene has been found to be correlated with the *ChREBP* transcription factor, and it has been observed that this complex is capable of modulating the transcriptional activity of genes involved in lipid metabolism, including *ACC1* and *FASN* (Donald, 2012). According to a model proposed by Ma et al. (2005), two ChREBP-Mlx heterodimers would bind to the two E boxes of the ChoRE to provide a transcriptional complex necessary for glucose regulation.

The Signal Transducer and Activator of Transcription 3 (*STAT3*) gene has also been identified in the *RXRA* eQTL. According to Wu et al. (2018), *STAT3* gene plays a crucial role in regulating the *FAS* and *CPT1α1b* lipid metabolism genes, as their promoter regions are associated with the binding locus of *STAT3* in yellow catfishes. Unfortunately, we were unable to locate any relevant literature concerning this transcription factor's association with the *RXRA* gene or its involvement with lipid metabolism genes in pigs.

On the other hand, the STAT5A/B family was also mapped at *RXRA* *trans*-eQTL. According to Kliewer et al. (1999), the *PPARγ* is a key member of the nuclear hormone receptor superfamily. It collaborates with *RXRA* to effectively regulate specific genes associated with adipocyte differentiation and insulin sensitisation. In relation with this, Meirhaeghe et al. (2003) indicated that *PPARγ3* could play a role in maintaining lipid balance in humans by influencing the Growth Hormone/*STAT5B* pathway. Hence, it suggests that the STAT proteins not only control the expression of genes specific to fat tissue but also serve as targets of regulation by tran-

scriptional factors such as *PPARγ*. In addition, an study conducted by Si & Collins (2002) on the regulation of haematopoiesis in humans and its association with transcription factors, it was discovered that the JAK2/STAT pathway, specifically the activation of *STAT5*, plays a crucial role in enhancing RAR transcriptional activity in cultured hematopoietic cells.

Trans-expression quantitative trait loci hotspot regions

Furthermore, we have detected two hotspot regions that modulate the expression of several genes. A *trans*-eQTL hotspot located on SSC13 and spanning 5.1 Mb (77.1–82.2 Mb) was associated with the expression of four genes: *APOE, ELOVL6, FASN* and *PLIN1*. The Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta (*PIK3CB*) gene was mapped in this region. *PIK3CB* participates in the PI3K-Akt signalling pathway and mTOR signalling pathway, and the regulation of the PI3K-Akt-mTOR signalling pathway on lipid metabolism has been mentioned by different studies (Dibble and Manning, 2013; Liu et al., 2016). Furthermore, inhibition of the PI3K-Akt-mTOR pathway leads to a decrease in intracellular lipid accumulation along with a reduction in mRNA expression and protein content of genes involved in *de novo* FA synthesis (Zhao et al., 2023). Hence, we can suggest that *PIK3CB* is involved in muscle lipid metabolism, being an interesting candidate gene to explain the differences in the expression of four genes associated with the SSC13 hotspot. Other potential genes related to lipid metabolism (*ESYT3*, *RBP1* and *RBP2*) were detected, but no evidence was found for their possible regulation of the four genes in the mentioned hotspot.

The second *trans*-hotspot region covering 1.74 Mb on SSC15 (93.1–94.9 Mb) was associated with the expression of *APOE* and *FASN* genes. In this region, we detected two candidate regulatory elements, *STAT1* and *STAT4*, which have been associated with lipid metabolism pathways. Zhang et al. (2019) suggested that *STAT1* may regulate the expression of genes associated with lipid metabolism and FA synthases (FASs), such as *FASN*, the key enzymes in *de novo* lipogenesis, which promotes the synthesis of long-chain FAs. A previous study has indicated that *STAT1* regulates adipogenesis and adipolysis (Stephens et al., 1996). In addition, the Myostatin (*MSTN*) gene was also mapped in this hotspot region. The *MSTN* gene has been described by Xin et al. (2020) as being involved as a regulator of AMP kinase activity in cattle. The down-regulation of *MSTN* triggers the activation of AMPK signalling pathways to regulate glucose and lipid metabolism, which highlights its possible role in lipid metabolism. Moreover, based on their findings, Pan et al. (2021) propose that *MSTN* exerts an inhibitory effect on adipogenesis and promotes lipolysis in the subcutaneous adipose tissue of pigs, primarily through the activation of ERK1/2 and PKA signalling pathways. These results indicate that *MSTN* may act as a powerful regulator of genes involved in lipid metabolism pathways.

The two trans hotspots detected in this study are associated with *APOE* and *FASN* gene expression, suggesting that different genetic variants are regulating the muscle expression of these genes, but further studies are required to identify these variants.

Muscle gene expression and fatty acid composition correlation networks

Our study investigates the relationship between specific gene expression and the quantities of different FAs in the porcine *longissimus dorsi* muscle. *SCD* and *PPARG* genes are found to play a crucial role in regulating C18:1n-9 levels, with a positive correlation observed between their expression and the abundance of this FA. Similarly, the genes *FABP5* and *SREBF1* are implicated in controlling C18:3n-3 levels, with a positive correlation for *FABP5* and a negative correlation for *SREBF1*. Furthermore, the study explores the association between C20:4n-6 and the *PPARG* gene, revealing a negative correlation and suggesting a regulatory role of *PPARG* in C20:4n-6 metabolism. Moreover, we want to highlight the importance of the *PPARG* gene due to its substantial number of connections with other genes and its association with FAs, suggesting a significant role of this gene in FA metabolism and with potential implications in pork quality. In addition, the genes *PDHX*, *HIF1AN*, *ACAA2*, and *NCOA6* display significant correlations with multiple genes, indicating their role as central regulators. The positive and negative correlations between the genes and the FAs are shown in Fig. 7.

Conclusion

In the present work, we discerned genetic variants linked to the expression of genes associated with lipid metabolism in muscle tissue. These genetic variants were clustered in 27 eQTLs from which three were described as *cis*-acting major regulators of *GPAT3*, *RXRA* and *UCP3* gene expression levels. The other 24 regions were identified as *trans*-acting regulators, which affect different lipid-related genes along the chromosomes. In addition, two *trans*-regulatory hotspots, which affect the regulatory control over the expression of multiple genes, were detected within the SSC13 and SSC15. Furthermore, the co-expression analysis revealed the influence of four regulatory genes modulating the levels of different FA levels within the 3BCs, concurrently affecting the expression of other genes associated with lipid metabolism. Our results increase the knowledge of the genetic basis of gene expression regulation in muscle lipid metabolism. Overall, the expression of genes related to lipid metabolism is regulated in a complex way and further validations are needed to corroborate our findings.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.101033.

Ethics approval

Animal care and procedures were carried out following the Spanish Policy for Animal Protection RD1201/05 and the European Union Directive 86/609 about the protection of animals used in experimentation.

Data and model availability statement

All relevant data produced or evaluated in this research are disclosed in the paper as well as its supplementary information files. Additional materials can be requested to JMF.

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

During the preparation of this work, the author used the ChatGPT tool in order to correct potential grammatical errors, search for synonyms, enhance the author's written sentences, as well as to translate different words related to this field.

Author ORCIDs

M. Passols: https://orcid.org/0000-0002-6853-4119.
F. Llobet-Cabau: https://orcid.org/0000-0002-2769-5741.
C. Sebastià: https://orcid.org/0000-0003-2250-6451.

J. Valdés: https://orcid.org/0000-0001-5314-0988.
A. Castelló: https://orcid.org/0000-0001-8497-6251.
L. Criado-Mesas: https://orcid.org/0000-0002-1115-4131.
J.M. Folch: https://orcid.org/0000-0003-3689-1303.
A. Sánchez: https://orcid.org/0000-0001-9160-1124.

Author contributions

JMF conceived the study and was the principal investigator of the project with the participation of AS. JMF collected the animal samples. MP, FLC and LCM performed the total RNA isolation. Real-time qPCR expression analysis was performed by MP, FLC, AC and LCM. MP, FLC and LCM performed the bioinformatic analysis to generate the gene expression data. MP, FLC, CS and JVH participated in data processing and bioinformatic methods. JMF, CS and MP proposed the statistical methodology and performed the global association study and gene functional analysis. JMF reviewed the statistical methodology and conceived the structure of the paper. MP and JMF wrote the paper. All authors critically revised and approved the final manuscript.

Declaration of interest

The authors declare no conflict of interest.

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