



Universitat de Lleida

## Genetic biomarkers for fat content and fatty acid composition in pork

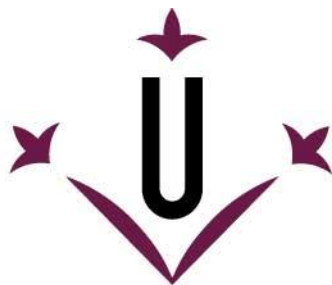
Sofia Gol i Parera

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**Universitat de Lleida**

**TESI DOCTORAL**

**GENETIC BIOMARKERS FOR FAT  
CONTENT AND FATTY ACID  
COMPOSITION IN PORK**

Sofia Gol i Parera

**Memòria presentada per optar al grau de Doctor  
per la Universitat de Lleida**

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Agrària i Alimentària**

Directors

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*“The key is man’s power of accumulative selection:  
nature gives successive variations; man adds  
them up in certain directions useful to him.”*

*-Darwin, p. 35, sixth edition of The Origin of Species. 1920.*



*A la memòria del meu avi; **Josep Gol i Tutusaus**,  
Per transmetre'm el seu amor a l'agricultura i a la ramaderia.*



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## SUMMARY

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This PhD dissertation is part of a research line on the genetic improvement of pork quality. One of the latest goals to be included in some sire lines selected for premium markets is to achieve an optimum level of intramuscular fat and fatty acid composition without penalizing lean growth performance. This thesis is comprised of four studies conducted on a purebred Duroc line used for producing high-quality meat products. The experiments were designed with the aim to better understand the genetic variability underlying fat content and fatty acid composition and to identify potential markers for breeding. The first study examined the genetic parameters of the linoleic acid (C18:2) to arachidonic acid (C20:4) pathway. In particular, it was showed that selection for the absolute value of C18:2 is expected to deliver a similar response outcome as selection for intramuscular fat at restrained backfat thickness. These findings led to investigate the fatty acid desaturase-2 (*FADS2*) gene, as a candidate gene for C18:2 metabolism route. Thus, the second study evaluated the effects of a variant (rs321384923 was used as a tag single nucleotide polymorphism) in the promoter of the *FADS2* gene. Results showed that this polymorphism affects the n-6 fatty acid profile by enhancing the desaturation efficiency of C18:2 to C20:4. Additionally, the association of perilipin (*PLIN*) genes and guanylate-binding protein-1 (*GBP1*) gene with growth and meat quality traits was assessed. Thus, the third study examined the effects of two polymorphisms in *PLIN1* and *PLIN2*, which have been related to lipid storage and mobilization. Results indicated that the rs333231747 polymorphism on *PLIN2* is associated to early growth and lean weight. The last study showed that *GBP1* has two active polyadenylation signals and that their usage depends on the rs80800372 genotype. The pigs carrying the G allele, which has been associated with lower viraemia after porcine reproductive and respiratory virus infection, had longer transcripts and lower gene expression. In non-epidemic conditions, the G allele increased intramuscular fat content but decreased lean weight. Linoleic acid content and the investigated genetic markers can be used to design appropriate selection strategies to enhance meat quality and lean growth.

## RESUMEN

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Esta tesis doctoral es parte de una línea de investigación sobre la mejora genética de la calidad de la carne en porcino. Uno de los recientes objetivos incluido en algunas líneas paternas seleccionadas para mercados de calidad es lograr un nivel óptimo de grasa intramuscular y de composición en ácidos grasos sin penalizar el crecimiento magro. Esta tesis comprende cuatro estudios realizados en una línea pura de cerdos Duroc destinados a la producción de cárnicos de alta calidad. Los experimentos se diseñaron con el objetivo de comprender mejor la variabilidad genética subyacente en el contenido de grasa y la composición en ácidos grasos e identificar marcadores potenciales a la selección. El primer estudio examinó los parámetros genéticos de la ruta del ácido linoleico (C18:2) al ácido araquidónico (C20:4). En particular, se demostró que se espera que la selección por el valor absoluto de C18:2 proporcione una respuesta similar a la selección por grasa intramuscular a grasa dorsal restringida. Estos hallazgos llevaron a investigar el gen de la desaturasa-2 de los ácidos grasos (*FADS2*), como gen candidato para la ruta del C18:2. Por lo tanto, el segundo estudio evaluó los efectos de una variante (el polimorfismo rs321384923 se usó como marcador) en el promotor del gen *FADS2*. Los resultados mostraron que este polimorfismo afecta el perfil de ácidos grasos n-6 al aumentar la eficiencia de desaturación de C18:2 a C20:4. Además, se evaluó la asociación de los genes de las perilipinas (*PLIN*) y el gen de la proteína de unión a guanilato 1 (*GBP1*) con caracteres de crecimiento y de calidad de la carne. De este modo, el tercer estudio examinó los efectos de dos polimorfismos en *PLIN1* y *PLIN2*, relacionados con la deposición y la movilización de lípidos. Los resultados indicaron que el polimorfismo rs333231747 en *PLIN2* se asocia con el crecimiento temprano y con el peso magro. El último estudio mostró que *GBP1* tiene dos señales de poliadenilación activas y que su uso depende del genotipo rs80800372. Los cerdos portadores del alelo G, asociado con una menor viremia después de la infección por el virus reproductivo y respiratorio porcino, tenían transcritos más largos y una menor expresión génica. En condiciones no epidémicas, el alelo G aumentó el contenido de grasa intramuscular pero disminuyó el peso magro. El contenido de C18:2 y los marcadores genéticos investigados se pueden usar para diseñar estrategias de selección adecuadas para mejorar la calidad de la carne y el crecimiento magro.

## RESUM

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Aquesta tesi doctoral forma part d'una línia de recerca sobre la millora genètica de la qualitat de la carn en porcí. Un dels recents objectius inclòs en algunes línies paternes seleccionades per mercats de qualitat és aconseguir un nivell òptim de greix intramuscular i de composició en àcids grassos sense penalitzar el rendiment magre. Aquesta tesi està formada per quatre estudis realitzats en una línia pura de porcs Duroc destinats a la producció de càrnics d'alta qualitat. Els estudis es van dissenyar amb l'objectiu d'entendre millor la variabilitat genètica subjacent al contingut de greix i a la composició en àcids grassos i d'identificar marcadors potencials a la selecció. El primer estudi va examinar els paràmetres genètics de la ruta de l'àcid linoleic (C18:2) a l'àcid araquidònic (C20:4). En particular, es va demostrar que s'espera que la selecció pel valor absolut de C18:2 proporcioni una resposta a la selecció similar a la selecció pel greix intramuscular a greix dorsal restringit. Aquests resultats van conduir a investigar el gen de la desaturasa-2 dels àcids grassos (*FADS2*), com a gen candidat per a la ruta metabòlica de C18:2. Així, el segon estudi va avaluar els efectes d'una variant (el polimorfisme rs321384923 es va utilitzar com a marcador) al promotor del gen *FADS2*. Els resultats van demostrar que aquest polimorfisme afecta el perfil d'àcids grassos n-6 millorant l'eficiència de desaturació de C18:2 a C20:4. Addicionalment, es va avaluar l'associació dels gens de les perilipines (*PLIN*) i del gen de la proteïna d'unió al guanilat 1 (*GBP1*) amb caràcters de creixement i de qualitat de la carn. Així, el tercer estudi va examinar els efectes de dos polimorfismes als gens *PLIN1* i *PLIN2*, relacionats amb l'emmagatzematge i la mobilització de lípids. Els resultats indiquen que el polimorfisme rs333231747 a *PLIN2* està associat al creixement primerenc i al pes magre. L'últim estudi va demostrar que *GBP1* té dos senyals de poliadenilació actius i que el seu ús depèn del genotip rs80800372. Els porcs portadors de l'al·lel G, associat a una menor viremia després de la infecció pel virus reproductiu i respiratori porcí, van tenir transcripcions més llargues i una menor expressió gènica. En condicions no epidèmiques, l'al·lel G va augmentar el contingut de greix intramuscular, però va disminuir el pes magre. El contingut de C18:2 i els marcadors genètics investigats es poden utilitzar per dissenyar estratègies de selecció adequades per millorar la qualitat de la carn i el creixement magre.



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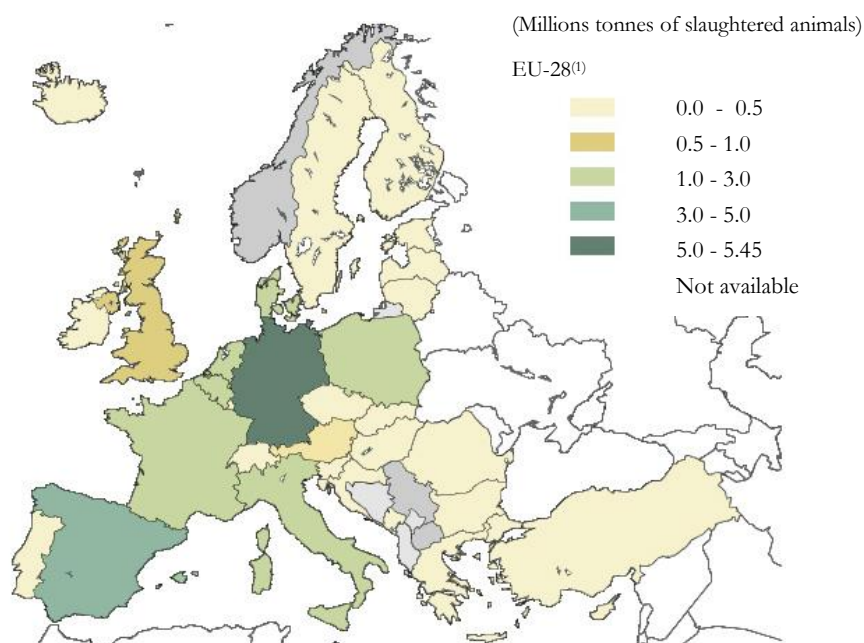
# **INTRODUCTION**

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## 1. Current state of swine production

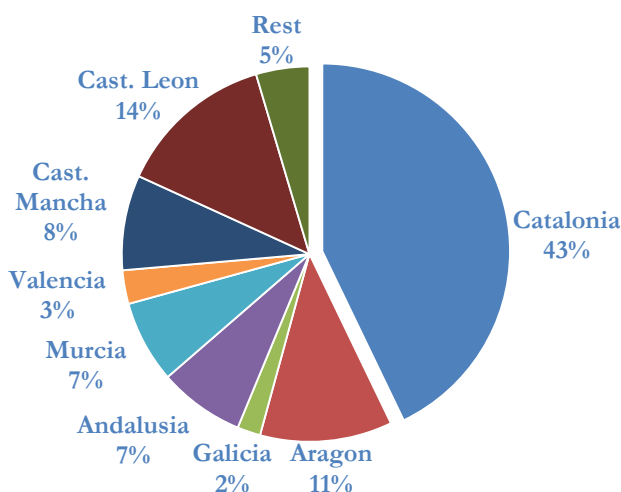
The increasing demand on food resources is an important challenge in which animal breeding plays an important role. Indeed, the high nutritional value of pork makes it one of the most complete foods for humans. Pork represents over 35% of the meat produced worldwide (FAO, 2017; <http://www.fao.org/>; accessed January 2019). In this regard, it is worth to mention that the high increase in swine productivity in the last 10 years, reaching 119 million tonnes in 2017, has been mostly due to the genetic improvement of the animals. Globally, the region with more production is Asia, accounting for 55.78% of pork production, followed by Europe (24.28 %) and America (18.23 %). In this context, Spain produces 3.9 million tonnes of meat, representing the 3.3% of world pork production (FAO, 2017; <http://www.fao.org/>; accessed January 2019). In the European Union (EU), Spain is the second largest pork producer, only overcome by Germany (Figure 1).



(1) Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden and United Kingdom.

**Figure 1.** Distribution of pork production on European Regions in 2017 (Millions of tonnes) (EUROSTAT, 2017; <https://ec.europa.eu/eurostat/>; accessed January 2019).

The economic importance of the pig industry is evident in Spain. Pig production represents the 37% of total livestock production and the 14% of total agricultural production (MAPAMA, 2018a). Since 2014 meat production in the EU has grown by 5.24%, while production in Spain has increased by 19.89% in the same period, which gives an idea of the enormous growth that the sector is experiencing. On the national level, Catalonia, with 42.9% of the Spanish swine production, remains as the main productive area of Spain (Figure 2). However, the fluctuation of various factors such as the price of industrial feed, which represents over 60% of total pig production costs, the emergence of specific swine diseases or the legal impositions, for example concerning animal welfare, have very important productive repercussions. Thus, the decrease in feed prices from 2013 has resulted on a higher market profitability (DARP, 2017).



**Figure 2.** Distribution of pork production on Spanish regions in 2016 (tonnes) (MAPAMA, 2017).

The Spanish exportations of pork in 2017 were of 2.2 million tons, 66% of which were exported within the EU (MAPAMA, 2018a). The main destination countries were France (22.2%), Italy (13.6%) and Portugal (13.9%). In the international scenario, China stands out as the main exporting destination, with 40.4% of exports followed by Japan, with a 15.7%. The commercialization of

pork in Spain differs between the European and the international market. On the latter, in addition to fresh meat, the market offers a rich variety of pork products including the traditional dry-cured hams. Remarkably, in Asian countries, offal represents 25.6% of exported tonnes. Currently, Spain is the world's leading producer (42.6 millions hams/year) and consumer (2.3 Kg/person/year) of dry-cured hams (MAPAMA, 2018b). These values double those of Italy, the second worldwide consumer country. In Spain, there are several types of dry-cured hams, being the Iberian one of the most differentiated. Cured hams can be produced from several pig breeds, typically Duroc, Pietrain, Landrace and Large White. On the other hand, Iberian hams are produced from Iberian pigs (pure or bred up to 50% with Duroc). Pigs for dry-cured ham production are culled at an approximate age of 6 months. In the case of the Iberian dry-cured ham, the current regulation stipulates that pigs must be culled at a minimum of 14 or 10 months of age, depending on whether they were raised under extensive or intensive systems, respectively (RDL 4/2014, 10 of January). The curing process also varies between both type of hams, being shorter in the case of cured hams, with a duration ranging between 9 and 15 months (RDL 474/2014, 13 of June), as compared to the Iberian ham, where this process lasts at least 19 months (RDL 4/2014, 10 of January). Regarding commercialization, for the cured ham there is only one Protected Designation of Origin (PDO Teruel) and two Protected Geographical Indications (GPI Trevelez and GPI Serón). Regarding Iberian ham, there are four protected designations of origin (PDO Guijuelo, PDO Dehesa de Extremadura, PDO Jabugo and PDO Los Pedroches).

## **2. Relevant traits in pig production**

The initial pig breeding programs consisted of mating among them the individuals showing the best traits in terms of productivity, rusticity and external appearance. In the latest decades of the XX century, reproduction traits, such as litter size, became of great interest to the industry because they were used by farmers as indicators of production efficiency (Distl, 2007). Regarding



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productivity, genetic improvement efforts focused on a number of economic traits such as growth rate, meat percentage and feed efficiency, achieving relevant progress on the production of lean meat and on reducing the time required to reach market weight (Dekkers et al., 2011). Thus, the continuous selection during decades against backfat thickness (BT) resulted in lean pig lines mostly used for the production of fresh pork. This also reduced the level of intramuscular fat (IMF) content, which resulted in a negative impact on the organoleptic and technological attributes required for high-quality dry-cured products (Hocquette et al., 2010). In response to this, the breeding goal of the pig lines used for dry cured markets, were redefined accordingly. Thus, in order to cope with this problem, but also to satisfy the increasing consumer concerns on health and sustainable production, breeding goals have started to shift towards including traits with a non explicit economic value. Some of them are related to meat quality, but not only, such as uniformity, pig welfare or disease susceptibility (Table 1; Kanis et al., 2005; Merks et al., 2012).

Meat quality is a complex and highly-subjective trait related to sensorial, nutritive and technological attributes. The aroma, the tenderness and the juiciness are englobed in sensory traits which makes the meat pleasant and appetizing (Aaslyng et al., 2007; Font i Furnols and Guerrero, 2014). From a technological point of view, pH and water-holding capacity are two current concerns for the retail industry, as they influence fresh meat colour and shelf life (Holmer et al., 2009; Hughes et al., 2014). In addition, the ultimate pH has been linked to sensorial quality of meat (Boler et al., 2010) and specifically to tenderness (Maltin et al., 2003). On our research group, emphasis has been made on the study of IMF and fatty acid composition, two of the most relevant nutritional parameters of meat quality which are explained in detail below.

**Table 1.** Main traits of interest in the porcine industry

Characteristics	Trait
Production	Growth rate
	Lean percentage
	Feed efficiency
Reproduction	Litter size
	Fertility
Body composition	Lean content backfat thickness
	Fat content
Meat quality	Sensorial: Colour
	Tenderness
	Juiciness
	Aroma
	Nutritional: Fat content
	Fatty acid composition
	Protein content
	Technological: pH
	Water-holding capacity
	Fat consistence
Oxidative Stability	
Welfare and health	Disease resistance
	Longevity
	Agressiveness

Adapted from Hovenier et al., 1993; Kanis et al., 2005 and Lawrie et al., 2014.

## 2.1 Intramuscular fat

Intramuscular fat content is a trait of major importance on meat quality because it affects sensorial attributes such as taste and flavour and therefore overall acceptability (Fernandez et al., 1999). The term IMF stands for the sum of the lipids located within the muscle, differing from fat which is located between muscles and known as intermuscular fat (Hocquette et al., 2010). Thus, IMF includes both triglycerides, the main form of energy reserves, and

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phospholipids, which are primarily located in the cell membrane (De Smet et al., 2004). Moreover, both hypertrophy and hyperplasia of adipocytes determine IMF (Shi-Zheng and Su-Mei, 2009).

On average, the recommended IMF for production of fresh meat is considered to be around 3%. Percentages below this threshold are associated with lower eating quality, mainly due to its effects on sensory attributes (Fernandez et al., 1999; Huff-Lonergan et al., 2002). The content of IMF can be measured on muscle samples by different methods, many of them based on the method described by Folch and co-workers (1957). Currently, there is an increasing interest on developing fast and cheap methods to estimate IMF, especially on-line at the slaughterhouse. Examples of the use of real-time ultrasounds (Newcom et al., 2002; Jung et al., 2015) reported a phenotypic correlation between measured and predicted IMF content ranging between 0.60-0.70.

Many factors influence the variation of IMF. Among non-genetic factors, stand out the gender, the age and the diet. Regarding genetics factors, IMF is influenced by the species, the breed, the individual and even the muscle within the same animal. The value of the heritability for IMF in pigs ranges from 0.39 to 0.65 (Newcom et al., 2004; Suzuki et al., 2005a) and it is favorably correlated with pH, cooking loss and drip loss (Suzuki et al., 2005a) but unfavourably with lean content. Recent selection efforts have been precisely focused on the development of methods to allow improving IMF and lean content independently (Estany et al., 2017).

## **2.2 Fatty acid composition**

In addition to total IMF content, the fatty acid composition is also closely related to pork quality. Fatty acids can be classified as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) according to the presence and number of double bonds between carbon atoms. Pork fat is high in MUFA (45-50%), followed by SFA (30-35%) and PUFA (10-

15%). While the latter are predominantly found in phospholipids, SFA and MUFA are mainly stored as triglycerides (De Smet et al., 2004).

The fatty acid composition has an important effect on the nutritional value of meat and, consequently, to human health. In this regard, both higher unsaturated to saturated fatty acids, and omega-3 to omega-6 fatty acid ratios are desirable and have been associated to lower risk of cardiovascular disorders (Astrup et al., 2011). Conversely, SFA and omega-6 have the opposite effect and are considered to increase the risk for cardiovascular diseases in humans (Ordovas, 2006). The fatty acid composition also affects technological attributes of meat. For instance, the variation between the number of double bonds on each type of fat influences the melting point and consequently the fat firmness (Wood et al., 2003). In this view, PUFA are less stable and can be responsible for higher drip loss and for rancidity due to oxidative processes (Wood et al., 2008).

Hence, that it would be advisable to approach the pork fatty acid profile to the recommendations concerning SFA, MUFA, n-3 and n-6 PUFA proportions. In swine, especially oleic acid (C18:1 (n-9)), as the main MUFA, but also palmitoleic acid (C16:1 (n-7)) and vaccenic acid influence the taste and aroma of cured products. Regarding SFA, the fatty acids with greater effect on the technological quality of meat are palmitic acid (C16:0), stearic acid (C18:0) and myristic acid. Among PUFA, the main linoleic acid (C18:2 (n-6)) has remarkable effects. The essential C18:2 is a major ingredient of feeds for all species. Among all fatty acids, it shows a greater incorporation into tissues in relation to the amount in the diet (Nguyen et al., 2003). However, as the *novo* synthesis of fatty acids progresses, the proportion of C18:2 declines and it is consequently considered as an indicator of fatness (Wood et al., 2008).

The fatty acid composition is affected by several factors, including fatness, dietary fatty acid composition, energy intake, the gender or the genetic background (Zhang et al., 2007; Nieto and Ros, 2012; Alonso et al., 2015). The fatty acid composition of fat is heritable, although reported estimates show great

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variation (heritability ranging from 0.15 to 0.57), with values depending on the investigated fat depot and the pig's genetic background (Sellier et al., 2010; Gjerlaug-Enger et al., 2011).

### **3. Pig breeding schemes**

The basic breeding scheme used in pig production is the three-way crossing. In this strategy, genetic progress is generated in nucleus farms, the top layer of the production pyramid, where purebred paternal and maternal lines (also known as F0) are selected. Sire lines are regularly selected for growth and carcass traits (such as weight at slaughter age and percentage of lean meat), robustness and meat quality (Oldenbroek and van der Waaij, 2015), while dam lines are also selected for reproduction traits (such as total live born). The dissemination of the genetic progress is carried out on multiplication farms in which F1 individuals are produced by crossing boars and sows from two distinct dam lines. In a second step, once already in the production farms, these F1 sows are crossed again with a boar from a sire line to produce the F2 offspring which will be used for fattening until slaughter (Dekkers et al., 2011).

The crossbreeding performed in these schemes includes genetic lines with complementary abilities in order to exploit both complementarity between traits and heterosis. Landrace and Large White breeds are commonly used for dam lines and Pietrain and Duroc for sire lines. The terminal sire largely influences carcass and meat quality attributes of F2 crossbred pigs (Suzuki et al., 2003; Mortimer and Przybylski, 2016). For this reason, Pietrain sires are very popular in breeding schemes aimed to produce carcasses with high lean percentage. In contrast, Duroc sires, where some lines are well known to show a good balance between growth rate and IMF, are increasingly used in markets, as in the dry-cured domain, where meat quality is a requirement. Moreover, the Duroc-based lines have also been favorably associated to higher ultimate pH, juiciness, tenderness, redness colour and lower drip loss as compared to other breeds (Suzuki et al., 2003; Meinert et al., 2008; Lee et al., 2012; Choi et al., 2016).

Duroc sows are also known to have high prolificacy and are consequently used in crosses as a maternal line (Alonso et al., 2015).

Traditionally, the genetic evaluation of pigs relied on phenotypic data and pedigree information only. In the late 80's, the BLUP (Best Linear Unbiased Prediction) methodology represented a breakthrough in the process of swine breeding (Hill, 2014; Jonas and de Koning., 2015). This approach takes advantage of using both own and all family records to predict the genetic merit of a candidate, known as the estimated breeding value (EBV). Hence, that in all livestock species BLUP has been the method of choice. However, with the development of molecular genetics from the 1990s, new opportunities arose to overcome the limitations to the improvement of traits showing low heritabilities or costly to record because either they can only be measured after slaughter (i.e. meat quality traits) or simply they are not feasible to measure in commercial conditions (i.e. disease resistance) (Dekkers, 2007). Also, molecular genetics may help to break the genetic correlation between antagonistic traits, as for instance the correlation between lean content and IMF.

### **3.1 Genetic markers and biomarkers selection**

The initial uses of molecular information on breeding programs were based on a few genetic markers or quantitative trait loci (QTL) underlying the traits of interest, the so-called marker-gene assisted selection (MAS or GAS, respectively). In its most refined version, the QTL information was included into BLUP jointly with phenotypic data (Meuwissen et al., 2016). The detection of the QTL used in MAS was done by means of ad hoc experiments. The QTL mapping experiments identified a large number of QTLs among all chromosomes, but only for some major QTLs the causative mutation was found. Several factors may have influenced on the low success of these studies, but likely the most important is that most QTL detection analyses were conducted on experimental crosses using reduced sample sizes and a low-density genetic maps. Furthermore, most of the important traits are regulated by many genes with small effects (Davoli and

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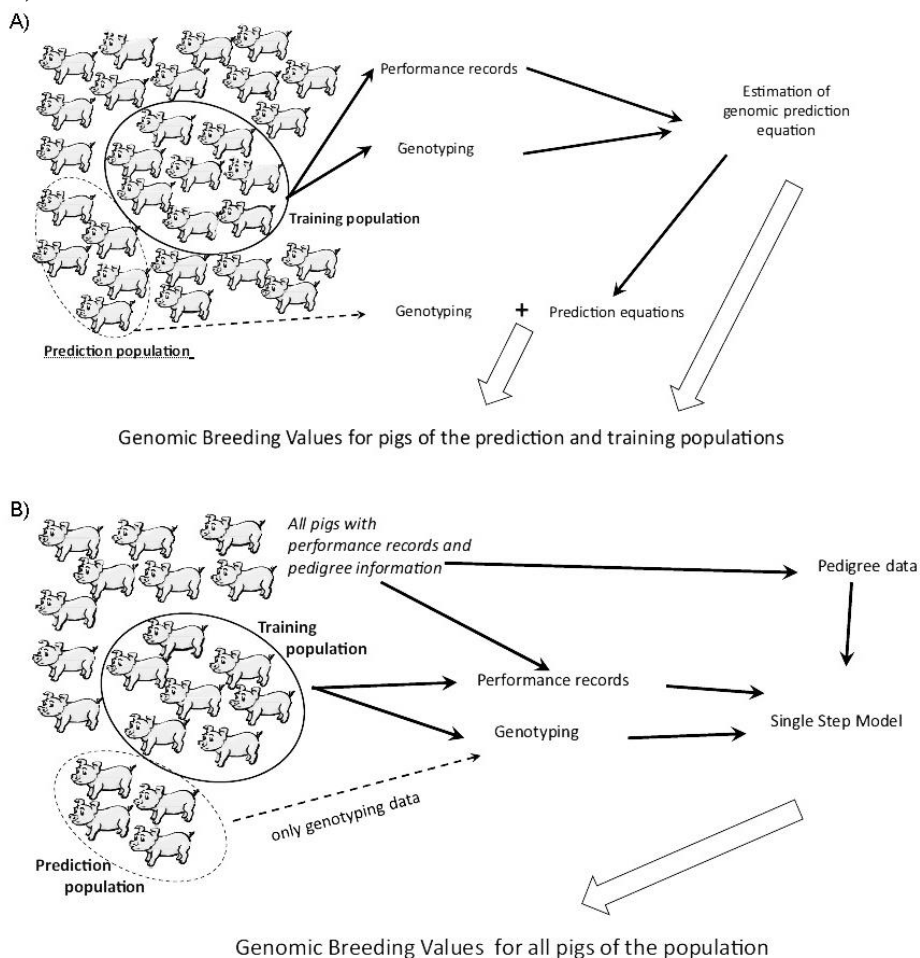
Braglia, 2007; Meuwissen et al., 2016), which makes more difficult to unravel the effect of individual genes or closely genetic markers in linkage disequilibrium. Given the difficulties of finding enough reliable markers, MAS has not reached the initial proposed achievements. However, the new throughput technologies allowed genotyping cost-effectively thousands of genetic markers at a time, especially single-nucleotide polymorphisms (SNP) and, as a result, the application of a genome-wide assisted selection.

In addition to genetic markers, selection can be performed based on any other piece of biological information. Biomarkers are body metabolites such as hormones, lipoproteins, carbohydrates or fatty acids that indicate a given state of a biological process. They can capture part of the variability of a trait of interest and, as such, they can be useful for understanding and selecting traits that are difficult to improve using regular phenotypic records. For instance, biomarkers associated specifically with IMF or BT can help improving one of them without altering the other or simply to have records on the own candidate that are difficult to obtain *in vivo*. In this thesis, the term genetic biomarkers has also been taken as synonymous of molecular markers in the sense that it includes both genetic markers and biomarkers subjected to genetic regulation.

### **3.2 Genomic selection**

Genomic Selection has undoubtedly been the second major conceptual change in pig breeding evaluation after BLUP. It was proposed as a way to predict the genetic merit of individuals using massive high density genotyping data (Meuwissen et al., 2001). Genomic selection has had a tremendous impact in the pig industry, so that in the last years traditional EBV have been replaced by genomic EBV (GEBV). In pure genomic selection, a training population is phenotyped and genotyped for thousands of SNPs in order estimate the individual SNP effects, which then are used to predict the GEBV of pigs that only are genotyped (Figure 3A). However, with this approach non-genotyped pigs are excluded as selection candidates even if they are phenotyped for some traits.

To solve this problem, a variation of the initial scheme, known as single-step BLUP, has been proposed with the purpose of calculating the GEBV from using all available data, whether genotypic or phenotypic (Legarra et al., 2009) (Figure 3B).



**Figure 3.** Schematic overview of the logistics behind genomic selection (Adapted from Samore and Fontanesi, 2016)

Genome-wide SNP panels are available for most of the livestock species. To date, four SNP panels have been commercialized in porcine (Table 2). The Porcine SNP60 BeadChip (Illumina, Santa Clara, CA, USA) was the first one to reach the market (Ramos et al., 2009) and covers 60K SNPs spread over all the chromosomes. A limitation of this array is that it does not include the most well-



known mutations affecting economic traits as well as sequence variations only observed in local breeds. To overcome these limitations, GeneSeek/Neogen company updated the Illumina array with a 70K SNP panel including relevant SNPs to the industry and removing SNP that had a very low minor allelic frequency or that generally genotyped badly (GGP-Porcine LD). With the purpose of reducing genotyping costs, 10K low-density panels have also been developed. On the other hand, Affymetrix has recently released a novel porcine SNP genotype array that covers 650K markers including the 56K already existing in the Illumina panel and some other from local European and Asian breeds (Groenen et al., 2015). Besides its use in genomic selection, the SNP panels have facilitated the detection of new QTLs through genome-wide association studies (GWAS), thereby providing new insights into the genetic basis of phenotypic variation of target traits (Plastow et al., 2005).

**Table 2.** SNP genotyping panels commercially available on pig.

Chip name	No. of SNPs	Company	Technology
PorcineSNP60 BeadChip v2 array	61.565	Illumina	Illumina Infinium chemistry
Genomic Profile for Porcine HD (GGp-Porcine LD)	10.241	GeneSeek/Neogen	Illumina Infinium chemistry
Genomic Profile for Porcine HD (GGp-Porcine HD)	68.528	GeneSeek/Neogen	Illumina Infinium chemistry
Axiom Genome-Wide Pig genotyping Array	~650.00	Affymetrix	Axiom assay

Adapted from Samore and Fontanesi, 2016

Genomic selection has been extremely effective in cattle, where it enables for a reliable genetic evaluation of young bulls and thus to decrease the generational interval to 1.5 years, as compared with the 5-6 years of traditional selection (Pryce and Daetwyler, 2012). As a result, approximately 2 million of dairy cattle worldwide have been genotyped for selection purposes (Meuwissen

et al., 2016). In contrast, the application of genomic selection in porcine is not so straightforward and likely less cost-effective, since in each generation there is a large number of individuals to genotype and the generation interval is much shorter. Moreover, the breeding schemes combine maternal and paternal lines, involving crossbred individuals at the commercial level.

In general, the linkage disequilibrium in pigs is higher as compared to other species. If linkage disequilibrium is high, then a lower number of markers are needed to capture genetic variation. In this situation, a representative tag-SNP per haploblock suffices for selection (Pena et al., 2016) and imputation purposes. An option to reduce the number of SNPs genotyped and thus genotyping cost is to impute a high-density genotype from a low-density panel (Huang et al 2012; Cleveland and Hickey, 2013). A simulation study on a sire line showed that an interesting strategy is to combine imputation with high-density genotyping of a limited number of pre-selected candidates (Tribout et al., 2013). Our research group compared different approaches for the genetic evaluation of IMF and fatty acid composition and found that results from genotyping a few set of animals with a high-density SNP panel were comparable, if not better, to those obtained with BLUP plus two markers of relevant effect (Pena et al., 2016). In overall, this result highlights the idea that a reasonable strategy for improving meat quality traits would be to develop a customized low-density SNP panel in conjunction with a cost-effective recording scheme.

### **3.3 Selection for IMF content and fatty acid composition**

Although farmers are mostly paid for lean weight, the growing importance of meat quality traits for consumers has led to incorporate them in the selection objective of some sire lines. This has been particularly true for IMF content and fatty acid composition, especially C18:1, in the Duroc lines used for the production of dry-cured products and in the Iberian breed, where these two traits can be critical to achieve the highest marketing standards. It is well known that IMF displays a slower relative growth than subcutaneous fat. The

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relationship between these two traits is positive although breed-dependent, with Duroc pigs having a greater IMF at a lower BT (Wood et al., 2004). According to this positive relationship, available studies show that selection for IMF is possible but at the expense of increasing fatness. Thus, on a six-generation experiment on Duroc pigs, Schwab et al. (2009) found that, selection for IMF measured with real-time ultrasounds, increased IMF but also BT while no major changes on growth performance were observed.

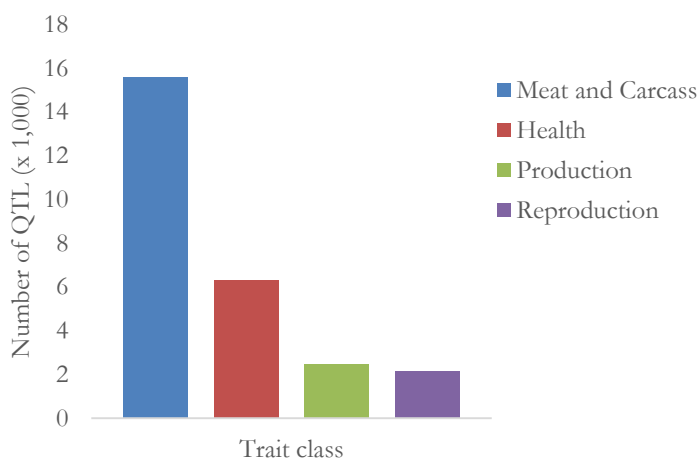
In the Duroc line used in this thesis, the genetic correlation between IMF and oleic acid is positive (0.47) and the genetic correlations between IMF and body weight and BT are 0.27 and 0.37, respectively (Ros-Freixedes et al., 2012). With these genetic parameters, it is shown that there are selection strategies where IMF, MUFA and lean weight could be improved simultaneously (Solanes et al., 2009; Ros-Freixedes et al., 2012). In Duroc breed, three selection experiments are available. On the former, performed by Suzuki et al. (2005b), pigs were selected during seven generations for an index which included body weight, BT and IMF. Results showed that IMF can be increased while BT is constrained, but not reduced (Suzuki et al., 2005). The second, performed by Ros-Freixedes et al. (2013), was designed to test the opposite, to reduce BT when IMF is fixed at the optimum value. In this experiment, consisting of three generations, selected pigs had increased lean weight but lower IMF, although a higher decrease was expected without the restriction. The last was designed to investigate the response of fatty acids to selection (Ros-Freixedes et al., 2014). In that study, pigs selected for increased C18:1 had more IMF, while no changes were found in body weight.

As a whole, these works evidence that although selection for IMF and lean growth can be feasible, it is not easy to achieve, particularly in lines of small size and with limited phenotyping for IMF and fatty acids. This can be partly overcome with the use of genetic biomarkers that specifically affect BT, IMF or fatty acid composition. Since increasing either phenotyping or genotyping is costly, new strategies should consider the number of animals to be phenotyped

and genotyped as well as the methods utilized. In addition, prospective investments in this area should be done in line with the price that consumers are willing to pay for premium products.

#### 4. Genetic markers affecting meat quality traits in pigs

The advancement of genetic maps has facilitated the identification of markers associated with many traits. Since 2004, all publicly available QTL of livestock animal species are stored in the Animal QTL database (QTLdb; <http://www.animalgenome.org/QTLdb>). The Pig QTL is a subset database within the QTLdb devoted to the description of QTL in the pig genome. It currently gathers 28,720 QTL extracted from 646 publications, representing 677 traits (accessed January 2019), classified in different categories as shown in Figure 4



**Figure 4.** Distribution of QTL by pig trait classes (thousands of QTLs) (PigQTLdb; <https://www.animalgenome.org/cgi-bin/QTLdb/index>; accessed January 2019).

Regarding carcass and meat quality traits, a large number of QTL have been described, which highlights the importance of these traits for researchers of the pig industry. The first QTL for abdominal and backfat was described by Andersson et al. (1994) on chromosome 4. It was designated FAT1 and replicated

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in many pig populations. However, the causal QTN mutation has yet to be identified. Currently, the 15,581 QTLs for these and other carcass and meat quality traits have been mapped on almost every chromosome. In particular, 3,027 QTLs have been identified for fat deposition and a total of 652 and 5,723 QTLs for IMF and fatty acid composition, respectively.

Regarding meat quality traits, multiple studies reporting QTLs with effect on IMF (Ovilo et al., 2002; Uleberg et al., 2005; Mohrmann et al., 2006; Quintanilla et al., 2011) and on fatty acid composition (Clop et al., 2003; Guo et al., 2009; Ros-Freixedes et al., 2016) have been carried out. As commented above, despite of the large number of QTL identified, only a few have been assessed for the identification of segregating genetic markers and its association with relevant traits, some of which are highlighted in Table 3.

In this context, several genes related to growth, fatness and carcass composition have been identified. Among these genes, stands out Insulin like growth factor 2 (*IGF2*) and Melanocortin 4 receptor (*MC4R*), responsible for mutations IGF2-G3072A and Asp298Asn, respectively, being the first polymorphisms with strong correlation with leanness and feed intake in the porcine species (Kim et al., 2000; Van Laere et al., 2003). The polymorphism on *MC4R* gene is included on the Axiom Genotyping array. Moreover, although *IGF2* is affected by imprinting, and only the paternal allele is active, what difficults its application on pig breeding schemes, a test has been developed by the Gentec company.

Within this group of genes, the leptin receptor (*LEPR*) should be also highlighted. The *LEPR* is a mediator of the satiety effect of the LEP (Barb et al., 2001) and thus, the corresponding gene is considered a candidate gene for traits related to growth and body composition. On an experimental Iberian x Landrace F2 population, a mutation located on *LEPR* (g.1987C>T) was associated to BT at different locations (Oviló et al., 2005). Posterior studies have reported effects of this polymorphism on traits like feed intake and average daily gain (Rodríguez

et al., 2010; Hirose et al., 2014). Works from our research group on Duroc pigs showed that the polymorphism is associated to carcass fattening in both BT and IMF (Gol et al., 2015; Ros-Freixedes et al., 2016).

Other important functional and positional candidate genes are those encoding the Perilipin (PLIN) family proteins. Perilipin proteins localize on the cytoplasmatic membrane of lipid droplets in all cells of the body and regulate the access of lipases to stored triacylglycerides (Kimmel et al. 2010). On porcine, the genes encoding for two members of this family, *PLIN1* and *PLIN2*, are located in regions where QTL for growth and quality meat have been reported (Geldermann et al., 2003; Kim et al., 2005). As in pig this family of genes and proteins have not been widely studied, one work included in this Thesis has analyzed the effect of the porcine PLIN on growth, carcass and meat quality traits to corroborate the little information available to date.

One of the most promising gene affecting meat quality is the gene encoding for the enzyme Stearoyl-CoA desaturase (*SCD*). In this line, our research group identified a haplotype of three polymorphisms in the proximal promoter of the pig *SCD* gene with impact on the percentage of SFA and MUFA without affecting the amount of PUFA (Estany et al., 2014). This effect is mainly due to a higher content of C16:1 and C18:1 synthesised from C16:0 and C18:0. Moreover, this mutation improves the proportion of C18:1 content without increasing fat deposition in BT or in IMF. Currently, this marker is being used for selective breeding in pigs.

Together with *SCD* and  $\Delta$ -5 desaturase, the  $\Delta$ -6 desaturase, encoded by fatty acid desaturase-2 (*FADS2*) gene, is other important desaturase in mammals. The  $\Delta$ -6 desaturase catalyses the limiting step on the synthesis of long-chain PUFA. In porcine, recent GWAS studies (Ros-Freixedes et al., 2016; Zhang et al., 2016) pigs have found evidence of association between markers in the region where *FADS2* is located with IMF content and the long chain PUFA. For this reason, in this thesis, a chapter is dedicated to explore in great detail the role of *FADS2* to the content and composition of fat. Specifically, we have investigated

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the association of *FADS2* variation with the fatty acids involved in the biosynthesis of C20:4 from C18:2.

Finally, from the candidate genes affecting disease resistance, a progress is being made in identifying genetic markers associated with reduced Porcine Reproductive and Respiratory Syndrome (PRRS). Among the genes proposed as responsible for lesser susceptibility to the virus, only some have been studied in detail (Ren et al., 2012; Wang et al., 2012). In 2012, the rs80800372 polymorphism on the Interferon-induced guanylate-binding protein 1 gene (*GBP1*), related to the control of the innate immune response, was associated to lower viremia and higher growth after infection (Boddicker et al., 2012). On a previous work, Abella et al. (2016) showed that the favorable allele at the rs80800372 SNP was associated to decreased average daily gain in uninfected pigs. Thus, in this thesis, we have investigated the impact of this polymorphism on the functionality of *GBP1* as well as its correlated effects on a range of meat quality and carcass traits.

Although the causality of rs80800372 SNP is not proven, the company Topics Norsvin has recently implemented the use of this polymorphism to select for increased natural resistance to PRRS (<https://topignorsvin.com>; accessed January 2019). Regarding the research against the PRRS, the major breakthrough has been the generation of gene-edited pigs lacking the cell receptor needed for the infection. This edited pigs have been generated through a long-standing collaboration between the Genus company and the University of Missouri (<http://www.genusplc.com>; accessed January 2019). The company is continuing to develop and trying to commercialize this technology.

**Table 3.** Main candidate genes whose mutations affect pig production.

<b>Trait</b>	<b>Candidate gene</b>	<b>Acronym</b>	
Coat Colour	Mast/stem cell growth factor	<i>KIT*</i>	
	Melacortin 1 receptor	<i>MC1R</i>	
Growth, fatness and carcass composition	Growth hormone	<i>GH</i>	
	Growth hormone-releasing hormone	<i>GHRH</i>	
	Insulin like growth factor 2	<i>IGF2*</i>	
	Leptin Receptor	<i>LEPR</i>	
	Leptin	<i>LEP</i>	
	Melacortin 4 receptor	<i>MC4R*</i>	
	Myostatin	<i>MSTN</i>	
	Perilipin	<i>PLIN</i>	
	Meat quality	Calpastain	<i>CAST*</i>
		Fatty acid elongase 6	<i>ELOVL6</i>
Phosphoenolpyruvate carboxykinase 1		<i>PCK1</i>	
Ryanodine receptor 1		<i>RYR1*</i>	
Fatty acid desaturase 2		<i>FADS2</i>	
Stearoyl CoA desaturase		<i>SCD*</i>	
Fatty acid binding protein3		<i>FABP3*</i>	
Protein kinase subunit gamma 3		<i>PRKAG3*</i>	
Litter size	Estrogen receptor 1	<i>ESR1</i>	
	Follicle-stimulating hormone beta	<i>FSHB</i>	
	Retinal binding protein 4	<i>RBP4</i>	
Disease resistance	Prolactin receptor	<i>PRLR</i>	
	Fucosyltransferase 1	<i>FUT1*</i>	
	Interferon-Inducible guanylate-binding protein	<i>GBP1*</i>	

\*Genetic markers used by the industry (adapted from Rothschild, 2010 and Ersnt and Steibel., 2013)



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## 5. Future trends

The rapid evolution of new genomic technologies has provided new opportunities to researchers, particularly for the investigation of genes affecting underlying molecular pathways affecting traits of interest. An important milestone in this direction has been the availability of the pig genome, first sequenced in 2009 (Archibald et al., 2010). The latest version of the pig genome (called Sscrofa 11.1) is available in the Ensemble database and comprises 22,452 encoding genes, 49,448 transcripts, 3,250 non-coding genes and 178 pseudogenes. Also, there is information about more than 64 million short variants, including SNPs, insertions and deletions (<http://www.ensembl.org>, accessed January 2019). Efforts from the Functional Annotation of Animal Genomes (FAAG) initiative are and will contribute to improve the annotation and description of variation on the pig genome and epigenome.

Apart from focusing on DNA markers linked to traits, attention has been paid to large-scale gene expression profiles, gene clusters and networks. For instance, one of the first methods for global gene expression analysis were the expression arrays, which allowed to quickly analyse the gene expression of many genes in one single reaction (Pena et al., 2014). A particularity of these arrays is the possibility to create customized gene expression experiments. Several studies used this technology to study the IMF content and composition in pigs (Cánovas et al., 2010; Hamill et al., 2013; Yu et al., 2013). The significant drop in next-generation sequencing costs has popularised transcriptome sequencing (RNA-seq) as the mean to describe expression and sequence variability in a single experiment. Future trends regarding this technology include the sequencing of full transcripts in one single read and the development of amplification-free libraries.

The OMICs sciences extend to other fields including epigenomics, proteomics and metabolomics (Mackay et al., 2009). They enable to measure more specific and underlying phenotypes, although the major challenge is still

how integrate them into new genetic knowledge and selection procedures for target traits (Civelek and Lusk, 2014). To ensure that this happens, appropriate computational and bioinformatics approaches need to be developed as well (Ritchie et al., 2015).

Finally, advances in technology should also result in new or more cost-efficient phenotyping methods. In the context of the traits investigated in this thesis, the use of near infrared spectroscopy (NIRS) or advanced imaging technologies are promising options for taking a step further on live (Matika et al., 2016) or on-line determinations at the slaughter chain (Prieto et al., 2017).

These technologies will be useful to face the increasingly demand for high quality protein. Tendencies highlight that consumers expect healthy meat produced by sustainably raised animals (Kristensen et al., 2014; Henchion et al., 2014). Regarding the nutritional value of meat, emphasis will be made not only on the composition of fatty acids but also on the contribution of meat on vitamins and micronutrients consumption, some of which, such as iron and zinc, are not dependent on animals diet (De Smet and Vossen, 2016).

## **6. Thesis dissertation**

The studies included in the present thesis are part of a line of research on the genetic improvement of pig meat quality, with particular interest to IMF content and composition. The main goal of this line of research is to find new selection criteria contributing to obtain pigs displaying an optimal balance among IMF, fatty acid composition and lean content.

Specific biomarkers for IMF, fatty acid composition or lean weight, and in particular genetic markers, can be a useful tool to refine the selection criteria and therefore to enhance meat quality without undesirable correlated responses. A handful of genetic markers associated with meat quality traits have already been identified, including some segregating in the Duroc line used in this thesis, such

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as those at the *SCD* and *LEPR* genes with effects on fat content and fatty acid composition.

The studies presented here are a continuation of previous work on the search for biomarkers associated with meat quality carried out by the group of Animal Breeding of the University of Lleida-Agrotecnio Center. Specifically, the research carried out has been developed in the framework of the research projects AGL2012-33529 and AGL2015-65846-R (Ministerio de Economía y Competitividad, Spain). In particular, DNA and tissue samples were from the UdLGIM biobank, which contains around 1,200 genealogically referenced Duroc pigs from the same line with records on body weight, fat content and fatty acid composition.

# **OBJECTIVES**

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This thesis has been developed in the frame of two research projects funded by the Spanish Ministry of Economy and Competitiveness and the European Union Regional Development Funds (AGL2012-33529 and AGL2015-65846-R grant).

**General goal:**

The main objective of this thesis was to identify genetic biomarkers associated with intramuscular fat content and fatty acid composition, and then to assess their potential as selection criteria in a purebred Duroc line used for producing high-quality meat products.

**Specific goals:**

- 1- To estimate the heritability of the fatty acids involved in the linoleic acid metabolism pathway and the genetic correlation amongst them and with intramuscular fat and lean growth.
- 2- To search sequence variations in the promoter of fatty acid desaturase-2 gene in order to investigate their association with fat content, fatty acid composition and lean growth.
- 3- To examine the effect of identified polymorphisms in the perilipin 1 and perilipin 2 genes on a range of performance, carcass and meat quality traits.
- 4- To determine the functionality of a polymorphism in the guanylate-binding protein-1 gene with effects on both the porcine reproduction and respiratory syndrome virus load and weight gain, assessing in particular whether it is also related to intramuscular fat content and composition.



## **ANIMALS AND PROCEDURES**





## 1. Animals and pork samples

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida (Agreement 2/01, March 2001). All pigs were raised and slaughtered in commercial units following applicable regulations and good practice guidelines on the protection of animals kept for farming purposes, during transport and slaughter.

Animals and meat samples belong to a purebred Duroc line (Selección Batallé, Riudarenes, Girona, Spain) closed in 1991 and selected for an index including body weight, BT and IMF with the primary objective of producing premium pork and high quality-dry cured hams. Pedigree-connected pigs from this line were performance-tested for BW and BT at different ages. Backfat thickness was ultrasonically measured at 5 cm off the midline at the position of the last rib (Piglog105, Fontmatek, Denmark). After slaughter at around 210 days, the carcass weight and length, the carcass backfat and loin thickness, and the ham weight were measured. Carcass backfat and loin thickness at 6 cm off the midline between the third and fourth last ribs, together with the carcass lean percentage, were estimated using an on-line ultrasound automatic scanner (AutoFOM; SFK-Technology, Denmark). Immediately after slaughter, samples of the semimembranosus muscle, subcutaneous adipose tissue and liver were collected, snap-frozen, and stored at -80°C. After chilling for about 24 h at 2°C, samples of the muscles gluteus medius (GM) and longissimus thoracis (LM) were collected, vacuum packaged, and stored at -20°C.

## 2. Determination of fat content and composition

A representative aliquot from a pulverized freeze-dried sample was used to determine, in duplicate, the individual fatty acid by gas chromatography (Bosch et al., 2009). In brief, fatty acids methyl esters were directly obtained by transesterification using a solution of 20% boron trifluoride in methanol (Rule, 1997). Methyl esters were determined by gas chromatography using a capillary column SP2330 (30 m × 0.25 mm; Supelco, Bellefonte, PA) and a flame

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ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225°C at 7°C/min and injector and detector temperatures were both 250°C. The quantification was carried out through area normalization with an external mixture of fatty acids methyl esters (Supelco 37 Component FAME Mix. Sigma, Tres Cantos, Madrid). The internal standard was 1,2,3-tripentadecanoylglycerol. Then, the amount of each fatty acid was expressed either in absolute (mg/g of dry muscle) or in percentage to total fatty acids. The complete profile for each sample included saturated (SFA: C14:0; C16:0; C18:0; and C20:0); monounsaturated (MUFA: C16:1n-9; C18:1n-7; C18:1n-9; and C20:1n-9); and polyunsaturated (PUFA: C18:2n-6; C18:3n-3; C20:2n-6; and C20:4n-6) fatty acids. The fat content was calculated as the sum of the individual fatty acids expressed as triglyceride equivalents (AOAC, 2000) on both wet and dry tissue basis.

### 3. Isolation of genomic DNA and genotyping

The isolation of genomic DNA was carried out from muscle samples stored at - 80°C. Samples were lysed in the presence of proteinase K and DNA was purified through extraction with phenol:chloroform, followed by ethanol precipitation. Finally, DNA was re-suspended and stored in TE buffer. The quantification and estimation of the quality and purity of genomic DNA was performed using a Nanodrop- 1000 spectrophotometer, considering an A260/A280 ratio of 1.8-2.2 as acceptable. DNA integrity was tested through electrophoresis in a 1% agarose gel. Pigs were genotyped for *FADS2* rs321384923A>G (Chapter II), *PLIN1* JN860199:g.173G>A and *PLIN2* GU461317:g.98G>A (also referred as rs333231747G>A) (Chapter III) and *GBP1* rs80800372G>A (Chapter IV). Moreover, pigs were genotyped for the *LEPR* NM\_001024587:g.1987C>T and the SCD AY487830:g.2228T>C SNP, which effects were considered when analyzing each SNP commented above.

The SNPs on *LEPR* and *PLIN2* were genotyped by High Resolution Melt analysis (Luminaris Color HRM Master Mix, Thermo Scientific) in a real time thermocycler (QuantStudio 3 qPCR, ThermoFisher) using 10 ng of genomic DNA and 0.4  $\mu$ M of primer mix in a final volume of 5  $\mu$ l (Table 1). The SNPs on *SCD* and *GBP1* were genotyped using an allelic discrimination assay in a reaction mix that contained 1x Universal TaqMan master mix (LifeTechnologies, USA), 0.9  $\mu$ M Primer mix, 0.2  $\mu$ M Probe mix and 10 ng of DNA. Cycling conditions were 95° 10 min and 40 cycles at 95°C 15 sec and 60°C 1 min. When performing High Resolution Melt, cycling conditions were followed by melt curve.

RFLP-PCR genotyping protocols were set up to genotype the SNPs on *FADS2* and *PLIN1*. PCRs were carried out in 13  $\mu$ L reactions containing 60 ng of genomic DNA, 1x buffer, 0.2  $\mu$ M of dNTP mix, 2 mM of MgCl<sub>2</sub>, 0.5  $\mu$ M of each primer and 1 U of Taq polymerase (Bioline). Thermocycling conditions were 95°C 10 min, 35 cycles at 95°C 20 sec, 56°C 20 sec for *FADS2* SNP or 60°C 20 sec for *PLIN1* SNP and 72°C 20 sec finishing with 72°C 5 min. Ten  $\mu$ l of PCR were digested with *Ava*I (*FADS2*) or *Hin*1III (*PLIN1*) and solved by electrophoresis in agarose gels.

**Table 1.** Primers used for the genotyping of the single nucleotide polymorphisms in the porcine genes analyzed in this thesis.

Gene	SNP	Primer name	Sequence 5'→3'
<i>FADS2</i>	rs321384923A>G	Forward	ACCCCCACCTTTATTTCCCTG
		Reverse	TTGCTTTCGGCTTTTGTCTT
<i>PLIN1</i>	JN860199:g.173G>A	Forward	AGGGAAGTGTATGGTGTAGAGG
		Reverse	CAGGCTGGGTATTTAAAGGCTAGAG
<i>PLIN2</i>	GU461317:g.98G>A	Forward	GAGCC TAGCCAGTTCTCTGTG
		Reverse	CATGCAATGTGAGACAAACC
<i>GBP1</i>	rs80800372G>A	Forward	AGACCTAGAATCTCCACAGAATTTCCA
		Reverse	GGAAAGGACAGTTTCGCTTCTCTAG
		Probe A allele	VIC-CTGGGTGATAAATAAAT-NFQ
		Probe G allele	FAM-TGGGTGATGAATAAAT-NFQ
<i>SCD</i>	AY487830:g.2228T>C	Forward	CCCTTCTTGGCAGCGAATAAAA
		Reverse	CAGGCTGGGTATTTAAAGGCTAGAG
		Probe C allele	VIC-CGACCGTGTCTCTGTATT-NFQ
		Probe T allele	FAM-CGACCGTATCTCTGTATT-NFQ
<i>LEPR</i>	NM_001024587:g.1987C>T	Forward	CAGAGGACCTGAATTTTGGAG
		Reverse	CATAAAAATCAGAAATACCTTCCAG

#### 4. Isolation of RNA and gene expression

Gene expression on Chapter II and Chapter IV was performed as follows. RNA was isolated with TRI-Reagent (Sigma-Aldrich) following the manufacturer's indications. Purity of the RNA was assessed by spectrophotometry with a Nanodrop-1000 and the integrity was tested by electrophoresis in agarose gels. *FADS2* or *GBP1* and two reference genes, *YWHAZ* and *RPL32*, were analyzed by a quantitative PCR assay (Table 2). Briefly, 2 µg of total RNA were reverse transcribed using SuperScript IV Reverse Transcriptase (Invitrogen) with oligo-dT and random primers. Real-time PCR assays were carried out in triplicate in 8 µl reactions, containing 1x iTaq Universal SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and 3 µl cDNA

template diluted 1:30 in water. Cycling parameters followed a melt curve. To quantify and normalize expression data, we used the  $\Delta\Delta C_t$  method against the geometrical mean of the two reference genes.

**Table 2.** Primers used in this thesis for the analysis of gene expression.

Gene	Primer name	Sequence 5'→3'
<i>YWHAZ</i>	Forward	TGATGATAAGAAAAGGGATTGTGG
	Reverse	G TTCAGCAATGGCTTCATCA
<i>RPL32</i>	Forward	CACCAGTCAGACCGATATGTCAA
	Reverse	CGCACCCCTGTTGTCAATGC
<i>FADS2</i>	Forward	GCTGGATTCCAACCCTCATG
	Reverse	AGCCTGGGCCTGAGAGGTA
<i>GBP1</i>	Forward	TGGCTGAGAAGATGGAGAAG
	Reverse	TCCTGAATTAGTCGGGCTTG



## CHAPTER I

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# LINOLEIC ACID METABOLIC PATHWAY ALLOWS FOR AN EFFICIENT INCREASE OF INTRAMUSCULAR FAT CONTENT IN PIGS

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## Abstract

### Background

Intramuscular fat (IMF) content is a relevant trait for high-quality meat products such as dry-cured ham, but increasing IMF has the undesirable correlated effect of decreasing lean growth. Thus, there is a need to find selection criteria for IMF independent from lean growth. In pigs, the proportion of linoleic (C18:2) and arachidonic (C20:4) acids decline with fat deposition and therefore they can be considered as indicators of fatness. The aim of this research was to estimate the genetic variation for C18:2 and C20:4 in IMF and their genetic correlations with IMF and lean growth traits, with the objective to assess their potential as specific biomarkers of IMF. The analysis was conducted using a full-pedigreed Duroc resource line with 91,448 records of body weight and backfat thickness (BT) at 180 days of age and 1,371 records of fatty acid composition in the muscle gluteus medius.

### Results

The heritability estimates for C18:2 and C20:4 in IMF, whether expressed in absolute (mg/g of muscle) or in relative (mg/g of fatty acid) terms, as well as for their ratio (C20:4/C18:2), were high ( $> 0.40$ ), revealing that the C18:2 to C20:4 pathway is subjected to substantial genetic influence. Litter effects were not negligible, with values ranging from 8% to 15% of the phenotypic variance. The genetic correlations of C18:2 and C20:4 with IMF and BT were negative (-0.75 to -0.66, for IMF, and -0.64 to -0.36, for BT), if expressed in relative values, but almost null (-0.04 to 0.07), if expressed in absolute values, except for C18:2 with IMF, which was highly positive (0.88). The ratio of C20:4 to C18:2 also displayed a high heritability (0.50) and a stronger genetic correlation with IMF (-0.59) than with BT (-0.10).

### Conclusions

The amount of C18:2 in muscle can be used as an IMF-specific biomarker. Selection for the absolute amount of C18:2 is expected to deliver a

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similar response outcome as selection for IMF at restrained BT. Further genetic analysis of the C18:2 metabolic pathway may provide new insights into differential fat deposition among adipose tissues and on candidate genes for molecular markers targeting specifically for one of them.

## 1. Introduction

Linoleic acid (C18:2) is a major ingredient of feeds and the most abundant PUFA in pig adipose tissue and muscle (Ros-Freixedes and Estany, 2014). Since pigs are not able to synthesize C18:2, its amount in tissue is highly correlated with dietary intake. Of all fatty acids, C18:2 shows the greatest tissue response to dietary levels (Nguyen et al., 2003). In the cells, C18:2 can be either elongated to eicosadienoic acid (C20:2) or transformed into arachidonic acid (C20:4) (Nakamura and Nara, 2004). Thus, although C20:2 and C20:4 are sourced from diet, they can also be endogenously synthesised. The proportion of C18:2 and C20:4 in pig adipose tissue and muscle declines with fat deposition (Ros Freixedes and Estany, 2014), a phenomenon that has been explained by the relative lower concentration of dietary fatty acids in adipose cells as *de novo* synthesis of FA progresses. For this reason, the content of C18:2 in adipose tissue content has been considered as an indicator of fatness (Wood et al., 2008).

Intramuscular fat (IMF) content and fatty acid composition are relevant traits for high-quality Mediterranean meat products such as dry-cured ham. Increasing IMF has the undesirable correlated effect of decreasing lean growth, so that, in this scenario, a common commercial target is to find selection criteria for IMF independent from lean growth (Estany et al., 2017). Although substantial genetic variation between (Wood et al., 2004) and within (Ntawubizi et al., 2009; Gjerlaug-Enger et al., 2011) genetic types for fatty acid composition has been reported, the potential of C18:2 and its long chain products as specific indicators of IMF has not yet been fully addressed. Thus, the aim of this study was to estimate the genetic relationships of C18:2, C20:2 and C20:4 in IMF with lean

growth traits in a purebred Duroc population and then to discuss their potential use as a means to direct selection solely for IMF.

## **2. Methods**

### **2.1 Animals and sample collection**

Data from a purebred Duroc line (Selección Batallé, Riudarenes, Girona, Spain) were used for the analyses (Solanes et al., 2009; Ros-Freixedes et al., 2012). The line was completely closed in 1991 and since then has been selected for an index including body weight (BW), backfat thickness (BT), and IMF. The data set used for the estimation of genetic parameters consisted of 162,494 pedigree-connected pigs, from which 91,525 had at least 1 recorded trait (Table 1). At about 75 days of age pigs were moved to the fattening units, where they were allocated by sex in pens of 8 to 12 individuals and were given ad libitum access to commercial diets. Pigs were performance-tested at an average age of 177 d for BW and BT. Backfat thickness was ultrasonically measured at 5 cm off the midline between the third and fourth last ribs using the portable equipment Piglog 105 (Frontmatec, Kolding, Denmark). Since 2002, 1,371 purebred barrows used for producing dry-cured ham were sampled to record IMF content and fatty acid composition in gluteus medius muscle. These barrows were raised in 23 batches to slaughter at around 215 days of age. From 160 days of age they were fed a finishing diet (Esporc, Riudarenes, Girona, Spain) including around 6.0% fat (27% C18:2 and 0.3% C20:4 of total FA). All barrows were slaughtered in a slaughterhouse equipped with a carbon dioxide stunning system (Butina ApS, Holbaek, Denmark). After chilling for about 24 h at 2°C, a sample of at least 50 g of the gluteus medius muscle was taken from the left side ham. Muscle samples were immediately vacuum packaged and stored at -20 °C until required for IMF and fatty acid determinations. The number of records, sires, dams, and litters used for each analyzed trait is given in Table 1.

**Table 1.** Description of the data set used in the analyses

Item	No. of pigs	No. of sires	No. of dams	No. of litters	Mean	Standard deviation
Pedigree	162,494	1,032	32,767	38,253	-	-
Trait <sup>a</sup>						
Body weight at test, kg	91,448	670	22,297	38,226	105.4	12.4
Backfat thickness at test, mm	91,061	670	22,256	38,109	16.1	3.4
Intramuscular fat, %	1,371	179	752	755	4.9	1.9
C18:2, mg/g FA	1,371	179	752	755	113.4	21.9
C20:2, mg/g FA	1,371	179	752	755	5.4	1.2
C20:4, mg/g FA	1,371	179	752	755	13.5	6.3
C18:2, mg/g	1,371	179	752	755	17.1	4.2
C20:2, mg/g	1,371	179	752	755	0.8	0.3
C20:4, mg/g	1,371	179	752	755	1.9	0.7
C20:2 / C18:2 (x100)	1,371	179	752	755	4.8	0.8
C20:4 / C18:2 (x100)	1,371	179	752	755	11.9	4.8
Covariates						
Age at test, days	91,448	670	22,297	38,226	177.2	10.6
Age at slaughter, days	1,371	179	752	755	213.5	9.9

<sup>a</sup> Intramuscular fat content and fatty acid composition in the muscle *gluteus medius*. Linoleic acid (C18:2), eicosadienoic acid (C20:2) and arachidonic acid (C20:4) are expressed in relative (mg/g of fatty acid (FA)) or in absolute value (mg/g of dry muscle).

## 2.2 Fatty acid analysis

Defrosted muscle samples were freeze-dried and pulverized prior to fat analysis. Intramuscular fat content and fatty acid composition was determined in duplicate by quantitative determination of the individual fatty acids by gas chromatography (Bosch et al., 2009). Total fatty acid methyl esters from both neutral lipids and phospholipids were directly obtained by transesterification

using a solution of 20% boron trifluoride in methanol (Rule, 1997). Methyl esters were determined by gas chromatography using a capillary column SP2330 (30 m × 0.25 mm; Supelco, Bellefonte, PA) and a flame ionization detector with helium as carrier gas. Runs were made with a constant column-head pressure of 172 kPa. The oven temperature program increased from 150 to 225°C at 7°C/min and injector and detector temperatures were both 250°C. The quantification was carried out through area normalization with an external mixture of fatty acid methyl esters (Supelco 37 Component FAME Mix. Sigma, Tres Cantos, Madrid) after adding into each sample 1,2,3-tripentadecanoylglycerol as internal standard. Fatty acids were identified by comparing their relative retention times with those of the external standard and confirmed by mass spectrometry. The amount of C18:2, C20:2 and C20:4 was expressed either in absolute (mg/g of dry muscle) or in relative (mg/g of total fatty acids) values. The total amount of fatty acids was calculated as the sum of C14:0, C16:0, C16:1 $n-9$ , C18:0, C18:1 $n-7$ , C18:1 $n-9$ , C18:2 $n-6$ , C18:3 $n-3$ , C20:0; C20:1 $n-7$ , C20:2 $n-6$  and C20:4 $n-6$ . Intramuscular fat content was calculated as the sum of each individual fatty acid expressed as triglyceride equivalents (AOAC, 1997) on a wet tissue basis. Means and standard deviations of the investigated fatty acids and their associated ratios (C20:2/C18:2 and C20:4/C18:2) are shown in Table 1.

### 2.3 Genetic parameters

Genetic parameters for BW, BT, IMF, C18:2, C20:2 and C20:4 (fatty acids alternatively expressed in relative or absolute values) were estimated fitting a 6-trait multivariate animal model. In matrix notation, the model used was  $\mathbf{y}_i = \mathbf{X}_i \mathbf{b}_i + \mathbf{Z}_i \mathbf{a}_i + \mathbf{W}_i \mathbf{c}_i + \mathbf{e}_i$ , where  $\mathbf{y}_i$  is the vector of observations for trait  $i$  (BW, BT, IMF, C18:2, C20:2 and C20:4);  $\mathbf{b}_i$ ,  $\mathbf{a}_i$ ,  $\mathbf{c}_i$ , and  $\mathbf{e}_i$  are the vectors of systematic, additive genetic, litter, and residual effects, respectively; and  $\mathbf{X}_i$ ,  $\mathbf{Z}_i$ , and  $\mathbf{W}_i$ , are the known incidence matrices that relate  $\mathbf{b}_i$ ,  $\mathbf{a}_i$ , and  $\mathbf{c}_i$  with  $\mathbf{y}_i$ , respectively. Systematic effects for BW and BT were the batch (1,032 levels), gender (3 levels; males, females, and barrows), and age at measurement as a covariate. Pigs tested at the same time and in the same farm unit were considered

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as one batch. The same model was used for IMF and fatty acid composition but with systematic effects only including the batch at slaughter (23 levels) and the age at slaughter as a covariate. Genetic parameters were estimated in a Bayesian setting, in line with the methodology described in Ros-Freixedes et al. (Ros-Freixedes et al., 2012), and using Gibbs sampling with the TM software (Legarra et al., 2011). The traits were assumed to be conditionally normally distributed as  $[\mathbf{y}_i | \mathbf{b}_i \mathbf{a}_i \mathbf{c}_i \mathbf{R}] \sim N(\mathbf{X}\mathbf{b}_i + \mathbf{Z}\mathbf{a}_i + \mathbf{W}\mathbf{c}_i, \mathbf{R})$ , where  $\mathbf{R}$  was the (co)variance matrix. Sorting records by trait, and pig within trait,  $\mathbf{R}$  could be written as  $\mathbf{R}_0 \otimes \mathbf{I}$ , with  $\mathbf{R}_0$  being the 6×6 residual (co)variance matrix between the six traits analyzed and  $\mathbf{I}$  an identity matrix of appropriate order. Flat priors were used for  $\mathbf{b}_i$  and residual (co)variance components.

Additive genetic and litter values, conditionally on the associated (co)variance components, were both assumed to be multivariate normally distributed with mean zero and with (co)variance  $\mathbf{G} \otimes \mathbf{A}$  and  $\mathbf{C} \otimes \mathbf{I}$ , respectively, where  $\mathbf{A}$  was the numerator relationship matrix,  $\mathbf{G}$  was the 6×6 genetic relationship matrix between the six traits, and  $\mathbf{C}$  was the 6×6 (co)variance matrix between litter effects. The matrix  $\mathbf{A}$  was calculated using all the pedigree information summarised in Table 1. Flat priors were used for additive and litter (co)variance components. Statistical inferences (means, standard deviations and HPD95) were derived from the samples of the marginal posterior distribution using a unique chain of 1,000,000 iterations, where the first 200,000 were discarded and one sample out of 100 iterations retained. Statistics of marginal posterior distributions and the convergence diagnostics were obtained using the BOA Package (Smith, 2005). Convergence was tested using the Z-criterion of Geweke and visual inspection of convergence plots. The genetic parameters for C20:2/C18:2 and C20:4/C18:2 were estimated separately using the same procedure but with a 4-trait multivariate animal model including BW, BT and IMF.

## 2.4 Expected Responses

Expected genetic responses in BW, BT and IMF were predicted after selecting for IMF, BT, IMF at constant BT and C18:2. A population with discrete generations was simulated in which 40 boars were randomly mated to 400 sows with a mating ratio of 1 boar to 10 sows. The breeding scheme consisted of one selection stage resulting in the top 25% males and 50% females. It was assumed that two males and two females from the offspring of each sow were performance-tested for BW and BT and three paternal half-sibs of different dams were used for IMF and C18:2 determinations. Pigs were assumed to be selected for one trait at a time, but using the records taken only on the traits included in the selection criterion or in all traits. Selection response was predicted by deterministic simulation of a one-stage selection scheme with discrete generations using the program SelAction (Rutten et al., 2002). The program accounts for reduction in variance due to selection (Bulmer, 1971) and corrects selection intensities for finite population size and for the correlation between index values of family members (Meuwissen, 1991).

## 3. Results

The content of C18:2, C20:2 and C20:4 in IMF is influenced by the pig's genetic background (Table 2). The estimates of the heritability for C18:2, C20:2 and C20:4, expressed in relative terms, were high, with values ranging from 0.43 (C20:2) to 0.72 (C18:2). Moreover, the proportion of the phenotypic variance due to litter effects was not negligible for these fatty acids, showing values around 0.10 (0.08, for C18:2 and C20:2, and 0.13, for C20:4, with a probability of 95% of being greater than 0.04, 0.03 and 0.08, respectively). The genetic correlations among them were all positive, high for those involving C18:2 ( $>0.60$ ), and low for that between C20:2 and C20:4 (0.19; HPD95 [-0.07, 0.49]). Litter correlations were in line with genetic correlations. If C18:2, C20:2 and C20:4 were expressed in absolute values, the heritabilities and litter variances displayed a similar pattern as for relative values. Thus, the heritabilities ranged from 0.42 (C20:4) to 0.61



(C20:2) and litter variances stayed around 10% of the phenotypic variance. The genetic correlation structure among them, however, differed when expressed in absolute terms. All of the genetic correlations were still positive, but only that between C18:2 and C20:2 remained high (0.96; HPD95 [0.94, 0.98]). The genetic correlations of C20:4, in absolute value, with C18:2 (0.15; HPD95 [-0.11, 0.41]) and C20:2 (0.12; HPD95 [-0.09, 0.48]) did not exceed 0.15.

**Table 2.** Posterior means (standard deviation) of heritability (bold diagonal), genetic correlations (above diagonal), litter correlations (under diagonal), litter variance in proportion to the phenotypic variance ( $l^2$ ), additive genetic variance ( $\sigma_a^2$ ), litter variance ( $\sigma_l^2$ ), and residual variance ( $\sigma_e^2$ ) for linoleic acid (C18:2), eicosadienoic acid (C20:2) and arachidonic acid (C20:4), expressed in either relative (mg/g of fatty acid) or absolute value (mg/g of dry muscle).

	Trait		
	C18:2	C20:2	C20:4
Relative value			
C18:2	<b>0.72 (0.09)</b>	0.71 (0.06)	0.61 (0.10)
C20:2	0.74 (0.12)	<b>0.43 (0.08)</b>	0.19 (0.15)
C20:4	0.41 (0.17)	0.04 (0.21)	<b>0.53 (0.08)</b>
$l^2$	0.08 (0.03)	0.08 (0.02)	0.13 (0.03)
$\sigma_a^2$	223.89 (35.14)	0.31 (0.07)	13.83 (2.43)
$\sigma_l^2$	25.93 (9.50)	0.06 (0.02)	3.42 (0.87)
$\sigma_e^2$	60.00 (20.48)	0.36 (0.04)	8.89 (1.82)
Absolute value			
C18:2	<b>0.58 (0.09)</b>	0.96 (0.01)	0.15 (0.15)
C20:2	0.95 (0.02)	<b>0.61 (0.08)</b>	0.12 (0.14)
C20:4	-0.02 (0.19)	-0.08 (0.19)	<b>0.42 (0.10)</b>
$l^2$	0.08 (0.02)	0.08 (0.02)	0.15 (0.03)
$\sigma_a^2$	9.41 (1.85)	0.04 (0.01)	0.10 (0.03)
$\sigma_l^2$	1.31 (0.39)	0.01 (0.00)	0.04 (0.01)
$\sigma_e^2$	5.31 (1.19)	0.02 (0.01)	0.10 (0.02)

The estimates of the genetic parameters for BW, BT and IMF showed little differences if fatty acids in the multivariate model were expressed in relative or absolute value. Because of this, only the estimates for absolute values are given (Table 3). As expected, the estimates were in close agreement with previous results in this Duroc line (Estany et al., 2017). The genetic correlation between IMF and BT was positive but moderate (0.32). The genetic correlations of C18:2, C20:2 and C20:4, in relative value, with BW, BT and IMF are shown in Table 4. All of them were negative, ranging from -0.75 (HPD95 [- 0.87, -0.60]), for C20:4 and IMF, to -0.17 (HPD95 [-0.46, 0.13]), for C20:2 and IMF.

**Table 3.** Posterior mean (standard deviation) of heritability (bold diagonal), genetic correlations (above diagonal), litter correlations (under diagonal), litter variance in proportion to the phenotypic variance ( $l^2$ ), additive genetic variance ( $\sigma_a^2$ ), litter variance ( $\sigma_l^2$ ), and residual variance ( $\sigma_e^2$ ) for body weight (BW), backfat thickness (BT) and intramuscular fat content (IMF).

	Trait		
	BW	BT	IMF
BW	<b>0.39 (0.01)</b>	0.65 (0.01)	0.22 (0.10)
BT	0.61 (0.02)	<b>0.53 (0.01)</b>	0.32 (0.08)
IMF	-0.28 (0.26)	0.27 (0.23)	<b>0.57 (0.10)</b>
$l^2$	0.09 (0.00)	0.06 (0.00)	0.08 (0.03)
$\sigma_a^2$	37.27 (1.30)	5.28 (0.14)	1.89 (0.43)
$\sigma_l^2$	8.61 (0.34)	0.55 (0.03)	0.25 (0.10)
$\sigma_e^2$	49.10 (0.74)	4.08 (0.08)	1.12 (0.27)

**Table 4.** Posterior mean (standard deviation) of the genetic correlation, litter correlation and residual correlation of linoleic acid (C18:2), eicosadienoic acid (C20:2) and arachidonic acid (C20:4), expressed in relative value (mg/g of fatty acid), with body weight (BW), backfat thickness (BT) and intramuscular fat content (IMF).

	Trait		
	BW	BT	IMF
Genetic correlation			
C18:2	-0.39 (0.08)	-0.64 (0.07)	-0.66 (0.10)
C20:2	-0.29 (0.11)	-0.48 (0.09)	-0.17 (0.15)
C20:4	-0.24 (0.12)	-0.36 (0.10)	-0.75 (0.07)
Litter correlation			
C18:2	0.20 (0.18)	-0.20 (0.20)	-0.53 (0.21)
C20:2	-0.19 (0.17)	-0.31 (0.24)	-0.10 (0.30)
C20:4	0.33 (0.18)	-0.16 (0.18)	-0.58 (0.15)
Residual correlation			
C18:2	-0.16 (0.09)	-0.20 (0.09)	-0.50 (0.20)
C20:2	-0.13 (0.06)	-0.15 (0.06)	-0.11 (0.12)
C20:4	-0.13 (0.08)	-0.22 (0.08)	-0.43 (0.11)

Interestingly, the genetic correlation of C20:4 with IMF was lower than with BT (-0.36; HPD95 [-0.56, -0.16]), while the opposite situation happened for C20:2, where the genetic correlation with IMF was greater than with BT (-0.48; HPD95 [-0.65, -0.28]). This was not the case for C18:2, which presented similar genetic correlations with IMF (-0.66; HPD95 [-0.84, -0.49]) and BT (-0.64; HPD95 [-0.77, -0.51]). A different genetic correlation structure emerged when fatty acids were expressed in absolute value (Table 5). In this case, all genetic correlations were very low (from -0.04, HPD95 [-0.26, 0.18], to 0.15, HPD95 [-0.02, 0.34]), except those of C18:2 and C20:2 with IMF, which were very high (>0.88). This result indicates that C18:2 and C20:2 in IMF, expressed in absolute terms, are highly specific to IMF.

**Table 5.** Posterior mean (standard deviation) of the genetic correlation, litter correlation and residual correlation of linoleic acid (C18:2), eicosadienoic acid (C20:2) and arachidonic acid (C20:4), expressed in absolute value (mg/g of dry muscle), with body weight (BW), backfat thickness (BT) and intramuscular fat content (IMF).

	Trait		
	BW	BT	IMF
Genetic correlation			
C18:2	0.07 (0.11)	0.07 (0.10)	0.88 (0.03)
C20:2	0.10 (0.11)	0.15 (0.10)	0.91 (0.03)
C20:4	0.00 (0.15)	-0.04 (0.11)	0.06 (0.13)
Litter correlation			
C18:2	-0.13 (0.24)	0.21 (0.26)	0.85 (0.07)
C20:2	-0.28 (0.26)	0.14 (0.27)	0.84 (0.08)
C20:4	0.01 (0.20)	-0.11 (0.21)	-0.03 (0.20)
Residual correlation			
C18:2	-0.03 (0.08)	-0.05 (0.10)	0.69 (0.12)
C20:2	-0.03 (0.09)	-0.04 (0.11)	0.65 (0.13)
C20:4	-0.12 (0.08)	-0.21 (0.07)	0.17 (0.12)

The estimates of the genetic parameters for the ratios related to the transformation efficiency of C18:2 into C20:2 and C20:4 confirmed that the linoleic to arachidonic acid pathway is subjected to genetic determinism (Table 6). Both ratios showed a high heritability, in the range of 0.40 to 0.50, and relevant litter effects, particularly for C20:4/C18:2, where they explained 15% of the phenotypic variance. Similarly to C20:2 and C20:4, the genetic correlation of C20:4/C18:2 with IMF (-0.59; HPD95 [-0.82, -0.33]) was stronger than with BT (-0.10; HPD95 [-0.32, 0.10]), while that of C20:2/C18:2 with IMF (0.76; HPD95 [0.61, 0.87]) was greater than with BT (0.36; HPD95 [0.15, 0.54]). Taken together, this correlation pattern corroborates the potential of the C18:2 metabolic pathway as a candidate route to hold molecular markers specifically targeting IMF.

**Table 6.** Posterior mean (standard deviation) of additive genetic variance ( $\sigma^2_a$ ), litter variance ( $\sigma^2_l$ ), and residual variance ( $\sigma^2_e$ ), heritability ( $h^2$ ) and litter variance in proportion to the phenotypic variance ( $l^2$ ) for the eicosadienoic acid (C20:2) to linoleic acid (C18:2) ratio (C20:2/C18:2) and the arachidonic acid (C20:4) to C18:2 ratio (C20:4/C18:2), and their genetic, litter and residual correlations with body weight (BW), backfat thickness (BT) and intramuscular fat content (IMF).

	Trait (x100)	
	C20:2/C18:2	C20:4/C18:2
$\sigma^2_a$	0.18 (0.03)	5.83 (1.48)
$\sigma^2_l$	0.02 (0.01)	1.72 (0.50)
$\sigma^2_e$	0.25 (0.02)	4.14 (0.84)
$h^2$	0.40 (0.06)	0.50 (0.11)
$l^2$	0.05 (0.02)	0.15 (0.04)
Genetic correlation		
BW	0.24 (0.12)	-0.09 (0.14)
BT	0.36 (0.10)	-0.10 (0.12)
IMF	0.76 (0.07)	-0.59 (0.12)
Litter correlation		
BW	-0.38 (0.24)	0.18 (0.19)
BT	-0.04 (0.28)	-0.19 (0.20)
IMF	0.64 (0.19)	-0.52 (0.19)
Residual correlation		
BW	-0.03 (0.06)	-0.10 (0.09)
BT	0.01 (0.06)	-0.16 (0.09)
IMF	0.27 (0.08)	-0.43 (0.10)

To illustrate and explore the potential of using C18:2 as a selection criterion for IMF, the expected genetic response on a basic breeding scheme was predicted using different selection criteria and data availability scenarios (Table 7). With the genetic parameters estimated here, it is shown that, in terms of expected response, selection for absolute values of C18:2 parallels selection for IMF at restrained BT. Although both criteria rendered similar results for IMF (from 80% to 92% of the direct response), selection for C18:2 led to higher

responses in both BW (32-35% vs 6% of the direct response) and BT (21-24% of the direct response vs no change). Thus, pigs selected for C18:2 (in absolute value) are expected to show at least the same lean growth and IMF than pigs selected for IMF at restrained BT. Results anticipate that the detrimental effect of selection for C18:2 on carcass lean content should be offset by the increase in body weight.

**Table 7.** Expected response per generation in body weight (BW), backfat thickness (BT) and intramuscular fat (IMF) to selection for BW, BT, IMF at restricted BT ( $\Delta BT = 0$ ) and C18:2 (mg/g of dry muscle) on a basic pig breeding scheme when records used for selection were taken only on the selected traits or on all traits <sup>a</sup>.

Selection criterion	Expected response		
	BW, kg	BT, mm	IMF, %
Records on selected traits <sup>b</sup>			
IMF	100	100	100
BT	644	681	70
IMF at $\Delta BT = 0$	6	0	92
C18:2 mg/g	32	21	87
Records on all traits <sup>c</sup>			
IMF	100	100	100
BT	156	165	61
IMF at $\Delta BT = 0$	6	0	80
C18:2 mg/g	35	24	83

<sup>a</sup> The breeding scheme consisted of one selection stage resulting in the top 25% males and 50% females of the offspring of 40 boars and 400 sows (ratio of 1 boar to 10 sows). Two males and two females from the offspring of each sow were performance-tested for BW and BT and three paternal half-sibs of different dams were used for IMF and C18:2 determinations. Pigs were selected for one trait at a time, but using the records taken only on the traits included in the selection criterion or in all traits.

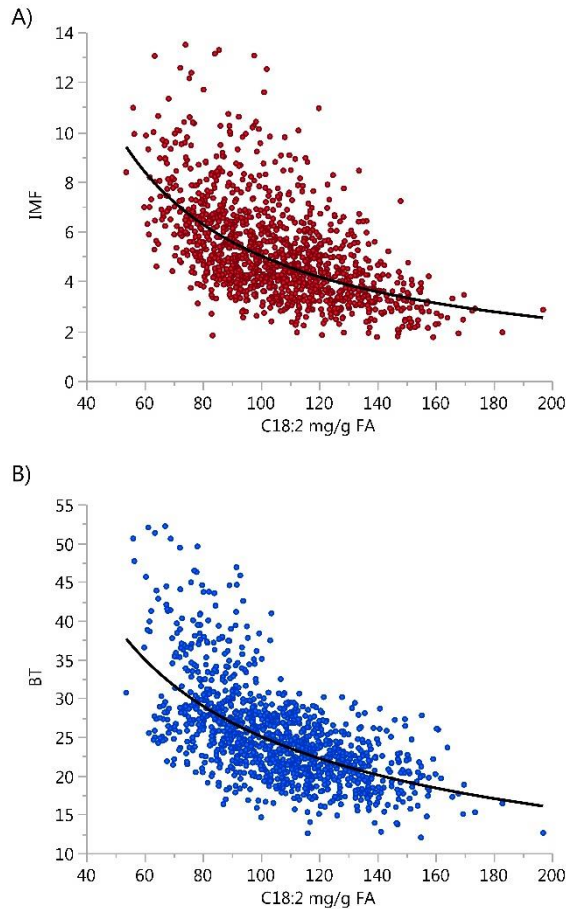
<sup>b</sup> Responses in percentage relative to responses to selection for IMF (0.39 kg, 0.21 mm and 0.40%, for BW, BT and IMF, respectively), which are set to 100.

<sup>c</sup> Responses in percentage relative to responses to selection for IMF (1.51 kg, 0.88 mm and 0.46%, for BW, BT and IMF, respectively), which are set to 100.

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## 4. Discussion

The C18:2 present in the adipose tissue of pigs derives from the diet, as mammals cannot synthesize this fatty acid. Linoleic acid is a major fat ingredient of commercial pig diets, mostly composed of grains and oils very rich in C18:2. Hence, C18:2 is relatively abundant in pigs, particularly as compared to ruminant species (Wood et al., 2008). Relative C18:2 percentages in muscle, depending on the breed, diet and muscle, vary from 5% to 20% of the total fatty acids (Wood et al., 2004). Values found in our experiment, of around 11%, fall within the average. Once in the tissue, C18:2 can be transformed to C20:4 by two metabolic routes. On one hand, C18:2 can be desaturated to  $\gamma$ -linolenic acid, and then successively elongated and desaturated to C20:4. On the other hand, C18:2 can be elongated to C20:2 and then desaturated twice to C20:4. As compared to reported values (2%-12%; Wood et al., 2004), we observed a relatively high C20:4 to C18:2 ratio (11.9%). This would point to a relatively active endogenous transformation of ingested fatty acids, since dietary C20:4/C18:2 was only about 1%. As a result of the de novo fatty acid biosynthesis, the relative amount of C18:2 declines, as also happened here (Figure 1). This has led to propose C18:2 as a candidate biomarker of both feed intake (Baylin and Campos, 2006) and fatness (Wood et al., 2008). But we can go a step further and hypothesize that, because the rate and timing of fat deposition differs between adipose tissues, with IMF developing later than subcutaneous fat (Du et al., 2013), C18:2 in IMF, and by extension the other fatty acids involved in its metabolism, could be IMF-specific enough to capture that part of the variability of IMF which is independent of BT (Figure 1). Previous results in this Duroc line have evidenced that the correlation pattern of fatty acid composition among different muscles and with subcutaneous fat is far from unity (Ros-Freixedes et al., 2014).



**Figure 1.** Relationship between the relative amount of linoleic acid in muscle and (A) intramuscular fat or (B) subcutaneous backfat thickness. The linoleic acid (C18:2, in mg/g of fatty acid (FA)) is negatively related to both intramuscular fat content (IMF, %;  $\log(\text{IMF}) = 6.26 - 1.00 * \log(\text{C18:2})$ ;  $R^2: 0.36$ ) and backfat thickness (BT, mm;  $\log(\text{BT}) = 6.24 - 0.65 * \log(\text{C18:2})$ ;  $R^2: 0.39$ ).

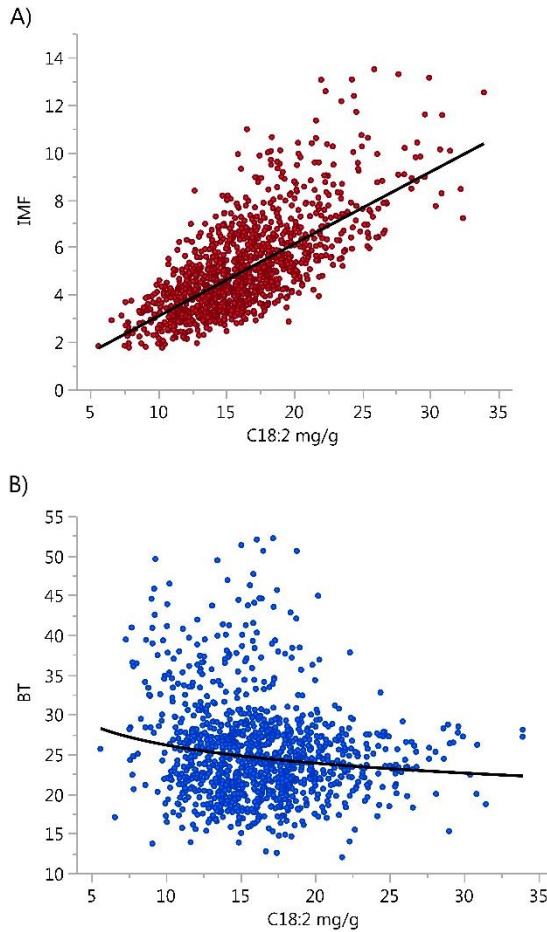
To examine this hypothesis we first have shown that C18:2 and C20:4 displayed genetic variability, whether expressed in relative or absolute values. In general, our estimates of the heritability for C18:2 and C20:4 were higher than others published so far, which ranged, for C18:2, from 0.24 to 0.55 (Fernández et al., 2003; Suzuki et al., 2006; Casellas et al., 2010; Gjerlaug-Enger et al., 2011; Ntawubizi et al., 2010; Sellier et al., 2010) and, for C20:4, from 0.15 to 0.56 (Casellas et al., 2010; Ntawubizi et al., 2010; Sellier et al., 2010). The estimate of the heritability for C20:2 was also relatively high and in line with the only one



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published so far (Ntawubizi et al., 2010). Most of the reported estimates, however, were adjusted either for carcass weight, IMF or total fatty acids, which may affect the estimates downwards (Ntawubizi et al., 2010). Interestingly, we have also found that C18:2, C20:2 and particularly C20:4 display a relevant litter effect. A similar effect has been reported by Ibáñez-Escriche et al. (2016) in Iberian pigs. Variation across litters for C18:2 and C20:4 can arise from maternal effects due to differential nutrient intake. Maternal nutrition has been seen to influence fetal programming (Du et al., 2015) and milk yield and composition (Jin et al., 2017), which are known to influence adipogenesis and therefore meat fatty acid composition. Altogether, these findings evidenced that the linoleic to arachidonic acid pathway has a strong genetic background and is not unresponsive to common environmental litter effects, which, as shown, can remain for a long time after weaning.

Secondly, the genetic correlation structure of C18:2, C20:2 and C20:4 with IMF and BT showed that these fatty acids have potential to be used as IMF- or BT-specific biomarkers, although this depends critically on how they are expressed. Thus, in line with the results in Suzuki et al. (2006), if expressed in relative value, all three fatty acids were negatively correlated with IMF and BT, whereas, if expressed in absolute value, only C18:2 and C20:2 were correlated with IMF, and positively. This discrepancy makes the absolute amount of C18:2 and C20:2 in IMF a criterion of choice for discriminating IMF against BT. Of these two fatty acids, C18:2 is a more feasible biomarker given its abundance, which makes determinations less sensitive to measurement errors. This dual relationship of C18:2 with IMF (positive) and BT (null) can be directly viewed upon depicting the raw phenotypes of IMF and BT against the absolute amount of C18:2 in IMF (Figure 2). We were unable to find in the literature other estimates of genetic parameters for fatty acids in absolute value. Efficiency ratios did not improve the potential of C18:2 for specific targeting of IMF. Although both C20:2/C18:2 and C20:4/C18:2 were also more linked to IMF than BT, their correlation structure with IMF and BT was less uneven than in C18:2.



**Figure 2.** Relationship between the absolute amount of linoleic acid in muscle and (A) intramuscular fat or (B) subcutaneous backfat thickness. The linoleic acid (C18:2, in mg/g of dry muscle) is positively related to intramuscular fat content (IMF, %;  $\log(\text{IMF}) = -1.17 + 0.99 * \log(\text{C18:2})$ ;  $R^2:0.50$ ) but not to backfat thickness (BT, mm).

Improving IMF without compromising lean growth is a common goal in pig lines for niche and quality markets where IMF is a valued feature. In practice, this is basically done by selecting for BW and IMF and against BT, but imposing some restrictions on either IMF or BT (Ros-Freixedes et al., 2013). However, since IMF and BT are positively correlated, undesirable changes in BT to selection for IMF and vice versa can easily happen. For this reason, there have been attempts to find indirect selection criteria, such as circulating lipid indicators (Estany et al. 2007; Muñoz et al., 2012), targeting specifically to one of them. The

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favourable genetic correlation pattern of C18:2 (in absolute value) with IMF and BT calls for exploring C18:2 as one of such criteria. Expected responses in IMF, BT and BW indicate that selecting for the absolute amount of C18:2 is at least as efficient to selecting for IMF at restrained BT. In other words, the absolute amount of C18:2 in IMF is able to capture most of the variance of IMF that is independent of BT and, in this way, it behaves as an IMF-specific biomarker. Nonetheless, the use of C18:2 as an indirect selection criterion for IMF presents several limitations. As happens for IMF, the most immediate is to have a feasible routine recording scheme. In this regard, the near infrared spectroscopy (NIRS) allows continuous non-invasive phenotyping of meat quality traits at a fair cost. This technology has been already used to determine the fatty acid composition of the subcutaneous fat in Iberian (Fernández et al., 2003) as well as in Duroc and in Landrace pigs (Gjerlaug-Enger et al., 2011). Furthermore, new portable NIRS-based equipment is becoming available to facilitate on-line recording at the abattoir. In this scenario, C18:2 can be interpreted as an endophenotype whose variants are indirectly captured by NIRS spectra (Rincen et al., 2018).

We have used the gluteus medius as the muscle of choice, as it is a representative muscle of the ham, the most valuable entire piece for the dry-cured meat product industry. Other reference muscles could have been used for this purpose, such as muscle longissimus thoracis. Although results may differ among them, at least for these two muscles the differences are not expected to be substantial (Ros-Freixedes et al., 2014). This has been confirmed using a subset of pigs having also data from longissimus thoracis. In this muscle, the genetic correlation pattern of C18:2 (in absolute value) with IMF (0.82; HPD95 [0.69, 0.88]) and BT (0.23; HPD95 [0.06, 0.41]) was in line with the observed in gluteus medius. Alternatively, the fatty acid composition in subcutaneous fat could work as a BT-specific biomarker. Current evidence indicates that the C18:2 content in subcutaneous fat is negatively correlated to BT (Suzuki et al., 2006) and uncorrelated to IMF (Fernández et al., 2003). If confirmed, this can be an option for pig lines already performing at an optimum level of IMF, where selection is

focused on lean growth at restrained IMF. The use of C18:2 as selection criterion may draw the idea that fat will become more polyunsaturated and with less oleic acid, thereby affecting adversely key attributes of dry-cured products. However, the exact opposite occurs. Estimates obtained in this Duroc population indicate that the absolute value of C18:2 is genetically positively correlated with the oleic acid content, regardless of how it is expressed, either in absolute (0.77; HPD95 [0.67, 0.86]) or in relative (0.15; HPD95 [-0.10, 0.41]) value. This provides evidence that selection for C18:2 in absolute value would not entail unfavorable correlated effects on fatty acid composition.

Over the last decades molecular markers have also raised interest as a tool to improve genetic analysis and selection. Several markers have been described to be associated with IMF, BT and fatty acid composition, although only one of them has proved to be IMF-specific (Pena et al., 2016). The distinct association of C18:2 and C20:4 with IMF and BT described here supports the search for molecular markers in genes encoding enzymes and transcription factors involved in the C18:2 metabolic pathway. One of them is the fatty acid desaturase-2 gene (*FADS2*), a rate-limiting enzyme in the conversion of C18:2 into C20:4. The activity of *FADS2* can be indirectly measured by C20:4/C18:2 and C20:2/C18:2. These two ratios are expected to decrease and increase, respectively, with IMF rather than with BT, thereby suggesting that *FADS2* could be a candidate gene to explore IMF-specific molecular markers. In this context, Gol et al. (2018) found a polymorphism in the promoter region of the *FADS2* gene that modifies C20:4/C18:2 and C20:2/C18:2. The correlated effects on IMF and BT were in line with the expected, i.e., the allele showing a positive effect on C20:4/C18:2 had less absolute C18:2 and IMF, while it did not alter BT. All in all, the results obtained would confirm that quantitative biological analysis is a good approach to find new traits and candidate markers for an efficient selection for IMF and lean growth.

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## 5. Conclusions

In conclusion, our work demonstrates that the C18:2 to C20:4 pathway is subjected to genetic variation. Also, we show that the genetic (co)variation structure of the fatty acids in this pathway with IMF and BT differs by fatty acid and on whether they are expressed in absolute (mg/g of muscle) or relative values (mg/g of fatty acid). In particular, the distinct genetic relationship of C18:2 and C20:2 (in absolute values) in IMF with IMF (positive) and BT (almost null) allow us to propose them as candidate IMF-specific biomarkers. In addition, we have proved that selection for the absolute amount of C18:2 in IMF is expected to deliver a similar genetic response outcome that selection for IMF at restrained BT. The quantitative genetic analysis of the C18:2 metabolic pathway has provided new insight into the relationship between IMF and lean growth, pointing to relevant candidate genes to search for potential IMF-specific markers.

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## CHAPTER II

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### A POLYMORPHISM IN THE *FATTY ACID DESATURASE-2* GENE IS ASSOCIATED WITH THE ARACHIDONIC ACID METABOLISM IN PIGS

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## Abstract

Arachidonic acid (C20:4) is related to a wide range of biological effects including lipid homeostasis. The fatty acid desaturase-2 (*FADS2*) gene encodes for the delta-6-desaturase, which is involved in the biosynthesis of C20:4 from linoleic acid (C18:2). The purpose of this study was to characterise mutations in the promoter of the porcine *FADS2*, evaluating in particular the effect of one haplotype tagging polymorphism (rs321384923A>G) on the biosynthesis pathway of C20:4. A total of 1,192 Duroc barrows with records on fatty acid composition in muscle and subcutaneous fat were genotyped.

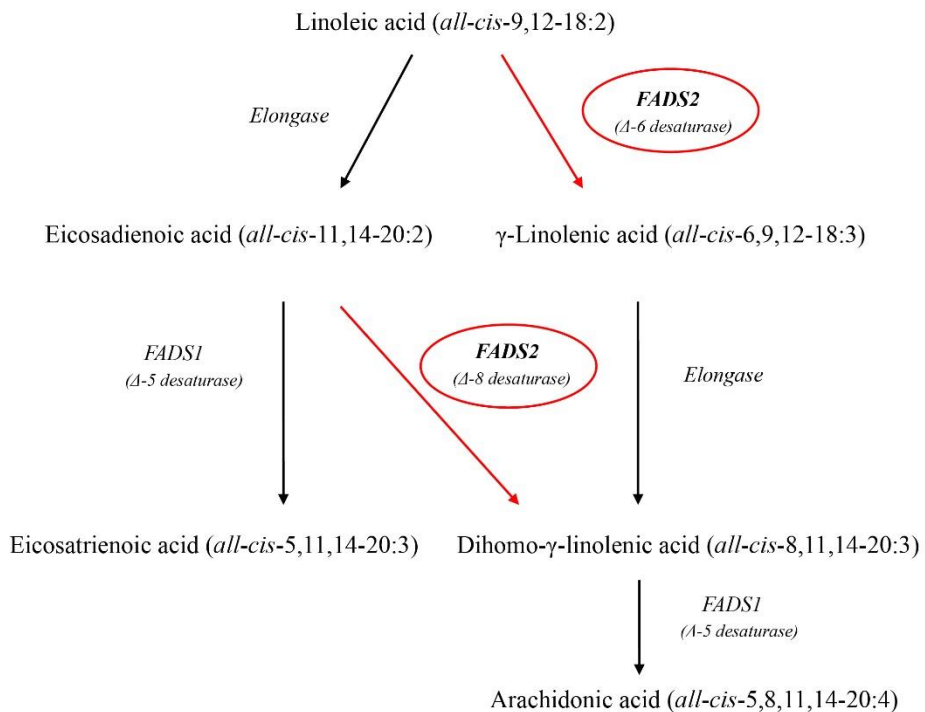
Pigs carrying the A allele showed, irrespective of fat content, both enhanced *FADS2* expression and higher C20:4 in muscle and exhibited increased ratios of C20:4 to C18:2 and of C20:4 to eicosadienoic acid (C20:2) in both muscle and adipose tissue. Despite the inverse relationship observed between C20:4 and fat content, the rs321384923 polymorphism had no impact on lean weight. It is concluded that the haplotype encompassing the rs321384923 polymorphism at the porcine *FADS2* affects the n-6 fatty acid profile by specifically modifying the desaturation efficiency of C18:2 to C20:4 rather than by concomitant variations in C18:2 following changes in fat content.

## 1. Introduction

Arachidonic acid (C20:4, *all-cis*-5,8,11,14–20:4) is the precursor of several bioactive lipid mediators of the eicosanoid family related to a wide range of biological effects including lipid homeostasis and inflammatory response. C20:4 is essential in many organs such as liver and brain, where it is one of the most abundant fatty acids. In skeletal muscle, C20:4 promotes myocyte growth both *in vitro* (Markworth and Cameron-Smith, 2013) and *in vivo* (Markworth et al., 2018) through the Akt/mTOR pathway. Mainly esterified into phospholipids, it also exerts a substantial contribution to maintaining membrane fluidity and in cell signalling. The C20:4 content differs between lipid classes, tissues and muscles and is influenced by both the diet and the individual's genetic background (Wood

et al., 2004; Wood et al., 2008). Diets rich in C20:4 or diets producing relatively high levels of linoleic acid (C18:2, *all-cis*-9,12–18:2) result in enhanced levels of C20:4 in plasma (Sinclair and Mantf, 1996; Markworth et al., 2018) and muscle (Warren et al., 2008; Markworth et al., 2018). Although C20:4 can be taken up from the diet, it can also be synthesised in the animal. There is evidence indicating that the biosynthesis of C20:4 is genetically mediated, notably in pigs, where substantial genetic variation between (Warren et al., 2008) and within genetic types (Ntawubizi et al., 2010) has been reported.

**Figure 1.** The role of FADS2 in the biosynthesis of arachidonic acid from linoleic acid.



The fatty acid desaturase-2 (FADS2,  $\Delta$ -6 desaturase) catalyses the first step for the biosynthesis of arachidonic acid (*all-cis*-5,8,11,14–20:4), in which linoleic acid (*all-cis*-9-12-18:2) is desaturated to  $\gamma$ -linolenic acid (*all-cis*-6,9,12–18:3) and then elongated into dihomo- $\gamma$ -linolenic acid (*all-cis*-8,11,14–20:3). Alternatively, linoleic acid is elongated into eicosadienoic acid (*all-cis*-11,14–20:2), which in turn can be either desaturated to eicosatrienoic acid (*all-cis*-5,11,14–20:3) via fatty acid desaturase-1 (FADS1,  $\Delta$ -5 desaturase) or to dihomo- $\gamma$ -linolenic acid (*all-cis*-8,11,14 20:3) via FADS2 ( $\Delta$ -8 desaturase). The arachidonic acid is finally synthesized by desaturating dihomo- $\gamma$ -linolenic acid via FADS1.

The delta-6-desaturase enzyme, encoded by the fatty acid desaturase-2 (*FADS2*) gene, is responsible for the first and rate-limiting step in the biosynthesis of C20:4 (Figure 1), where C18:2 is desaturated to  $\gamma$ -linolenic acid (*all-cis*-6,9,12–18:3) (Nakamura and Nara, 2004). The lack of *FADS2* leads to obesity resistance and, as reported by Stoffel et al. (2014) using auxotrophic mice mutants, it may activate a surrogate reaction in which C18:2 is elongated to eicosadienoic acid (C20:2, *all-cis*-11,14–20:2) and then to eicosatrienoic acid (*all-cis*-5,11,14–20:3) but not to C20:4. In pigs, *FADS2* is located in chromosome 2 (2:9632454–9667044:-1) as a part of a cluster including *FADS1* and *FADS3*. The genomic structure of the gene is comprised of 12 exons and 11 introns (assembly Sscrofa11.1), which produce three protein-coding splice variants. The activity of *FADS2* in the synthesis of long chain fatty acid is enhanced by an alternative transcript of pig *FADS1* (Taniguchi et al., 2015), as also reported in baboons and humans (Park et al., 2012). In a recent genome-wide association study with five divergent pig populations, Zhang et al. (2016) provide evidence that in Erhualian pigs the region containing *FADS2* was associated with the C20:4 content in muscle. Although an uncharacterised polymorphism in exon 3 of the pig *FADS2* has been associated with C20:4 and intramuscular fat (IMF) content (Renaville et al., 2013), the sequence variation of *FADS2* has not been otherwise investigated. *FADS2* is a TATA-less gene and such genes are often subjected to complex transcription mechanisms. This makes the promoter region of *FADS2* a sensible location for screening for DNA polymorphisms. The aim of our study was to describe genetic variants in the promoter of the porcine *FADS2* and then to further investigate their association with C20:4 and fat content in the main lipogenic tissues. To this end, we made use of a biorepository of fat, muscle and liver specimens from a high-fat Duroc pig line where at least two relevant genes for fatty acid composition are also segregating (Estany et al., 2014; Ros-Freixedes et al., 2016).

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## 2. Material and methods

### 2.1 Ethics Statement.

All pigs used in the study were raised and slaughtered in commercial units following applicable regulations and good practice guidelines on the protection of animals kept for farming purposes, during transport and slaughter. The experimental protocol was approved by the Ethical Committee on Animal Experimentation of the University of Lleida.

### 2.2 Animals and phenotypes.

A total of 1,192 barrows from 159 sires and 590 dams of the same Duroc line were used in this experiment. Pigs were raised in 20 batches between 2002 and 2016 following a similar standard protocol for data recording and tissue sampling (Ros-Freixedes et al., 2012). In each batch, pigs were raised from 75 days of age until slaughter at 210 days in the same farm under identical conditions. Pigs had ad libitum access to commercial feed (Esporc, Riudarenes, Girona, Spain). From 160 days of age onwards they were fed a finishing diet including around 6.0% fat (27% C18:2 and 0.3% C20:4 of total fatty acids). All pigs were slaughtered in the same abattoir, where carcass weight and carcass backfat and loin thickness at 6 cm off the midline between the third and fourth last ribs were measured by an on-line ultrasound automatic scanner (AutoFOM, SFK-Technology, Denmark). Immediately after slaughter, samples of semimembranosus (SM, n = 187) muscle, subcutaneous adipose tissue (n = 388) and liver (n = 118) were collected, snap-frozen, and stored at  $-80^{\circ}\text{C}$ . After chilling for about 24 h at  $2^{\circ}\text{C}$ , samples of the muscles gluteus medius (GM, n = 1,179) and longissimus thoracis (LM, n = 548) were collected, vacuum packaged, and stored at  $-20^{\circ}\text{C}$ . Intramuscular and liver fat content, as well as fatty acid composition, were determined in duplicate by quantitative gas chromatography (Bosch et al., 2009). The proportion of C18:2, C20:2 and C20:4 were expressed as percentages relative to total fatty acid content (14:0; 16:0; *cis*-9–16:1; 18:0; *cis*-

11–18:1; *cis*-9–18:1; C18:2, all-*cis*-9,12,15–18:3; 20:0; *cis*-13–20:1; C20:2; and C20:4) and their ratios calculated as indicators of FADS2 activity.

### 2.3 Genotyping.

Genomic DNA was isolated from GM muscle samples using a standard protocol. The proximal promoter of the *FADS2* (~1 Kb) was amplified and sequenced in a subset of 14 pigs with high or low C20:4 content in SM (Table S1) with primers and conditions detailed in Table S2. An RFLP-PCR genotyping protocol was set up to genotype the rs321384923A>G substitution. PCRs were carried out in 13 µL reactions containing 60 ng of genomic DNA, 1x buffer, 200 nM of dNTP mix, 2 mM of MgCl<sub>2</sub>, 500 nM of each primer and 1 U of Taq polymerase (Bioline). Thermocycling conditions were 95°C 10min, 35 cycles of 95°C 20sec, 56°C 20sec and 72°C 20sec finishing with 72°C 5min. Ten µl of PCR were digested with *Ava*I (37°C × 3h) and solved by electrophoresis in agarose gels. Additionally, two other SNPs known to influence fat content and composition in our resource Duroc line (the *AY487830:g.2228T>C* SNP at the *stearoyl-CoA desaturase (SCD)* gene on chromosome 14 and the *NM\_001024587:g.1987C>T* SNP at the *leptin receptor (LEPR)* gene on chromosome 6) were genotyped as described in Ros-Freixedes et al. (2016).

### 2.4 FADS2 expression.

Total RNA from 70 SM samples from two batches (AA, n = 14; AG, n = 26; GG, n = 30) and 31 livers from one batch (AA, n = 2; AG, n = 15; GG, n = 14) was isolated with TRI-Reagent (Sigma-Aldrich) following the manufacturer's indications. Purity of the RNA was assessed by spectrophotometry with a Nanodrop-1000 and the integrity was tested by electrophoresis in agarose gels. *FADS2* and two reference genes, *YWHAZ* and *RPL32*, were analysed by a quantitative PCR assay (Table S3). Briefly, 2 µg of total RNA were reverse transcribed using SuperScript IV Reverse Transcriptase (Invitrogen) with oligo-dT and random primers. Real-time PCR assays were



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carried out in triplicate in 8  $\mu$ l reactions, containing 1x iTaq Universal SYBR Green Supermix (Bio-Rad), 200 nM of each primer and 3  $\mu$ l cDNA template diluted 1:30 in water. Cycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melt curve analysis. To quantify and normalise the *FADS2* expression data, we used the  $\Delta\Delta$ Ct method against the geometrical mean of the two reference genes (Yuan et al., 2006).

## 2.5 Statistical analyses.

The association analysis of *FADS2* genotypes with C18:2, C20:2, C20:4 and their ratios was performed using a mixed model including the batch (20 levels for fatty acids), the *FADS2* genotype (3 levels), the *SCD* genotype (3 levels) and the *LEPR* genotype (3 levels) as fixed effects and the sire and the dam as random effects, with fat content as a covariate (IMF for muscle, backfat thickness for subcutaneous fat and fat content for liver). The same model was used for gene expression (without the covariate) and for carcass traits (with the age at slaughter as a covariate instead of fat content). Additivity was tested replacing the genotype effect by the covariate [1, 0, -1] for the AA, AG, and GG genotypes, respectively. The effects of the *FADS2* genotype and additivity were tested using the F-statistic while the pairwise differences among *FADS2* genotypes were contrasted with the Tukey-HSD test. Results are presented as least-square means  $\pm$  standard error and were considered statistically significant at  $P < 0.05$ . The non-linear relationship of IMF with C20:4 was assessed regressing the reciprocal term of IMF on C20:4. All models were solved using the JMP Pro 12 package (SAS Institute Inc., Cary, NC).

### 3. Results

#### 3.1 Sequence variability at the *FADS2* promoter.

A total of 5 SNP polymorphisms and one 12 bp insertion were segregating in the proximal promoter of the pig *FADS2* gene (Figure S1). Among them, three SNPs (rs336076510, rs321384923 and rs331050552 at positions -676, -706 and -798 bp upstream the ATG codon, respectively) were fully linked forming two haplotypes (AAT and GGC). The stability of the two haplotypes was confirmed by genotyping the three SNPs in a subset of 51 pigs evenly distributed across haplotypes (data not shown). The middle SNP, rs321384923A>G substitution, was selected for further analysis as it modified a potential retinoic acid/oestrogen related receptors (TGCCCG) binding site while no potential transcription factor binding sites were detected in the other two SNP sites. While human and rat *FADS2* expression responds to oestrogen hormone (Kitson et al., 2013) and vitamin A (Dziedzic et al., 2018), identification of causal mutations has been hindered by the presence of clusters of polymorphisms in strong linkage disequilibrium both upstream and downstream of the translation start site (Lattka et al., 2010).

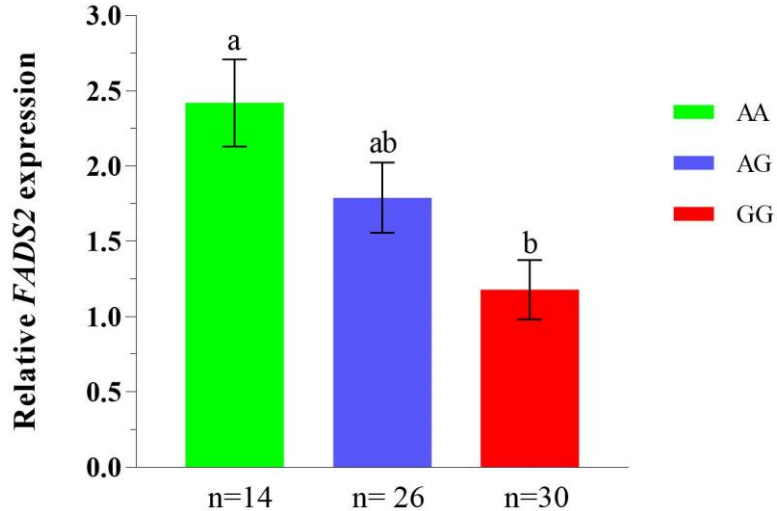
#### 3.2 *FADS2* rs321384923 genotype frequencies.

The frequencies of the *FADS2* rs321384923 genotypes by *SCD* and *LEPR* genotypes are given in Supplementary Table S4. The A allele was the minor allele (frequency of 30.9%). The *g.2228T>C* *SCD* and the *g.1987C>T* *LEPR* SNPs were both segregating at intermediate frequencies (46.1% and 43.5% for the T allele, respectively). All possible genotypes for the three SNPs were observed and, as expected for genes in different chromosomes, they were in linkage equilibrium ( $r^2 < 0.005$ , for all pairwise linkage disequilibrium between SNPs).

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### 3.3 *FADS2* genotype and *FADS2* expression.

*FADS2* expression was determined in SM on pigs of the three *FADS2* rs321384923 genotypes (Figure 2). The expression analysis was only performed in SM, since it was not possible to obtain GM and LM samples immediately after slaughter. The relative gene expression of *FADS2* in muscle was 2-fold higher in the AA genotype as compared to the GG genotype (2.34 vs 1.10,  $P < 0.01$ ). As evidenced by the allele substitution effect ( $0.63 \pm 0.18$ ,  $P < 0.01$ ), heterozygous pigs displayed intermediate levels of gene expression. Results of *FADS2* expression in liver confirmed the same trend, with pigs carrying the A allele (AA and AG) showing higher expression than the GG pigs (2.83 vs 1.38,  $P < 0.05$ ). From this foundation, we proceed to explore the possible functional consequences caused by the higher expression of the A allele at the porcine *FADS2*.



**Figure 2.** Relative *FADS2* mRNA expression in muscle by rs321384923 genotype. The *FADS2* gene expression in the semimembranosus muscle was around two-fold higher for the AA genotype as compared to the GG genotype. The number of pigs (n) per genotype ranged from 14 to 30. Error bars represent standard errors. Means with different superscripts differ significantly ( $P < 0.05$ ).

**Table 1.** Effect of the *FADS2* rs321384923 genotype on n-6 fatty acid composition. As compared to the GG pigs, the AA pigs showed a higher content of arachidonic acid (C20:4) and a lower content of eicosadienoic acid (C20:2) in muscle because they were more efficient transforming linoleic acid (C18:2) into C20:4. Subcutaneous fat was measured in terms of backfat thickness and intramuscular fat was determined in gluteus medius muscle. The proportion of each fatty acid is expressed as a percentage relative to total fatty acid content and, as well as ratios, adjusted for intramuscular fat (IMF) content. <sup>a</sup> P-value associated with the effect of the *FADS2* genotype; <sup>b</sup> Pairwise comparisons of *FADS2* genotypes. <sup>c</sup> Within row, means with different superscripts differ significantly ( $P < 0.05$ ).

Trait	P-value <sup>a</sup>	<i>FADS2</i> <sup>b</sup>		
		AA	AG	GG
Backfat, mm	0.99	26.3 ± 0.4	26.3 ± 0.3	26.3 ± 0.2
IMF, % dry matter	0.10	18.0 ± 0.5	18.7 ± 0.3	19.1 ± 0.3
C18:2, %	0.13	10.06 ± 0.13	10.28 ± 0.07	10.33 ± 0.07
C20:2, % (x10)	<0.001	4.65 ± 0.07 <sup>a</sup>	4.85 ± 0.04 <sup>b</sup>	4.95 ± 0.04 <sup>b</sup>
C20:4, %	<0.001	1.62 ± 0.04 <sup>a</sup>	1.55 ± 0.02 <sup>a</sup>	1.44 ± 0.02 <sup>b</sup>
C20:4/C18:2 (x10)	<0.001	1.59 ± 0.03 <sup>a</sup>	1.52 ± 0.02 <sup>a</sup>	1.42 ± 0.02 <sup>b</sup>
C20:2/C18:2 (x100)	0.004	4.70 ± 0.04 <sup>a</sup>	4.77 ± 0.02 <sup>a</sup>	4.84 ± 0.02 <sup>b</sup>
C20:4/C20:2	<0.001	3.51 ± 0.07 <sup>a</sup>	3.25 ± 0.04 <sup>b</sup>	2.98 ± 0.04 <sup>c</sup>

### 3.4 *FADS2* genotype and arachidonic acid.

The effect of the *FADS2* rs321384923 was first assessed in GM (Table 1). The results are presented adjusted for IMF, but those unadjusted led to similar conclusions. AA pigs had 12.5% more C20:4 and 6.1% less C20:2 in GM than GG pigs. As suggested by the expression data, the *FADS2* SNP displayed an additive behaviour, with a G to A allele substitution effect of  $0.09 \pm 0.02$ , for C20:4 ( $P < 0.001$ ), and  $-0.13 \pm 0.03$ , for C20:2 (values x 10;  $P < 0.001$ ). The same trend was observed when fatty acids were quantitatively expressed in mg/g of muscle. These values, although only accounting for about 2% of the total variance of these two fatty acids (2.2% for C20:4 and 1.7% for C20:2),

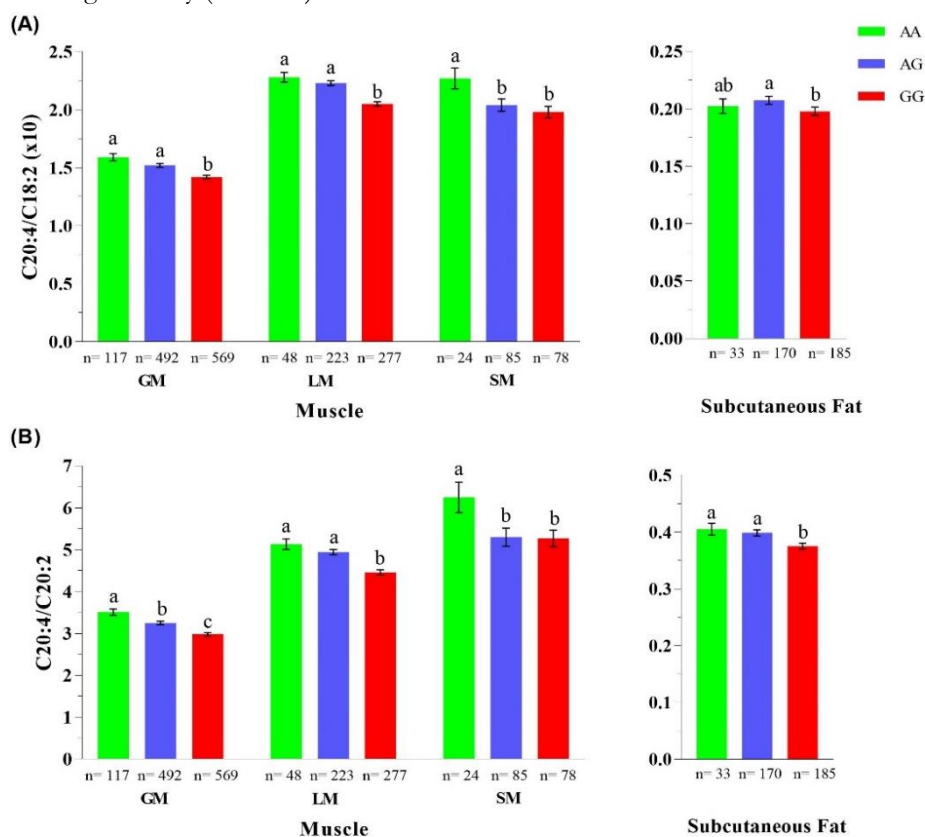
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provide support to the hypothesis that there exists genetic variation in the promoter region of the *FADS2* that impacts n-6 fatty acid biosynthesis in pigs. This was confirmed by analysing the indicator ratios of *FADS2* activity (Table 1). Regarding the C20:4 to C18:2 ratio, which can be interpreted as the overall efficiency of transforming C18:2 to C20:4, AA pigs were 12.0% more efficient than GG pigs. Interestingly, for the alternative route that converts C18:2 into C20:2 (C20:2/C18:2 ratio), AA pigs were 2.9% less efficient than GG pigs. However, since *FADS2* also acts in the desaturation pathway from C20:2 to C20:4, a supplementary effect of the *FADS2* SNP on C20:4 is expected to occur over this route, with the A allele further enhancing the synthesis of C20:4 and the G allele accumulating more C20:2. This effect was highlighted by the relatively greater differences by genotype for the C20:4/C20:2 ratio, which was 17.8% higher in AA pigs than in GG pigs, explaining up to 5.8% of the total variance of the ratio. On the other hand, C18:2, the primary substrate in the endogenous metabolism of C20:4, should decrease with increased *FADS2* activity. We only were able to detect this effect when C18:2 was expressed in mg/g of muscle instead of as a percentage of total fatty acids. Then, as expected, AA pigs showed the lowest value of C18:2 (15.4 mg, 16.0 mg and 16.0 mg for AA, AG and GG, respectively,  $P < 0.05$ ).

The association of *FADS2* rs321384923 genotypes with fatty acid composition was investigated in two other muscles (LM and SM), subcutaneous fat and liver. The effect of *FADS2* SNP in LM and SM were in line with those observed in GM, particularly for the C20:4 to C18:2 and C20:4 to C20:2 ratios (Figure 3). As compared to GG pigs, AA pigs had a greater proportion of C20:4 in relation to C18:2, both in LM (2.28 vs 2.05, values  $\times 10$ ) and in SM (2.27 vs 1.98, values  $\times 10$ ; Figure 3A) and to C20:2, also both in LM (5.13 vs 4.46) and in SM (6.25 vs 5.27, Figure 3B). The C20:4 to C18:2 and the C20:4 to C20:2 ratios gave similar results in subcutaneous fat, albeit around twenty times smaller in magnitude (Figure 3B). However, we did not find differences across genotypes for these two ratios in liver (Figure S2). Regarding C20:4 content, the effect of

the *FADS2* genotype in LM was fully consistent with the results in GM, with AA pigs performing better than GG pigs (1.84% vs 1.62%, with a substitution effect of  $0.12 \pm 0.02$ ,  $P < 0.001$ ). Likely due to the limited data size, this effect was not evident in SM (3.12%, for AA, and 2.74%, for GG, with a substitution effect of  $0.13 \pm 0.11$ ,  $P = 0.24$ ). No difference between *FADS2* genotypes was detected for C18:2 and C20:2 in both LM and SM. The results in LM and SM were the same when individual fatty acids were expressed in mg/g of muscle.

**Figure 3.** Efficiency of arachidonic acid biosynthesis by *FADS2* rs321384923 genotype in muscle and subcutaneous fat. (A) The AA genotype of *FADS2* was more efficient than the GG genotype in transforming linoleic acid (C18:2) into arachidonic acid (C20:4) both in muscle (GM: m. gluteus medius; LM: m. longissimus thoracis muscle; and SM: m. semimembranosus muscle) and in subcutaneous fat, around 12% and 2%, respectively. As a result, (B) C20:4 to eicosadienoic acid (C20:2) ratio in muscle and in subcutaneous fat was, respectively, 18% and 8% greater in AA pigs as compared to GG pigs. The number of pigs (n) genotyped per tissue and genotype ranged from 24 to 569. Error bars represent standard errors. Within tissue, means with different superscripts differ significantly ( $P < 0.05$ ).



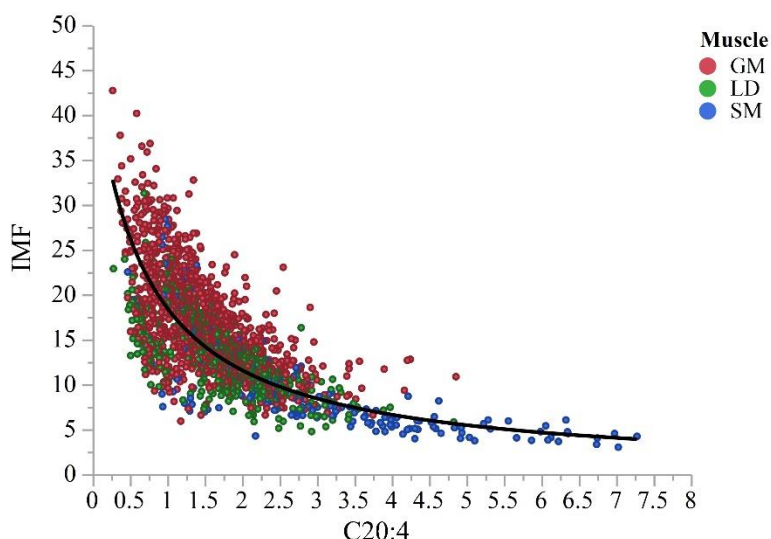
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### 3.5 Relationship of *FADS2* and *LEPR* genotypes with fat content.

We did not find evidence of association of *FADS2* rs321384923 with carcass weight and lean percentage and, as a result, with lean weight (Table S5). The *FADS2* genotype was not associated with IMF either (Table S5), although the A allele showed a negative trend towards decreasing IMF in GM, where the allele substitution effect was  $-0.49 \pm 0.23$  ( $P < 0.05$ ). This finding contrasts with the clear-cut negative relationship between C20:4 and muscle fat content (Figure 4). However, two overlapping phenomena should be considered when accounting for C20:4 in relation to total fatty acid content: the efficiency in transforming C18:2 into C20:4 and the dilution of C20:4 as overall endogenous fat synthesis progresses, two effects which in turn are not fully independent. As indicated by the covariate adjustment, IMF showed a negative relationship not only with all investigated n-6 fatty acids (beta:  $-0.07\% \pm 0.01$ ,  $P < 0.001$ , for C20:4) but also with all proxy ratios (beta:  $-0.04 \pm 0.01$ ,  $P < 0.001$ , for C20:4/C18:2, values x10) except C20:2/C18:2 (beta:  $0.06 \pm 0.01$ ,  $P < 0.001$ , values x 100), thereby indicating that increased fat content, in addition to diluting C20:4, involves a decline in the biosynthesis efficiency of C20:4. Both effects are taken into account by adjusting *FADS2* genotype comparisons for fat content. Therefore, the differences in C20:4 between *FADS2* genotypes should be attributed to differential efficiency performance.

To validate the specificity of the *FADS2* genotypes on C20:4 biosynthesis efficiency we made use of the *LEPR* *g.1987C>T* SNP as an internal control gene for fatness, provided that this polymorphism, which co-segregates with *FADS2* SNP in this population, is known to affect lipid accumulation. The *LEPR* TT pigs used here produced around 5% and 11% more backfat and IMF, respectively, than *LEPR* CC pigs (Table 2) at no significant change in carcass weight. Whether adjusted for IMF or not, the results for the *LEPR* SNP on C20:4 were in line with the expected from Figure 4, with the C allele affecting negatively IMF and positively C20:4 (Table 2). However, in contrast to the *FADS2* SNP, the favourable effect of the *LEPR* C allele on C20:4 was

accompanied by concomitant increases in C18:2 and C20:2. Hence, the *LEPR* SNP had no effect on the ratios associated with *FADS2* activity (C20:4/C18:2, C20:2/C18:2 and C20:4/C20:2) when adjusting for IMF (Table 2). The dissimilar behaviour of *FADS2* and *LEPR* SNPs in relation to these ratios substantiates the two paths by which C20:4 can be modified. Thus, while the effect of the *LEPR* SNP on C20:4 is to a great extent a matter of scale, a result of variations in fat content, the effect of the *FADS2* polymorphism is based on changes in efficiency, which does not necessarily mean variations in fat content. No interaction between *LEPR* and *FADS2* genotypes was observed for these fatty acids. The effect of the *SCD* genotype was neutral for n-6 fatty acid composition and fat content.



**Figure 4.** Relationship of arachidonic acid in muscle with intramuscular fat content. The arachidonic acid (C20:4) content, expressed as a percentage of total fatty acids, is negatively related to intramuscular fat (IMF- 1 = 0.0218 + 0.0323\*C20:4; R<sup>2</sup>:0.66). The regression was obtained across muscles using 1,912 datapoints from gluteus medius (GM; n = 1,177), longissimus thoracis (LM; n = 548) and semimembranosus (SM; n = 187) muscles. The GM showed the lowest C20:4 content (raw mean of 1.47%, 1.72% and 2.95%, for GM, LM and SM, respectively) and the highest level of IMF (raw mean of 17.2%, 13.3% and 9.6% for GM, LM and SM, respectively).



**Table 2.** Effect of the *LEPR* *g.1987C>T* SNP genotype on n-6 fatty acid composition. As compared to TT pigs, the CC pigs showed a higher content of arachidonic acid (C20:4) and eicosadienoic acid (C20:2) in muscle because they were fatter and not because they were more efficient transforming linoleic acid (C18:2) into C20:4 and C20:2. Subcutaneous fat was measured in terms of backfat thickness and intramuscular fat was determined in gluteus medius muscle. The proportion of each fatty acid is expressed as a percentage relative to total fatty acid content and, as well as ratios, adjusted for intramuscular fat (IMF) content. <sup>a</sup> P-value associated with the effect of the *LEPR* genotype; <sup>b</sup> Pairwise comparisons of *LEPR* genotypes. Within row, means with different superscripts differ significantly (P < 0.05).

Trait	P-value <sup>a</sup>	<i>LEPR</i> <sup>b</sup>		
		CC	CT	TT
Backfat, mm	0.003	25.8 ± 0.3 <sup>a</sup>	26.1 ± 0.3 <sup>a</sup>	27.0 ± 0.3 <sup>b</sup>
IMF, % dry matter	<0.001	17.9 ± 0.3 <sup>a</sup>	18.1 ± 0.3 <sup>a</sup>	19.8 ± 0.4 <sup>b</sup>
C18:2, %	<0.001	10.49 ± 0.09 <sup>a</sup>	10.29 ± 0.08 <sup>a</sup>	9.89 ± 0.10 <sup>b</sup>
C20:2, % (x10)	<0.001	4.94 ± 0.05 <sup>a</sup>	4.84 ± 0.04 <sup>b</sup>	4.65 ± 0.06 <sup>b</sup>
C20:4, %	0.01	1.59 ± 0.03 <sup>a</sup>	1.54 ± 0.02 <sup>ab</sup>	1.48 ± 0.03 <sup>b</sup>
C20:4/C18:2 (x10)	0.44	1.53 ± 0.02	1.50 ± 0.02	1.49 ± 0.02
C20:2/C18:2 (x100)	0.85	4.77 ± 0.03	4.76 ± 0.03	4.78 ± 0.03
C20:4/C20:2	0.64	3.28 ± 0.05	3.24 ± 0.04	3.21 ± 0.06

#### 4. Discussion

We report here a SNP in the promoter region of the pig *FADS2* (rs321384923) with effect on the desaturation pathways leading to C20:4 biosynthesis. The polymorphism is a tagging SNP for a 3-SNP haplotype in the *FADS2* promoter and it is situated -706 bp upstream from the ATG codon and -294 bp upstream from the start of transcription of the closest *FADS2* transcript (ENSSSCT00000014289.3). Two previous genome-wide association studies have identified markers around this haplotype region associated with *FADS2* expression and fatty acid metabolic traits. In the first one, in an Iberian x Landrace backcross, Revilla et al. (2018) found that the most significant cis-SNP for *FADS2* gene expression (rs81474400) was located 230 Kb upstream of the *FADS2*. In this study, the three SNPs of the haplotype were found to be segregating in the Iberian founders, but no association was observed between the most proximal of them (rs331050552) and *FADS2* mRNA expression in the backcrossed pigs. The low frequency of the minor allele and/or different haplotype structure in the backcrossed individuals could have interfered with the expected results in an experimental population of limited size. In the second genome-wide association study, a SNP (rs81360272) located in the fifth intron of *FADS2*, was reported to be associated in Erhualian pigs with proxy ratios of *FADS2* activity (Zang et al., 2016). SNPs from both studies are from the Illumina's pig genotyping array and encompass a region containing at least four additional SNPs from this array. Using data on 272 Duroc pigs from our line genotyped with this chip, we found that these two SNPs were in low linkage disequilibrium with our tag SNP ( $r^2 = 0.10-0.15$ ) and had no effect on C20:4 and associated ratios. In contrast, the two nearest upstream SNPs to our haplotype (rs343441264 and rs81360470) were almost fully linked with our tag SNP ( $r^2 = 0.88-0.92$ ) and parallel their effects. Overall, this genomic pattern would confirm that our tag SNP is capturing a functional variant in the promoter region of *FADS2* influencing C20:4 content in pigs.

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The presence of the A allele of the rs321384923 SNP additively enhances *FADS2* expression and, as a result, the desaturase activity in both muscle and subcutaneous fat. *FADS2* is the rate limiting enzyme in the conversion of essential fatty acids C18:2 and  $\alpha$ -linolenic to long-chain polyunsaturated fatty acids. The knock out of this gene results in mice lacking polyunsaturated fatty acids beyond eicosatrienoic acid (Stoffel et al., 2008) (*all-cis*-5,11,14–20:3; Figure 1), indicating that there are no other enzymes with a redundant activity. *FADS2* participates in two well-characterized steps in the biosynthesis of n-6 polyunsaturated fatty acids: the desaturation of (i) C18:2 to  $\gamma$ -linolenic acid (*all-cis*-6,9,12–18:3) and (ii) C20:2 to dihomogamma-linolenic acid (*all-cis*-8,11,14–18:3). These are critical steps to produce C20:4. Thus, the presence of the more active A allele accelerates the production of C20:4 through both routes, as seen by significant changes in the C20:4/C18:2 and C20:4/C20:2 ratios, resulting in 12–14% more C20:4 in the three muscles tested (GM, LM and SM) regardless of IMF. Furthermore, this was a consistent additive effect, paralleling the gene expression results. In agreement with this, there was a small correlated decrease in the amount of C18:2 in the genotypes carrying the A allele. These results are in line with previous findings (Stoffel et al., 2014) indicating that a lack of *FADS2* triggers an alternate reaction where C18:2 is diverted to C20:2 instead of *all-cis*-6,9,12–18:3, the first intermediate fatty acid in the canonical endogenous synthesis of C20:4. In the absence of *FADS2*, C20:2 cannot be transformed to *all-cis*-8,11,14–20:3, the last precursor of C20:4, and thus it is further desaturated via *FADS1* to *all-cis*-8,11,14–20:3, an aberrant fatty acid which is incorporated as a surrogate of C20:4 in the diacylglycerol-backbone of membrane phospholipids. Unfortunately, we have no available data to test whether *all-cis*-8,11,14–20:3 declines as expected with the presence of the A allele.

Despite the fact that, in pigs, liver and subcutaneous fat express 8–10 times more *FADS2* than muscle (Taniguchi et al., 2015), the effect of the *FADS2* rs321384923 on the accumulation of C20:4 is more evident in muscle than in subcutaneous fat and liver. Apart from having more statistical power in muscle,

the functional approach in this tissue can be more accurate for the reason that it shows a relatively higher de novo fatty acid synthesis (Bosch et al., 2012). In this regard, the case of the Duroc pigs is particularly interesting because they present a higher proportion of C18:2 in IMF relative to other breeds (Wood et al., 2004). Moreover, an alternative transcript of *FADS1*, particularly enriched in subcutaneous fat and liver (Taniguchi et al., 2015), has been shown to regulate FADS2 activity in humans (Park et al., 2012). In this line, in a previous expression genome-wide association study (Revilla et al., 2018), the correlation between *FADS1* and *FADS2* mRNA expression was higher in liver than in subcutaneous fat ( $r = 0.92$  and  $0.63$ , respectively) while *FADS2* expression showed the lowest correlation ( $r = 0.23$ ) between these two tissues. Thus, the interaction between these two genes and tissue-specific mechanisms of regulation is a question worth exploring in the future.

Many human and mouse studies positively correlates  $\Delta$ -6 activity with metabolic syndrome, insulin resistance and obesity (Naughton et al., 2016). Diets rich in C18:2 result in an increase in C20:4, which can then be converted into prostacyclins and endocannabinoids, both of them with a strong pro-adipogenic activity (Naughton et al., 2016). Indeed, although many of these studies report weight gain and increased adipose inflammation, the final outcome is highly dependent on the whole diet and other external factors such age or physical activity. Some research even showed that dietary supplements with C18:2 increase lean mass (Belury et al., 2016). In our study with pigs none of the carcass traits analyzed were affected by the higher FADS2 activity of the A rs321384923 allele. Even though C20:4 was negatively correlated with carcass weight ( $r = -0.39$ ,  $p < 0.01$ , for GM) and positively with lean content ( $r = 0.22$ ,  $p < 0.01$ , for GM), the effect of the *FADS2* SNP on n6-fatty acid composition did not per se modify these traits. Our results also indicate that, although unevenly across muscles, the tag SNP could exert some influence on IMF, especially in muscles displaying lower C20:4 content (i.e. GM, with 15% and 50% less C20:4 than LM and SM, respectively; Figure 4). Using data from four commercial genetic types, Renaville

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et al. (2013) found that a SNP in the exon 3 of *FADS2* was associated with contents of C20:4 and IMF, but only in LM and not in muscle biceps femoris. In line with our results, the favorable effect on IMF was associated with the negative allele for C20:4 and evidenced in the muscle with the lowest C20:4. Moreover, the potential distinct effect of the *FADS2* SNP on IMF in different muscles could be attributed to the differential partitioning of C20:4 between neutral lipids and phospholipids across muscles. Thus, Wood et al. (2004) found that the proportion of C20:4 in phospholipids as compared to neutral lipids was higher in LM (around 50 times) than in psoas major (around 10 times), even when both muscles were compared at a similar level of IMF. In a limited subset of pigs, we obtained that in GM C20:4 was around 35 times more abundant in phospholipids than in neutral lipids. These results indicate that the ability of C20:4 to get incorporated into membrane phospholipids, and likely to mediate in cell signaling events, could be muscle-specific. Results in mice evidenced that *FADS2* deficiency alters the membrane phospholipidomic profiling, affecting the maturation of transcription factor sterol-regulatory- element-binding protein and therefore lipid homeostasis (Stoffel et al., 2014). In this sense, an interesting piece of research to address the nuances of functionality of C20:4 in pig muscle is to determine how differently the *FADS2* genotypes affect phospholipid and neutral lipid fatty acid composition and whether their allocation relates with fat content. IMF is a relevant trait for the pig industry in general, but particularly in Duroc lines used in premium quality meat markets, where pigs are raised to display a high level of IMF. For this reason, rs321384923 cannot be discarded as a candidate marker to increase IMF without altering lean weight.

In line with findings in humans (Merino et al., 2010), our results confirm that the porcine *FADS2* is subjected to functional genetic variation while providing evidence that the rs321384923 SNP in its promoter region impacts gene expression. We showed that there is an haplotype tagging SNP in the promoter region of *FADS2* that results in a more efficient transformation of C18:2 into C20:4. However, we were not able to observe any consistent

implication of this on the traits usually selected for in pig populations. Evidence in humans indicates that fatty acid desaturases affect plasma and tissue lipid profiles and therefore associated disease risk factors. Recent findings in humans suggest that *FADS* genes have been subjected to strong positive selection in response to C18:2 consumption and that this event is not neutral in relation to plasma cholesterol levels (Buckley et al., 2017) and to chronic and inflammatory disorders (Merino et al., 2010). Further studies are needed to identify the molecular mechanisms by which variation in *FADS2* modulates gene expression and functional phenotypes. In this regard, the present work confirms that selected pig populations can be an interesting genetic resource.

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## Supplementary Information

**Table S1.** Description of the animals used to sequence the *FADS2* promoter. From the resource Duroc population, a subset of 14 animals from two batches were selected based on their intramuscular fat (IMF, in % dry matter) and arachidonic acid (C20:4, in % of total fatty acids) content in the gluteus medius muscle (GM), longissimus thoracis (LM) and semimembranosus muscle (SM). On average, pigs in group A had about twice as much C20:4 than pigs in Group B.

Group	No of pigs	IMF, %			C20:4, %		
		GM	LM	SM	GM	LM	SM
A	7	13.4	9.6	5.6	2.5	3.0	5.9
B	7	20.7	17.2	13.3	1.3	1.3	2.3

**Table S2.** Primers (A), reagents (B) and (C) cycling PCR conditions used to sequence the pig *FADS2* proximal promoter.

A. Primers used to amplify the proximal promoter of the pig *FADS2* gene.

Primer	Sequence (5' → 3')	T <sub>m</sub>	Expected size
FADS2_Pf1	ACCCCCACCTTTATTTTCCTG	59.7 °C	1097 bp
FADS2_PR1	TTGCTTTTCGGCTTTTGTCTT	55.7 °C	

B. Reagents used to set up the *FADS2* promoter PCR.

Reagents	Volume per PCR reaction
H <sub>2</sub> O	9.5 µl
10x Buffer	1.5 µl
50 mM MgCl <sub>2</sub>	0.75 µl
5 mM dNTP	0.6 µl
Primer mix (Fw+Rv) 10 µM each	0.6 µl
Taq polymerase, Bioline (5 U/µl)	0.06 µl
Genomic DNA (30 ng/µl)	2 µl
Total	15 uL

C. Cycling conditions used to PCR the pig *FADS2* promoter.

PCR Program		
	Temperature	Time
Initial DNA denaturing	94°C	4 min
DNA denaturing	97°C	15 sec
35 cycles	Primer annealing	60°C
	Extension	72°C
Final extension	72°C	5 min

**Table S3.** Primers used to analyse *FADS2* gene expression.

Primer	Sequence (5' → 3')	Tm	Expected size
qFADS2_F	GCTGGATTCCAACCCTCATG	57.9 °C	56 bp
qFADS2_R	AGCCTGGGCCTGAGAGGTA	59.8 °C	
qYWHAZ_F	TGATGATAAGAAAGGGATTGTGG	59.4 °C	134 bp
qYWHAZ_R	G TTCAGCAATGGCTTCATCA	61.3 °C	
qRPL32_F	CACCAGTCAGACCGATATGTCAA	61.1 °C	70 bp
qRPL32_R	CGCACCCCTGTTGTCAATGC	61.1 °C	

**Table S4.** Genotype distribution of the *FADS2* single nucleotide polymorphism rs321384923A>G. *FADS2* genotypes are presented across stearoyl-CoA desaturase (*SCD*, AY487830:g.2228T>C) and leptin receptor (*LEPR*, NM\_001024587:g.1987C>T) genotypes. The *SCD* and *LEPR* SNPs, which have been shown to exert a substantial influence on fatty acid composition, were also segregating in the resource Duroc pig line used in this study. A total of 1,192 samples have been genotyped for the three polymorphisms.

<i>FADS2</i>									
<i>SCD</i>	AA (n=120)			AG (n= 497)			GG (n=575)		
	<i>LEPR</i>			<i>LEPR</i>			<i>LEPR</i>		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
CC	14	10	4	61	73	33	55	74	30
CT	21	30	22	78	103	39	93	134	57
TT	11	5	3	23	61	26	34	77	21

**Table S5.** Carcass weight and composition of pigs by *FADS2* rs321384923 genotype. There was no evidence that the A allele at the *FADS2* genotype had a consistent impact on growth, lean content and fat distribution.

Trait	P-value <sup>1</sup>	<i>FADS2</i> genotype <sup>2</sup>		
		AA	AG	GG
Carcass weight, kg	0.14	96.4 ± 1.0	98.3 ± 0.6	97.6 ± 0.5
Backfat thickness, mm	0.99	26.3 ± 0.4	26.3 ± 0.3	26.3 ± 0.2
Loin thickness, mm	0.21	43.3 ± 0.8	44.5 ± 0.5	43.7 ± 0.4
Lean, %	0.83	40.9 ± 0.5	41.3 ± 0.3	41.1 ± 0.3
Lean weight, kg	0.11	39.0 ± 0.6	40.2 ± 0.3	39.8 ± 0.3
Intramuscular fat, % dry matter				
m. gluteus medius	0.10	18.0 ± 0.5	18.7 ± 0.3	19.1 ± 0.3
m. longissimus thoracis	0.82	14.1 ± 0.5	13.7 ± 0.3	13.8 ± 0.3
m. semimembranous	0.80	10.3 ± 1.1	10.6 ± 0.6	10.1 ± 0.6
Liver fat content	0.12	15.1 ± 0.9	15.9 ± 0.4	14.8 ± 0.4

<sup>1</sup>P-value associated with the effect of the *FADS2* genotype;

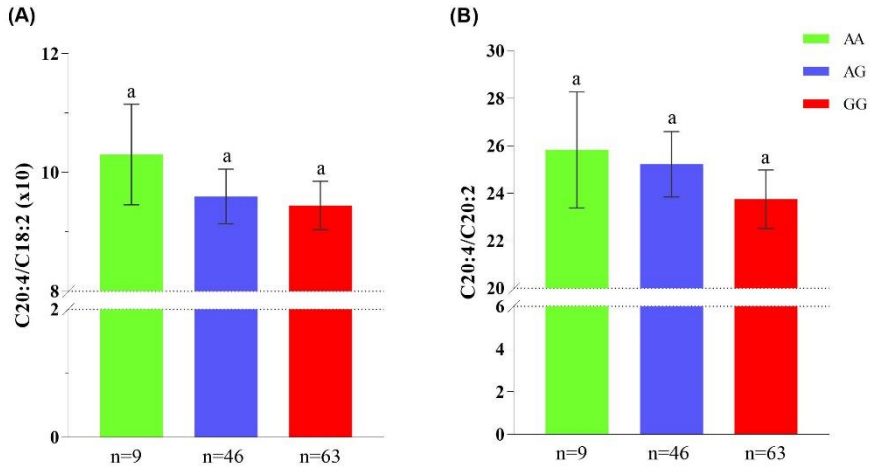
**Figure S1.** Sequence of the pig *FADS2* proximal promoter. The sequence corresponding to the primers used for sequencing is underlined and highlighted in yellow. In bold, coding region. The arrow indicates the start of the first exon in the closest transcript described in Ensembl Sscrofa 11.1 (ENSSSCT00000014289.3). Positions are referred to the ATG codon. In pink, polymorphisms identified during the sequencing process. The SNP selected for this study correspond to rs321384923.

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AGGCCTATCCTTTTGTACCCCCACCTTTAATTCCTGGAGTCTCTACAGCATCACTGAACA -1111
ATTGATGGTATGAGGTCCCGTATTGCTTTCCATGTTCTCCCTCTCCCAACTAGGTTGTGAGA -1050
TTCCTGAGTTCAGGGCTAGAACCAAGTCTTGTTCACCCTGGGCCCCCCTCTCCCCACC -989
ACGGCGCCCCCTCTCCACCACCATGGCGCCTAACACAGGAAAGCACATCAGAGGTTCT -927
GCAATTTTCTCCTAAGATCCACTGATTACAGGCTTCAGAGTCYTGAGCCGTCGGGAGGAGG -866
GCTCCTTTCCGAACCAGGGAGGCTGCAGGAGGGCGCCAATGGGGTAAAGCGGACAATGCG -805
GATCCTRAAGTCTCGATTCCAGCGGGTCAGGGACGGGCGCCACCTATCCAGGCCGGTCCCA -744
GAGTGGAACCTCGCCAGCCCCCAGCTCCGCGCTGCCRAGCACCCGCCAGCCTGTCTTCCG -683
GCCGGTYGCCACCGTAAAGCAAGCGGGACCTCTGGGCGCCAGCWTCAGGTGCAAACCCCG -622
GAAGCGCGGGGATGGGGGCGCGCACGCCAAGGAACCTCAGCCACCGCCCCCTCTCGCGG -561
GCCGCGCTCCCTGAGATCCCCTCCCGCGGCGCGGCGCCGGAGCGGGGGCGGAGGAGTTC -500
GGACACGTAACCTGCCTCCCTGCCTGGCTCGACTCCGCGTGGCGGGCAGGCGGGGAGCC -439
GGGACCGCTGCTCCAGCCCGCTGCCTTCGAAAGATCCTCCTGGGCCAATGGCAGGCGGG -378
CGACGCGCCCGATTGGTGCAGAAGCTCTGCTGATCACTGTGAAACCCAGGCGGAGGGGA -317
ACGCGGGAGGATGCAGAGCCCTGGGCGGGGGAGTCCGAGGGGCGGGCACAGGAGCCCTGG -256
AGGCCCTGAGCCTACCGGGGAGTTTAGCT◆GGAGGCAAAGTCCATTGCGGGCTGGCGG -197
GCGGAGGGAGGGGCGGAGGGAGGGGACCGTTTGGGGCCACTGGGAAGCCTGGAGGAAAGGC -136
AAGGATACTCCCAGCGGAGGCGAGGAGGCTGGGGGAGGGGCGCGGTGGGAGGAGGAGAA -75
GACAAAAGCCGAAAGCAAGGAGGGCCGAGCGGCACAGACCGCAGTGACCCGGGCAACCTG -14
GTCAGCGGGCAGCATGGGGAAGGGGGGAACCAGGGCGAGGGGGCCACCAGCGCGAGGCC +48

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**Figure S2.** Efficiency of arachidonic acid biosynthesis by *FADS2* genotype in liver. The presence of the A allele was not seen to be associated neither with (A) the arachidonic acid (C20:4) to linoleic acid (C18:2) ratio nor with (B) C20:4 to eicosadienoic acid (C20:2) ratio in the liver. Error bars represent standard errors. Within trait, means with the same superscript do not differ significantly ( $P < 0.05$ ).



## CHAPTER III

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### RELATIONSHIP BETWEEN *PERILIPIN* GENES POLYMORPHISMS AND GROWTH, CARCASS AND MEAT QUALITY TRAITS IN PIGS

Published as:

Gol, S., Ros-Freixedes, R., Zambonelli, P., Tor, M., Pena, R.N., Braglia, S., Zappaterra, M., Estany, J. and Davoli, R. (2016). Relationship between perilipin genes polymorphisms and growth, carcass and meat quality traits in pigs. *Journal of Animal Breeding and Genetics*, 133, 24-30. DOI: 10.1111/jbg.12159





**Abstract**

The perilipins (PLIN) belong to a family of structural proteins that play a role regulating intracellular lipid storage and mobilization. Here, *PLIN1* and *PLIN2* have been evaluated as candidate genes for growth, carcass and meat quality traits in pigs. A sample of 607 Duroc pigs were genotyped for two single-nucleotide polymorphisms, one in intron 2 of the *PLIN1* gene (*JN860199:g.173G>A*) and the other at the 3' untranslated region of the *PLIN2* gene (*GU461317:g.98G>A*). Using a Bayesian approach, we have been able to find evidence of additive, dominant and epistatic associations of the *PLIN1* and *PLIN2* polymorphisms with early growth rate and carcass length. However, the major effects were produced by the dominant A allele at the *PLIN2* polymorphism, which also affected the carcass lean weight. Thus, pigs carrying an additional copy of the A allele at the *g.98G>A PLIN2* polymorphism had a probability of at least 98% of producing carcasses with heavier lean weight (+0.41 kg) and ham weight (+0.10 kg). The results obtained indicate that the *PLIN2* polymorphism could be a useful marker for lean growth. In particular, it may help to reduce the undesired negative correlated response in lean weight to selection for increased intramuscular fat content, a common scenario in some Duroc lines involved in the production of high quality pork products.

**1. Introduction**

Growth rate and carcass lean content are crucial characteristics for the economic viability of pork production. Selection emphasizing lean content has led to reduce some pork quality attributes, including the intramuscular fat (IMF) content. The use of molecular markers may be useful to improve the genetic progress in traits that are difficult and expensive to measure (Dekkers, 2004), but also to break down unfavourable genetic correlations between antagonistic traits, such as those between lean growth rate or carcass lean content and IMF content (Ros-Freixedes et al., 2012; 2013). In this scenario, performing association studies

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with candidate genes related to proteins affecting fat metabolism is of particular interest.

The perilipins (PLIN) belong to a family of structural proteins that coat intracellular lipids into cytosolic droplets (Kimmel et al., 2010), where they regulate intracellular lipid storage and mobilization by finetuning the activity of lipases (Bickel et al., 2009). The composition of PLIN changes as lipid droplets enlarge and mature. Perilipin 2 (PLIN2) is the most prominent PLIN protein in most adult cell types and in immature adipocytes. In contrast, the large central mature lipid droplets of mature adipocytes are largely coated by perilipin 1 (PLIN1). Recently, PLIN1 and PLIN2 have been shown to colocalize in the skeletal muscle of pigs (Gandolfi et al., 2011).

Mutations in the *PLIN* genes have been associated to body fat mass in mice (Saha et al., 2004) and humans (Qi et al., 2004; Corella et al., 2005; Ruiz et al., 2011). So far, only two reports in pigs have investigated the association of PLIN1 and PLIN2 polymorphisms with a limited number of production traits. In the first report, two synonymous single-nucleotide polymorphisms (SNPs) in exons 3 and 6 of *PLIN1* showed suggestive associations with average daily gain (ADG) and backfat thickness in large white pigs (Vykoukalová et al., 2009). In a second study, a 3' untranslated region (UTR) SNP at the *PLIN2* gene (GU461317:g.98G>A) was found to be associated to lean growth and content but not to visible intermuscular fat (Davoli et al., 2011). The aim of this study was to further investigate the contribution of *PLIN1* and *PLIN2* genes to a wider range of performance, carcass and meat quality traits in pigs and, in particular, to confirm whether PLIN1 and PLIN2 genotype variants exert a differential effect on lean growth and IMF content.

## 2. Material and methods

### 2.1 Animal, traits and sample collection

A panel of 20 unrelated pigs from three Italian heavy breeds was used for the SNP screening of *PLIN1* gene, including eight Italian Large White, four Italian Duroc and eight Italian Landrace pigs. A total of 607 Duroc barrows from 88 sires and 348 dams of a Spanish commercial Duroc line (Ros-Freixedes et al., 2012) were used for the association analyses. These pigs were randomly sampled in seven batches and performance tested from 75 to 210 days of age under commercial conditions. During the test period, they had ad libitum access to commercial diets. A complete description of the line and of the procedures followed for testing and sample collection is given in Ros-Freixedes et al. (2012). The traits recorded included live body weight (BW), backfat thickness and loin thickness at 120, 180 and 205 days. Backfat and loin thickness was ultrasonically measured at 5 cm off the midline at the position of the last rib (Piglog 105; SFK-Technology, Herlev, Denmark). After slaughter at 210 days, the carcass weight and length, the carcass backfat and loin thickness, and the ham weight were measured. Carcass backfat and loin thickness at 6 cm off the midline between the third and fourth last ribs, together with the carcass lean percentage, were estimated using an on-line ultrasound automatic scanner (AutoFOM; SFK-Technology, Herlev, Denmark). After chilling for approximately 24 h at 2°C, the pH was measured in the longissimus dorsi and in the semimembranosus muscles. Samples of at least 50 g of gluteus medius muscle and longissimus dorsi were taken, immediately vacuum packaged, and stored in deep freeze until required for IMF content and fatty acid determination (Bosch et al., 2009).

### 2.2 Single-nucleotide polymorphism discovery and genotyping

To search for sequence variation in the pig *PLIN1* gene, the genomic, cDNA and EST sequences available in the GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) and in the Ensembl databases

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(<http://www.ensembl.org>) were compared for an in silico variability analysis. Italian heavy pigs were used to validate the in silico-identified SNPs. Seven primer pairs (Table S1) were designed using PRIMER3 v.0.4.0 software (<http://frodo.wi.mit.edu/primer3/>) to amplify seven porcine *PLIN1* gene fragments. The PCR products were sequenced on both strands. The sequences obtained were compared with MEGA v4.0 ([www.megasoftware.net/](http://www.megasoftware.net/)). The *JN860199:g.173G>A* *PLIN1* SNP was genotyped by PCR-restriction fragment length polymorphism assay by restricting the ‘P2’ PCR product (Table S1) with Hin1II (Fermentas, Vilnius, Lithuania). For *PLIN2*, the *GU461317:g.98G>A* SNP was genotyped by High Resolution Melting PCR in a Rotor-Gene™ 6000 (Corbett Research, Mortlake, NSW, Australia) following the protocol described in Davoli et al. (2011).

### 2.3 Association analysis

The additive, dominant and epistatic effects of the *PLIN* genotypes were estimated independently for each trait using a Bayesian setting, in line with the methodology described in Ros-Freixedes et al. (2012). A two-generation pedigree was used for the analyses. In matrix notation, the model used for the *i*th trait was  $\mathbf{y}_i = \mathbf{X}_i\mathbf{b}_i + \mathbf{Z}_i\mathbf{a}_i + \mathbf{e}_i$ , where  $\mathbf{y}_i$  is the vector of observations for trait *i*;  $\mathbf{b}_i$ ,  $\mathbf{a}_i$  and  $\mathbf{e}_i$  are the vectors of systematic, polygenic and residual effects, respectively; and  $\mathbf{X}_i$  and  $\mathbf{Z}_i$  the known incidence matrices that relate  $\mathbf{b}_i$  and  $\mathbf{a}_i$  with  $\mathbf{y}_i$ , respectively. The systematic effects were the batch (seven levels), the age at test as a covariate, and orthogonal coefficients for additive (a), dominance deviation (d) and first-order epistatic effects (aa: additive x additive; ad: additive x dominance; da: dominance x additive; and dd: dominance x dominance) for *PLIN1* and *PLIN2* SNPs. Pigs in a given batch were contemporaneous pigs tested at the same unit and slaughtered in the same abattoir. The litter effect was not included because, on average, there were <2 piglets per litter. The orthogonal coefficients for the genetic effects were calculated using the algorithm proposed by Alvarez-Castro and Carlborg (2007).

The models were solved using Gibbs sampling with the TM software (Legarra et al., 2008). The traits were assumed to be conditionally normally distributed as  $[y_i | \mathbf{b}_i, \mathbf{a}_i \mathbf{I} \sigma_{ei}^2] \sim N(\mathbf{X}\mathbf{b}_i + \mathbf{Z}\mathbf{a}_i; \mathbf{I}\sigma_{ei}^2)$ , where  $\sigma_{ei}^2$  is the residual variance and  $\mathbf{I}$  the appropriate identity matrix. The animal effects conditionally on the additive genetic variance  $\sigma_{ei}^2$  were assumed multivariate normally distributed with mean zero and variance  $\mathbf{A}\sigma_{ei}^2$ , where  $\mathbf{A}$  was the numerator relationship matrix. The matrix  $\mathbf{A}$  was calculated using 1043 animals in the pedigree. Flat priors were used for  $\mathbf{b}_i$  while the variance components were set to the values obtained by Ros-Freixedes et al. (2013) with data and pedigree from 1996 onwards (Table S3). Statistical inferences were derived from the samples of the marginal posterior distribution using a unique chain of 500,000 iterations, where the first 100,000 were discarded and one sample out of 100 iterations retained. The additive, dominance and epistatic effects were assessed by calculating both the probability of each of these components being greater or lower than zero. Convergence was tested using the Z-criterion of Geweke (1992) and visual inspection of convergence plots.

### 3. Results and discussion

#### 3.1 Polymorphisms and sequence variation of PLIN genes

The in silico analysis revealed ten SNPs (detected at least twice) within the coding sequence of *PLIN1*, located in the exons 1, 2, 5 and 8 (data not shown) and five SNPs in intronic regions. Seven genomic regions, covering the positions of the 10 putative SNPs, were subjected to direct sequencing in 20 animals from Italian heavy pig breeds. The fragment on exon 8 was not analysed due to the unsuccessful amplification of this region. Four SNPs (two intronic and two exonic) out of the ten SNPs discovered in silico were detected by sequencing Italian heavy pig breeds (Table 1). The other six polymorphisms identified in silico were not detected during the sequencing. The two intronic SNPs were novel [JN860199; SNP *g.173G>A* and *g.3484C>G*], while the two exonic SNPs were both synonymous and had been reported before (GenBank: AM931171; SNP

*g.4119A>G* and *g.7966T>C*; Vykoukalová et al., 2009). The four SNPs were in complete linkage disequilibrium in the initial panel of 20 pigs. The intronic *JN860199 g.173G>A* SNP was selected for subsequent analyses because a restriction enzyme was available to analyse this mutation. In this SNP, the G allele was the less frequent (0.38) while the alleles G and A in *GU461317:g.98G>A* *PLIN2* SNP showed identical frequencies (Table S2).

**Table 1.** Single nucleotide polymorphisms (SNP) detected by sequencing the porcine *PLIN1* gene in Italian heavy pigs.

SNP <sup>1</sup>	Gene position <sup>2</sup>	Gene location	Amino acid change
JN860199 <i>g.173G&gt;A</i>	691	Intron 1	-
JN860199 <i>g.3484C&gt;G</i>	4,004	Intron 1	-
AM931171 <i>g.4119A&gt;G</i>	4,119	Exon 2	Synonymous <sup>3</sup>
AM931171 <i>g.7966T&gt;C</i>	7,966	Exon 5	Synonymous <sup>3</sup>

1 GenBank accession number is indicated.

2 Position from the start codon as referred to the entry [Ensembl:ENS-SSCG00000001844; assembly Sscrofa10.2: chromosome 7; 60,126,614:60,139,897:-1]

3 These SNPs are also reported by Vykoukalová et al., 2009

### 3.2 Effect of *PLIN* genotypes

The additive, dominant and epistatic effects of *PLIN1 g.173G>A* and *PLIN2 g.98G>A* SNPs associated to BW and growth rate at different ages during the fattening period are given in Table 2. The substitution of A for G in *PLIN1* showed some evidence of a negative additive effect on BW (-0.66 kg at 120 days and -0.68 kg at 180 days, with a probability of 6 and 10% of being greater than zero, respectively), but a strong evidence of a positive additive effect in *PLIN2*, with values of +0.95 kg, +1.19 kg, and +1.08 kg at 120, 180 and 205 days, respectively, with an associated probability of being greater than zero superior to 95% in the three ages. The substitution effect of A for G for BW was similar at 120, 180 and 205 days, thereby indicating that the beneficial effect of allele A on

BW was due to increased growth at early stages. In concordance, the effect of allele A at *PLIN2* for ADG was evident up to 120 days (+7.26 g/day, with a probability of being positive of 98%) but not thereafter, both from 120 to 180 days (+4.15 g/day) and from 180 to 205 days (-0.42 g/day). Consequently, the variance associated to the additive effects of *PLIN2* *g.98G>A* SNP (Falconer and Mackay, 1996) is able to capture a greater proportion of the additive variance of BW (Ros-Freixedes et al., 2013) at 120 days (1.49%) than at 205 days (1.12%). Regarding the dominant effects, a negative dominant effect for BW at 120 and 180 days in *PLIN1* (-1.04 and -1.56 kg, respectively) and a positive dominant effect for BW at 180 days in *PLIN2* (+1.17 kg) were observed (Table 2). No clear evidence of epistasis between *PLIN1* and *PLIN2* SNPs was observed for BW and ADG, with the exception of an additive x additive effect for BW at 120 days (-0.88 kg, with a probability of being positive of 6%) and for ADG up to 120 days (-7.94 g/day, with a probability of being positive of 4%).

The additive, dominant and epistatic effects of *PLIN1* *g.173G>A* and *PLIN2* *g.98G>A* SNPs associated to backfat and loin thickness at 120, 180 and 205 days of age are given in Table 3. The *PLIN1* *g.173G>A* SNP did not show a clear pattern of association with fatness traits, but results for the *PLIN2* *g.98G>A* SNP indicated that A allele is positively associated to backfat thickness at early ages (+0.17 mm and +0.19 mm, at 120 days and at 180 days, respectively, with a probability of being positive of 91 and 98%) and negatively to backfat thickness at 205 days (-0.22 mm, with a probability of being positive of 10%). The effect of the *PLIN2* *g.98G>A* SNP on backfat thickness followed a similar pattern as for ADG, with the positive effect of allele A at 120-day vanishing at later ages.

In agreement with these results, no strong evidence of association of *PLIN1* and *PLIN2* SNPs with carcass backfat thickness, and carcass loin thickness was observed (Table 4). However, allele G at *PLIN1* and allele A at *PLIN2* had some beneficial effects on other carcass traits. Thus, pigs carrying an additional copy of allele G at *PLIN1* and allele A at *PLIN2* had longer carcasses (+0.62 and +0.43 cm, with a probability of being positive >96 and 99%,



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respectively) and, more interestingly, those carrying allele A at *PLIN2* showed a higher carcass lean weight (+0.41 kg, with a probability of being positive of 99.9%). This latter effect should be interpreted as a result of a moderate but favourable change in both carcass weight (+0.58 kg), mostly as a consequence of increased growth rate at early ages, and carcass lean percentage (+0.23). As a result, the *PLIN2* *g.98G>A* SNP reached to explain 0.59% of the additive variance of lean weight. Moreover, a positive effect of allele A at *PLIN2* on ham weight was also detected (0.10 kg, with a probability of being positive of 94%). No evidence was found indicating that meat quality traits (pH and IMF) were additive by *PLIN1* and *PLIN2* SNP, although some minor changes were observed for IMF fatty acid composition (Table S4). In particular, allele A at *PLIN1* decreased PUFA (-0.20%) and increased MUFA (0.20%) while allele A at *PLIN2* decreased SFA (-0.24%).

Evidence supporting the existence of dominant and epistatic effects associated to carcass and meat quality traits was mostly circumscribed to traits where the additive effects were more evident (carcass length and carcass lean weight), hereby suggesting that the mode of action of *PLIN1* and *PLIN2* on the traits that they are influencing is subjected to complex regulations. As for BW and ADG, the dominant effect associated to lean weight was negative in *PLIN1* (-0.19 kg, with a probability of 2% of being positive) but positive in *PLIN2* (0.41 kg, with 99.9% probability of being positive). These dominant values were around twofold higher than their respective additives, a result which supports for an underdominant *PLIN1* and overdominant *PLIN2* gene action for lean weight. To assess the stability of the estimates to model overparameterization, the additive and dominance effects were also estimated ignoring the epistatic effects. The estimates obtained (results not shown), although slightly higher, were in line with those reported with the model that included epistasis, thereby confirming the favourable effects of allele G at *PLIN1* and allele A in *PLIN2* on growth and carcass traits.

**Table 2.** Mean (standard deviation) and additive, dominant, and epistatic effects of *PLIN1* JN860199:g.173G>A and *PLIN2* GU461317:g.98G>A polymorphisms associated to live body weight and growth rate at different ages.

Trait	Mean SD	Additive (a) and dominant (d) effects <sup>1</sup>															
		PLIN1, g:173G>A					PLIN2, g:98G>A										
		a <sub>1</sub>	P(>0)	d <sub>1</sub>	P(>0)	a <sub>2</sub>	P(>0)	d <sub>2</sub>	P(>0)	a <sub>1a1</sub>	P(>0)	a <sub>1d2</sub>	P(>0)	d <sub>1a2</sub>	P(>0)	d <sub>1d2</sub>	P(>0)
Body weight, kg																	
120days	61.28 (12.13)	-0.66	<b>0.06</b>	-1.04	<b>0.05</b>	0.95	<b>0.99</b>	0.77	0.89	-0.88	<b>0.06</b>	0.47	0.71	-0.51	0.29	1.35	0.86
180days	107.32 (11.01)	-0.68	<b>0.10</b>	-1.56	<b>0.03</b>	1.19	<b>0.98</b>	1.17	<b>0.94</b>	-0.78	0.14	0.64	0.73	0.13	0.55	0.59	0.65
205days	122.15 (11.33)	-0.42	0.27	-0.51	0.29	1.08	<b>0.96</b>	1.03	0.87	-1.01	0.12	0.19	0.56	0.46	0.63	0.18	0.55
Daily gain, g/d																	
0-120days	500.77 (80.94)	-4.76	<b>0.09</b>	-6.93	<b>0.09</b>	7.26	<b>0.98</b>	5.51	0.86	-7.94	<b>0.04</b>	4.70	0.76	-4.59	0.27	12.04	0.88
120-180days	766.88 (112.88)	-1.95	0.38	-6.83	0.29	4.15	0.74	4.37	0.69	2.26	0.60	1.10	0.54	15.38	0.87	-10.22	0.30
180-205days	596.23 (193.43)	5.72	0.70	22.65	<b>0.94</b>	-0.42	0.48	-9.57	0.48	-8.23	0.28	-3.27	0.41	20.03	0.82	-22.91	0.24

<sup>1</sup> The numbers 1 and 2 refer to *PLIN1* and *PLIN2*, respectively, with the additive effects expressed as A-G. P (>0): Posterior probability of a value being positive. In bold, probabilities above 0.90 or below 0.10.

**Table 3.** Mean (standard deviation) and additive, dominant, and epistatic effects of *PLIN1* JN860199:g.173G>A and *PLIN2* U461317:g.98G>A polymorphisms associated to backfat and loin thickness at different ages.

Trait	Mean SD	Additive (a) and dominant (d) effects <sup>1</sup>						Epistatic effects <sup>1</sup>									
		a <sub>1</sub>	P(>0)	d <sub>1</sub>	P(>0)	a <sub>2</sub>	P(>0)	d <sub>2</sub>	P(>0)	a <sub>1a1</sub>	P(>0)	a <sub>1d2</sub>	P(>0)	d <sub>1a2</sub>	P(>0)	d <sub>1d2</sub>	P(>0)
Body thickness, mm																	
120d	11.05 (2.72)	-0.07	0.29	-0.18	0.17	0.17	<b>0.91</b>	-0.07	0.33	-0.23	<b>0.07</b>	0.03	0.55	-0.14	0.29	0.59	<b>0.95</b>
180d	17.76 (3.74)	-0.06	0.27	-0.15	0.14	0.19	<b>0.98</b>	-0.10	<b>0.31</b>	-0.76	0.16	0.54	0.69	0.15	0.56	0.79	0.68
205d	20.66 (4.15)	0.01	0.52	-0.24	0.16	-0.22	<b>0.10</b>	-0.03	0.46	-0.41	<b>0.03</b>	0.06	0.58	0.12	0.63	0.05	0.54
Loin thickness, mm																	
120d	40.38 (3.25)	0.33	<b>0.92</b>	-0.40	0.15	-0.42	<b>0.04</b>	-0.59	0.04	0.07	0.59	-0.23	0.31	-0.91	0.04	0.31	0.66
180d	45.04 (3.97)	0.26	0.85	-0.56	0.20	-0.05	0.41	-0.63	0.03	0.23	0.75	1.51	0.93	0.49	0.82	-0.42	0.28
205d	48.57 (4.49)	0.00	0.51	0.11	0.61	0.02	0.52	-0.08	0.42	-0.46	0.09	-0.33	0.25	-0.47	0.19	0.31	0.65

<sup>1</sup> The numbers 1 and 2 refer to *PLIN1* and *PLIN2*, respectively, with the additive effects expressed as A-G. P (>0): Posterior probability of a value being positive. In bold, probabilities above 0.90 or below 0.10.

**Table 4** Mean (standard deviation) and additive, dominant, and epistatic effects of *PLIN1* JN860199:g.173G>A and *PLIN2* U461317:g.98G>A polymorphisms associated to carcass traits.

Trait	Mean SD	Additive (a) and dominant (d) effects <sup>1</sup>															
		PLIN1, g.173G>A					PLIN2, g.98G>A										
		a <sub>1</sub>	P(>0)	d <sub>1</sub>	P(>0)	a <sub>2</sub>	P(>0)	d <sub>2</sub>	P(>0)	a <sub>1a<sub>2</sub></sub>	P(>0)	a <sub>1d<sub>2</sub></sub>	P(>0)	d <sub>1d<sub>2</sub></sub>	P(>0)		
Carcass weight, kg	93.69 (9.28)	-0.20	0.36	0.41	0.70	0.58	0.86	-0.95	0.11	1.09	<b>0.94</b>	0.19	0.57	-0.07	0.47	-0.50	0.36
Carcass backfat, mm	22.59 (3.68)	-0.09	0.33	0.02	0.52	-0.15	0.24	0.10	0.65	0.32	0.88	0.41	0.85	0.19	0.69	-0.21	0.36
Carcass loin, mm	45.25 (7.23)	0.23	0.69	-0.19	0.39	0.28	0.73	-0.52	0.22	0.58	0.83	0.69	0.78	-0.74	0.22	-0.70	0.31
Carcass lean, %	43.77 (4.96)	0.08	0.62	-0.01	0.50	0.23	0.80	-0.47	0.11	-0.17	0.32	-0.20	0.36	-0.14	0.41	0.20	0.59
Carcass length, cm	86.58 (2.96)	-0.62	<b>0.04</b>	0.81	<b>&gt;0.99</b>	0.42	<b>0.99</b>	-0.82	<b>&lt;0.01</b>	0.92	<b>0.98</b>	-0.22	0.24	-0.45	0.11	-0.14	0.39
Lean weight, kg	40.73 (5.29)	0.07	0.85	0.19	<b>0.98</b>	0.41	<b>&gt;0.99</b>	-0.72	<b>&lt;0.01</b>	0.30	<b>&gt;0.99</b>	-0.11	0.20	-0.37	<b>&lt;0.01</b>	-0.06	0.38
Ham weight, kg	12.09 (1.16)	0.00	0.51	-0.04	0.34	0.10	<b>0.94</b>	-0.05	0.28	0.09	0.86	0.20	<b>0.95</b>	-0.04	0.39	-0.10	0.28

<sup>1</sup> The numbers 1 and 2 refer to *PLIN1* and *PLIN2*, respectively, with the additive effects expressed as A-G. P (>0): Posterior probability of a value being positive. In bold, probabilities above 0.90 or below 0.10.

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Our findings are consistent with the results in Vykoukalová et al. (2009), who found suggestive associations of the two exonic *PLIN1* SNP with ADG in large white pigs, and, particularly, with those in Davoli et al. (2011), who reported a favourable effect of allele A at *PLIN2* on ADG, feed conversion ratio, lean cuts and ham weight estimated breeding values in Italian Duroc. The five members of the PLIN family have been studied in depth in humans and model animals. Most reports have focused on PLIN1, the main perilipin protein in mature adipocytes, particularly in relation to BW and obesity-related phenotypes (Smith and Ordovás, 2012), but results do not show a consistent trend across them. It must be taken into account that, depending on the energy state of the organism, PLIN1 either limits lipase access to stored triglycerides (in the fed state) or facilitates hormonally stimulated lipolysis (in the fasted state). This dual activity is illustrated by the fact that both PLIN1-null and PLIN1-overexpressing mice are protected from diet-induced obesity (Saha et al., 2004). In our pig population, mutations in the *PLIN1* did not correlate with growth or fat deposition traits. This indicates that genes other than *PLIN1* are the main players of fat deposition in pig, or that other mutations outside the transcribed sequence, for instance in the 5' or 3' regulatory regions, might have a more relevant effect over the expression of the gene. In contrast, only few reports in humans and mice have focused on *PLIN2* gene. Our results indicate that allele A at the *PLIN2* g.98G>A SNP has beneficial effects on early growth, lean growth and prime retail cuts. In agreement with this, the genomic position of *PLIN2* on chromosome 1 colocalizes with quantitative trait loci for ADG (Liu et al., 2007), BW at birth (Guo et al., 2008) and daily feed intake (Kim et al., 2000) (Table S5). Of the five PLIN proteins, PLIN2 and 3 are by far the most prominent in human skeletal muscle (Gjelstad et al., 2012), with PLIN2 accounting for >60% of total perilipin content. It has been shown that PLIN2 is also the main perilipin in pig muscle (Gandolfi et al., 2011). Therefore, it is not surprising that *PLIN2* is related to growth and lean weight, as perilipins regulate not the deposition of fat per se, but more

importantly, the accessibility of lipases to the stored fats in response to the energy demands of the cells.

Our results indicate that *PLIN2* *g.98G>A* SNP could be a useful marker for lean growth, which is a relevant trait for the pig industry in general, very interested in fast-growing lean animals. Although results are encouraging for Duroc, further association studies are needed to confirm whether this polymorphism similarly affects other pig breeds. However, it is in this breed where it can be of particular interest. Duroc lines are the most used in premium quality markets, where pigs are raised to heavy weights and IMF becomes a key trait. In such scenario, it is very convenient to find selection criteria addressed to reduce the undesired negatively correlated response on lean weight to selection for IMF.

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## Supplementary Information

**Table S1.** Primers used for single nucleotide polymorphism discovery in *PLIN1* gene.

Primer	Sequence (5'-3')	Gene regions	Product size (bp)	Annealing Temperature (°C)
P1	F GTCAAATAACCATAGCAACCAAC R ATTCCCAGAAGACCCTAACC	partial promoter; exon 1; partial intron 1	253	61
P2	F AGGGAAGTGTGGTGAGAGG R TCCGCAAGAAGGAGTGAGG	partial intron 1; exon 2, partial intron 2	306	60
P3	F AGAGCCAAGGTTGTGACCAG R CAGGCAGTGAACGAGCAAG	partial intron 2; exon 3, partial intron 3	415	61
P4	F ATCTGCACGCCTGACTCC R TGGTGGCCTCTTGTAATTC	partial intron 4; exon 5; partial intron 5	375	60
P5	F CGGGATGACCACTTTCTAACC R GCTCAGGGCAGACACTCAC	partial intron 5; exon 6	289	60
P6	F AGGTGCTGTGAAGTCAGTGG R TGTTCCAGGGTGAGGTGAAG	partial intron 6; exon 7; partial intron 7	368	61
P7	F GGATAGTGAGGAGGGGAAGG R CAGGAGACTGGGGAAGGAG	partial intron 7; exon 8; 3'downstream genomic region	431	63

**Table S2.** Number of pigs (N), frequency of the allele G (f(G)), and number of pigs per *PLIN1* and *PLIN2* genotypes by batch.

	N	<i>PLIN1</i> (JN860199:g.173G>A)				<i>PLIN2</i> (GU461317:g.98G>A)			
		f(G)	GG	AG	AA	f(G)	GG	AG	AA
Batch 1	108	0.51	36	38	34	0.49	23	60	25
Batch 2	102	0.51	31	42	29	0.37	16	44	42
Batch 3	66	0.35	13	20	33	0.50	15	36	15
Batch 4	69	0.33	6	34	29	0.43	16	27	26
Batch 5	84	0.26	6	32	46	0.60	31	39	14
Batch 6	95	0.31	8	42	45	0.61	37	42	16
Batch 7	83	0.32	8	37	38	0.48	19	42	22
Total	607	0.38	108	245	254	0.50	157	290	160

**Table S3.** Variance components used in the analyses by trait.

Trait <sup>1</sup>	Genetic variance	Residual variance	Heritability
Body weight, kg			
120 days	30.26	28.86	0.51
180 days	29.76	66.41	0.31
205 days	52.18	7378	0.71
Daily gain, g/d			
0-120 days	2,396	1,602	0.60
120-180 days	5,659	4,576	0.55
180-205 days	8,804	23,102	0.28
Backfat thickness, mm			
120 days	2.83	1.93	0.59
180 days	4.11	5.06	0.45
205 days	9.10	5.99	0.60
Loin thickness, mm			
120 days	4.21	8.67	0.33
180 days	7.08	11.40	0.38
205 days	9.04	13.59	0.40
Carcass weight, kg	39.77	47.89	0.45
Carcass backfat, mm	6.73	5.66	0.54
Carcass loin, mm	19.77	41.05	0.33
Carcass lean, %	13.00	10.21	0.56
Carcass length, cm	5.05	3.72	0.58
Lean weight, kg	12.34	14.14	0.47
Ham weight, kg	0.49	0.71	0.41
pH24 LM	0.02	0.02	0.43
pH24 SM	0.03	0.03	0.51
IMF, %	1.77	1.45	0.55
SFA, %	2.25	2.81	0.44
MUFA, %	2.78	2.46	0.53
PUFA, %	2.85	2.06	0.58

1 pH24 LM (SM): pH at 24 h post mortem at muscle longissimus dorsi (semimembranosus); IMF: intramuscular fat in muscle gluteus medius; SFA: saturated fatty acids (C14:0+C16:0+C18:0) in muscle gluteus medius; MUFA: monounsaturated fatty acids (C16:1+C18:1+C20:1) in muscle gluteus medius; PUFA: polyunsaturated fatty acids (C18:2+C18:3+C20:2+C20:4) in muscle gluteus medius.

**Table S4** Mean (standard deviation) and additive, dominant, and epistatic effects for *PLIN1* JN860199:g.173G>A and *PLIN2* U461317:g.98G>A polymorphisms associated to meat quality traits.

Trait	Additive (a) and dominant (d) effects <sup>1</sup>																
	PLIN1, g.173G>A						PLIN2, g.98G>A										
	Mean SD	a <sub>1</sub>	P(>0)	d <sub>1</sub>	P(>0)	a <sub>2</sub>	P(>0)	d <sub>2</sub>	P(>0)	a <sub>1</sub> a <sub>2</sub>	a <sub>1</sub> d <sub>2</sub>	P(>0)					
pH24 LM	5.71 (0.25)	0.00	0.58	0.01	0.61	-0.01	0.23	0.02	0.86	-0.01	0.24	0.03	<b>0.90</b>	0.00	0.47	-0.03	0.20
pH24 SM	5.72 (0.25)	0.01	0.79	0.00	0.52	0.00	0.43	0.03	<b>0.92</b>	-0.02	0.12	0.00	0.57	0.01	0.61	-0.03	0.22
IMF, %	4.50 (1.66)	0.10	0.85	-0.07	0.32	0.04	0.67	0.06	0.67	-0.16	0.11	0.05	0.59	0.11	0.70	0.18	0.73
SFA, %	34.99 (3.68)	0.01	0.53	0.01	0.53	-0.24	<b>0.04</b>	0.07	0.66	-0.15	0.19	-0.22	0.19	-0.08	0.40	-0.08	0.41
MUFA, %	50.54 (3.11)	0.20	<b>0.94</b>	-0.05	0.40	0.30	0.99	-0.17	0.17	0.04	0.59	-0.15	0.29	-0.06	0.42	0.74	<b>0.98</b>
PUFA, %	14.47 (2.75)	-0.20	<b>0.06</b>	0.04	0.59	-0.06	0.32	0.10	0.73	0.12	0.77	0.40	<b>0.95</b>	0.15	0.71	-0.60	<b>0.05</b>

<sup>1</sup> The numbers 1 and 2 refer to *PLIN1* and *PLIN2*, respectively, with the additive effects expressed as A-G. P (>0): Posterior probability of a value being positive. In bold, probabilities above 0.90 or below 0.10.

**Table S5.** Quantitative trait loci (QTL) co-localizing with the porcine *PLIN2* mapping position (SSC1q2.3-2.7; 227.3 Mb on SSC assembly 10.2)1.

QTL Trait	QTL (cM)	Reference <sup>2</sup>
Abdominal fat	107.6	Geldermann et al. (2010)
Adipocyte diameter	94.3-122.6	Geldermann et al. (2003)
Average daily gain	3.0-140.5	Liu et al. (2007)
Average daily gain	42.36-134.76	Onteru et al. (2013)
Average daily gain	49.4-79.4	Rückert & Bennwitz (2010)
Average daily gain	73.0-140.5	Harmegnies et al. (2006)
Average daily gain	100.8-118.5	Mohrmann et al. (2006)
Average daily gain	127.1-140.5	Evans et al. (2003)
Backfat thickness	80.0-110.5	Liu et al (2007)
Body weight at birth	16.4-132.0	Guo et al. (2008)
Daily feed intake	78.7-79.4	Kim et al. (2000)
Ham weight	94.3-122.6	Geldermann et al. (2003)
Lean meat percentatge	94.3-122.6	Geldermann et al. (2003)
pH48 hours post mortem (loin)	102.9-119.5	Thomsen et al. (2004)

1 Source: Animal Genome GBrowse ([www.animalgenome.org/cgi-bin/gbrowse/pig/](http://www.animalgenome.org/cgi-bin/gbrowse/pig/)), accessed on 22 November 2014.

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## CHAPTER IV

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### EXPRESSION PROFILING OF THE *GBP1* GENE AS A CANDIDATE GENE FOR PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME RESISTANCE

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## Abstract

A genomic region in pig chromosome 4 has been previously associated with higher viraemia levels and lower weight gain following porcine reproduction and respiratory syndrome virus (PRRSV) infection. The region includes the marker *WUR1000125*, a G>A polymorphism next to a putative polyadenylation site in the 3'-untranslated region (3'-UTR) of the guanylate-binding protein 1, interferon-induced (*GBP1*) gene. The protein encoded by *GBP1* is a negative regulator of T-cell responses. We show here that *GBP1* expression is lower in liver and tonsils of pigs carrying the *WUR1000125-G* allele due to differential allele expression (allele A expression is 1.9-fold higher than for allele G). We also show that the *GBP1* gene has two active polyadenylation signals 421 bp apart and that polyadenylation usage is dependent on the *WUR1000125* genotype. The distal site is the most prevalently used in all samples, but the presence of the A allele favours the generation of shorter transcripts from the proximal site. This is confirmed by a differential allele expression study in *AG* genotype liver and tonsil samples. The interaction between *WUR1000125* and other mutations identified in the 5'- and 3'-UTR regions of this gene needs to be studied. In conclusion, our study indicates that the *WUR1000125* mutation is associated with changes in the expression of the negative T-cell regulator *GBP1* gene. However, the chromosome 4 locus for PRRSV viraemia levels and weight gain contains a cluster of four other GBP genes that remain to be studied as candidate genes for this QTL.

## 1. Introduction

The porcine reproduction and respiratory syndrome virus (*PRRSV*) is one of the main clinical problems in pig production. Vaccines have proved to be an unreliable consistent approach to control the disease due to the high mutational rate of this virus. Consequently, awareness has been raised that selection for resistant or tolerant pigs can be a solution to mitigate the negative impact of this virus on pig production. Recently, a major QTL at porcine

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chromosome SSC4 has been reported to affect both pig growth rate and response to American strains of PRRSV (Boddicker et al., 2012). A group of six SNPs in perfect linkage disequilibrium captured 15.7% genetic variance for viraemia levels and 11.2% for body weight gain after experimental infection.

These polymorphisms are located in the guanylate-binding protein 1, interferon-induced (*GBP1*) gene, which has been associated with the control of the immune innate response to bacterial and viral infections in other species (Kim et al., 2011; Pan et al., 2012; Selleck et al., 2013). Among these six SNPs, *WUR1000125* was selected as a tag SNP to evaluate the effect of alternate haplotypes. *WUR1000125* is a G>A polymorphism which lies next to a putative polyadenylation site (*AATAAA*) in the 3'-untranslated region (3'-UTR) of *GBP1*. Mutations in the 3'-UTR region can potentially affect transcript stability, thus influencing protein synthesis rate. Moreover, alternative usage of polyadenylation sites is a well-reported regulator of protein expression, influencing mRNA stability, transport and translation, generally through the loss and gain of regulatory motifs, including microRNA-binding sites (Barrett et al., 2012; Sun et al., 2012). In the present work, we have analysed whether the *WUR1000125* mutation affects *GBP1* mRNA expression by analysing total expression levels, allele-specific expression and polyadenylation site usage rate in liver and tonsils from pigs of different *WUR1000125* genotypes.

## **2. Material and methods**

### **2.1 DNA and RNA samples**

Pig tissue samples were available in the laboratory at the time of this experiment. We used liver (n = 42) and tonsil (n = 13) samples from 42 Duroc pigs, collected upon slaughter and stored at -80 °C until analysis (Table 1). Animals were all males from two batches of previous experiments developed under commercial conditions. Pigs in the same batch were of the same sex, from the same unit, and slaughtered at the same age (100 days of age for batch 1 and

180 days of age for batch 2). Within batch, pigs were selected from different litters to minimise parental relationship. Liver samples were collected from both batches and tonsils were collected only from pigs in batch 1. Genomic DNA was isolated from liver using standard protocols (Sambrook and Russell, 2001). Total RNA was isolated with TRI-Reagent (Sigma-Aldrich) following the manufacturer's indications. Nucleic acid concentration and purity was assessed by spectrophotometry with a Nanodrop-100, and the integrity was tested by electrophoresis in agarose gels.

**Table 1.** Pig tissue samples used in the experiment, distributed by *WUR1000125* genotype.

Tissue	N	WUR1000125 genotype		
		AA	AG	GG
Liver	42	20	16	6
Tonsils	13	8	5	-

## 2.2 Retrotranscription

Prior to retrotranscription, 1  $\mu\text{g}$  of total RNA was digested with Turbo DNA-free DNase (Ambion, Life Technologies), as indicated by the manufacturers, to eliminate any traces of genomic DNA. First-strand cDNA synthesis was performed with RevertAid reverse transcriptase (Fermentas, Thermo Fisher) in 20- $\mu\text{l}$  reactions containing 1 x buffer, 1 mM of dNTPs, 50 pmol of random hexamers, 1  $\mu\text{l}$  of RiboLock (Fermentas), 100 U of enzyme and 1  $\mu\text{g}$  of RNA. Reactions were incubated 10 min at 25 °C, 1 h at 42 °C and 10 min at 70 °C.

## 2.3 *GBP1* expression levels by real-time quantitative PCR (qPCR)

cDNA was diluted 1:10 in DEPC-treated H<sub>2</sub>O prior to qPCR analysis. Primers (Table S1) for *GBP1* and two reference genes, *YWHAZ* and *RPL32*,

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were designed with PRIMER3PLUS using the qPCR default parameters (Untergasser et al., 2007). For each gene, a standard curve was generated by amplifying serial dilutions of a control cDNA to check for linearity between initial template concentration and cycle threshold (Ct) values. qPCR assays were carried out in triplicate in an ABI-7500 device (Life Technologies) in a final volume of 5 $\mu$ l containing 1 x Maxima SYBR green/ ROX Master mix (Fermentas) and 200 nM of each primer. The following thermal profile was used for all reactions: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a slow denaturation ramp from 60 °C to 95 °C to generate a dissociation curve to control the specificity of the amplified product. Ct values were used as quantitation units. To quantify and normalise the expression data, we used the  $\Delta\Delta$ Ct method (Yuan et al., 2006) using the geometric mean Ct value from the two reference genes and the *GBP1* Ct values.

### **2.3 *WUR1000125* genotyping and allele-specific expression assays**

The *WUR1000125* SNP was genotyped with a custom allelic discrimination assay (Life Technologies). Primers and probes are given in Table S1. We followed the manufacturer's protocol to set up the PCRs. SEQUENCE DETECTION SYSTEMS software (SDS 2.0) was used to automatically collect and analyse the data and to generate the genotype calls. Allelespecific expression was analysed as in Lo et al. (2003). Briefly, genomic DNA from two pigs homozygous for *WUR1000125*, one with genotype *AA* and the other with genotype *GG*, was mixed at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (*AA*: *GG*). Allelic discrimination assays were conducted as above, and the Ct data were used to calculate, for each mixing ratio, the Ct(VIC)/Ct(FAM) ratio, where the VIC signal corresponds to the detection of the *A* allele and FAM to the detection of *G*. These data were used to generate a standard curve. The allelic discrimination assay was then run in heterozygous *AG* cDNA samples (nine liver samples from batch 1 and batch 2; five tonsil samples from batch 1), and the gene expression allele ratio was extrapolated by intercepting the Ct(VIC)/Ct(FAM) ratio of each

sample on the standard curve. Genomic DNA samples from the same heterozygous *AG* pigs were assayed in parallel as a control.

## 2.4 3'-rapid amplification of cDNA ends (3'-RACE)

3'-end characterisation of the *GBP1* mRNA was carried out as follows from the total RNA of tonsils (*AA*,  $n = 4$ ; *AG*,  $n = 4$ ) and liver (*AA*,  $n = 4$ ; *AG*,  $n = 4$ ; *GG*,  $n = 4$ ), selected at random within each genotype from pigs in batch 1 (tonsils) and batch 2 (liver). Retrotranscription was performed from total mRNA as above but using an anchored oligo(dT) primer (Table S1) that included an extended adaptor sequence (UAP, universal amplification primer). Samples were incubated at 42 °C for 1 h, and the reaction was terminated at 70 °C for 10 min. All primers used in this study are detailed in Table S1 and Figure S1. To amplify the 3'-ends, PCRs were performed in 20  $\mu$ l containing 0.4  $\mu$ M of each primer (3'-RACE\_1/UAP) and 1  $\mu$ l of cDNA. After an initial denaturation step at 94 °C for 5 min, the reaction was performed for one cycle at 94 °C for 30 s, 60 °C for 2 min and 72 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 90 sec. The final extension was carried out at 72 °C for 10 min. A nested reaction was performed using 1  $\mu$ l from the first reaction (1:100 dilution) with 0.4  $\mu$ M of each primer (3'-RACE\_2/UAP) under the same cycling conditions. Amplified PCR products were subjected to 1.2% agarose gel electrophoresis and visualised with ethidium bromide staining. Distinct PCR bands were excised from the gel, purified using a QIAquick Gel Extraction Kit (QIAGEN) and sequenced.

## 2.5 *GBP1* polyadenylation usage ratio

A PCR was conducted with a FAM-labelled forward primer (*GBP1\_fam*) and two reverse primers (Table S1 and Figure S2), one located just downstream of the first polyadenylation site and which included a string of seven T's (*GBP1-SHORT\_R*) and a second one just downstream the first primer, which should only be present in transcripts using the second polyadenylation site (*GBP1-*

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LONG\_R). Samples included total RNA from liver (AA, n = 4; AG, n = 4; GG, n = 4) and tonsils (AA, n = 3; AG, n = 3), selected at random within each genotype from pigs in batch 1 (tonsils) and batch 2 (liver). The PCR was performed with 0.3  $\mu$ l of cDNA retrotranscribed with an anchored oligo(dT) (50 pmol), diluted 1:10 in DEPC-treated H<sub>2</sub>O using the same cycle conditions as described in the 3'-RACE section. The expected sizes for the two PCR products were 725 and 745 bp respectively. Given the small length differences, the two products were expected to be amplified with the same efficiency. After PCR, 1  $\mu$ l of each reaction was mixed with 10  $\mu$ l of HI-DI Formamide (Life Technologies), denatured at 95 °C for 5 min and incubated on ice for 2 min. The products were then separated in an ABI-3100 capillary electrophoresis system (Life Technologies). Peak identification and area under the curve were calculated with the DAX data acquisition and data analysis software.

Additionally, in the heterozygous samples (liver, n = 4; tonsils, n = 3), the transcripts resulting from the alternative polyadenylation sites were amplified separately with primers qGBP1\_F/GBP1-SHORT\_R and qGBP1\_F/GBP1-LONG\_R. The A and G allelic contribution to each of the transcripts was measured using an allelic discrimination assay from PCR template diluted 1:100, as explained in the allele-specific expression section.

## 2.6 *GBP1* promoter and full 3'-UTR amplification and sequencing

Primers were designed with PRIMER3PLUS (Untergasser et al., 2007) to amplify and sequence the *GBP1* proximal promoter (up to 1100 bp upstream from +ATG) and the full exon 11, which includes the STOP codon and the 3'-UTR sequence (Table S1). PCRs were carried out in a Veriti thermocycler (Life Technologies) in a volume of 25  $\mu$ l containing 1x buffer, 200  $\mu$ M of dNTP mix, 2.0 mM of MgCl<sub>2</sub>, 400 nM of each primer, 1 U of Taq polymerase (BIOTOOLS) and 60 ng of genomic DNA. The thermal profile was as follows: initial denaturing step for 5 min at 95 °C and then 35 cycles of 20 s at 95 °C, 30 s at 60 °C and 1.30 min at 72 °C, finishing with 5 min at 72 °C. PCR products

were separated by electrophoresis in a 1.2% agarose gel, and positive bands were purified with an ExoSAPit enzyme kit (USB) and sequenced with the BigDye Terminator Sequencing kit v3.1 (Life Technologies). Sequences obtained were edited using the SEQUENCING ANALYSIS software (Life Technologies) and aligned with the CLUSTALW program (Chenna et al., 2003).

Prediction of potential transcription factor-binding sites was performed with the TRANSFAC 8.3 database with the PROMO v2.0.3 tool (Messeguer et al., 2002) and the 2014 JASPAR-CORE repository (Mathelier et al., 2014). The 3'-UTR region was scanned for RNA structural and regulatory motifs using RegRNA (Huang et al., 2006).

## **2.7 Statistical analysis**

Variation of *GBP1* expression was analysed in each tissue separately with a model including the batch and the *WUR1000125* genotype. The least squares means of the genotypes were separated using the Tukey test. Allele specific expression in AG samples was analysed within tissue as above, in a model that included the batch, on the logit transformation of the data, as these were closed percentages (Ros-Freixedes and Estany, 2014). Comparison of polyadenylation usage by genotype and of allele contribution per transcript was performed within tissue using a t-test based on the logit transformation of the data. All the analyses were performed with JMP PRO 11 (SAS Institute Inc.), and differences were considered significant at  $P < 0.05$ .

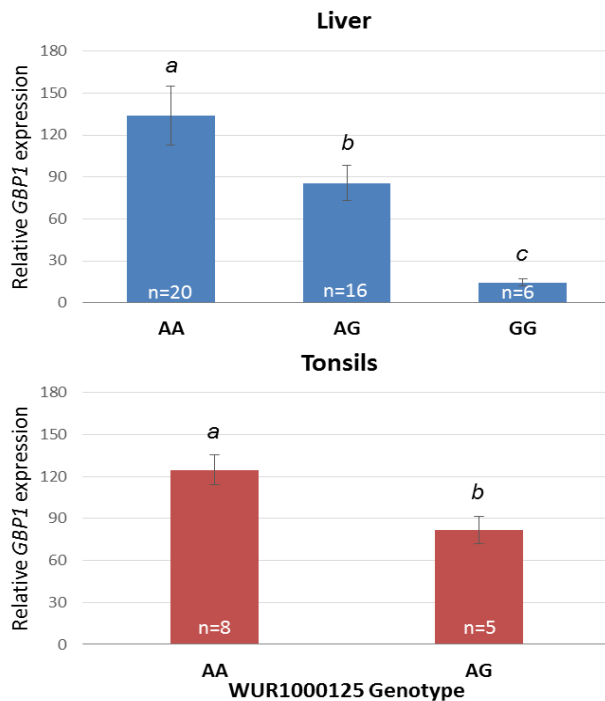


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### 3. Results

#### 3.1 Genotype of the *WUR1000125* mutation is associated with *GBP1* expression

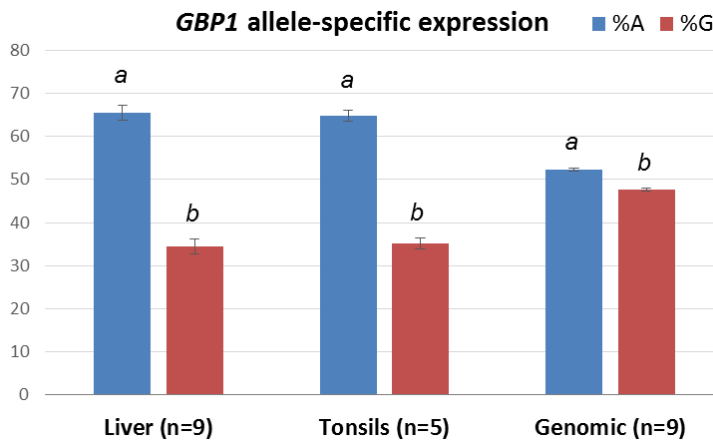
*GBP1* expression levels were measured in liver and tonsils of pigs with genotypes AA, AG and GG for the *WUR1000125* SNP marker, which is located at the 3'-UTR of the *GBP1* gene. Expression of *GBP1* was lower in the liver of pigs with the GG genotype as compared to AA pigs ( $P < 0.01$ ) (Figure 1). Expression of heterozygous AG liver samples was intermediate to the alternative homozygotes. In a subset of these animals for which tonsils were available, we also observed higher expression of *GBP1* in AA pigs than in AG animals ( $P < 0.05$ ). No GG pig could be analysed for this tissue.



**Figure 1.** Least squares means of *GBP1* gene expression in liver and tonsils of Duroc pigs by *WUR1000125* genotype. Error bars represent standard errors. Within tissue, different letters indicate significant differences ( $P < 0.05$ ).

### 3.2 Expression of the *WUR1000125* marker is allele specific

Differential allele expression was assessed in liver and tonsils of heterozygous AG animals (Figure 2). In both tissues, allele A was expressed at higher levels (about 1.9 fold) than was allele G ( $P < 0.001$ ). Presence of A and G alleles in controls of genomic DNA from AG pigs were approximately at a 50:50 ratio, as expected.



**Figure 2.** Allele-specific expression at the *WUR1000125* polymorphism of the *GBP1* gene measured in total RNA from liver and tonsils of heterozygous pigs and in genomic DNA as a control. Error bars represent the standard error of each mean. Within tissue, different letters indicate significant differences ( $P < 0.05$ ).

### 3.3 *GBP1* gene has two active polyadenylation signals

Amplification of the 3'-end of the *GBP1* mRNA indicated the presence of two main transcripts differing at approximately 400 nt (Figure S2). The two transcripts were detected in all samples analysed, which included liver from AA, AG and GG pigs and tonsils from AA and AG animals. PCR bands corresponding to the short and long transcript were subsequently sequenced to investigate the size differences. Sequence alignment and analysis indicated that the two transcripts differed in length due to the alternative use of two active

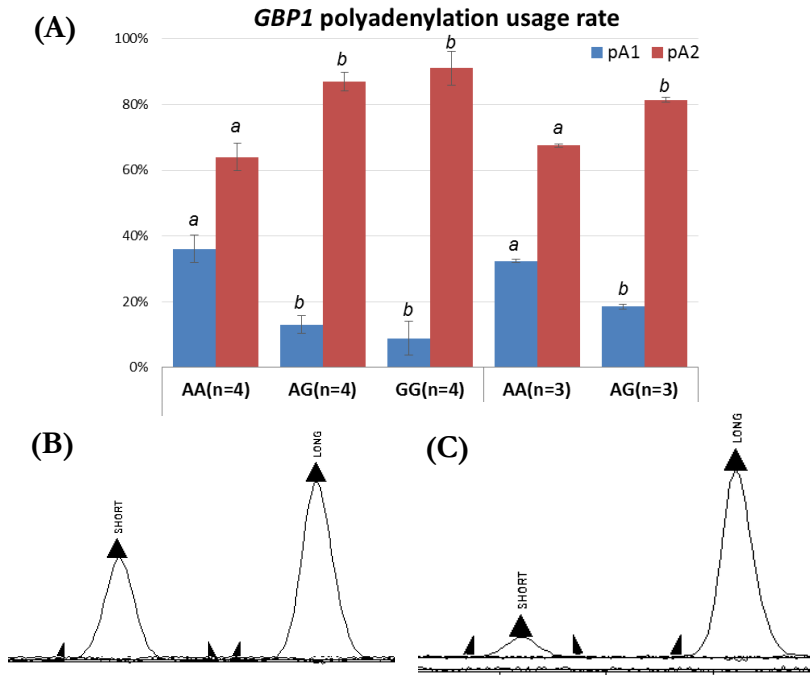
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polyadenylation sites 421 bp apart. The full 3'-UTR of the two transcripts were 603 and 1029 nt long. A and G alleles were identified in the sequences of both the long and short transcripts.

Transcription termination and start of polyadenylation took place 17 and 16 nt after the proximal and distal polyadenylation sites respectively (Figure S2). Analysis of the sequence also revealed the presence of other polymorphisms in phase with the *WUR1000125* SNP in this fragment of the 3'-UTR of the gene (Figure S2) which are explained in more detail below.

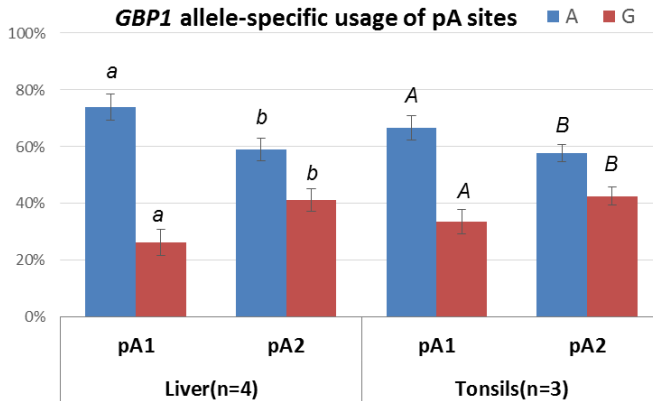
### **3.4 Polyadenylation usage is dependent on the *WUR1000125* genotype**

The *WUR1000125* polymorphism lays 1 bp upstream of the first polyadenylation site. We next questioned whether the *WUR1000125* polymorphism affected the rate of polyadenylation site usage. To test this, we carried out a modification of the 3'-RACE protocol with a common FAM-labelled forward primer in exon 10 and two reverse primers in exon 11 that were specific for the short or the long transcripts (Figure S1). After amplification, fragments were separated by capillary electrophoresis and quantified as FAM fluorescent units (Figure 3). The distal polyadenylation site exhibited the highest usage rate in all samples analysed. On the other hand, the prevalence of the short transcript generated from the proximal polyadenylation site differed across genotypes ( $P < 0.05$ ). In AA pigs, short transcripts represented one-third of all the *GBP1* mRNAs, both in liver and tonsils. The use of this proximal site was lower in AG pigs, representing 13% and 18% of total *GBP1* mRNAs in liver and tonsils respectively. The lowest usage rate of the proximal polyadenylation site was observed in the liver of GG pigs, resulting in approximately 10-fold higher expression of long vs. short transcripts (Figure 3).



**Figure 3** (a) Production of short and long transcripts by alternative use of proximal (pA1) and distal (pA2) polyadenylation sites in tonsil and liver samples from pigs with different genotypes on the *WUR1000125* marker of the *GBP1* gene. Error bars represent the standard error of each mean. Within tissue and transcript, different letters indicate significant differences between genotypes ( $P < 0.05$ ). Quantification of short and long transcripts after capillary electrophoresis of FAM-labelled 3'-UTR PCR products in an AA tonsil (b) and a GG liver (c) sample.

To further investigate this matter, the relative contribution of A and G alleles to the total amount of short and long transcripts was investigated in liver and tonsil samples from AG pigs (Figure 4). Regarding the long transcripts, the ratio of A:G expression was similar to the allele-specific expression levels measured on total *GBP1* mRNA (Figure 2). The A allele contributed to ~60% of the transcripts in all samples. In contrast, the short transcripts had a higher representation of the A allele ( $P < 0.05$  in liver;  $P < 0.10$  in tonsils), particularly in liver where it accounted for 74% of the mRNA polyadenylated at the proximal site (Figure 4).



**Figure 4.** Contribution of *WUR1000125* alleles to the short and long transcripts of *GBP1*, generated by alternative use of proximal (pA1) and distal (pA2) polyadenylation signals, in liver and tonsil heterozygous AG pigs. Data represent percentage of allele contribution to each transcript. Within each tissue and allele, different letters indicate significant differences in transcript distribution (lowercase,  $P < 0.05$ ; uppercase,  $P < 0.01$ ). Error bars represent the standard error of each mean.

### 3.5 Description of other polymorphisms in the *GBP1* regulatory regions

The expression of *GBP1* is induced by interferon (IFN)- $\alpha/\beta$  and IFN- $\gamma$ , as well as tumour necrosis factor (TNF)- $\alpha$  and interleukin-1 (Naschberger et al., 2004). In the human gene, several cytokine response elements have been described in the proximal promoter region as well as in the 5'-UTR sequence in exon 1. These include GAS ( $\gamma$ -IF activation sites), ISRE (IFN- $\alpha$  stimulated response element) and an NF $\kappa$ B-binding motif. Additional in silico analysis of potential transcription factor-binding sites identified two putative overlapping sites for interferon regulatory factor-1 (IRF-1) and NF $\kappa$ B (Figure S3). *GBP1* is a TATA-less promoter and, therefore, has several transcription start sites. We sequenced 1100 bp upstream of the ATG signal, which included the entire exon 1 and approximately 300–400 bp of the promoter, in samples of pigs with AA and GG genotypes for the *WUR1000125* polymorphism. We identified a total of seven polymorphisms (six SNPs and a 1-bp INDEL) (Table S2 and Figure S3). None of the mutations changed the cytokine response elements described in this

region, but a SNP mutation at –631 bp co-localised with the putative overlapping IRF-1/NF $\kappa$ B regulatory elements (Table S3).

We next sequenced the entire 3'-UTR region to describe any additional mutations that might affect the stability of this region. We identified 10 SNPs (including the *WUR1000125* marker). We searched for 3'-UTR structural and regulatory elements colocalising with these mutations. The *GBP1* 3'-UTR contains four interferon response elements, three of which are common to the short and long transcripts (Table S4). Three of the mutations identified lay in two of these regulatory elements (Figure S4). In our sequencing data, the three mutations were fully linked to the *WUR1000125* polymorphism.

#### 4. Discussion

Several studies have reported differences in susceptibility to PRRSV infection and disease development between pig breeds (Reiner et al., 2010; Pena et al., 2013) and also within lines (Lewis et al., 2009). The genetic component of this has been confirmed by several authors (Lewis et al., 2009; Seroo et al., 2014). Functional candidate genes for PRRSV susceptibility have been listed through global transcriptomics (Xiao et al., 2010; Arceo et al., 2012; Jiang et al., 2013), but only a few of them have been looked into in some more detail (Ren et al., 2012; Wang et al., 2012a,b). The first genomic region associated with response to PRRSV infection was reported in 2012 (Boddicker et al., 2012). Working with American commercial pigs, the authors identified a region on chromosome 4 with six markers in perfect linkage associated with PRRSV viraemia levels and weight gain. One of these markers, *WUR1000125*, was selected as a tag SNP to further characterise the influence of this region on viraemia profiles of experimentally infected pigs. The *WUR1000125* marker (SNPdb accession number rs80800372) is a G>A SNP polymorphism that lays in the 3'-UTR of the *GBP1* gene. Allele A, the unfavourable allele, is associated with higher PRRSV viraemia levels and lower weight gain following infection (Boddicker et al., 2012). Conversely, the favourable G allele promotes lower plasma PRRSV titre levels and favours weight

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gain following a PRRSV challenge. In a previous study, we have shown that the WUR1000125 marker segregates in European pig lines at allele frequencies similar to those described in American populations (Pena et al., 2013).

The *GBP1* gene encodes for an interferon-induced guanylate-binding protein belonging to the dynamin superfamily of large GTPases. This protein is an important player in cell-autonomous immunity (MacMicking, 2012), displaying antiviral, antimicrobial and antiparasitic activity (Kim et al., 2011; Selleck et al., 2013; Zhu et al., 2013). Although interaction with several viral and microbial proteins has been postulated, the molecular mechanisms of action of *GBP1* remained unknown until recent reports linking the action of this protein with the cytoskeleton remodelling that takes place in interferon-activated cells (Ostler et al., 2014). Moreover, recently, GBP1 has been recognised as a negative regulator to T-cell activation (Forster et al., 2014), interfering with the early stage of T-cell receptor signalling through interaction with structural proteins.

Given all the above, it is likely that the haplotype described by Boddicker et al. (2012) might affect the functionality of this gene. Using *WUR1000125* as a tag SNP, we show here that pigs carrying the favourable G allele have lower expression levels of *GBP1* in liver and tonsils. The lower mRNA levels are due to allele-specific differences in expression, with the expression of the A allele exceeding the expression of the G allele by approximately 1.9 fold. The differences of expression between alleles are comparable to the differences in expression between genotypes. Overall, the lower expression of the favourable G allele agrees well with the recent finding that *GBP1* assists in tuning down T-cell responses (Forster et al., 2014). Genotypes associated with lower *GBP1* expression are expected to exhibit more effective T-cell responses. It is well-reported that a PRRSV-specific T lymphocyte IFN- $\gamma$  response does not develop until at least 2 weeks after infection (Cecere et al., 2012). This cellular immune response is associated with an efficient immune response against this virus (Mateu and Diaz, 2008). This fact highlights the relevance of efficient T-cell responses regarding the outcome of PRRSV infection.

The length of the 3'-UTR is a major determinant in mRNA expression (Barrett et al., 2012). In general, longer 3'-UTRs correlate with a relatively lower expression level, as longer 3'-UTRs are more likely to possess miRNA binding sites and AU-rich elements that have the potential to promote mRNA decay and inhibit translation (Barrett et al., 2012). We show here that, in pigs, the *GBP1* gene has two active polyadenylation signals. Alternative usage of polyadenylation sites is one of the mechanisms leading to changes in the 3'-UTR length of mRNA transcripts. The *WUR1000125* mutation is next to the more proximal polyadenylation site; therefore, we tested whether *WUR1000125* could be causal to the drop of expression by affecting the usage rate of the two polyadenylation signals. The usage of both signals was confirmed by 3'-RACE characterisation of *GBP1* in AA, AG and GG pigs. The three genotypes favoured the usage of the distal site both in liver and tonsils. Polyadenylation from the proximal signal was promoted mainly by the A allele, increasing the proportion of the shorter, potentially more stable, transcripts by fourfold in AA (36% of short transcripts) with respect to GG liver samples (~9%). The distribution of A and G alleles among the longer transcripts paralleled the allelespecific expression results observed in the total *GBP1* mRNA. However, and in agreement with the polyadenylation usage results, the shorter transcripts had a larger representation of A alleles both in liver and tonsils of heterozygous pigs. These shorter transcripts have potentially less capacity to be regulated by trans-factors in response to internal cues, such as changes in the immune state of the pig. Conversely, the G allele tends to accumulate in longer transcripts, originating from the second polyadenylation site. The potentially lower stability of these mRNAs agrees with the lower *GBP1* expression levels in pigs carrying the favourable G allele and is consistent with a model promoting the fine regulation of the GBP1 protein production.

Taken together, these results strongly indicate that the *WUR1000125* mutation changes the processability of the proximal polyadenylation signal of the *GBP1* gene. This can potentially be the cause of the differences in expression



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observed between genotypes, as the length of the 3'-UTR influences the stability and the translation rate of the transcripts (Sun et al., 2012). Mutations in the gene promoter also can be responsible for differences in transcription levels. *WUR1000125* is in total linkage disequilibrium with other markers in the SSC4 chromosome (Boddicker et al., 2012). Our analysis of the *GBP1* gene has identified 16 other polymorphisms in the 5'-UTR and 3'-UTR of this gene, most of which are also linked to this tag SNP. Although none of the three functional IFN and NF $\kappa$ B response elements known in the promoter are affected by these mutations, a putative IFN regulatory element overlaps a SNP mutation at -631 bp from the start ATG codon. Moreover, three mutations in the 3'-UTR of the *GBP1* gene co-localise with two other IFN- $\gamma$ -response elements. The potential effect of these mutations on the expression of *GBP1* cannot be overlooked, and their possible interaction with the *WUR1000125* marker should be explored in more detail.

In conclusion, our study indicates that the QTL for PRRSV viraemia levels and weight gain described by Boddicker et al. (2012) is associated with changes in the expression of the negative T-cell regulator *GBP1* gene. However, although the causal mutation responsible for this remains unknown, the results reported call attention to the transcriptional regulation of *GBP1*, a negative regulator of T-cell responses. Nevertheless, in the pig genome, *GBP1* is within a cluster of five GBP genes (*GBP1*, *GBP2*, *GBP4*, *GBP5* and *GBP6*). Further study of the other GBP genes might be of interest in helping to position the causal mutation. Moreover, it would be interesting to measure T-cell-related cytokines in animals with AA and GG *WUR1000125* genotypes to confirm the involvement of this genomic region with T-cell activation and response. A further question that remains unanswered is how the function of this gene family relates to the enhanced growth rate reported in PRRSV-challenged pigs (Boddicker et al., 2012). Our own preliminary data (Pena et al., 2013) suggest that the relationship of *GBP1* with growth rate depends on the epidemic phase of PRRS. Although allele A is positively associated with growth in non-epidemic phases, in contrast,

allele G is associated with enhancing weight gain during the epidemic phase or in challenged pigs. This hypothesis is further supported by the fact that the A allele, unfavourable for PRRSV viraemia levels, is the most frequent in commercial populations selected for increased growth rate (Boddicker et al., 2012; Pena et al., 2013), with allelic frequencies in the range of 0.8–0.9. The contribution of this gene to growth gain in non-epidemic phases will need to be assessed in the future.

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## Supplementary Information

**Table S1.** Primers and probes used in this study.

Primer	5'→3' sequence	T <sub>m</sub>	Position	Amplicon size
Gene expression experiment				
qGBP1_F	TGGCTGAGAAGATGGAGAAG	58.1	E10	97 bp
qGBP1_R	TCCTGAATTAGTCGGGCTTG	60.2	E11	
qYWHAZ_F	TGATGATAAGAAAGGGATTGTGG	59.4	E3	134 bp
qYWHAZ_R	GTTTCAGCAATGGCTTCATCA	61.3	E4	
qRPL32_F	CACCAGTCAGACCGATATGTCAA	61.1	E1	70 bp
qRPL32_R	CGCACCCCTGTTGTCAATGC	61.1	E2	
Genotyping and allele-specific assay				
gGBP1_F	AGACCTAGAATCTCCACAGAATTTCCA	64.2	E11	105 bp
gGBP1_R	GGAAAGGACAGTTCGCTTCTCTAG	62.7	E11	
Probe allele A	VIC-CTGGGTGATAAAATAAAT-NFQ		E11	
Probe allele G	FAM-TGGGTGATGAATAAAT-NFQ		E11	
3'RACE				
Oligo(dT)	ACTGGAAGAATTCTCGGCCGCAG(T) <sub>30</sub> VN			
UAP	ACTGGAAGAATTCTCGGCCGCAG	70.3		
3'RACE_1	CTTCAGGAACAAGCCCGACT	62.8	E10-E11	1200-770 bp
3'RACE_2	<i>GACCAGAAGACCCTGAGCAC</i>	61.2	E11	750-350 bp
Polyadenylation usage ratio				
GBP1-fam	FAM-CTTCAGGAACAAGCCCGACTA	65.1	E10	
GBP1-SHORT_R	TTTTTTTTTCGCTTCTCTAGCCCATT	64.8	E11	725 bp
GBP1-Long_R	<i>TCGAGCAGGAAAGGACAGTTC</i>	62.9	E11	740 bp
Promoter sequencing				
prmGBP1_F	CCGGGATCTGGAGAGAACCT	63.9	Promoter	1029 bp
prmGBP1_R	TTCACGGGAGGGTTTACTG	64.5	E1	
Exon 11 sequencing				
GBP1e11_F	GCCCGACTAATTCAGGAAGGA	63.6	E11	1078 bp
GBP1e11_R	AGGGCTTTTGACAACTGCAA	61.9	E11	

**Table S2.** List of polymorphisms identified during the sequencing of the *GBP1* promoter and 5' and 3'UTR. Positions in promoter and 5'UTR are given in relation to the ATG START codon. Positions in the 3'UTR are counted from the TAA STOP codon.

Polymorphism	Change	Position	WUR100125 AA pigs	WUR1000125 GG pigs
5'UTR				
rs335275118	G>T	-748	G	T
novel	G>C	-651	G	G/C
novel	C>A	-608	C	C/A
novel	T>C	-311	T	C
novel	T>C	-310	T/C	C
novel	INDEL	-307	delC	C
3'UTR				
rs339886073	G>T	*445	G	T
rs80863339	G>A	*540	A	G
rs342010514	C>T	*544	T	C
novel	A>G	*545	G	A
rs80800372**	A>G	*580	A	G
rs324386096	T>C	*610	T	C
novel	G>C	*718	C	G/C
novel	G>T	*742	G	G/T
novel	A>T	*743	A	A/T
rs323595619	T>C	*859	T	C

\*\*rs80800372 = WUR1000125



**Table S3.** In silico analysis of putative transcription factor-binding sites in the proximal promoter and exon 1 of the *GBP1* gene, using PROMO and JASPAR tools. Sites potentially affected by polymorphisms found in the promoter region are highlighted.

Please find the table on the attached link:

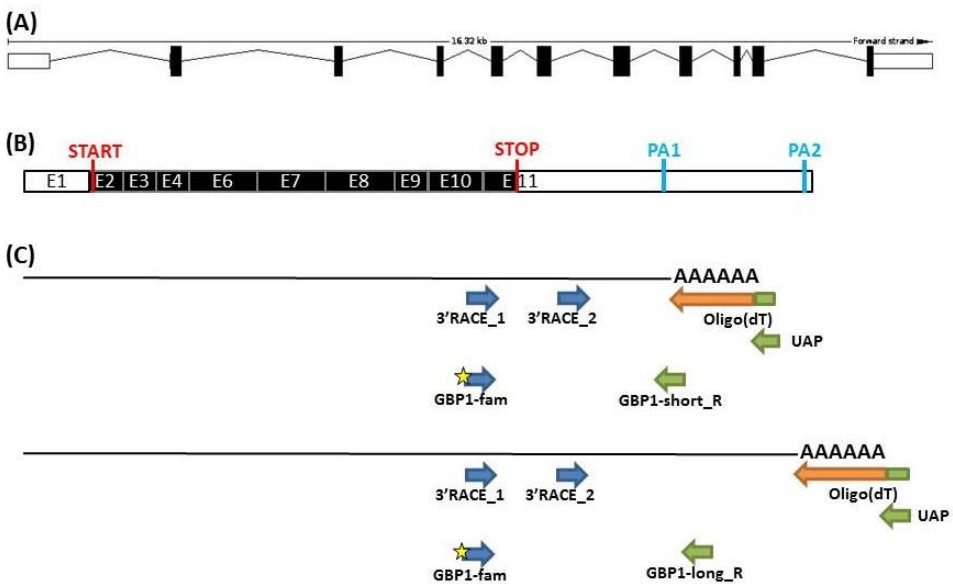
<https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fage.12347&file=age12347-sup-0007-TableS3.pdf>

**Table S4.** In silico analysis of the *GBP1* 3'-UTR region for RNA-regulatory motifs, using the Reg-RNA tool.

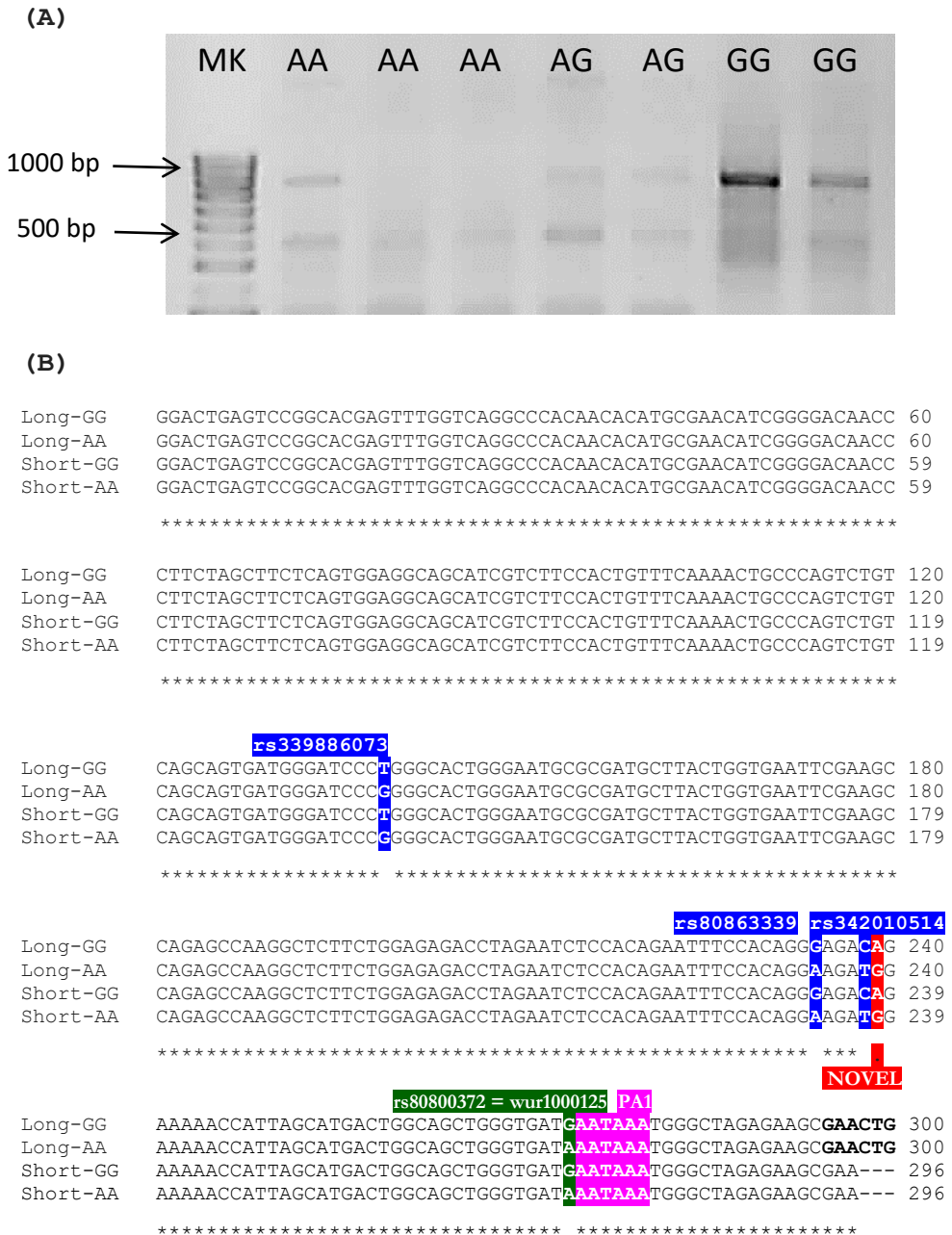
Please find the table on the attached link:

<https://onlinelibrary.wiley.com/doi/full/10.1111/age.12347>

**Figure S1.** Location of primers used for the characterisation of *GBP1* 3'UTR and polyadenylation usage rate experiments. (A) Structure of *GBP1* in exons (boxes) and intron (lines) according to Ensembl (ENSSSCT00000007584). Coding sequences are filled in black and 5' and 3' regions in white. (B) Situation of START and STOP codons and proximal (PA1) and distal (PA2) polyadenylation signals in the mRNA. (C) Location of primers in relation to the short and long transcripts. Primer sequence and description is detailed in Supplementary Table S1.



**Figure S2.** Characterisation of the 3'UTR region of the pig *GBP1* gene. (A) The 3'RACE experiment resulted in the amplification of two 3'UTR products in AA, AG and GG animals. MK – 100 bp ladder (HyperLadder, Bioline) (B) PCR fragments were sequenced and aligned. The two transcripts differed in the length of the 3'UTR by the alternative use of two polyadenylation signals (in pink). Ten SNP polymorphisms (described in SNPdb, in blue; novel, in red) were also identified.



**rs324386096**

Long-GG TCCTTCCTGCTCGATTTCGCGCAGATTCTAACTTTACTAGGTGGGACTCTCTGGAATTTT 360  
 Long-AA TCCTTCCTGCTCGATTTCGCGCAGATTCTAACTTTACTAGGTGGGACTCTCTGGAATTTT 360  
 Short-GG -----  
 Short-AA -----

Long-GG AGGTTACAGTGGACTACACAGTGACCTGAAAAAGTTTCCCATGGCGTTTCGGCAATTT 420  
 Long-AA AGGTTACAGTGGACTACACAGTGACCTGAAAAAGTTTCCCATGGCGTTTCGGCAATTT 420  
 Short-GG -----  
 Short-AA -----

Long-GG ACAGTCTGCAAAGAAATATGTGAAATGACAACAGAAACTGTGTTCGAAAAGTCTGAGCTAAC 480  
 Long-AA ACAGTCTGCAAAGAAATGATGTGAAATGACAACAGAAACTGTGTTCGAAAAGTCTGAGCTAAC 480  
 Short-GG -----  
 Short-AA -----

Long-GG TTAAGCGGCTAGACGGTTTAAACCTAGAGTTTAAAGCTATCTTTTCCAAATTCCTTCGCCAT 540  
 Long-AA TTAAGCGGCTAGACGGTTTAAACCTAGAGTTTAAAGCTATCTTTTCCAAATTCCTTCGCCAT 540  
 Short-GG -----  
 Short-AA -----

**rs323595619**

Long-GG CATAcataaaaaCTTATTTTTGCCCTAGAGAATATGAATTGCTTTTGACATTTTTGCCCA 600  
 Long-AA CATAcataaaaaCTTATTTTTGCCCTAGAGAATATGAATTGCTTTTGACATTTTTGCCCA 600  
 Short-GG -----  
 Short-AA -----

Long-GG GTTAAATAATGCTCTTGCTATTACTTAGTATATAGACTTTATTGCAGTTGTCAAAGCCC 660  
 Long-AA GTTAAATAATGCTCTTGCTATTACTTAGTATATAGACTTTATTGCAGTTGTCAAAGCCC 660  
 Short-GG -----  
 Short-AA -----

Long-GG TAGGTAATGGGAAGACGATTAAGAGTATTTTCGAGCTGGAAATAAACTGTGCTTCACTG 720  
 Long-AA TAGGTAATGGGAAGACGATTAAGAGTATTTTCGAGCTGGAAATAAACTGTGCTTCACTG 720  
 Short-GG -----  
 Short-AA -----

Long-GG AGCAAAAAAAAAAAAAAAAAAAAAA...CTGCGGCCGAGAATCTTCCAGT  
 Long-AA AGCAAAAAAAAAAAAAAAAAAAAAA...CTGCGGCCGAGAATCTTCCAGT  
 Short-GG ---AAAAAAAAAAAAAAAAAAAAA...CTGCGGCCGAGAATCTTCCAGT  
 Short-AA ---AAAAAAAAAAAAAAAAAAAAA...CTGCGGCCGAGAATCTTCCAGT

**Figure S3.** Characterisation of sequence variation in the promoter (*in italics*) and 5'UTR (exon 1) of the pig *GBP1* gene. Underlined: In pink, two putative overlapping sites for interferon regulatory factor (IRF-1) and NFκB; in red, a putative p53 binding element (human); in blue, a NFκB-binding motif; in green, ISRE (IFN-α stimulated response element). Polymorphic sites identified during the sequencing experiment are highlighter in black. Number on the right-hand side indicate bp from the ATG START codon in exon 2.

*CCGGGATCTGGAGAGAACCTTGTAAACCATCTCCCTTCTCCTCACTCCCCTCTTCTCTCGT* -1034  
*CCAGGGCGAGAAAAAGCAGTGAGCTTAAGGGTAAACAGAGAATCAAATCTGTATCCACCT* -974  
*CTGACGAGCTTGGTTGACAGGAACGGGCATCATCACCCACCCTCCTCATCATCACCCACC* -914  
*CTCCACTGTAAGATGGAGACAGTCCCTCTTCTTTTGCCTGGTTCCGGTCAGGACTGAATT* -854  
*GAACATTAACACAGGTAACTCTTAGAAAACAATCAAGACATAAAGCTGTGACTTCCCCTG* -794  
*CTCTTTGGCAGACACAGAAAGGCAGTACAGTGAAGTCAGAAGGGT***K***TGAGATCAAGGATG* -734  
*ATTTGGTTCAGATTATTTACAGGGCTGCGATGTGGACAAGAGGGTACATTTTCTGTGCC* -674  
*TTAGTTTCTACAAAACG***A***ATGGG***S***AAATGTT**TGTTCTAAGTTATTGAACGCATGTAGAGCG* -614  
*GTCAG***M***ACAGAGCCTGGAACTGAGCACAGGACATGCAGCCCCTTCTGCGTGTGGGAAT* -554  
*TAAGTTCAGGGACTGT**GTGTTTCATGAAAGCGCCAGATCTGTCC**TCTTCTTCACTGACCC* -494  
*CACACATAAAGGAGGAGCCTGGTTCAGTGTGAGCCGTTTTTAAGGAAATTAACCTTAA* -434  
*GATGAGGAGGCTTCTCATTCTAAATAGTTTTTCAAACAGACCCCAATCCTGAGATATAG* -374  
*TCCATTATATTTAAACATCTAGTAGACATGTTTTTAAAACAGCAAATGATCTCTAAGATCT* -314  
*CT***YS****CC***CCCCCCCCAAAAAGAACGTGAAGATCATGCCAAATCCATTTACCTTCTCCCA* -254  
*GGATGGCTTTT**AGAAATTCCT**TTTATGGTTGTTGAGTCATTGCTTTGTATTCAATT**GCTTT* -194  
*CAGTTTCAT**ATTTATTCTAAGTCTATTACAGAGGTTGCTTTGCTTCTGACTCGGCTCTAG* -134  
*AGGGAATCAGTAAAGCTCCTCGACACTGGCTGTGTGGACTAACAGTCAAACCCTCCCGTG* -74  
*AAACAGAGAAGTTACAGAGAAGTCCACTCGTCTCACTGAGAAGAGGAAAGAACTCTCAAT* -14  
 GAG -11 <[INTRON 1]

**Figure S4.** Regulatory, structural and sequence variation analysis of the pig *GBP1* 3'UTR region. The sequence from the STOP codon (boxed in grey) in exon 11 is indicated. Four interferon-response elements are underlined. The 10 SNP polymorphisms identified during the sequencing of the 3'UTR region are highlighted in black except the WUR1000125 mutation, which is highlighted in green. Numbers on the right-hand side indicate nucleotide positions from the TAA STOP codon.

**EXON11** <TAA>AGAACTGGAGAAGAGCGCTTCCCGGTCCCCTTAGCCATGGTCTTGCTCA \*51  
 AGTAGTTTAGAATTAAGGAAAATGTTGCCAAACCTGATGATAATTACATTTACATTGGT \*111  
 ATTACACAAAGAACTCGCACATCACGCAGCAGGGTACCTGAAATCATCTCGACCTTCCTC \*171  
 ACCACACCAAAGGGGGACAGGATACGCATTTACCTCTGCACCCGCCAGATGGCACCA \*231  
 CGGTCTGGTTCCAATCAGGAGCTTCTCTTCCAGATGACCGCCAGCTAGACCAGAAGACC \*291  
 CTGAGCACCGTCTCGGGACTGAGTCCGGCAGAGTTTGGTCAGGCCACAACACATGCGA \*351  
 ACATCGGGGACAACCCTTCTAGCTTCTCAGTGGAGGCAGCATCGTCTTCCACTGTTTCAA \*411  
 AACTGCCAGTCTGTGCAGCAGTGATGGGATCCCCGGGCACTGGGAATGCGCGATGCTTAC \*471  
 TGGTGAATTCGAAGCCAGAGCCAAGGCTCTTCTGGAGAGACCTAGAATCTCCACAGAATT \*531  
TCCACAGGAAGATTGAAAAACCATTAGCATGAACTGGCAGCTGGGTGATAATAAATGGGC \*591  
 TAGAGAAGCGAACTGTCCTTTCCCTGCTCGATTTCGCGCAGATTCTAACTTTACTAGGTGGG \*651  
 ACTCTCTGGAATTTTAGGTTACAGTGGACTACACAGTGACCTGAAAACAGTTTCCCATGG \*711  
 CGTTTGCGGCAATTTACAGTCTGCAAAGAACAATGTGAAATGACAACAGAAACTGTGTTT \*771  
 GAAAACCTGAGCTAACTTAAGCGGCTAGACGGTTTAACCCTAGAGTTTAAGCTATCTTTTC \*831  
 CAAATCTTCGCCATCATAATAAAAACTTATTTTTGCCCTAGAGAATATGGAATTGCTTT \*891  
TGACATTTTTGCCAGTTAAATAATGCTCTTGCTATTACTTAGTATATAGACTTTATTGC \*951  
 AGTTGTCAAAGCCCTAGGTAAATGGGAAGACGATTAAGAGTATTTTCGAGCTGGAAATA \*1011  
 AACTGTGCTTCACTGAGCTTTA \*1071

## **GENERAL DISCUSSION**



In the last decades, the quality of meat has taken importance due to the increasing concern on the part of consumers. Meat quality encompasses sensory, nutritional and technological attributes. The complex nature of meat quality traits together with the fact that industry does not explicitly pay for them, delayed their inclusion in the selection objectives of breeding programs (Kanis et al., 2005). However, especially in Duroc, a breed associated with high quality pork, meat quality traits can reach a considerable share (20 to 37%) of the breeding goal of the line (Knap, 2014). New technologies such as NIRS should accelerate this trend, since they would allow obtaining phenotypic records at low cost and therefore increase both selection accuracy and intensity (Araus et al., 2018). Meat quality traits should also take advantage of advanced genomic tools such as high-throughput chips or whole-genome sequencing platforms to gain knowledge on their underlying biological mechanisms, where to search for and identify new molecular markers. The research undertaken in this thesis provides additional insight into the genetic basis of lipid deposition and metabolism in relation with the quality of meat.

The results in Chapter I provide a specific selection criterion for IMF based on the absolute amount of C18:2. A limitation of this approach is phenotyping, since recording fatty acid composition, as well as IMF content, is costly and time consuming. For this reason, several studies have assessed the use of NIRS technology to determine these traits, either from minced and homogenized samples (Cheng et al., 2015) or even directly on the raw meat. In fact, portable devices have been developed to allow for continuous and non-invasive collection of phenotypic data. These equipments have been used for evaluating both carcass (Zamora-Rojas et al., 2012) and meat quality traits including IMF (Roza Delgado et al., 2014) and its composition on fatty acids (González-Martín et al., 2005) with promising results. Recent studies on this area propose to use this technology to classify pork carcasses or even live animals based on low/high SFA, MUFA and PUFA content (Prieto et al., 2015) or as a high-throughput tool to capture endophenotypic variants and compute



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relationship matrices for predicting complex traits (Rincent et al., 2018). Paralleling genomic selection, this approach has been called phenomic selection, where NIRS signatures, now considered as potential biomarkers, are used instead of SNP to predict the heritable variation of quantitative traits.

The use of the absolute amount of C18:2 as selection criteria could give the impression that fat will become more polyunsaturated and therefore softer, less-firm, and more prone to rancidity. Also, it might conclude that it will decrease the content of oleic acid. However, what happens is the opposite. Since during fattening C18:2 accumulates at a lower growth rate than MUFA including oleic acid, absolute C18:2 correlates negatively with relative C18:2 (-0.17) and positively with relative C18:1 (0.15). Thus, selection for the absolute amount of C18:2 is not expected to increase C18:2 or decrease C18:1 with respect to total fatty acids. This response structure might be different in other muscles. However, the same pattern was observed in LM, the most commonly referenced muscle, where absolute C18:2 also correlates negatively with relative C18:2 (-0.27) and positively with relative C18:1 (0.26). Consequently, correlation for absolute C18:2 between LM and GM muscle is expected to be positive (0.45). In contrast, absolute C18:2 in GM and in subcutaneous fat are almost independent (-0.04), and selection for absolute C18:2 in subcutaneous fat is expected to affect similarly the BT (-0.16) and the IMF content (-0.13).

The results in Chapter II would confirm that the C18:2 to C20:4 fatty acid modification is a good pathway where to search for molecular markers related to lipid deposition and fatty acid composition. Amongst all the enzymes involved in the route (FADS1, FADS2 and ELOVL5), *FADS2* was chosen to be sequenced, given its rate-limiting role and because there is evidence indicating the existence of a QTL for C20:4 content in the genomic region where it is annotated (Zhang et al, 2016). Porcine *FADS2* is located on a gene cluster on chromosome 2, together with two genes of the same family, *FADS1* and *FADS3*. Similarly, in human, these genes are located in the same region, showing in 75% of the

sequence identity and homology in the organization of the exons and introns (Guillou et al., 2010).

In order to characterize this gene, the promoter region was sequenced in pigs with low and high C20:4 content. Among all detected mutations, the one investigated (rs321384923A>G) was selected based on its putative effect on a retinoic acid/oestrogen receptor binding site. This SNP is located in the middle of a haplotype of three fully linked SNP. Our results demonstrated that the A allele, the one showing higher *FADS2* expression in muscle, is also the most efficient transforming C18:2 into C20:4. In the investigated Duroc line, two other polymorphisms, g.2228T>C and the g.1987C>T, localizing in the *SCD* and *LEPR* genes, respectively, segregate at intermediate frequency. These genes have known effects over fat content and fat composition (Estany et al., 2014; Ros-Freixedes et al., 2016). Neither of the two genes affected the efficiency of transformation of C18:2 into C20:4. Although the *LEPR* gene affected C18:2 and C20:4, their ratio was not modified, thereby indicating that observed changes were just the result of concomitant variations in IMF. The *SCD* gene did not affect IMF, C18:2 and C20:4 or their ratio.

Although *FADS2* rs321384923 affected the n-6 fatty acid profile, it did not impact carcass traits including lean weight. However, there was evidence that the G allele, which led to lower relative C20:4 and higher absolute C18:2 also showed increased IMF, in line with the positive (negative) genetic correlation of IMF with absolute C18:2 (relative C20:4) estimated in Chapter 1. This effect was only observed in GM, the muscle with less C20:4. Thus, although results are encouraging, the use of *FADS2* rs321384923A>G as a specific genetic marker for IMF needs to be confirmed. Other biological implications and roles and this polymorphism also need to be further investigated. For instance, in humans, genes on the *FADS1/FADS2* cluster have been shown to influence lipidic metabolism and inflammation by affecting the C20:4 levels (Naughton et al., 2016). In addition, through the supply of C20:4 in breast milk, genetic variants of maternal *FADS1/FADS2* are associated to the immunologic response of the

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progeny (Muc et al., 2015). *FADS2* is involved in the biosynthesis of some minor fatty acids of the n-3, n-6, n-9 and n-10 families (Guillou et al., 2010). On the other hand, our results indicate that *FADS2* activity is tissue-specific, since the effect over fatty acid composition was more evident in muscle than in subcutaneous fat and liver. Reported values in pigs would confirm that *FADS2* expression can greatly differ among tissues, but contrarily to what was found here, they show lower expression in muscle than in adipose tissue and liver (Taniguchi et al., 2015). Several factors may influence gene expression, including the interactions with other genes. In human, an alternative transcript of *FADS1* has been shown to regulate *FADS2* expression (Park et al., 2012). Hence, it would be interesting to explore whether the interaction between *FADS1* and *FADS2* varies by tissue or some epigenetic mechanisms beyond gene sequence. *FADS2* has a TATA-less promoter that may be regulated by methylation of CpG islands. On a recent work in humans, a variant of *FADS1/FADS2* gene cluster has been shown to exert an effect on gene expression by changing DNA methylation levels (He et al., 2018). Therefore, an interesting experiment could be to study the methylation pattern in pigs with low and high C20:4 content in muscle.

In Chapter III we investigated the impact of two markers located in *PLIN1* (JN860199:g.173G>A) and *PLIN2* (GU461317:g.98G>A, also referred as rs333231747) genes. In pig, the PLIN family, both genes and proteins, have not been widely studied and there is little information on their genetic variability. As reported on Chapter III, the polymorphism in *PLIN2* gave consistent results on BW, BT and loin thickness at different age time-points and on carcass traits (carcass length, ham weight and lean weight). The favorable effects of the A allele on early growth and lean weight are in line with the fact that PLIN2 is found typically in myocytes and immature adipocytes. Although further validation studies are needed, the results indicated that selection in favour of the A allele could help to improve lean growth in Duroc with little or no effect on IMF content and composition. Although PLIN1 is the most abundant PLIN protein in mature adipocytes, we did not find any effect of the *PLIN1* polymorphism on

fat deposition. Conversely, in a recent transcriptome analysis of Iberian pigs, *PLIN1* was overexpressed in pigs with high IMF (Muñoz et al., 2018). Hence, it is possible that mutations other than the investigated, probably located on the 5' or 3' regions, may impact *PLIN1* gene expression.

The association analysis of rs333231747G>A *PLIN2* was assessed without considering the effects of the *SCD* and *LEPR* genes on fat content and composition. Thus, the effect of the *PLIN2* polymorphism was reexamined adjusting for the effect of the *SCD* and *LEPR* genes following the model as in Chapter II. Unlike model in Chapter III, the one used here did not take into account the full pedigree information but only the sire and the dam. The results were in line with those reported in Chapter III, with the exception of BT at different ages and ham weight, where the effect of the *PLIN2* genotype was less evident (Table 1). This makes sense, since the main effect of *LEPR* is on fatness and some confounding between both genes cannot be discounted. Importantly, however, the effect of the *PLIN2* genotypes on growth and lean weight was maintained. As expected, AA pigs showed higher body weight at 120 and 180 days. The substitution effect of allele A was similar for both ages ( $1.16 \pm 0.42$ ;  $P < 0.01$  for 120 days and  $1.37 \pm 0.57$ ;  $P = 0.02$  for 180 days). Similarly, AA pigs had higher lean weight when compared to GG pigs (allele A substitution effect of  $0.50 \pm 0.17$ ;  $P = 0.04$ ). Results regarding IMF and its fatty acid composition were also in agreement with those reported in Chapter III. Thus, no evidence of association was found for IMF and PUFA with *PLIN2* genotype. However, AA pigs had less SFA and more MUFA than GG pigs, with allele A showing a substitution effect of  $-0.31 \pm 0.11$  ( $P < 0.01$ ) and  $0.26 \pm 0.11$  ( $P = 0.02$ ), respectively. To assess the stability of the estimates to the model used on Chapter III, the effects on fatty acid composition were also estimated without adjusting for IMF content. The estimates obtained remained practically unchanged. No relevant changes were found concerning *PLIN1* after adjusting for *SCD* and *LEPR* genotypes.

**Table 1.** Effect of the *PLIN2* rs333231747G>A genotype on lean growth and intramuscular fat content and fatty acid composition after adjusting for the effect of the *SCD* and *LEPR* genes.

Trait <sup>a</sup>	P-value <sup>b</sup>	<i>PLIN2</i> <sup>c</sup>		
		AA (n=157)	AG (n=292)	GG (n=164)
Body weight, kg				
120 days	0.02	62.75 ± 0.66 <sup>a</sup>	62.15 ± 0.49 <sup>ab</sup>	60.51 ± 0.64 <sup>b</sup>
180 days	0.04	108.87 ± 0.92 <sup>a</sup>	108.54 ± 0.71 <sup>ab</sup>	106.27 ± 0.90 <sup>b</sup>
Backfat thickness, mm				
120 days	0.19	11.49 ± 0.21	11.14 ± 0.16	11.08 ± 0.21
180 days	0.66	17.82 ± 0.33	17.98 ± 0.25	18.19 ± 0.32
Carcass weight, kg	0.13	94.48 ± 0.83	94.59 ± 0.63	92.86 ± 0.81
Carcass backfat, mm	0.78	22.59 ± 0.34	22.57 ± 0.26	22.81 ± 0.33
Carcass lean, %	0.68	43.77 ± 0.45	43.85 ± 0.35	43.44 ± 0.43
Lean weight, kg	0.03	41.31 ± 0.34 <sup>a</sup>	41.03 ± 0.44 <sup>ab</sup>	40.05 ± 0.42 <sup>b</sup>
Ham weight, kg	0.14	12.23 ± 0.11	12.14 ± 0.08	11.97 ± 0.10
IMF, %	0.50	15.90 ± 0.50	15.30 ± 0.35	15.34 ± 0.36
SFA, %	0.02	34.89 ± 0.17 <sup>b</sup>	35.20 ± 0.13 <sup>ab</sup>	35.51 ± 0.17 <sup>a</sup>
MUFA, %	0.04	50.80 ± 0.17 <sup>a</sup>	50.38 ± 0.13 <sup>ab</sup>	50.26 ± 0.17 <sup>b</sup>
PUFA, %	0.43	14.31 ± 0.15	14.42 ± 0.12	14.21 ± 0.15

<sup>a</sup> IMF: intramuscular fat expressed as percentage of dry muscle; SFA: saturated fatty acids (C14:0+C16:0+C18:0); MUFA: monounsaturated fatty acids (C16:1+C18:1+C20:1) and PUFA: polyunsaturated fatty acids (C18:2+C18:3+C20:2+C20:4) in muscle gluteus medius.

<sup>b</sup> P-value associated with the effect of the *PLIN2* genotype.

<sup>c</sup> Pairwise comparisons of *PLIN2* genotypes. Within row, means with different superscripts differ significantly (P<0.05).

Recent publications have studied other members of the PLIN family. Zappaterra et al. (2018) reported that transcription levels of *PLIN5* and

hormone-sensitive lipase (*LIPE*) gene are positively correlated. Moreover, they showed that a SNP on *PLIN5* was associated to MUFA content on BT. In a GWAS study in Iberian pigs, Pena et al. (2019) found that *PLIN3*, *PLIN4* and *PLIN5* map in a region strongly associated to C16:1 on IMF. However, there is still scarce information on how the members of the PLIN family interact. The mechanisms regulating their expression is a question worth studying in the future.

Finally, Chapter IV dealt with a genetic marker (*WUR1000125*, also known as rs80800372 A>G) that has been proposed as a potential genetic marker for PRRS virus susceptibility (Boddicker et al., 2012). It is known that this SNP segregates in the Duroc line used here as well as in commercial crossbreds, both in Spain and in United States (Pena et al., 2013; Abella et al., 2016). Results in Boddicker et al. (2012) indicate that pigs carrying the G allele were more resilient to PRRSV infection and had less viraemia and higher growth during the challenge. Interestingly, Abella et al. (2016) found that in non-infected pigs the A allele was the one which was associated positively with daily gain. We showed that the G allele downregulates *GBP1* expression and that this, in epidemic situations, would make pigs carrying this allele more effective to T-cell responses. In a free-environment, this advantage may turn into disadvantage in terms of growing capacity. However, no evaluation has been done so far on the impact on fat content and composition. As can be seen in Table 2, the rs80800372 polymorphism also affects fat deposition, with the G allele increasing carcass BT and decreasing lean content (allele G substitution effect of  $0.79 \pm 0.24$ ,  $P < 0.01$  for BT and  $-1.09 \pm 0.32$ ,  $P < 0.01$  for lean content). As a result, pigs carrying the G allele have less lean weight, being the substitution effect of  $-0.94 \pm 0.33$  ( $P < 0.01$ ). In contrast, in comparison to AA pigs, GG pigs have increased IMF and less PUFA (allele G substitution effect of  $0.78 \pm 0.32$ ,  $P < 0.01$  for IMF and  $-0.28 \pm 0.01$ ,  $P < 0.01$  for PUFA). These results stress that *GBP1* is subjected to pleiotropic effects as well as to genetic-environment interactions.

**Table 2.** Effect of the *GBP1* rs80800372G>A genotype on lean growth and intramuscular fat content and fatty acid composition

Trait <sup>a</sup>	P-value <sup>b</sup>	<i>GBP1</i> <sup>c</sup>		
		AA (n=553)	AG (n=259)	GG (n=26)
Body weight, kg				
120 days	0.83	62.01 ± 0.47	61.84 ± 0.63	60.98 ± 1.74
180 days	0.38	109.44 ± 0.59	109.65 ± 0.78	106.67 ± 2.11
Backfat thickness, mm				
120 days	0.39	11.15 ± 0.15	11.33 ± 0.20	11.76 ± 0.52
180 days	0.38	18.50 ± 0.20	18.55 ± 0.26	19.47 ± 0.70
Carcass weight, kg	0.90	96.48 ± 0.53	96.76 ± 0.71	96.09 ± 1.92
Carcass backfat, mm	<0.01	22.63 ± 0.20 <sup>b</sup>	23.43 ± 0.27 <sup>a</sup>	24.18 ± 1.12 <sup>ab</sup>
Carcass lean, %	<0.01	41.78 ± 0.27 <sup>a</sup>	42.68 ± 0.36 <sup>b</sup>	41.66 ± 0.96 <sup>ab</sup>
Lean weight, kg	0.02	41.93 ± 0.29 <sup>a</sup>	41.04 ± 0.38 <sup>ab</sup>	39.87 ± 1.01 <sup>b</sup>
Ham weight, kg	0.71	12.22 ± 0.07	12.27 ± 0.09	12.40 ± 0.23
IMF, %	0.02	16.71 ± 0.27 <sup>b</sup>	17.25 ± 0.36 <sup>ab</sup>	19.12 ± 0.95 <sup>a</sup>
SFA, %	0.23	36.70 ± 0.10	36.87 ± 0.14	37.23 ± 0.38
MUFA, %	0.80	49.17 ± 0.11	49.16 ± 0.14	49.42 ± 0.39
PUFA, %	0.02	14.14 ± 0.09 <sup>a</sup>	13.94 ± 0.12 <sup>ab</sup>	13.30 ± 0.33 <sup>b</sup>

<sup>a</sup> IMF: intramuscular fat expressed as percentage of dry muscle; SFA: saturated fatty acids (C14:0+C16:0+C18:0); MUFA: monounsaturated fatty acids (C16:1+C18:1+C20:1) and PUFA: polyunsaturated fatty acids (C18:2+C18:3+C20:2+C20:4) in muscle *gluteus medius*.

<sup>b</sup> P-value associated with the effect of the *GBP1* genotype.

<sup>c</sup> Pairwise comparisons of *GBP1* genotypes. Within row, means with different superscripts differ significantly (P<0.05).

The genetic markers at *FADS2*, *PLIN2* and *GBP1* contribute to explain part of the genetic variance (Table S3 Chapter III) associated to fat content and composition. Thus, markers on *PLIN2* and *GBP1* captured, respectively, 1.02% and 2.17% of the additive variance of lean weight. Regarding IMF, *GBP1* marker

explained 1.44% of the additive variance. All of these genetic markers are in different chromosomes and therefore segregate independently. There is no evidence of linkage disequilibrium and of relevant epistatic interactions among them. At the very most, some indications of additive per dominant epistasis between *SCD* and *LEPR* for carcass weight (-2.21 kg) and carcass BT (-0.78 mm) and for SFA and PUFA content on IMF (-0.38 % and 0.52 %, respectively) were found (Gol et al., 2015). Additive per additive epistasis between *PLIN1* and *PLIN2* (Chapter III) for weight (-0.88 kg for body weight at 120 days and 1.09 kg for carcass weight), BT at different ages (-0.23 and -0.41 mm for BT at 120 and 205 days, respectively), carcass length (0.98 cm) and lean weight (0.30 kg) were found. Regarding functional gene characterization, we conducted a study for the haplotype of three SNPs previously found on *SCD* (Gol et al., 2017). Results were in agreement with association and gene expression analysis indicating that the named H1 haplotype was the responsible of an enhanced expression. Hence, it would be interesting to use this approach to determine the causality of the markers reported here. Overall, the results obtained provide new knowledge on the genetic background affecting lipid metabolism and new clues on how to independently modify lean weight and IMF.





## **CONCLUSIONS**

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**General conclusion:**

The results of this doctoral thesis have shown that the linoleic acid content and three polymorphisms on *FADS2*, *PLIN* and *GBP1* can be useful to select for fat content and fatty acid composition in order to obtain pork with more favourable nutritional and technological profiles.

**Specific conclusions:**

1- The linoleic to arachidonic acid pathway is subjected to substantial genetic influence. More than half of the observed variance for linoleic acid content in muscle is due to genetics. The genetic correlation of the amount of linoleic acid in muscle with intramuscular fat content is close to unity while with lean weight is almost null. Selection for linoleic acid content in muscle is expected to deliver a similar response outcome as selection for intramuscular fat at restrained backfat thickness. Therefore, the amount of linoleic acid in muscle can be considered as an intramuscular-specific biomarker.

2- The haplotype encompassing the rs321384923A>G polymorphism in the promoter of the porcine fatty acid desaturase-2 gene affects the biosynthesis of arachidonic acid from linoleic acid. The G allele acts additively to decrease arachidonic acid content in muscle, with evidence showing that this may lead to increased intramuscular but not subcutaneous fat deposition.

3- The polymorphism rs333231747G>A in the 3' untranslated region of the porcine perilipin-2 gene is associated with lean weight but not with intramuscular fat. The pigs carrying an additional copy of the A allele have heavier lean weight and hams. The selection in favour of the A allele could be used to reduce the undesired negatively correlated response on lean weight to selection for intramuscular fat and vice versa.

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4- The guanylate-binding protein-1 gene expression is lower in pigs carrying the G allele in the rs80800372G>A polymorphism next to a polyadenylation site in the 3'-untranslated region. Low expression is related to the prevalence of longer transcripts from the distal of two active polyadenylation sites. The G allele, which has been associated with lower viraemia after porcine reproductive and respiratory virus infection, in non-epidemic conditions, spurs increased intramuscular fat content but decreased lean weight.

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## **RESEARCH OUTPUTS**

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## Scientific publications

**Gol, S.**, González-Prendes, R., Bosch, L., Tor, M., Reixach, J., Pena, R.N. & Estany, J. 2019. Linoleic acid metabolic pathway allows for an efficient increase of intramuscular fat content in pigs. *Journal of Animal Science and Biotechnology*. 10.1 (2019): 33.

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Tor, M., Vilaró, F., **Gol, S.**, Bosch, L., Reixach, J., Pena, R.N. & Estany, J. 2019. Cuantificación de la enzima estearoyl-CoA desaturasa hepática en cerdos Duroc. *XVIII Jornadas sobre Producción Animal AIDA*, Zaragoza, Spain, May 7-8.

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**Gol, S.**, Ros-Freixedes, R., Tor, M., Pena, R.N., Rothschild, M. & Estany, J. 2015. Efectos aditivos, dominantes y epistáticos de los genes *SCD* y *LEPR* sobre el contenido y la composición de la grasa en cerdo. *XVI Jornadas sobre Producción Animal AIDA*, Zaragoza, Spain, May 19- 20.

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