Università degli Studi della Tuscia

Dipartimento di Scienze Ecologiche e Biologiche

Dottorato di Ricerca in

ECOLOGIA E GESTIONE DELLE RISORSE BIOLOGICHE
XXIV CICLO

Functional analysis of candidate genes for meat quality in *Sus scrofa*

AGR/17

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1. Introduction

Since ancient times, pigs have been farmed for the consumption of their meat and some European breeds were crossed with other ones to influence and modify their fertility and docility (Ojeda et al. 2006). Many commercial breeds have inherited genetic features from other lines, while local breeds have preserved some special traits.

Nowadays, the improvement of commercial breeds, like Large White, with determinate features has become dependent on their “meat quality traits”. These are a very important group of properties and perceptions that determine meat eating quality and healthiness, including animal welfare and environmental impact (Maltin et al. 2003). The meat quality traits are the results of multi factorial and complex mechanisms operating together, such as genetic background, animal nutrition and post mortem period (Ferguson *et al*. 2001). The meat quality traits are measured through quality standards, including biological, chemical and physical aspect of meat, such as intramuscular fat (IMF), cholesterol, ultimate pH, water-holding capacity (drip loss), and sensory traits such as taste, tenderness or sight (colour) (Malek et al. 2001). Among sensory and healthy standards, such as tenderness, colour and leanness of meat, the genetic background can make the difference, both for meat quality and for the conservation of these traits from the producer to the consumer.

Leanness is today considered one of the most important traits, and in the recent past, modern breeding strategies have been targeted to achieve the maximum lean muscle mass. Recently, it has been showed that lean meat isn’t always associated with good meat quality and that the selection of one trait may lead to the loss of another one. Individual phenotype, in fact, is the result of many different molecular processes that modulate gene expression. Malek et al. (2001) suggest that, to improve pork quality, breeding strategies must consider many traits together. For this reason the identification, mapping and function of the genes responsible for quality traits are very crucial

Several studies identified some genes that can be “candidate” for meat quality, because they show a critical role in metabolic or cell differentiation processes. The expression of the genes coding for specific muscle proteins during myogenesis can influence the resulting muscle body (Bains et al. 1984) also by the action of other factors. The activity and the regulation of these genes can determine an increase in myofiber diameter (hypertrophy) or in the number of myofibers (hyperplasia) (Klosowska et al. 2005).

Also the fatty-acid composition is a key aspect in pig meat quality; for example an increase in polyunsaturated lipid fraction in muscle and fat tissues, although favourable for human nutrition, can be undesirable for sensory qualities of pig meat products (Munoz et al.

2007). Lipid accumulation in porcine adipocytes seems to influence leanness, suggesting that the expression of key genes involved in lipid metabolism during porcine adipogenesis (Samulin et al. 2009), and their regulation must be precisely coordinated (Chmurzyñska 2006).

1.1. Expression pathways of candidate genes for meat quality

1.1.1 Genes belonging to the differentiation process: the formation of meat

The influence of genetic factors on the meat quality trait is related to the prenatal formation of muscle tissue (myogenesis), such as determination of precursor cells (myoblasts), proliferation (cell division), and differentiation into multinucleated myofibers. Myogenesis is a prenatal process that forms primary and secondary muscle fiber (Te Pas et al., 2004). Postnatal growth of muscle tissue is characterized by growth of myofibers without the formation of new myofibers. During the multiplication of muscle cells, many muscle fibres are formed, and the number of muscle fibres is determined by prenatally genetic factors and those environmental factors influencing myogenesis. In addition, differences among animals of different breeds can be evidenced by the influence of growth selection on muscle fibre number or size (Rehfeldt et al. 2000).

In the adult, muscle fibres can be classified on the basis of their contractile and metabolic activities. The most basic classification involves slow-twitch oxidative (SO or Type I), fast-twitch oxidative glycolytic (*red muscles*, FOG or Type IIa) and fast-twitch glycolytic (*white muscles*, FG or Type IIb) fibre types (Maltin et al. 2003). Both the contractile and metabolic nature of the fibres may relate to eating quality. Fast-twitch fibres tend to have higher levels of stored glycogen, a more extensive sarcoplasmic reticulum with higher amounts of Ca^{2+} activate myosin ATPase than slow-twitch fibres which have higher numbers of mitochondria, higher concentration of myoglobin and thicker Z lines. Fast and slow fibres have different abilities to generate ATP anaerobically and to sequester and release Ca^{2+}, which may have implications for the onset of cold shortening and rigor.

1.1.1.1. *MYOD* gene family: the *MYOD1* and *MYF5* genes

The *MYOD* gene family regulates the embryonic process of mammalian myofiber formation and consists of four structurally related genes, *MYOD1*, *MYOGENIN*, *MYF-5*, and *MYF-6*. *MYF-5* and *MYOD1* are expressed during proliferation of myoblasts, *MYOGENIN* is

expressed during terminal differentiation, and *MYF-6* is mainly expressed during postnatal life (Te Pas et al. 1999).

The expression of *MYOD* genes takes place exclusively in skeletal muscles and their products are specific transcription factors with the basic helix-loop-helix (bHLH) domain, which participate in muscle development from muscle cell determination and proliferation through fibre formation up to their postnatal maturation and function (Te Pas et al. 1999). In particular, the products of *Myogenic Factor 3* (*MYF3*) and *Myogenic Factor 5* (*MYF5*) genes are transcription factors, such as MYOD1 (MYF3) and MYF5, that, control the processes of myogenesis (Urbański P. & Kuryl J. 2004). Soumillion (1997) and then Urbański (2005) mapped the porcine *MYOD1* and *MYF5* genes to chromosomes 2 and 5, respectively. Stratil and Cepica (1999) reported seven mutations in the porcine *MYF5* gene, one of them was detected in exon 2 (position 2368).

In the promoter region of *MYF5* gene Urbański & Kuryl (2004) identified, three mutations at positions A65C, C580T and C613T. The A65C transversion doesn't influence gene expression (Urbański et al. 2006) and the relative content of *MYF5* mRNA in pig *Longissimus dorsi* muscle does not significantly across *MYF5* genotypes, although this mutation abolishes a putative binding site for two transcription factors (CDP and HSF1) and creates a putative binding site for Sp1.

1.1.1.2 Growth and Differentiation factors: the *GDF8* gene

The *transforming growth factor β* (*TGF-β*) gene super family encodes for growth factors that regulate embryonic development. The most famous gene member is the *myostatin* (*MSTN*), or *Growth and Differentiation Factor 8* (*GDF-8*) that negatively regulates skeletal muscle mass development (McPherron *et al*.1997).

Some studies had verified that a mutation in *GDF8* gene is linked to Double Muscled (DM) phenotype in cattle (Grobet *et al*.1997; Kambadur *et al*. 1997). This DM phenotype is present in several breeds of cattle such as Belgian Blue, Piedmontese and Asturiana de los Valles, and it is characterized by a visible and generalized increase in muscle mass due primarily to hyperplasia (Taketa et al. 2008), such as a change in number of cells caused by an excessive proliferation. This muscle condition is opposite to hypertrophy, where cells increase in size and not in number. All the studies about this condition in cattle, suggest that any allelic variation in *myostatin* can affect muscle mass and/or fat deposition in porcine too (Sostengard et al. 1998).

1.1.1.3. *Post-mortem* period: the Calpain-Calpastatin system

With respect of meat quality traits, the natural tenderization of meat depends on a post-mortem degradation process of muscle proteins. In mammal skeletal and cardiac muscle, calpains are implicated in processes regulating proteolysis of myofibrillar and cytoskeletal proteins. Their influence on these processes is regulated by its specific inhibitor, the calpastatin.

Recent studies have shown that calpain activity is required for myoblast fusion and cell proliferation in addition to cell growth. Parr et al. (2001) suggested that *calpastatin* gene expression in porcine, is probably regulated by multiple promoters which encode for several transcripts. These transcripts maybe control the type, amount and tissue specific gene expression.

1.1.2. Genes belonging to the lipid metabolism: adipose tissue formation

Lipid metabolism consists of fatty acid oxidation to produce energy or to synthesize lipids, and it is closely connected to the metabolism of carbohydrates, which may be converted to fats. The Fatty Acids (FAs), produced in muscles and liver, are also used for the formation of complex lipids, such as phospholipids and cholesterol, hormones and signalling compounds. Lipid metabolism is in a constant state of dynamic equilibrium and some lipids must be constantly oxidized to supply energy needs, while others are synthesized and stored.

It has been revealed that the increased of fatty acids concentration triggers the process of preadipocyte differentiation and the expression of terminal differentiation-related genes (Amri et al.1994). Metabolism of fatty acids into cells is divided into several steps, such as synthesis, storage, transport and mobilization crossing the membrane. Each step must be catalysed by proteins able to effective fatty acids movement (Hamilton 1998).

1.1.2.1. Biosynthesis of fatty acids: the *Acetyl Coenzyme A Carboxilase Alpha* (*ACACA*) gene

The sequence of reactions involved in the formation of lipids is known as Lipogenesis, and this process occurs in the cytoplasm, in contrast to the degradation (oxidation) which occurs in the mitochondria. Lipogenesis requires ATP and all the reactions, which are reductions, need addition of H^+ and use of NADPH.

A group of enzymes for the fatty acid synthesis, called fatty acid synthetase (FAS), catalyzes the synthesis of long-chain fatty acid, palmitate, by using acetyl-Co A as a primer,

malonyl-Co A as a two-carbon donor for chain elongation, and NADPH for the reduction reactions (Mao et al. 2006). In addition, malonyl-CoA might act as an inhibitor of the import of FAs into the mitochondria for ß-oxidation (Barber et al. 2005). Therefore, malonyl-CoA participates in two opposing pathways, a substrate for fatty acid synthesis and also as a regulator in fatty acid oxidation in the mitochondria (Mao et al. 2006).

The Acetyl-Coenzyme A Carboxylase α (ACACA) enzyme is involved in the biosynthesis of long-chain fatty acids by playing a key role in the conversion of acetyl-CoA to malonyl-CoA (Abu-Elheiga et al. 1995). The *ACACA* gene has been completely sequenced in human, and it is organized in 54 exons, encoding protein of 2346 amino acids (Barber et al. 2005). The porcine *ACACA* gene has been mapped to chromosome 12 (SSC12) by Calvo et al. (2000) and most of its coding sequence has been characterized by Muñoz (2007) revealing 15 synonymous SNPs, two of them were genotyped across the pedigree. Muñoz have verified also the presence of associations between the polymorphism of the *ACACA* gene and FA composition. In fact, the genomic location of this gene coincides with the confidence interval for a quantitative trait locus (QTL) for FA composition and this SSC12 QTL has been independently confirmed in an Iberian-Landrace resource population by Muñoz (2007) and in the Duroc commercial line by Gallardo (2009).

1.1.2.2. Storage of fatty acids: the *DiacylGlycerol AcylTransferase* (*DGAT1*) gene

In eukaryotic organisms the major form of stored energy is that of Triacylglycerols (TGs), esters of fatty acids and glycerol. Triacylglycerols are necessary components of mammalian energy homeostasis, and an imbalance between energy intake and expenditure can lead to the excessive accumulation of these components in tissues with pathological consequences.

The synthesis of TriAcylGlycerols (TAGs) has a key role in several physiological processes, such as intestinal fat absorption, intracellular energy storage, lactation and lipid transport. There are two different pathways for triacylglycerol synthesis, one that begins with the acylation of monoacyl-glycerol with a fatty acyl-Coenzyme A by monoacylglycerol acyltransferase, and the other that begins with the acylation of glycerol-3-phosphate with a fatty Acyl-Coenzyme A, producing lysophosphatidic acid to produce Diacylglycerol (DAG) (Bagnato et al. 2003)). Diacylglycerol (DAG) is a versatile molecule that participates as substrate in the synthesis of structural and energetic lipids, and acts as the physiological signal that activates protein kinase C.

The Diacylglycerol acyltransferase (DGAT) is the last committed enzyme in triacylglycerol synthesis, participates in the regulation of membrane lipid synthesis and lipid

signaling, thereby playing an important role in modulating cell growth properties (Bagnato et al. 2003). DAG is a common intermediate for both triacylglycerol and phospholipid synthesis and its segregation toward different metabolic routes seems to occur according to the need of the cell. For instance, when phospholipid synthesis is inhibited, DAG originally destined to form phospholipids is re-directed toward triacylglycerol. DAG regulates cell growth and differentiation by activating several isoforms of protein kinase C (PKC); recently a study shows that persistent accumulation of intracellular DAG is linked to oncogenic transformation. DAG released from plasma membrane may also be directly incorporated into triacylglycerol, suggesting the presence of a novel mechanism for terminating DAG signals based on the synthesis of a storage lipid (Bagnato et al. 2003).

The acyl-CoA-diacylglycerol acyltransferase 1 (DGAT1) is a key enzyme involved in DAG and triacylglycerol metabolism. DGAT1 and the recently discovered DGAT2, catalyze the last committed step in mammalian triacylglycerol synthesis by esterifying DAG. Thus, DGAT1 catalyses the final committed step in the formation of triglycerides using Diacylglycerol and acyl CoA as substrates (Cases et al. 1998). The *DGAT1* gene is widely expressed in most tissues, with the highest expression levels in small intestine and adipose tissue (Chen & Farese 2000; Buhman et al. 2002). Because both *DGAT* genes are widely expressed in mouse and human tissues, the presence of two DGAT enzymes in non-adipose tissues suggests that the synthesis of triacylglycerol might be linked to cell functions other than energy storage (Bagnato et al. 2003). Mercadè et al. (2005) verified that *DGAT1* deficiency modifies the fatty acid composition in adipose tissue and skeletal muscle, increasing saturated fatty acids and decreasing monounsaturated fatty acids (Chenet al. 2002). Moreover the *DGAT1* deficiency alters the triglyceride metabolism in tissues as mammary gland causing the absence of milk production (Chen & Farese 2000). Grisart et al. 2004 identified a non conservative substitution (K232A) in the bovine *DGAT1* gene and proposed this substitution to be the causal mutation underlying a quantitative trait locus (QTL) with effect on milk composition.

The porcine *DGAT1* gene comprises 17 exons and the 1935 bp-long cDNA codes for a protein of 489 amino acids with 85, 86, and 92% identity to mouse, human, and bovine proteins, respectively (Nonneman & Rohrer 2002). Mercadè et al. (2005) described a QTL located on chromosome 4 for fatty acid composition in an Iberian -Landrace cross (Pérez-Enciso et al. 2000; Clop et al. 2003) and comparative mapping aligns this region to the human *DGAT1* gene on chromosome 8q24.3 (Goureau et al. 1996), making the *DGAT1* gene a suggestive positional candidate gene in the QTL for fatty acid composition.

1.1.2.3. Fatty acids transport and mobilization: the *FABP* gene family

Several studies revealed that the increased of fatty acids concentration triggers the process of preadipocyte differentiation and the expression of terminal differentiation-related genes (Amri et al.1994). Transport of fatty acids into cells is divided into several steps, however there are 2 types of fatty acids translocation across plasma membranes, namely simple diffusion and protein-mediated translocation.

The many different types of fatty acid-binding proteins (FABPs) can be divided into 2 main groups (Glatz and van der Vusse 1996) between those associated with the plasma membrane (FABPPM) and those intracellular or cytoplasmic proteins (FABPC). Clarke et al. (2004) provided the first piece of evidence that over expression of FABPPM, a putative translocases, in a mammalian tissue can increase fatty acids transport. The fatty acids are desorbed from the plasma membrane into the cytoplasm and the FABPC proteins may accelerate fatty acids uptake, enhancing their solubility (Vork et al. 1993), or improving their transfer to acceptor membranes (Hsu and Storch 1996). FABPC proteins stimulate not only fatty acids desorption, but also cytoplasmic diffusion (Mc Arthur et al. 1999), thus FABPs can be defined as transport proteins.

One group of the transcription factors cooperating with FABPs is the PPAR family (peroxisome proliferator-activated receptor). PPAR family members are nuclear receptors known to regulate the transcription of many genes involved in lipid metabolism (Desvergne and Wahli 1999).

For example, PPAR is a key transcription factor in preadipocyte differentiation (Gregoire et al. 1998). Comparison of the aminoacid sequences of different FABPs revealed a wide variance in primary structure; therefore FABPs can be divided into 3 groups reflecting the binding abilities of the proteins:

(1) proteins capable of binding fatty acids and bulky ligands, such as bile salts, cholesterol (L-FABP and Il-FABP);
(2) proteins capable of binding fatty acids and additionally retinoids and eicosanoids (H-FABP,B-FABP, E-FABP, M-FABP, A-FABP and T-FABP);
(3) I-FABP which binds solely fatty acids, but in a different conformation than other FABPs (Chmurzyñska 2006; Sacchettini et al. 1988).

The overall gene structure is conserved between all family members and consists of 4 exons separated by 3 introns (Hayasaka et al.1993; Treuner et al. 1994; Hertzel and Bernlohr 2000). The *FABP* genes contain the canonical TATA box located around 23–30 nucleotides upstream the transcription start site (Sweetser et al. 1986 ,1987; Kurtz et al. 1994) and the expression of the *FABP* genes is tissue-specific (**Table 1**).

Gene name	Common name	Alternative name
FABP1	Liver FABP	*L-FABP*
FABP2	Intestinal FABP	*I-FABP*
FABP3	Heart FABP	*H-FABP*
FABP4	Adipocyte FABP	*A-FABP*
FABP5	Epidermal FABP	*E-FABP*
FABP6	Ileal FABP	*Il-FABP*
FABP7	Brain FABP	*B-FABP*
FABP8	Myelin FABP	*MP2*
FABP9	Testis FABP	*T-FABP*

Table 1- FABP gene family

Glatzand van der Vusse 1996 suggested that tissue content is mostly regulated at the transcriptional level and this seems to be a result of the synergistic interactions between conserved motifs of gene promoters. The two members of the FABP family (*A-FABP (FABP4)* and *H-FABP (FABP3)*) have been considered as candidate genes for pig fatness traits (Chmurzynska 2006) and studies revealed that both genes are located within the regions containing QTLs for these traits.

The porcine *H-FABP (FABP3)* gene was mapped to chromosome 6 by Gerbens et al. (1997) and this chromosomal region harbours QTL for fatness traits (Ovilo et al. 2002; Yue et al.2003). The effects of some candidate SNPs on these traits were tested by Chmurzynska (2006), but all of them are silent mutations: one is located in the 5' regulatory region and two in the second intron. In Duroc pigs, Gerbens et al. (1999) detected a significant contrast for intramuscular fat (IMF) and backfat thickness (BFT) between the homozygous genotypes of all 3 SNPs and in the 5'-flanking region described motifs recognized by activator protein 1 (AP-1), activator protein 2 (AP-2), CCAAT/enhancer-binding protein (C/EBP), mammary active factor (MAF), and Stat-5. Chmurzynska (2006) suggested that these elements could be responsible for the constitutive expression, as well as for pregnancy and lactation-dependent expression in mammary epithelium.

The porcine *A-FABP (FABP4)* gene was mapped to chromosome 4 and in that region a QTL for IMF, was suggested by de Koning and Rattink (2000). Hunt et al. (1986) compared the 5'-flanking region of the *A-FABP (FABP4)* gene with the promoters of two other genes activated during adipogenesis (glycerol-3-phosphate dehydrogenase and adipsin) and revealed multiple copies of a 13-nucleotide element within the 200bp of the TATA boxes of the all 3 genes. *A-FABP (FABP4)* gene contains three direct copies and one inverted copy of these sequences, called fat-specific element 1 (FSE1), and additionally other 15 bp element (FSE2).

FSE1 and FSE2 were found also in *glycerol-3-phosphate dehydrogenase* gene. FSE1 presumably activates developmental transcription, while FSE2 modulates the level of transcription, because it binds a nuclear complex containing c-*fos* and c-*Jun*-related proteins through the sequence recognized by transcription factor AP-I (Distel et al. 1987; Rauscher et al. 1988). The AP-I site acts as a positive regulator of gene expression but is silenced by the adjacent upstream sequence, which binds the CCAAT/enhancer-binding protein – C/EBP (Herrera et al. 1989).

These mentioned above have not clarified the problem of QTLs on chromosomes 4 and 6, and Chmurzyñska (2006) suggested the use of bioinformatic tools to find causative mutations directly responsible for the variability of fatness traits.

1.1.2.4. Fatty acids transport and mobilization control: the *hormone sensitive lipase (LIPE)* gene

The hormone sensitive lipase (LIPE) plays a key role in mobilization of fatty acids by controlling the hydrolysis of triglycerides in adipose tissue (Harbitz et al. 1998). Expressed in several tissues, LIPE has been also suggested to mediate lypolisis, cooperating with lipoprotein lipase (Oscai et al. 1990).

The porcine *LIPE* gene has been assigned to chromosome 6 in a region studied for the presence of the porcine malignant hyperthermia locus, and for the association with backfat thickness and the lean and fat content in pig muscles. For its role in fat metabolism, *LIPE* has been suggested as a candidate gene for these important quality traits (Otsu et al. 1991).

2. Aim of the work

The aim of this study was to analyse the activity of promoter regions of genes, which show a key role in meat quality. We have chosen some genes belonging to the *Myogenic Regulatory Factors* (*MRF*) gene family (*MYF5* and *MYOD),* to *Growth and Differentiation Factors* (GDF) gene family (*GDF8),* to the calpain-calpastatin system (*CALP* and *CAST),* to adipose tissue formation genes (*ACACA, DGAT1, LIPE*) and to the FABP family (*FABP3* and *FABP4*) as the literature demonstrated their importance for improving pork quality.

In particular, we have chosen to analyse only the promoter region of these genes, because this is an important part of it, harbouring several motifs binding to transcription regulatory factors. Mutations, as Single Nucleotide Polymorphisms (SNP) in the promoter region can influence the transcription process. SNPs in this region are likely to deeply affect RNA levels and consequently protein synthesis, and so they can be responsible of differences on meat quality traits among breeds.

We studied the promoter region of candidate genes for meat quality, in two different pig breeds, *Large White* (LW) and *Casertana* (CA), two very different pig breeds, in order to analyse the effects of promoter SNPs.

2.1. Features of *Large White* and *Casertana* Pig Breeds

The Casertana breed is a very ancient and an endangered pig breed, raised for centuries in semi wild conditions in the forests near Caserta and Benevento (**Figure 1**).

Figure 1- The Casertana breed

Chemical composition of CA muscles is characterized by a high lipid and low protein content. Studies revealed that CA pigs have a strong aptitude for fat deposition and produce more double backfat thickness than LW pigs. (Pietrolà et al. 2006). Because of its slow growing, this breed has not been selected at least in recent years, so CA pigs retain the traits of high fat depositing pigs, compared to the lean genetic lines actually exploited in the pig industry. Comparison with LW pigs demonstrated that the CA is less competitive regarding growth performance, reaching a commercial slaughter weight at a considerably greater age and with a 2-fold increase in backfat thickness (Pietrolà et al. 2006). For all these features, CA pig breed is certainly the most precious among Italian breeds, being able to provide large amounts of fat.

The Large White breed (**Figure 2**) is characterized by large size, and represents the most farmed breed in Italy. LW pigs' ability to cross with and improve other breeds, has given them a leading role in commercial pig production systems and breeding pyramids around the world. LW pigs excel in growth rate and lean meat percentage.

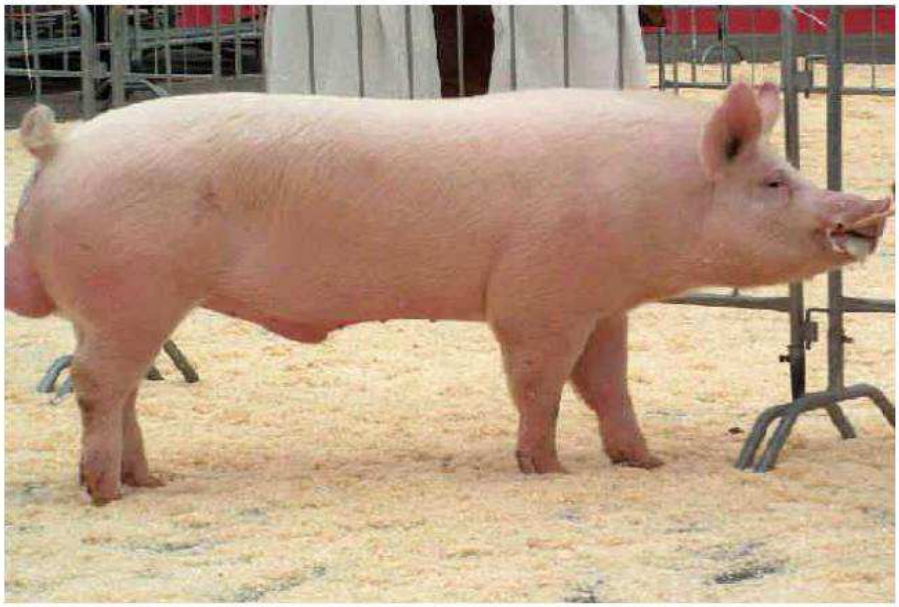

Figure 2- The Large White breed

These main characteristics were reflected in LW molecular proteomics and transcriptional profiles, as emerged from pathway, functional and network analyses (Murgiano et al. 2010). Besides, considering the semiwild rearing environment of CA, the expression of a fast energy source could be related to a greater need of energy compared to a static lifestyle of LW. In LW, the presence of proteins related to mitochondrial activity, lipid mobilization, insulin resistance, and antioxidant protein and transcripts are suggestive of a likely constant

mitochondrial activity consuming the smaller fat mass. LW pigs clearly showed a tendency to muscular growth. Most of the over expressed proteins and transcripts belonged to protein groups composing the fiber and the sarcoplasmic reticulum, as well as with antiproteolytic enzymes, as expected from a breed selected to develop lean muscular mass (Murgiano et al. 2010).

2.2. The workflow for a functional analysis of candidate genes for meat quality

This work was organized with several steps, listed below and explained in particular in Material and Methods:

1. Selection of gene affecting meat quality traits.
2. DNA extraction from liver of 10 animal of each breeds (LW and CA breed).
3. Primer design to amplified 500-2000 bp sequence fragments from promoter region.
4. Polymerase Chain Reaction (PCR).
5. Sequencing analysis for SNP discovery.
6. Cloning and screening of variants.
7. Insertion of variants in pGL4.17 vector and cloning for transfection experiments.
8. *in vitro* CHO cell transfection by liposomes.
9. Bioluminescence analysis and statistical analysis to evaluate differences in gene expression.

3. Materials and methods

3.1 Pig samples and DNA extraction

A total of 20 animals were analysed, namely, 10 Casertana and 10 Large White, from both sexes. The animals, kindly provided by Prof. Pilla, University of Molise, were raised outdoors in the same environmental conditions and fed using the same diet in order to detect only the genetic differences, as much as possible. All animals were reared and slaughtered at same age, according to European directives and laws. Genomic DNA was extracted, using Promega kit, according to manufacturer's instructions, from liver tissue collected at slaughter and stored at –20°C. DNA was checked for quality on agarose gel and quantified using a DTX microplate reader (Beckman Coulter) after staining with Picogreen (Invitrogen).

3.2 Polymerase Chain Reaction (PCR) conditions

Primer pairs for all tested genes were designed on sequences available at NCBI, using Blast software and synthesized by Sigma-Aldrich. The primer sequences and amplicon sizes are reported in **Table 2**. Each polymerase chain reaction (PCR) was performed in a total volume of 25 μl containing about 20 ng of genomic DNA, 1.0 pMol of each primer, 10 μl of BioMix (Bioline) composed by BIOTAQ^{TM} DNA Polymerase and 2mM of dNTPs, on a PTC-100^{TM} Peltier Thermal Cycler (MJ Research). The amplification was carried out on a PTC-100 (MJ Research) following standard conditions.

3.2.1 High fidelity PCR

To sequence PCR fragments and identify new SNP, the High Fidelity PCR system (Roche) was used following standard conditions by Roche (Buffer 1x, dNTP mix 20μM, Primer F 0.4 μM , Primer R 0.4 μM, DNA template variable, water to volume).

Gene	Name	Ref. Genebank	Primer		Size (bp)	T_a (°C)
ACACA	*Acetyl-Coenzyme A Carboxylase Alpha*	FJ263680	F	GGGGAATTTGAAAAAGAGCC	1607	58
			R	CTCGTTTGTCAGGAAGAGGC		
CALP	*Calpain*	U23954	F	GGAGCTTGATCTTAAACCTGAGGGTGG	677	58
			R	TCAGTCCTAGGGAAGACAAGGGGA		
CAST	*Calpastatin*	AJ583410	F	GGCGTGGCTGCCTGGAGAAA	380	62
			R	TGGCCTGCACGCCCAGAAAG		
DGAT1	*Diacylglycerol acyltransferase*	AY116586	F	CGGCGACCACAATTCCCAGG	1119	58
			R	TCCTTCAGCCCAACCAGGGG		
FABP3	*Heart Fatty Acid Binding Protein 3*	X98558	F	TGTTCGGACCCCAGGCTCGT	450	61
			R	CGCGGCCAACCTCTTCCCAG		
FABP4	*Adipocyte Fatty acid Binding Protein 4*	EF061476	F	ACTGTTCCCCAAAGCCTCTGCAA	700	60
			R	AAGCAGTACTTCATGTCTGGCTGGT		
GDF8	*Myostatin*	AY208121	F	TCTCTCAGACAGTGCAGGCATTAAA	200	58
			R	TTTTCCTTTTGCTCGCTGTT		
LIPE	*Hormone sensitive lipase*	AJ000483	F	TTTGGAAAGGATTCCACAGC	1352	58
			R	CTGAGTCCCAGACAGCTTCC		
MYF	*Myogenic Factor 5*	Y17154	F	CGGGGTTTCTTAGAACACACAGCTT	1244	64
			R	TCCGCCGATCCATGGTGGTG		
MYOD	*Myogenic Differentiation 1*	U12574	F	CCCGTCAGTCAGGAGGGACAG	609	61
			R	CTTGGGCAGCCGCTGATTCG		

Table 2- Primer sequences, Product size(bp) and Annealing Temperature(°C)

3.3 Sequencing analysis and SNP discovery

PCR products were purified through ExoSap-IT (USB Corporation) to remove residual primers and dNTPs and used as templates for forward and reverse sequencing reactions. Sequencing was performed using a Ceq8800 sequencer using DTCS Quick Start Kit and purifying with Agencourt CleanSEQ 96 (Beckman Coulter), according to manufacturer instructions. As an alternative, purified PCR products were outsourced to Macrogen Inc. (www.macrogen.com) for sequencing. Sequence analysis and alignments were performed using BLAST (Altschul et al. 1997; http://www.ncbi.nlm.nih.gov/BLAST) and BioEdit software (Hall 1999).

3.4 Cloning of allelic variants

Amplified fragments of selected genes were inserted in TOPO vectors and cloned with TOPO Cloning® technology. TOPO Cloning® technology allows to ligate PCR product into pCR®2.1- TOPO ® and transform it into competent cells (JM109). The transformation protocol was carried out following standards conditions. Plasmid mini preparations, for plasmid sequencing and transfection, were performed with the PureYield™ Plasmid Miniprep System (Promega).

3.4.1 pGL4 constructs

In order to exclude the complex interactions among genes, the effects of the SNP have been assessed in a genome-free environment *in vitro*, consisting of only the study promoter and a reporter gene. pGL4.17 vector (Promega) was used for testing the efficiency of study reporter (**Figure 3**).

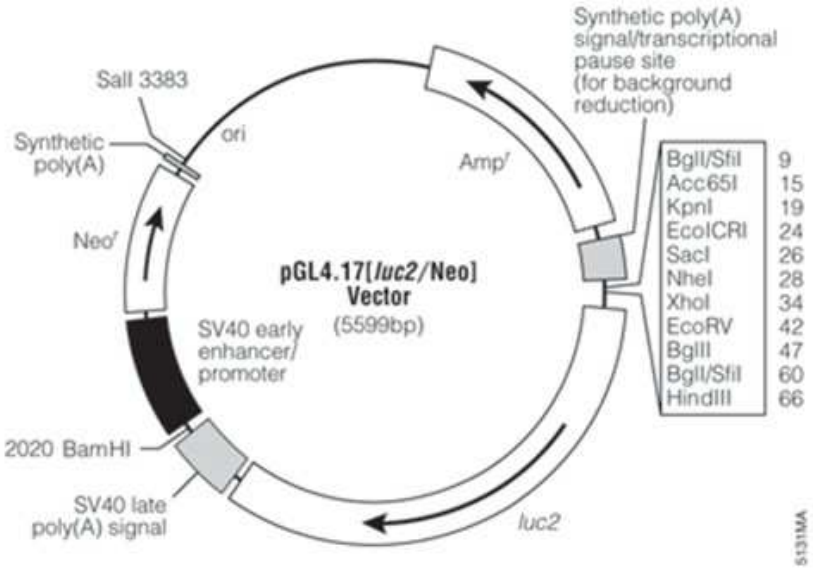

Figure 3- The pGL4.17 (Promega)

To normalize the transfection process pGL4.75 vector (Promega) was co-transfected (**Figure 4**). pGL4.75 brings a constitutive promoter and codifies for the *hRluc* fluorescent protein.

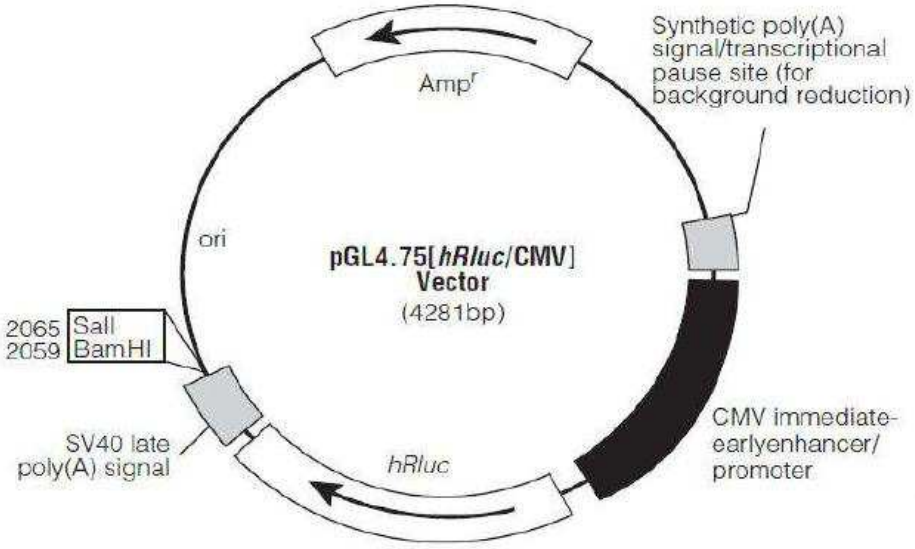

Figure 4- The pGL4.75 vector circle map (Promega)

The pGL4.17 constructs (**Table 3**) were designed and prepared by Biofab Research (www.biofabresearch.it) or, alternatively, by GeneCust (www.genecust.com).

Gene	*MYF5*	*GDF8*
SNP	C802G	G449A
construct A	C	G
construct B	G	A

Table 3- Constructs of pGL4.17

3.5 Transfection design experiment

The transfection experimental protocol was designed to make possible a significative statistical analysis. Different kind of cell wells were prepared: three cell wells with construct A, three cell wells with construct B, three cell wells with pGL4.17 without the insert construct (positive control), and two cell wells without DNA (negative control). These eleven cell samples were replicated in the same 24 well plate, changing the mix; moreover the entire experiment was replicated.

3.5.1 Cell Growth and *in vitro* transfection

Chinese Hamster Ovary (CHO) cells were grown in monolayer culture at 37° for 48h in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fatal bovine serum 1x, 2% Penicillin/Streptomycin 50x, 1% Glutamine 100x, 1% non essential amino acids. When cell confluence achieves about 100%, grown culture is removed and 3 ml of culture without antibiotic was put to make the count of the cells.

For transient transfection, cells were seed into 24 multiwell plates at a density of $8x10^5$. After a 24 h attachment period the cells were cotransfected, using lipofectamine (Lipofectamine 2000, Gibco-BRL), with 237.5 ng of promoter-Firefly luciferase plasmid (pGL4.17) and 12.5 ng phRG-TK vector containing Renilla luciferase plasmid (pGL4.75), to normalize for the efficiency of transfection and to achieve a ratio of 20:1. Usually, two kind of mix were prepared: one with total DNA and Opti-MEM broth, the other one with lipofectamine and Opti-MEM. After 5 minutes, these two mixes were combined, incubated for 20 minutes and then distributed 100 µl at each well containing the cells. The cultures were incubated at 37°C for a further 24 h, then rinsed with PBS and lysed in 100 µl of 1x lysis buffer at room temperature for 15 min on a rocking platform. In turn, whole cell extracts were analyzed for Firefly and Renilla luciferase activities using a Dual-Luciferase Reporter Assay System (bioluminescence analysis).

3.5.2. Bioluminescence analysis

The functional activity of promoter variants was analysed *in vitro* with the Dual-Luciferase Reporter Assay System™ (DLR™, Promega). In the DLR™ Assay, the activities of Firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferase were sequentially measured from a single sample. The Firefly luciferase reporter was measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the Firefly luminescence, this reaction was quenched, and the Renilla luciferase reaction was simultaneously initiated by adding Stop & Glow® Reagent into the same tube.

To read the Firefly luminescence (the first read) 20µl of cell lysate were added to 100 µl of LAR II into the tube of TD-20/20 Luminometer by Turner Designs. Then, the sample tube was removed from the luminometer and 100µl of Stop & Glo® Reagent were added to the mix to turn off Firefly luminescence and activate Renilla, luciferase activity (the second read).

3.6 Statistical analysis

Transfection results are used in the Student's t-test to support the hypothesis of significant differences of expression between variants. The t-test was calculated with R-software (http://www.r-project.org/)

3.7. Real time PCR

3.7.1 RNA extraction

Total RNA was extracted from the same 20 pig samples (10 Casertana and 10 Large White) using RNeasy Lipid Tissue kit (Qiagen) according to manufacturer's instructions, and stored at –80°C. RNA quality was checked on agarose gel and quantified using a DTX microplate reader (Beckman Coulter) after staining with Quant-iT protocol (Invitrogen).

3.7.2 Primer design and quantitative Real Time PCR

RNA from the same animals and tissue samples were used in quantitative Real Time PCR to confirm the transfection data. Two variants of total RNA, belonging one to LW and one to CA individuals, were reverse transcribed in cDNA using a blend of oligo-dT and random hexanucleotides and the Quantiscript Reverse Transcriptase (Quiagen). A standard curve was

generated using serial dilutions of the cDNA to calculate the efficiency of amplification. Real-time quantitative PCR was set up for *MYF5*. Primer pairs, designed using BLAST, are listed in **Table 4** and are derived from pig sequences publically available at NCBI. qRT-PCR was performed with the Brilliant II SYBR Green qRT-PCR kit (Stratagene, Agilent technologies) and using a Stratagene Mx3005P (Stratagene).

Amplification results of variants samples were calibrated against those of LW samples to obtain the differences in gene expression. All results were normalized to *ß-actin* (*ACTB*) and *Ribosomal Protein L4* (*RPL4*) housekeeping genes (Nygard et al. 2007). The *ACTB* and *RPL4* genes were found to have a high stability among the seven genes (*ACTB*, *RPL4*, *TBP*, *HPRT1*, *HMBS*, *YWHAZ*, *SDHA*) described by Nygard et al. Primer sequences of five housekeeping gene tested are showed in **Table 5**. In the qRT-PCR, three biological replicates for each variant and two technical replicates were used and performed for each gene investigated, including at least one housekeeping gene.

Gene	Name	Ref. Genebank	Primer		Size (bp)	T_a (°C)
RT *MYF5*	*Myogenic Factor 5*	AY609670	F1	CCCGGGCTTCTCCCCGATCT	160	63
			R1	TCGTCCCCGAACTCGCCCTC		
			F2	AGGGCGAGTTCGGGGACGAG	154	63
			R2	TTGCACGCTTTGCAGGCCCA		
			F3	TGCCTCATGTGGGCCTGCAA	161	62
			R3	TTGGGCAGCCTCTGGTTGGG		
			F4	ACCCCAACCAGAGGCTGCCC	150	63
			R4	AGCTGGAGGTGGGGCTGGTG		
			F5	CTCTGAGCCCACCAGCCCCA	191	61
			R5	AGTTGCTGATCCGATCCACTATGCT		

Table 4- MYF5 primer sequences performed in Real Time PCR

Gene	Name	Ref. Genebank	Primer		Size (bp)	T_a (°C)
ACTB	*Beta-Actin*	DQ845171	F	GGCCGGGACCTGACCGACTA	130	60
			R	GCTCGAAGTCCAGGGCGACG		
GAPDH	*Glyceraldehyde-3-Phosphate Dehydrogenase*	DQ845173	F	ACACTCACTCTTCTACCTTTG	90	60
			R	CAAATTCATTGTCGTACCAG		
HPRT1	*Hypoxanthine Phosphoribosyltransferase 1*	DQ845175	F	GGACTTGAATCATGTTTGTG	91	60
			R	CAGATGTTTCCAAACTCAAC		
RPL4	*Ribosomal Protein L4*	DQ845176	F	CAAGAGTAACTACAACCTTC	122	60
			R	GAACTCTACGATGAATCTTC		
TBP1	*TATA Box Binding Protein*	DQ845178	F	AACAGTTCAGTAGTTATGAGCCAGA	153	62
			R	AGATGTTCTCAAACGCTTCG		

Table 5- Primer sequences of housekeeping genes used in Real Time experiments

4. Results

In this study, we compared two breeds, Large White and Casertana, in order to analyze how single nucleotide polymorphisms in the promoter region of gene candidate for meat quality, can modulate gene expression. We have analysed the promoter region of ten candidate genes for meat quality traits (**Table 6**). The functional activity of promoter variants was analysed *in vitro* with the Dual-Luciferase Reporter Assay System™ (Promega). Using the standard protocol, the luminometer showed the results of Firefly luciferase activity, Renilla luciferase activity and the ratio between them (see Materials and Methods).

Gene	Name	Pathway	Transcript function	References
ACACA	*Acetyl-Coenzyme A Carboxylase Alpha*	Biosynthesis of long chain in fatty acids	Catalizator of reduction reaction	*Gallardo et al. 2009, Mao et al. 2006,Muñoz et al. 2007*
CALP	*Calpain*	Post mortem proteolisis	Enzyme	*Ciobanu et al. 2011, Suzuki et al. 1987*
CAST	*Calpastatin*	Post mortem proteolisis	Enzyme	*Ciobanu et al. 2011, Geesink et al. 1998, Meyers & Beever 2008, Parr et al. 2001*
DGAT1	*Diacylglycerol acyltransferase*	Storage of glycerolipids	Catalizator of oxidation reaction	*Mercadé et al. 2005, Bagnato et al. 2003, Shi & Cheng 2009*
FABP3	*Heart Fatty Acid Binding Protein 3*	Mobilization of fatty acids	Membrane Protein	*Chmurzynska 2006, Gerbens et al. 1997, Samulin et al. 2009,*
FABP4	*Adipocyte Fatty acid Binding Protein 4*	Mobilization of fatty acids	Membrane Protein	*Chmurzynska 2006, Gerbens et al. 1997, Samulin et al. 2009,*
GDF8	*Myostatin*	Cell differenziation	Regulatory Factor	*Guimaraes et al. 2007, Klosowska et al. 2005, Sostengard et al. 1998*
LIPE	*Hormone sensitive lipase*	Mobilization of fatty acids	Enzyme	*Harbitz et a. 1998*
MYF5	*Myogenic Factor 5*	Cell differenziation	Regulatory Factor	*Soumillon et al. 1997;Te Pas et al. 1999; Urbanski & Kuryl 2004, 2006*
MYOD1	*Myogenic Differentiation 1*	Cell differenziation	Regulatory Factor	*Robakowska et al. 2010; Urbanski & Kuryl 2004, 2007*

Table 6- Genes and pathways

4.1. *MYF5* gene

4.1.1. *MYF5* gene sequencing

In order to study the promoter region of *MYF5* gene, firstly we sequenced all the region carrying the polymorphisms previously described in literature.

In particular, in the promoter region of the *MYF5* gene we sequenced Urbański's mutations: A65C, C580T and C613T (*Genbank acc.no. Y17154*). In our samples we found the A65C SNP, with differences on the genotypes frequencies (A/A, A/C, and C/C) between the two breeds, like showed in **Table 7**. About the C580T transition, our samples are all homozygous CC, while we weren't able to sequence the region carrying the C613T mutation. As concerning the transition (C71T) described by Maak et al. (2006), our samples showed only the C/T and C/C genotypes, while individual was without great differences between the breeds. In our sample the transversion described by Liu et al. (2008) at position A1205C, was not present since all the individuals are homozygous A/A.

SNP	**A65C**			**C71T**		
Genotypes	AA	AC	CC	CC	CT	TT
Large White	0,56	0,33	0,11	0,44	0,56	0,00
Casertana	0,00	0,4	0,6	0,90	0,10	0,00

Table 7- Genotypes of *MYF5* gene mutations

Sequencing analysis of our samples has identified three new SNPs, two in the promoter region (T149C and C802G) and one in the first exon (G1288C) of *MYF5* gene. Sequencing results showed that, related to the T149C transition, Casertana individuals are characterized only by the C/C homozygous genotype with a frequency of 70%, while in Large White individuals the most present homozygous genotype is the T/T genotype with a frequency of 56%. Instead, heterozygous genotype is present with about the same frequency in both breeds (30%) (**Table 8**).

SNP	T149C			C802G			G1288C		
Genotypes	TT	TC	CC	CC	CG	GG	GG	GC	CC
Large White	0,56	0,33	0,11	1,00	0,00	0,00	1,00	0,00	0,00
Casertana	0	0,3	0,7	0,60	0,3	0,1	0,60	0,3	0,1

Table 8- Genotypes of new *MYF5* gene mutations

Concerning the C802G transversion, the C/C genotype is the most frequent in both the breeds, but the Large White breed showed only this genotype with a frequency of 100%. Instead, Casertana breed showed also the C/G heterozygous genotype with a frequency of 30%, and G/G homozygous genotype with a frequency of 10% (just one individual). The same genotype frequencies are showed as regards to the G1288C transversion in the first exon (**Table 8**).

For testing the SNP influence on promoter region, we have chosen to analyse the effect of C802G transversion. The G1288C transversion in the first exon doesn't cause any amino acid shift (silent mutation) and so doesn't seem to influence the expression of *MYF5* gene.

We were able to define 6 haplotypes for A65C, T149C, C802G, G1288C mutations, showing different frequencies between the two breeds (**Table 9**).

Genotype combinations	SNP on *MYF5* gene				Haplotype frequencies	
	A65C	**T149C**	**C802G**	**G1288C**	**LW**	**CA**
1	AA	TT	CC	GG	0,56	0
2	CC	CC	CC	GG	0,11	0,10
3	A/C	C/T	CC	GG	0,33	0,30
4	CC	CC	C/G	C/G	0	0,40
5	A/C	CC	CC	GG	0	0,10
6	CC	CC	GG	CC	0	0,10

Table 9- Haplotype frequencies of *MYF5* gene mutations

The *(A/A, T/T, C/C, G/G*) homozygous haplotype of the **first combination** is the most frequent, and it's present only in the Large White breed. In opposition, the (*C/C, C/C, G/G, C/C*) homozygous haplotype of the **sixth combination** is a characteristic of only one individual of Casertana (CA7).

Another (*C/C, C/C, C/C, G/G*) is that of **second combination**, that is rare and it's represented by only an individual for breed (LW7 and CA2).

The most frequent heterozygous haplotype is that of the **third combination** (*A/C, C/T, C/C, G/G*): three individuals of each breed show it. The other heterozygous haplotypes of **forth** (*C/C, C/C, C/G, C/G*) **and fifth** *(A/C, C/C, C/C, G/G*) **combinations** are present only in Casertana breed and in anyone individuals of Large White breed.

In the graphic below (**Figure 5**), we can see how these haplotypes are distributed among our individuals, and the differences between the breeds. LW breed shows only three combinations, instead CA individuals shows all combinations.

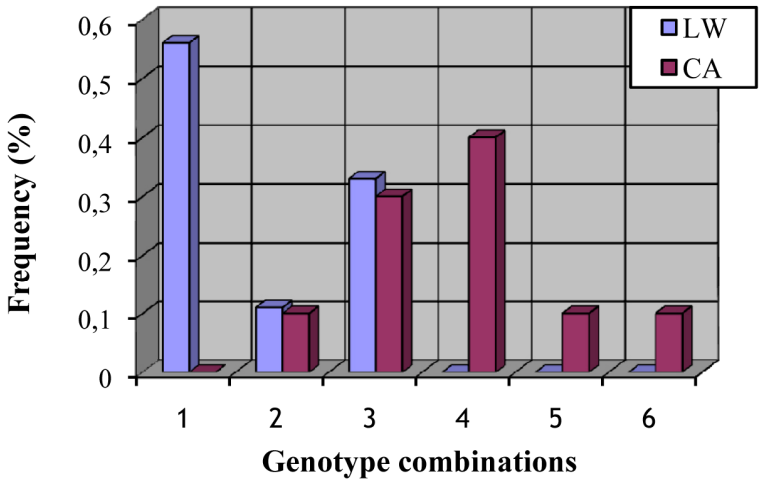

Figure 5- Genotype combinations on *MYF5* gene

4.1.2. *MYF5* transfection results

In order to evaluate the efficiency of *MYF5* promoter, the C802G SNP was tested with transfection experiments. The result of Firefly luciferase activity, Renilla luciferase activity and the ratio between them are showed in the **Table 10**.

	Samples		Day 1			Day 2		
	Mix	Replicate	FF	R	Ratio	FF	R	Ratio
Positive control	A	1	1464	3557	0,41	3313	4849	0,68
	A	2	1211	3079	0,39	6135	8492	0,72
	A	3	1122	3141	0,36	5205	8374	0,62
	B	1	775,2	2120	0,37	2495	3311	0,75
	B	2	816,7	2173	0,38	3625	5211	0,70
	B	3	590,5	3075	0,19	4984	6725	0,74
***MYF5* -G**	A	1	3059	2380	1,29	3180	2143	1,48
	A	2	3130	2121	1,48	4661	2350	1,98
	A	3	3146	2122	1,48	2744	1298	2,11
	B	1	1530	584,4	2,62	941,3	388,5	2,42
	B	2	1590	617,6	2,57	1093	398,3	2,74
	B	3	2377	1092	2,18	1343	484,9	2,77
***MYF5* -C**	A	1	4585	2059	2,23	3333	1269	2,63
	A	2	2658	1420	1,87	941,4	427,2	2,20
	A	3	3119	1736	1,80	1046	338,9	3,09
	B	1	4021	2085	1,93	3409	1520	2,24
	B	2	593,8	448,1	1,33	600,6	220,1	2,73
	B	3	2492	1286	1,94	809,5	308,5	2,62
Negative control	A	1	0,013	0,069	0,19	0,023	0,057	0,40
	A	2	0,01	0,092	0,11	0,028	0,064	0,44
	B	1	0,551	0,336	1,64	0,046	0,125	0,37
	B	2	0,264	0,364	0,73	0,057	0,081	0,70

Table 10- Transfection and Bioluminescence results of *MYF5* gene

Transfection results for C802G mutation on promoter region seem to prove differences between the promoter variants. The Student's t-test, calculated with R-software, on the ratio values of *MYF5*-C against *MYF5*-G, shows a p-value of 0.013. This result points to a possibility of 5% to make a mistake rejecting the zero hypotheses of no differences between these two groups of values. We can affirm that there is a significant difference of gene expression between the two variants.

4.1.3. Validation of data

The different expression of promoter SNPs discovered on *MYF5* gene was tested with comparative Real Time PCR to validate transfection results. Total RNA from LW6 and CA7 homozygous samples for the C802G SNP, was reverse transcribed and used for the standard curves. The comparative RT-PCR was performed for three fragments of exon 1 of *MYF5* gene in three-replicates of both samples (LW6 and CA7). All results were normalized to *ACTB* and *RPL4* housekeeping gene (see Table 4 and 5). Results confirmed that the C802G brings down the expression of *MYF5* gene in the Casertana breed, like showed in **Figure 6**.

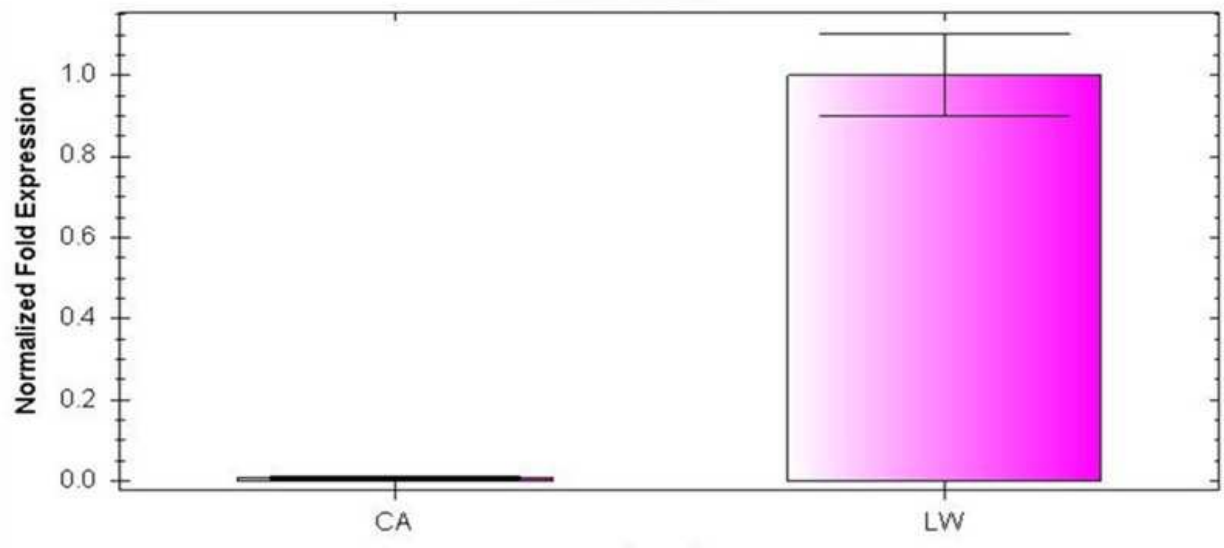

Figure 6- MYF5 gene expression results by Real Time PCR

4.2. Growth and Differentiation factors: *GDF8*

4.2.1. *GDF8* gene sequencing

In the promoter region of *GDF8* gene, we have analysed the presence of Guimaraes's SNPs at positions G398A and A409G (Guimaraes et al 2007). Sequencing analysis of our samples showed that all individuals were homozygous A/A for these two mutations.

In addition, we were able to find a new G->A transition at position 449. For this mutation, all Casertana samples were homozygous G/G (genotype frequencies of 100%), while almost all the Large White samples showed the G/A heterozygous genotype with a frequency of 67%, and all the rest were homozygous G/G (22%) and A/A(10%) (**Table11**).

SNP	**G449A**		
Genotypes	GG	GA	AA
Large White	0,22	0,67	0,11
Casertana	1	0,00	0,00

Table 11- Genotypes of *GDF8* gene mutation

4.2.2. *GDF8* transfection results

We tested the G796A effect on the promoter region of *GDF8* by transfection experiments. The **Table 12** shows the transfection results of variants.

The Student's t-test, calculated with R-software, on the ratio values of *GDF8-G* against *GDF8-A,* shows a p-value of 0.164. This result indicates a possibility greater than 5% to make a mistake rejecting the zero hypotheses of no differences between these two groups of values. We can affirm that there isn't a significant difference of gene expression between the two variants.

	Samples		Day 1			Day 2		
	Mix	Replicate	FF	R	Ratio	FF	R	Ratio
Positive control	A	1	5638	7435	0,76	2670	4089	0,65
	A	2	5239	6651	0,79	2978	5018	0,59
	A	3	3916	3091	1,27	3259	5966	0,55
	B	1	4671	5859	0,80	2477	2933	0,84
	B	2	4962	6346	0,78	2550	3993	0,64
	B	3	4982	6158	0,81	1770	2730	0,65
***GDF8*-G**	A	1	4704	1142	4,12	5610	1833	3,06
	A	2	1479	414,2	3,57	2012	642,5	3,13
	A	3	4366	1321	3,31	6684	2365	2,83
	B	1	2976	1350	2,20	2952	912,2	3,24
	B	2	360,6	106,2	3,40	1032	154,4	6,68
	B	3	320,2	68,02	4,71	1987	615,1	3,23
***GDF8*-A**	A	1	1902	1040	1,83	2404	2593	0,93
	A	2	1766	845,8	2,09	3066	2367	1,30
	A	3	1801	1045	1,72	1992	2088	0,95
	B	1	153,3	107,9	1,42	2401	1921	1,25
	B	2	325,8	104,3	3,12	1892	1536	1,23
	B	3	850,1	430,3	1,98	763,5	320,6	2,38
Negative control	A	1	0,005	0,065	0,08	0,08	0,098	0,82
	A	2	0	0,088	0,00	0,205	0,246	0,83
	B	1	0	0,121	0,00	0,016	0,051	0,31
	B	2	0,039	0,228	0,17	0,274	0,36	0,76

Table 12- Transfection and Bioluminescence results of *GDF8* gene

4.3. *MYOD1* gene

4.3.1. *MYOD1* gene sequencing

Looking for the presence of new SNP in the promoter region of *MYOD1* gene, firstly we tested our samples for the presence of the Urbański's SNPs.

They identified a *G*302*A* transition in the promoter region and two SNPs in the first exon of the *MYOD1* gene. The latter two SNPs are a transition C489T that doesn't change the amino acid sequence (Leu->Leu), and a transversion G566C that leads to a replacement of Arg with Pro at position 76. Our sequencing analysis showed the same genotype frequencies for all the Urbański's SNPs: all Casertana individuals are heterozygous (genotype frequencies of 100%), while the largest part of Large White individuals is homozygous with genotype frequencies of 89%, and one individual is heterozygous (**Table 13**).

SNP	**G302A**			**C489T**			**G566C**		
Genotypes	GG	GA	AA	CC	CT	TT	GG	GC	CC
Large White	0,89	0,11	0,00	0,89	0,11	0,00	0,89	0,11	0,00
Casertana	0,00	1	0	0,00	1	0	0,00	1	0

Table 13- Genotypes of *MYOD1* gene mutations

4.4. *FABP3* gene

The effects of some candidate SNPs on the porcine *H-FABP (FABP3)* gene were tested by several authors (Chmurzyñska 2006; Gerbens et al.; Li et al. 2010). Li et al. (2010) have studied the T103C variation in the 5'-upstream region, showing significant association with moisture and tenderness.

Sequencing analysis of our samples hasn't identified any new mutations on promoter regions, as well as the Li's SNP.

4.5. Other genes

Furthermore, our sequencing analysis was addressed to other genes like *ACACA, A-FABP (FABP4), CAST, CALP, DGAT1* and *LIPE*. Unfortunaly, we haven't identified any SNPs in the promoter region of these genes. We aim at discovering further polymorphisms in these promoters, exploring the region further extending the 5'.

5. Discussion

Meat quality traits take account of essential properties and features for the meat eating quality and healthiness. Recently, some studies have established a connection between these quality traits and the genetic background of different pig breeds. Therefore, several genes, belonging to particular metabolic or cell differentiation pathways, have been selected as candidates for meat quality traits.

The aim of this work was to study several candidate genes affecting quality traits and analyze if in their promoter regions, any SNP can be responsible of differences in gene expression. This study has been conducted on two Italian pig breeds, *Casertana* (CA) and *Large White* (LW), to verify if their phenotypical differences are expression of some mutations in the promoter regions.

The Casertana breed, in fact, is a very ancient and an endangered pig breed, raised for centuries in semi wild conditions; while Large White breed is the first breed in Italy for intensive livestock farming. CA muscles is characterized by an higher lipid content and a lower protein content, that determine CA pigs aptitude for fat deposition and produce more double backfat thickness than LW pigs (Pietrolà et al. 2006). Because of its slow growing, Casertana breed has not been subject of selection programmes and retain the traits of a high fat depositing pig, compared to the genetic lines actually exploited in the pig industry. Comparison with LW pigs demonstrated that the CA is less competitive regarding growth performance, reaching a commercial slaughter weight at a considerably greater age and with a 2-fold increase in backfat thickness (Pietrolà et al. 2006).

The Large White breed, instead, is characterized by large size, excelling in growth rate and lean meat percentage, and represents a rugged and hardy breed that can stand variations in climate and other environmental factors. LW pig ability to cross with and improve other breeds has given them a leading role in commercial pig production systems and breeding pyramids around the world. For all these features, CA pig breed is certainly the most precious among Italian breeds, being able to provide large amounts of fat.

In these two breeds, we have analysed a total of ten genes, which show a key role in meat quality. In particular we studied the expression of some genes belonging to the *Myogenic Regulatory Factors* (*MRF*) gene family (*MYF5* and *MYOD),* to *Growth and Differentiation Factors* (*GDF*) gene family (*GDF8),* to the calpain-calpastatin system (*CALP* and *CAST),* to adipose tissue formation genes (*ACACA, DGAT1, LIPE*) and to the *FABP* family (FABP3 and *FABP4*).

We have discovered new SNPs in the promoter regions of *MYF5* gene (T149C, C802G, and G1288C) and of *GDF8*gene (G449A). *MYF5* gene plays a critical role in skeletal myogenesis. MYF5 protein belongs to a family of myogenic regulatory factors (MFRs) which includes also MYOD, the myogenin and MRF4. These are all basic helix loop helix (bHLH) transcription factors that act sequentially in myogenic differentiation. In fact, *MYOD* expression is absent during G_0 phase of the cell cycle, while is highly expressed during mid-G_1 phase and between S to M phase. *MYF5* is highly expressed during G_0 and decreases during G_1 phase (Kitzmann M. et al., 1998). In particular, *MYOD* was found to promote cell cycle arrest by inducing cyclin dependent kinase (CDK) inhibitor p21, cyclin D3 and retinoblastoma (Rb) tumour suppression protein, all of which have important functions towards cell cycle withdrawal (Mastroyiannopoulous et al. 2012). Over expression of *MYOD* is able to promote myoblasts to differentiate, while by over expression of *MYF5* fail to differentiate. *MYOD* is also expressed in myotubes and collaborates with myogenin to regulate the expression of genes necessary for terminal differentiation (Blais et al. 2005).

As reviewed by Mastroyiannopoulous et al. (2012), down-regulation of myogenin causes cleavage of terminally differentiated myotubes into mononucleated product cells, which can go through into the cell cycle, altering gene expression which is involved both in muscle cell differentiation and in the cell cycle. The fusion of myoblasts is one of the key steps of muscle cell differentiation, expressing *Myogenin*, *MYOD* and *MYF5*, with each having as targets a distinct subset on muscle specific genes. Down regulation of these MRFs during fusion period showed that particularly *myogenin* significantly inhibited the fusion of myoblasts (Dedieu et. 2002).

Therefore the identification of molecular signals that control the *MYF5* expression and maintenance is central to understanding skeletal myogenesis (Zammit et al. 2004). Urbański et al. (2004; 2006) identified three new mutations in the promoter region of the *MYF5* gene: *A*65*C*, *C*580*T* and *C*613*T*. In their studies in Polish Landrace breed, Urbański and Kuryl (2004) evaluated the influence of the mutation *A*65*C* on *MYF5* gene expression, for all of the three genotypes (*AA*, *AC, and CC*). Even though this *A*65*C* transversion creates a binding site for Sp1, a very important transcription factor for several cell processes, the authors did not observe any significant influence on its expression in *Longissimus dorsi*.

Liu et al. (2008) investigated the association of several polymorphisms in the *MYF5* and *MYOD1* genes with meat quality traits in a series of three Large White X Meishan F2 populations, founding a novel A->C SNP within exon 1 of the *MYF5* gene, resulting in a Met-> Leu amino acid substitution in the basic region of the bHLH domain, also showing a significant effect on moisture content, IMF, and semispinalis capitis pH. Pigs with the CC

genotype had higher intramuscular fat and lower moisture content and semispinalis capitis pH at the same time. In the pig breeds tested by Liu et al., allele C was present only in the Meishan breed, which was known to have a high IMF. Intramuscular fat is a key factor for meat quality and has a high heritability and it's also closely related to a special flavour, juiciness, and tenderness (Pang et al. 2006). Therefore, breeding for higher IMF is a major consideration in porcine breeding programs. Identification of the genes and the underlying causal mutation that affects meat quality traits will greatly enhance the progress toward this goal.

Guimaraes et al. (2007) identified two SNPs, G398A and A409G, in the promoter region of the *Myostatin* gene in complete linkage disequilibrium, and investigated allele frequencies, gene expression pattern in the longissimus dorsi muscle. The authors also performed statistical associations with performance, carcass composition and meat quality traits in different breeds and commercial lines pigs, but none of these traits have been associated to the SNP in the EBV commercial cross analysis and this may indicate that the effect of these nucleotide changes are affected by background line development and perhaps other phenotypic factors.

Li et al. (2010) found four genetic variations in *FABP3* gene included one in the 5'-upstream region (T103C), showing significant association with moisture and tenderness but not with the IMF content.

In our samples, we could identify the A65C transversion. The *AA* genotypes showed a 56% frequency in LW breed against the 0% in CA breed. The heterozygous genotypes (*A/C*) showed in LW individuals a frequency of 33% against that of 40% in CA individuals. The other homozygous genotype instead is more frequent in CA breed with a frequency of 60%, than the frequency of about 10% of LW breed.

Close to this transversion, we analysed the Maak's et al. (2006) transition (C71T), and we found that the genotype frequencies don't show significative differences between our two breeds; therefore we can't tell if this transition presents some relationship to the muscle phenotype for Large White and Casertana, as described by Maak in wild boars and Pietrain pigs.

Sequencing analysis of *MYF5* gene in our samples let us identified three new mutations, two in promoter region (T149C and C802G) and one in exon 1 (G1288C). The genotype frequency analysis suggests an association within the breeds. For example, the *T/T* genotype, for the T149C transition, is present only in LW breed with a frequency of 56%. Instead, the *C/C* homozygous genotype is present in CA breed with a frequency of 70%, and in LW with a frequency of 10%.

The effect of C802G mutation in the promoter of *MYF5* gene has been analyzed by transfecting plasmid constructs carrying the allelic variants into cultured cells. The *G/G* genotype causes a decreasing in *MYF5* gene expression (decreasing confirmed by quantitative real time PCR). We can suppose that, the lessening of gene expression of the *MYF5*, triggered by the C802G SNP, can be correlated with the fat accumulation of Casertana individuals and with its adaptability to free-ranging.

The C802G and G1288C transversions seem to be in linkage disequilibrium, as suggested by the same genotype frequencies: LW individuals are characterized for both SNPs by a frequency of 100% of homozygous genotypes (*C/C* and *G/G*). Instead, in CA breed, these homozygous genotypes achieve only the 60%, while the heterozygous individuals show a frequency of 30%. Only a CA individual shows the other homozygous genotype combination (GG-CC). Haplotype analysis shows that genotype variants (C/G and *GC* heterozygous genotypes, *GG* and *CC* homozygous genotypes) are a feature of the Casertana breed, since only these individuals show all the genotypes (*CC-GG, CG-GC,* and *GG-CC*). Even if the G1288C mutation doesn't cause any aminoacid substitution (Leu->Leu) and doesn't seem to have any influence on the expression of *MYF5* gene, it can play a role in the *MYF5* gene regulation activity in combination with the C802G SNP.

We were able to define 6 haplotypes for A65C, T149C, C802G, G1288C mutations, showing different frequencies between the two breeds. LW breed shows only three combinations in total, two homozygous and one heterozygous (*AA, TT, CC, GG; CC, CC, CC, GG; A/C, C/T, CC, GG*). CA individuals shows, instead, five combinations, two homozygous and three heterozygous (*CC, CC, CC, GG; A/C, C/T, CC, GG; CC, CC, C/G, C/G; A/C, CC, CC, GG; CC, CC, GG, CC*).

The *(A/A, T/T, C/C, G/G*) homozygous haplotype is the most frequent, and it's present only in the Large White breed. In opposition, the (*C/C, C/C, G/G, C/C*) homozygous haplotype is a characteristic of only one individual of Casertana. The third (*C/C, C/C, C/C, G/G*) homozygous haplotype is rare and it's represented by only an individual for breed: probably this haplotype combination can be the result of a crossbreed.

The (*A/C, C/T, C/C, G/G*) heterozygous haplotype seem to reveal the point of contact of these two breeds: three individuals of each breed show it, and this is the most frequent heterozygous haplotype. The other heterozygous haplotypes (*C/C, C/C, C/G, C/G* and *A/C, C/C, C/C, G/G*) are present only in Casertana breed and in anyone individuals of Large White breed.

All these haplotype combinations let us to affirm that CA breed preserves more variability than LW breed. In fact, this breed is more heterozygous than LW breed, especially

for these MYF5 SNPs. Further studies and experiments will be direct to confirm these dataset and to find out how all these polymorphisms determine the MYF5 protein in the different breeds.

The *MYOD1* gene sequencing analysis also reveals a great genetic variability of Casertana breed. All Casertana individuals are heterozygous for all the SNPs identified by Urbański & Kuryl (2004) (G302A, C489T, G566C) in Polish Large White pigs. Instead, only an individual of Large White breed is heterozygous, and all the other ones are homozygous. The genotype frequencies confirm the Urbański's hypothesis of a linkage disequilibrium, since each breed shows the same genotype frequencies for these three SNPs (10% in LW and 90% in CA), suggesting that Large White breed had lost variability, probably for the intensive livestock farming.

As concerning the *GDF8* gene sequencing analysis, all the Casertana samples are homozygous *G/G* for the new identified G449A transition, instead the Large White samples are most heterozygous, but three individuals are homozygous. Testing the G796A transition influence on the promoter region of *GDF8* gene with the *in vitro* analysis, it seems that there aren't any differences between the promoter variants. Therefore, in LW the individual variability doesn't seem to affect differences between these breeds for this gene. Looking at genotypes, CA breed is characterized by only one genotype (*C/C*) with a frequency of 100% against the 22% of LW breed.

These data seem to show that LW breed have more variability than CA breed, in very contrast with all *MYF5* and *MYOD1* gene data. *GDF8* gene influences the muscle mass development, while *MYF5* and *MYOD1* belong to the muscle differentiation, a process that happens earlier than the other one in the muscle cells. We can presume that the great variability of CA breed for the *MYF5* and *MYOD1* genes is due to its adaptability to free ranging, and the low variability in the *GDF8* gene can be assigned to the mild exploitation of this breed for the pork production. In fact, LW breed during the time has been inbreeded with other breeds for improving quality meat traits. This caused the lost of genetic variability for differentiation genes as *MYF* and *MYOD*, while variability increased for in those genes, like *GDF8*, important for the pork production.

6. Conclusion and Further purposes

The present work is one of the few in genetic evaluation of economical livestock traits that tries to explore the effect of promoters variants as controllers of transcripts and therefore phenotype.

Many studies have been focused on the SNP detection in candidate genes for meat quality traits, sometimes associating also these mutations with phenotypical features of different pig breeds. Our results, however, don't always confirm the analysis on other breeds, as showed by *FABP3* results. We couldn't identify differences between CA and LW individuals for the T103C mutation described by Li et al. (2010) as associated with moisture content and tenderness.

In the promoters of *MYF5*, *MYOD1* and *GDF8* gene, we discovered SNPs that seem to explain some differences between Casertana and Large White individuals. Genetic results, however, must be completed with phenotypical data, and they must be correlated with meat quality traits. However, our results allow us to affirm that Casertana individuals preserve more genetic variability than Large White individuals, probably because this breed wasn't used for intensive pork production.

Further studies can go into a more depth about the role of these important genes in myogenesis of pigs and focused on this particular Italian breed, not studied a lot, to know its quality and to preserve its peculiarities.

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