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Genomic and proteomic approaches in pig meat quality research field

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Contents

Pig meat and carcass quality traits:

Pig meat and carcass quality is a complex concept of instrumentally measures of compositional and physiochemical characteristics, that covers different processing yields, traits relating to human health, safety or nutritional needs and sensory properties, responsible to the consumer's choices and acceptability of fresh pork or processed pork products. Quality is determined *in primis* on product characteristics and how these are perceived and evaluated by the local consumers preferences, thereby quality varies in different countries according to cuisine and different utilization of fresh pork or processed pork products (Russo V., 1988). Indeed, every culture have particular traditions, alimentary customs and it's possible to affirm that there are several quality pork meat parameters and not only one concept to resume it (Sellier P., 1998). At first, the meat quality is determined by environmental and genetic factors, that together define the quality of fat and muscle components. In fresh pork and processing pork the principals phenotypic characteristics are described like visual and sensory traits that include color and texture of meat, distribution of visible fat (marbling) and amount of water released (drip loss), eating quality (evaluation of meat texture, including tenderness and juiciness), pork flavor intensity (including taste and smell), and postmortem proteolysis. These characteristics are influenced by several factors, such as breed, genotype, feeding, fasting, pre-slaughter handling, stunning, slaughter methods, chilling and storage condition (Gao Y. *et al.*, 2007). Moreover meat and carcass quality are determined by technological properties that can influenced transformation, conservation, packed and cooked steps. In pork meat quality analyses, the changes in organoleptic properties are monitored through assaying of specific parameters (water holding capacity, pH and color), that are intrinsically related to muscle proteins composition. Water holding capacity (WHC) is one of the most important parameters for pork quality and its inadequately could be a signal that indicated PSE meat. Inadequately WHC in meat, involves reduction of proteolysis rate of three structural proteins (troponin T, myosin light chain and α-crystallin) and the total absence of heat shock protein 27, chaperone of the sHsp (small heat shock protein) that lead thermo tolerance, inhibition of apoptosis, regulation of cell development, and cell differentiation (D'Alessandro A. & Zolla L., 2013). The color is another important phenotypic character probably the first perceived from the consumer: dark or pale meat aspect is negative signal about quality, it could represent signal to identify DFD or PSE meat.

Water-holding capacity, pH, color, content and fatty acid composition, purge, star probe measurement, postmortem proteolysis, oxidative stability and uniformity, cooking loss and various processing yields, are the main interesting attributes measured (Sellier P., 1998).

The quality is defined by genetics and management systems, two different aspects to describe the muscle components (fiber size and type, fat and connective tissue) and production results. Although consumer and industry requirements vary in different countries, it is possible to pick out some universally valid requirements. In the actual society the most important requisite of carcass quality is high lean meat content and in the second hand, the carcass weight. Every country has traditions and the pigs are slaughtered at the weight which promotes the best commercial use of the carcass. Usually the desired weight is between 75 and 90kg with the exception of Italy, where for seasoning hams, carcasses of over 120kg are required and preferably ranging from 140 to 160kg (Russo V. *et al*., 1988). Therefore, growth rate and carcass fat content are two important characteristics that have to be improved through the pork meat selection. Some decades ago pig's meat presented more fat content than now. Especially during the seasoning, fat coverage lets the seasoning processes to proceed and let ham to preserve the quality proprieties. Moreover, the ham has to be covered by layer of fat to contain the "weight loss" during the seasoning and don't let to dry the muscle under it.

In the traditional selection the improvement of meat and carcass quality traits is more difficult than in other traits. In the last decades, the selection breeding program has played an important role to improve quality traits, in particular in pigs where the generation interval is short. The strong selection that was applied, has resulted in accumulation of new mutation with favorable phenotypic effects (Gao Y *et al.*, 2007). The meat quality traits are determined by several genes (polygenes) and by environment. The heritability in meat and carcass traits shows medium/high values (Tab.1). The traits utilized for selection in pigs concern not only carcass quality as back fat thickness (BFT), lean cuts (LC), hams weight (HW), but also meat quality traits as the processing yield CALO (indicating the weight loss during salting).Moreover average daily gain (ADG), feed conversion ratio (FCR) are included in genetic evaluation .Only in the Italian Duroc breed, the visible intramuscular fat (VIF) are evaluated only after slaughter, so it isn't possible to measure directly them on the selected candidate pigs, but only on their relatives (by sib-test) and late in life.

	pH1	pHu	Drip loss	WHC	IMF	Reflectance	Cooking loss	Tenderness	Carcass leanness	Carcass fatness
pH1	**0.16**									
pHu	0.75§	**0.21**								
Drip loss	-0.27	-0.71	**0.16**							
WHC	-0.65	0.45	-0.94	**0.15**						
IMF	0.06§	-0.51* /0.17§	-0.08	0.12	**0.50**					
Reflectance	-	-0.10*	0.49	-0.39	0.01	**0.28**				
Cooking loss	-0.14	-0.68	0.66	-0.25	0.07	0.26	**0.16**			
Tenderness	0.27	0.49	-0.16	0.23	0.15	-0.16	-0.46	**0.26**		
Carcass leanness	0.10	-0.13	0.05	-0.19	-0.34	0.16	-0.07	-0.20	**0.48/0.54**	
Carcass fatness	0.26	0.15	-0.10	0.02	0.30	-0.21	0.12	0.24	-0.65	**0.41/0.45**

* data from Suzuki *et al.* (2005)

§ data from Habier *et al.* (2007)

Tab. 1: Average heritability (h^2, on diagonal) and genetic correlation (r_G) among various meat quality and carcass composition traits. (Adapted from Sellier, 1998)

Muscle structure and metabolic changes in postmortem phase:

Muscle structure and fat composition, are considered the most important components to estimate variation in organoleptic and technological characteristics of meat (like tenderness, juiciness, flavor and odor) and to determinate the variation in meat and carcass quality. To evaluate these characteristics, the Italian breeding selection scheme of pig breeds utilizes a genetic multiple trait index. The traits considered for selection include back fat thickness (BFT), lean cuts (LC), hams weight (HW), the processing yield CALO (indicating the weight loss during salting), average daily gain (ADG), feed conversion ratio (FCR) and only in the Italian Duroc breed, visible intramuscular fat (VIF) (Bosi P. & Russo V., 2004; Russo V. & Nanni Costa L., 1995). From 1974 the "BLUP" model is utilized. This procedure can estimate in more animals, on the same time, the genetic predicted value and environmental effects.

Muscle structure and physiology are fundamental to maintaining body heat and movement of blood and lymph. The muscle organization and metabolism concur to maintain muscle integrity during contraction and during early postmortem period (Huff-Lonergan E. *et al*., 2010). Indeed, the biochemical processes responsible on the transformation of muscle to meat after slaughter, are complex and influence the meat quality. The quickly exsanguination causes a gradual loss of oxygen and nutrients and a shift from aerobic to anaerobic pathways, thereby the consequence is the production of lactate. In this step pH is one of the main intrinsic factors to determine meat quality characteristics. While the normal situation is characterized by pH with gradual decline (from approximately 7.4 to 5.6 over a period of 6-12 hours) and a modest downfall of water-holding capacity, in particular conditions the pH usually starts quickly to decrease and it causes extensive denaturation of many proteins including the pigment in meat, myoglobin. Actually, during postmortem phase a continuum in the phenotypic variability of meat quality and kinetics about pH fall exist and is common to recognize three distinct pH-related abnormalities (Fig.1) (Sellier P. 1998). Meat associated with pH_1 (pH measured at 45 min after slaughter) between 5.7 – 5.3 value, showed PSE quality characteristics (pale, soft and exudative), while meat with the pH_u (pH measured at 24 hour after slaughter) higher than 6.0-6.2 value, is termed DFD (dark, firm, dry) and "acid meat" if meat presents pH_u value lower than 5.4-5.5 (Huff-Lonergan E. & Page J., 2001).

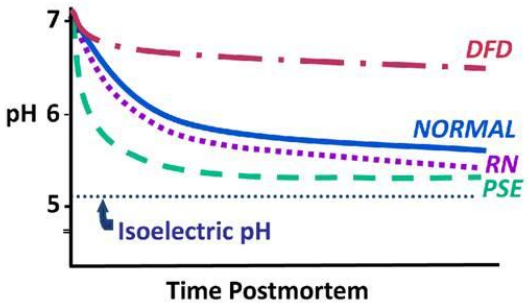

Fig.1: This graphic shows the measures of the three distinct pH-related abnormalities compared to the normal pH condition, during postmortem

Genetic factors and environmental circumstances are strongly related to these situations: DFD defect is essentially due to adverse external factors (e.g. long transportation and/or fasting times causing muscle glycogen depletion), while PSE and acid meat are determined by genes (Halothane, *HAL* and Rendement Napole, *RN* are two genes that determinate the PSE and acid meat respectively) (Sellier P., 1998). Thereby is possible to improve these traits by genetic selection programs.

The processes occurring during slaughtering are subjected to several variation factors that determinate the final quality. A number of physical and chemical changes take place over time as muscle is converted to meat. The muscle doesn't stop immediately its functions after pig slaughtered, but it remains efficient and metabolically active, enveloping changes in biochemical pathways and proteins metabolism that influence the conversion from muscle to meat. The blood circulation is depleted so the oxygen and nutrients transportation ways are stopped and waste products and heat can't be removed from the tissue. The direct consequences of oxygen depletion in the muscle is its change in metabolic system from aerobic pathways (where oxygen is required) to anaerobic pathways (where oxygen isn't required), continuing to produce ATP. These variations in the metabolic pathways, are the causes for many consequences in muscles that can define the quality of the meat. In particular, in the next Fig.2 the steps characterizing the tenderization in the muscle during postmortem, are schematized (D'Alessandro A. & Zolla L., 2013).

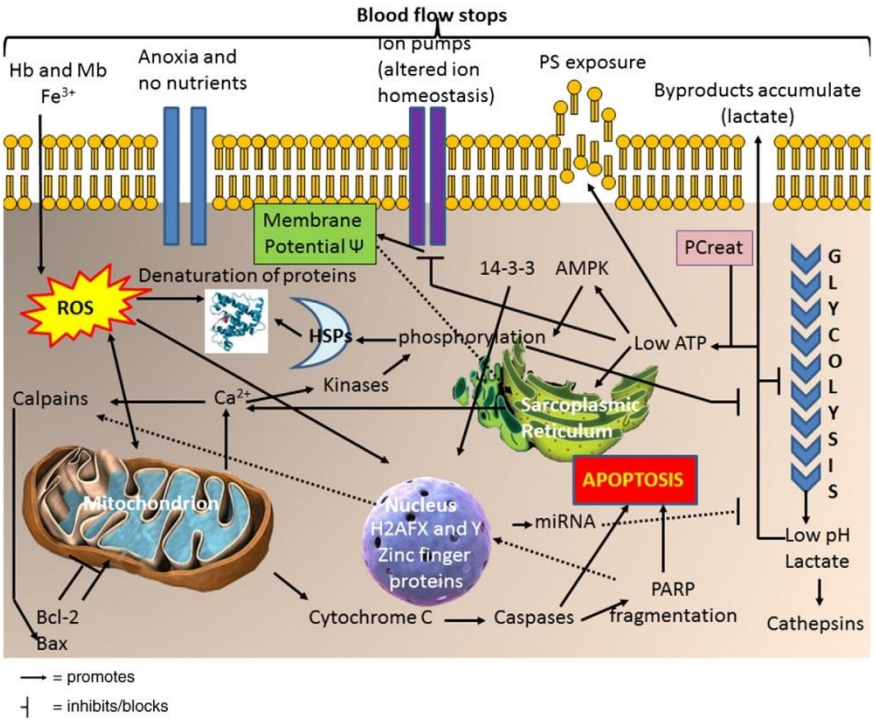

Fig.2: an overview of the intricate pathways leading tenderization of the muscle through apoptosis. The interplay between blood flow arrest (causing anoxia, accumulation of oxidized iron and of byproducts of metabolism, such as lactate and nutrient deprivation) is linked to the increase in reactive oxygen species (ROS) within the cell. Altered metabolism leads to the arrest of mitochondrial metabolic activity, to the alteration of ion homeostasis modulation and the calcium intracellular accumulation activates calpains, which activate Bax and promote apoptosis through the mitochondrial intrinsic pathway.
(Adapted from D'Alessandro & Zolla, 2013)

A by – product of anaerobic metabolism is acid lactic, that is removed from muscle via circulatory system in living muscle, gradually builds up in the postmortem muscle, leading the pH and water decline in muscular tissue (Huff-Lonergan E. & Page J., 2001). In this particular phase the meat color has to be checked carefully because it could change and so determines consumers' appraisal. Meats that appear so pale, dark or brown are often considered as a synonymous to bad quality, and not appropriate to the sale. The meat color is especially defined from the levels of hemoglobin and myoglobin molecules and this concentration is related at residual oxygen in the muscle. These two molecules have a strong affinity with oxygen and when their levels are high in the muscle, the meat appears red colored. On the other hand, in absence of oxygen the meat appears dark red (D'Alessandro A. & Zolla L., 2013). Another important visible characteristic that determines the quality of meat, is the presence of water in the meat packaging and its release while cooking meat (purge).

The ability of pork to retain its moisture is indicated as water holding capacity (WHC) and this is associated with specific changes that occur in early postmortem biochemical and biophysical processes, in muscle. The structural muscle organization is responsible about the distribution of water in the muscle. Muscle is composed of approximately 75% water and as much as 85% of this

water is held in the myofibrils. The space and consequent available water, between thick and thin filaments, are influenced by state of rigor, sarcomere length, intracellular pH and ionic strength. Well-known that drip loss increases in a linear fashion as the length of sarcomeres decreases, indeed it is higher in muscles that have very short sarcomeres. Interesting is to study the role of cellular proteins (present in cytoskeletal as microtubules, microfilaments and intermediate filaments proteins) because they can regulate retention/loss of moisture and the specific changes that occur in early postmortem muscle, furthermore their postmortem degradation is related with improvement in meat tenderness. Is important to consider that every proteins that play a role in muscle system, have different functions and different effects on it, to define WHC. Indeed, the degradation of cytoskeletal and intermediate filaments proteins (like desmin) early in postmortem, could limit the space available for water/fluid that has been forced from the myofibril during rigor; conversely degradation of some membrane proteins (like integrin) could contribute to the formation of drip channel and improve the ability of moisture to escape from the muscle cell. (Huff-Lonergan E. & Lonergan S.M., 2007).

Another important consequence of lactate production and accumulation in muscle, is the acidification in the muscle cellular environment, that involves changes in ionic composition and consequently alters the pH value. pH is one of the most important intrinsic factors that influence meat quality, a technological parameter that could be an indicator of metabolic status of pre-rigor meat and in some cases also of the metabolic "history" of fresh, post-rigor pork. Its value in activation of metabolic processes is fundamental, actually it is in relationship with water holding capacity (WHC) and at particular values, it determines the denaturation of principal muscle proteins that can define the ultrastructure of muscle. WHC, pH value and temperature are connected and have fundamental importance to characterize the meat quality. During the postmortem anoxic phase, one molecule of glucose is transformed in two molecules of pyruvate, obtained lactic acid as final product. Here anaerobic glycolysis is the principal source of energy, while in living muscle lactic acid isn't accumulated and cleared from the muscle via the circulatory system. The different conditions and the absence of ATP, contribute to the accumulation and buildup of lactate, and consequently the fall down of pH. The stress before slaughter, the metabolic rate of postmortem muscle and thus the rate of pH decline, can also affect the water-holding capacity. The muscle with normal water-holding capacity typically spends 6 – 8h to decrease its pH from 7.0 – 7.02 to 5.3 – 5.8, resulting very close to the isoelectric point of many of the major proteins in muscle (5.3 – 5.5). In pork meat with abnormally low pH_u values, the electrostatic forces that help to maintain proteins attraction and bind water are reduced more than in normal situation, so the water in these intracellular spaces is expelled and easily lost from the muscle cells. The muscle is still very warm and the combination between

acid conditions and warm muscle temperatures exaggerates the denaturation of muscle proteins, thus further limiting their ability to bind water, so comport an exudative meat. The muscle temperature is an important parameter to consider during postmortem phase, because it is directly links to many biochemical reactions that happened in postmortem muscle structure through its conversion in meat and it can influence meat and carcass quality.

Moreover the increased postmortem degradation of specific muscle cell proteins is associated with an improvement in meat tenderness and plays a significant role in the development of water-holding capacity (E. Huff-Lonergan & S.M. Lonergan, 2007).

Molecular genetic tools utilized for QTL research to improve meat and carcass quality in pigs:

Analysis and studies on animal genome are necessary to improve researches on meat quality (Hamill R.M. *et al*, 2013). Genome is composed by genes and DNA sequences (that define the genotype) located in the nucleus, that are transcribed to RNA and translated in proteins, that affect cellular functions and determine the phenotype. While genomics is the study of an organism's entire genome, proteomics is the global study of the whole set of proteins encoded by a genome under certain conditions and at certain time (Chen G and Zhang X., 2013). These two research fields are important to study biological markers of meat quality, including DNA polymorphism, transcript signatures and protein markers, to determine the influence of the animal genetic makeup, physiology in life and *postmortem* muscle metabolism on ultimate functionality and quality of meat produced (Hamill R.M. *et al*, 2013).

Genomic approach consists of three different application area: structural genomics, functional genomics, and comparative genomics that converge together to give the most completely analyses (Davoli R. *et al.*, 2012).

Structural genome investigates about genome sequences and genome variation, two themes enhanced in the last 30 years, with the develop of DNA sequencing techniques. In these years the improvement of sequencing technical innovations and the lowering of the sequencing price, allowed to obtain more than 1100 complete genome sequences (http://www.genomesonline.org/cgibin/GOLD/index.cgi?page_requested=Statistics#aname), so innovative bioinformatics tools are required to organize, analyse and investigate this amount of sequencing data. Functional genomics approaches analyze at the genome level, the interactions between genes and their products. It works on the interactions between the various systems of a cell on a large – scale basis, including interrelationship of its DNA, RNA, and synthesized proteins as well as metabolites, and explains how these interaction are regulated. Comparative genomics differences organisms and identifying functional sequences in livestock genome that could enrich genome information in human, to improve livestock maps and to identify new genes (Davoli R. *et al.*, 2012).

Polygenic meat quality traits are controlled by an unknown number of different genes that are placed in determinate area of the chromosomes. *Quantitative trait locus* (*loci*, plural) *"QTL",* is a stretch of DNA on chromosome where genes that encode for proteins responsible to determine the same

phenotypic characteristic, are present. Them identification, mapping and characterization on chromosomes, is the main aim of genomic research.

In these years, large variety of approaches have been developed especially to improve studies of model species (e.g. cattle, swine, chickens) for elucidating the mechanisms underlying complex disease, behavioral traits and physiological processes. From the first methodologies that utilized genome scan approach and candidate gene approach, nowadays the researches are focused on Chip-DNA technique and Genome Wild Association Studies (GWAS).

The candidate gene approach allows to investigate the association between the animal trait of interest and mutations on the known genes (usually is a mutation in *Single Nucleotide Polymorphism, SNP*), that may be associated with the physiological pathways underlying the trait, in specific QTL regions. Different steps characterize the candidate gene approach. The first important point of action consists to collect phenotypic data or breeding value relative to the traits of interest from a population of adequate size; in the second time is necessary to select genes or polymorphisms on genes that could affect the traits.

Furthermore, is important to proceed with the genotyping of population samples and to calculate the statistical analysis, essential to detect association between polymorphisms on selected gene and trait designated. One limit to the application of candidate gene method in experimental design, could be when is necessary to analyze a long list of possible genes for one interesting trait. In this case could be required to spend more time to find some relations between traits and gene analyzed; moreover could be presents gaps in known gene function and to obtain spurious results about linkage disequilibrium (Gao Y. *et al*, 2007).

The genome scan approach studies the relationship between a trait and anonymous DNA markers across the genome, to identify chromosomal locations associated between that trait DNA. In regard to QTL region spans about 5 – 30 cM, there are many problems to detect trait loci with limited effect due to the stringent significance threshold, so to obtain a fine mapping of a specific QTL is the main goal in this approach (Davoli & Braglia, 2008). The approaches that identified chromosomal locations associated with the qualitative trait locus, studying the relationship between the trait and a short DNA sequence that hosts a polymorphic site (*marker*), permit the genes identification and mapping. Indeed if gene is located in a *locus* (with quantitative effect) and if it's genetically associated with *marker*, this gene could be transfer to the next generation (this gene have an additive effect). Utilization of *markers*, associated with some favorable alleles, let the selection of carrier animal for these particular alleles and can be applied in breeding programs by using the Marker-Assisted Selection (MAS). This kind of selection is possible only by phenotypic analysis (especially to check production dates) or parental studies.

Carcass and meat quality traits are amenable to MAS because is possible the identification of a marker-set near genes that control a specifically characteristic, that will be transmitted to next generations. Unfortunately, the implementation of MAS in commercial breeding is limited, because only a few QTLs have been characterized at the gene level (Davoli & Braglia, 2008).

Nowadays, association and genome wide are based on the analysis about thousands SNPs in the same experiment. Modern technological platforms (e.g. "*Illumina"* http://www.illumina.com/) can permit to genotype until 5 million SNPs (on human genome) and until 50,000 SNPs in zootechnic species. This kind of platform let to increase the automation holding high levels of specificity and sensibility in the analysis. This technology is based on the propriety of a short DNA sequence, 20-100 bp, (called *probe*), to identify its complementary sequence (called *target*) and annealing with it, following an *hybridation* process. Every *probe* is connected to a microsphere (*bead*) and everyone is situated in a specific hole on the chip surface. This is the core of this technology, called "*beadchip"*. Each *bead* harbors a specific kind of *probe* that identifies a specific DNA *locus* where is present a specific SNP. *Beads* are mixed and obtained a solution of million probes lay down on chip surface in random way. To determine the position of each SNP, every *bead* will be target with a different fluorophore and combining these colors will be possible to determine a SNPs map present on chip, therefore to know the specific position of each SNPs on the silica support. Every chip is an unique product and in this way it will be possible to connect its result to a specific map and to decode the information obtained.

The silica support chips (its measure is similar to a microscope glass) permits the analysis of different numbers of samples: 8 samples on each silica support for 800,000 SNPs research (in Bovine – HD); 32 samples on each silica support for 3,000 SNPs research (in Bovine-3K). A high-density porcine SNP *Bead* chip has been realized by *Illumina*, which contains probes to genotype 62,163 SNPs, covering the whole genome. This platform has an average distance between SNPs of 39.61 kb in autosomes and 81.28 kb in chromosome X, so it is reasonable to study pig genetic variability and the molecular dissection of complex traits of economic importance (Ramayo-Caldas Y. *et al.* 2010)

The completed genome sequencing projects in many species and newly developed high density SNP arrays, have permitted to conduct genome-wide association (GWA) and genomic selection studies for several species of food producing animals. Therefore the availability of whole genome sequences (WGS) for individual studied species have change the landscape for livestock genomic research. These studies and applications of dense SNP arrays in livestock, focus on genomic selection in order to improve selection accuracy to accelerate genetic improvement for economically important performance traits in breeding animals. (Fan B. *et al.*, 2011).

Up to now, only a limited number of genes associated with meat quality was been identify: (*RYR*/Halothane, that regulates Ca^{++} transport across muscle cell membranes; *PRKAG3 (Adenosine Monophosphate (AMP) Activated Protein Kinase Complex)*, plays a role in the regulation of energy metabolism in skeletal muscle; *RN (Rendement Napole)* affects glycogen content of muscle; *MC4R (Melanocortin-4 Receptor)* coded by G protein that as transmembrane receptor check the energetic balance; *IGF2 (Insuline Like Growth Factor 2)*, is a complex of peptidic hormone that stimulates the growth in body fatness, *FABP3 (Fatty Acid Binding Protein 3)*, to regulate intramuscular fat content and FTO (*Fat Mass and Obesity Associated*), one of the most important gene implicated in human obesity and in pig association studies with deposition of IMG in Italian Duroc. These genes affecting meat quality traits in pig, were discovered and identified in these years and are considerated a part of current selection programs to improve meat quality traits. They are utilized in meat pork industry/pork chain, to obtain improvement in both quality and production traits.

Today to investigate on the genome sequence in databases, is the main start point for researches in animals genetic, to improve cross-species comparison of the effects of candidate gene allelic polymorphisms on meat quality (Davoli & Braglia, 2008).

The genome sequence and especially SNP map would solve a lot of questions on the selection of candidate genes (Gao Y. *et al*, 2007).

An important web database to find genetics information on pig and some other animals, is the *Animal Quantitative Trait Locus database (Animal QTLdb)* http://www.animalgenome.org/cgi-bin/QTLdb/index), a free web database that with its peripherial tools make it possible to compare, confirm and locate on animal's chromosomes, the most feasible location for genes responsible for qualitative traits. For example, in *Pig Quantitative Trait Locus database* (*Pig QTLdb*) all curated pig QTL and association data in the public domain are contains. At *December 24, 2013* were listed 9,862 pig QTLs, located into the Pig QTL db, from 391 publications representing 653 different pig traits. This database is organized to permit different kind of researches and allows the user to search, by chromosome, trait or key words from the publications (Davoli & Braglia, 2008). The number of QTLs in *Sus scrofa* genome counts in Meat & Carcass Quality traits, amounts around 6,114 QTLs, located on almost every porcine chromosome. Nine chromosomes were identified as being of most interest with regard to meat quality traits: SSC2, SSC4, SSC5, SSC6, SSC7, SSC11, SSC14, SSC15 and SSC17. From literature and *Pig QTLdb*, is known that chromosome 2 is one of the main chromosomes in pig where is possible to find QTLs associated for quality traits.

Actually the SSC2 count 1,014 QTLs and harbors many QTLs for meat and carcass quality. However only a limited number of the found QTLs has been implicated with a known causative mutation.

While the genetic information is the same in every cell of an organism, the expression of different RNAs (or different amount of RNAs) and proteins, varies in the different tissues and it determines the identity of the cell. RNA-seq analysis is a recent technology that permits to catalog and compare gene expression and to investigate all of the RNAs belonged to the cell (its transcriptome) (Korf I., 2013).

Proteomic is the research area that investigates on the protein sequences, quantity (presence and abundance), modification state, interaction partners, activity, sub cellular localization and structure. Genes are transcribed and translated to proteins which have a greater impact on the phenotype, that is determinated by the complex interaction between the proteins (network) and the metabolites. Phenotype is the complex of visible characteristics in a subject that depends by its genotype, proteome, interactions between genes, time or period of animal development and environmental in which the animal lives (Boggess M.V. *et al* 2013; Sellner E.M. *et al*, 2007). This first statement is extended in complexity including variation in coding and regulation DNA sequence, alternative splicing from RNA to mRNA and determination of phenotype, defined during translation. In these steps, non-coding small RNA (microRNAs or miRNA) constituted by fragments of 18 – 26 nucleotides long, regulates through the transcript degradation and the posttranslational modification of proteins, the gene expression (D'Alessandro & Zolla, 2013). This process happens by altering the translation of protein – encoding transcripts, on the basis of multiple mechanisms that involve both in degradation of target mRNA, blocking of initiation of mRNA translation and blocking of translocation of mRNA to processing bodies. The miRNAome indicated the whole miRNA complement of a given cell/tissue, an important intermediate actor in the genome-to-proteome cascade. Furthermore recently review by other authors, have come to light the miRNAs role in cell cycle regulation and apoptosis, a biological process underpinning postmortem muscle biochemistry, in which specific miRNAs affect gene expression of glycolytic enzymes and result in suppression of their activity; this is an important regulation both in the frame of cancer cells and in postmortem muscle, which rely on glycolysis for energy production. Moreover it's very important in pigs' researches, because have been demonstrated to play a role in porcine pre- and post- natal development, intestinal development, growth performances and fat accumulation (D'Alessandro & Zolla, 2013).

Sometimes, genes may be present but not transcribed and the number of mRNA copies can't always reflect the number of functional proteins present. The aim of proteomics studies is to obtain information about cellular protein expression and hence to reveal the function of genes, with the ultimate goal of explaining how heredity and environment interact could control cellular functions (Hamill R.M., *et al*., 2013). The variety and complexity of proteins studies, are summarize especially

in the numerous functions that characterize these macromolecules. Proteins could be expressed like enzymes, hormones, transport proteins, immunoglobulin and antibodies, structural proteins, motor proteins, receptors, signaling and storage proteins. Proteomics science is applied in farm animal science to monitor *in vivo* performances of livestock (growth performance, fertility, milk quality etc.) and during *post mortem* process both, where the alterations in the muscle proteome and biochemistry reflect the biology and complexity of this point. Therefore, proteomics has achieved a fundamental role over the last two decades, in which technological innovations of proteins separation (chromatography, electrophoresis), identification (mass spectrometry) and the bioinformatics approaches, had support the development of this new discipline. Moreover studies in pure pig breeds utilizing electrophoresis and microarray analyses, have considerated the possibility to utilize proteins like biomarkers, for breed classification and utilization in pigs traceability, an economy – relevant pig farming issue. Proteomic science is considerated also to check the effects of animal handling prior to slaughter, including the analyses of proteomics profiles in the animals transported to the slaughterhouse by comparison to animals under fattening conditions. Indeed it has been demonstrated that the stress levels in pig transported to the abattoir resulted in a significant increase in the levels of the pig major acute-phase protein (D'Alessandro & Zolla, 2013).

These modern biotechnological applications are identify as "the OMIC sciences" (genomics, transcriptomics, proteomics and metabolomics) and allowed the identification of parts of genes directly associated (*markers*) with the improved characteristics or with diseases and defects that you wish to remove in the next generations. Bioinformatics science gave to the research an essential contribution in these years, especially with the creation of public domain web sites, important founts of consultation, updates and revisions about analysis dates (Davoli & Braglia, 2008).

Genomics, proteomics and other "omics" sciences could work together in the field of system biology, but they needed integrations of disciplines that could be realized with interdisciplinary collaborations, to give contribute in the context of meat science. Moreover, these researches can help to predict genes and regulatory elements involved in biological pathways in human and other species (Davoli R. *et al*, 2012).

Fat deposition in pork and associated genes:

The principal tissue involved in fatty acids synthesis in swine, is the adipose tissue.

The principal kinds of fat deposition in carcass pig are indicated considering the fat position in tissue: subcutaneous-fat is located between the internal *epithelium* side and the muscular mass; intermuscular and intramuscular fat indicate the adipose tissue position in respect to its location around the muscle tissue.

Triglycerides, phospholipids and cholesterol are the main constituents of intramuscular fat (IMF) and they are principally located in the adipocytes placed between fibers (85-95%) and in minor percentage, inside the muscular fibers near the mitochondria (5-20%) (Pethick D.W. *et al.,* 2005; Gondret F. *et al.,*1998). The fatty acids have to be transported inside the cell to contribute at metabolic pathway. Membrane *carrier proteins* play this important role and lead the fatty acids, as FABP (fatty acid-binding protein), FATP (fatty acid transport protein) and FAT (fatty acid translocase), inside the designated cell.

These proteins are important regulatory elements in the fatty acids metabolism and deposition (especially in the intramuscular tissue). The fatty acids absorbed could be converted in molecular signals (e.g. sfingolipids) or transformed and stored in particular intracellular lipid droplets. The amount and type of fats in the diet have a direct impact on human health. In recent decades the life quality, the standard of living and diet custom in Italy and others countries, are changed and nowadays the people prefer meat with high lean percentage than meat rich of fat. Recent studies have demonstrated that high levels of total fat intake can be risk factors for cancer and obesity. Indeed high consumption of saturated fatty acids (SFA) raises plasma LDL-cholesterol, which is a major risk factor for atherosclerosis and coronal cardiophaty (CHD). In contrast, the mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) are beneficial for human health.

The cellular lipid metabolism and homeostasis are regulated by different mechanisms and organelles, but *in primis* by cytosolic lipid storage droplets, complexes of lipids and proteins that are present in the cytosol of all body cells. The deposition of lipids is an important function in the organism, regulated by metabolic systems and physiological processes that have to preserve the balance, because the excess of lipid storage is linked to adaptive (fasting and chronic exercise) and mal-adaptive (obesity and related health complication) body conditions (Sztalryd C. & Kimmel A.R., 2013). For this reason to find a connection of mammalian lipid droplet function and lipid homeostasis body system could be important.

The genes encoding lipogenic enzymes expression, play a key role in the regulation of fat deposition in pig.

Up to now, only a limited number of genes associated with fat traits has been identified. *MC4R (Melanocortin-4 Receptor)* coded by G protein that is a transmembrane receptor regulates the energetic balance; *IGF2 (Insuline Like Growth Factor 2)*, is a complex of peptidic hormone that stimulates the growth in body fatness, *FABP3 (Fatty Acid Binding Protein 3)*, to regulate intramuscular fat content and FTO (*Fat Mass and Obesity Associated*), one of the most important gene implicated in human obesity was found associated in pigs with deposition of IMG in Italian Duroc breed.

Among the genes candidate for fat deposition reported in literature there is an important set of genes represented by the Perilipin gene family that can have an important role on fat traits.

These genes have been recently identified and studied also in pigs, in particular Perilipin 1 and 2.

When food is scarce, animals depend on stored fatty acid for energy. These deposits are in form of fats (triacylglycerols "TAG" and other neutral lipids) surrounded by amphipathic lipids and proteins. In the majority of eukaryotic cells, a core of neutral lipids (TAG and/or cholesteryl ester "CE") is synthesized and stored into cytosolic lipid droplets (CLD). Lipid droplets are complexly dynamic organelles characterized by specific lipids and proteins composition, that guarantee lipid homeostasis and energy storage in the cell. This family is arranged by members characterize by varying levels of sequence similarity, that are able to bind lipid droplets and functions, in stabilizing lipid droplets (Skinner J.R., *et al.*, 2013; Bickel P.E., *et al.*, 2009; Braesaemle D.L., 2007). These proteins have a role in regulation of basal and hormonally stimulated lipolysis and they can govern lipids storage and hydrolysis, promoting triacylglycerol storage and in time of energy deficit, the maximal lipolysis by hormone-sensitive lipase and adipose triglyceride lipase. These roles are important to supervise distinct metabolic pathways, control CLD tissue – specific adaptation to lipids utilization and the relationship with obesity (Sztalryd C. & Kimmel A.R., 2013). Nowadays the term *"perilipin"* has been selected as a unifying nomenclature for the mammalian PAT family of intracellular lipid storage droplets proteins.

The Perilipins ("surrounding lipid", by Greek,) is the most represented family of proteins on the surfaces of adipocyte lipid droplets. Perilipin are synthesized on free ribosomes and are constituted by N-terminal sequence, similar within and across species.

In mammalian genome five different perilipin genes are encode, characterized by mRNA splice variants and an individual tissue-dependent expression patterns (Kimmel A.R. *et al.*,2010).

- Perilipin1 (*Plin1*) is expressed in WAT (white adipose tissue), BAT (brown adipose tissue) and steroidogenic tissue;
- Perilipin2 (*Plin2*, previously called adipophilin or ADRP) and Perilipin3 (*Plin3*, previously TIP47) are expressed all over;
- Perilipin4 (*Plin4*, previously S3-12) is highly expressed in adipocytes;
- Perilipin5 (*Plin5*, previously LSDP5, OXPAT, MLDP, PAT1) is expressed in oxidative tissue including heart, liver, BAT and skeletal muscle.

Perilipin1 (Plin1) coats the lipid storage droplets in adipocytes, involving the lipids regulation and fat hydrolysis. This protein is coded by PLIN1 gene, that maps on SSC7 (Nowacka-Woszuk J. *et al.*,2008) and HAS 15, on swine and humans, both. It is the only mammalian perilipin related protein whose function is acutely regulated during lipolysis, by its phosphorylation state. This protein is also considered an important differentiation marker in adipocytes and to identify regulation factors in adipogenesis. When energy isn't required Plin1 is unphosphorylated and it blocks the fat hydrolysis with a barrier to lipases; when stored energy is required, Plin1 is phosphorylated by protein kinase A (PKA) and the hyper –phosphorylated perilipin recruits and organizes the activation of the lipolytic machinery (Skinner J.R. *et al*., 2013). PKA activation leads Plin1 regulating substrate/CLD (access of adipose lipolytic enzymes) and it permitted the coordination of TAG and DAG (diacylglycerol) hydrolysis. Upon phosphorylation its role shifts from to storage, to mobilization of stored neutral lipids. Plin1 is dephosphorylated by protein phosphatase 1. Plin1 is more expressed in brown and white adipocytes than in steroidogenic cells of adrenal cortex, testes and ovaries (Gandolfi G. *et al,* 2011*)*.

Unphosphorylated Plin1 has a role in systemic glucose and lipid homeostasis, preventing lipases from gaining access to neutral lipids in the droplet core and reducing TAG hydrolysis during hypophosphorylated state and under basal conditions.Vice versa phosphorylated Plin1 easily can activate the lipase action, releasing the lipids in circle (Bickel P.E. *et al*.,2009; Wang H. *et al*., 2009).

Nowadays PLIN1 is considerated a possible candidate gene for meat quality in pig and especially to determine the IMF pig meat content. Interesting were the results showed in the recent study performed by Gandolfi G. *et* al., (2011)on the expression of PLIN1 in *Semimembranosus* muscle. In that work the relation between PLIN1 and IMF content in pig muscle was studied and a high PLIN1 expression in samples characterized by high IMF content, in contrast to samples characterized by low IMF content, was observed and validated.

Another considered protein, belonging to Perilipin family, is Perilipin 2 (called previously ADRP, ADFP or Adipophilin) a cytosolic protein with molecular weight of 50 kDa. The respective gene

PLIN2 was mapped on *Sus scrofa* chromosome 1 (SSC1) in a region where are located different QTLs for meat quality and fat deposition traits.

PLIN2 promotes the formation and stabilization of the intracellular lipid droplets, organelles involved in the storage of lipid depots (Davoli R. *et al,* 2011).

The PLIN2 function is different and opposite in contrast to PLIN1. PLIN2 protein decreases as adipocyte differentiation progresses, while PLIN1 mRNA and protein levels increase. In early adipocytes are placed smaller PLIN2-coated lipid droplets, while in mature adipocytes there are larger PLIN-1 coated lipid droplets (Nagayama M. *et al*., 2007). The exactly role of PLIN2 in the cell is unknown but test *in vitro* have demonstrated that its presence in the lipid droplets membrane, contrast the adipose triglyceride lipase (ATGL) activity, blocking the enzyme contact with triglycerides inside the droplets and promoting the increase of lipids in the cell (as happen when PLIN1 isn't phosphorylated).

Nowadays, the biological role and mapping localization suggest that porcine PLIN2 gene could represents a biological and positional candidate for intra muscular fat deposition (Kim T.H. *et al.,* 2005). Gandolfi G. *et al.* (2011) found a positive and significant correlation among PLIN2 gene expression and quantitative IMG content in *Semimenbranosus* pig muscle. Therefore Davoli R. *et al.*(2011) performed an association study considering a mutation site in PLIN2 (SNP 98G>A) in Italian Duroc pig breed. The results showed a positive association between the SNP and the Average Daily Gain (ADG), Feed Conversional Rate (FCR), Lean Cuts (LC) and Ham Weight (HW).

The biological role and mapping localization suggest that porcine PLIN2 gene could represents a positional candidate-gene for fat deposition in pigs.

Aim of the studies:

This thesis discusses and shows the results of different investigations aimed to study and to analyze pig meat and carcass quality focusing mainly on genomic; moreover proteomic approach has been also used.

The aim of this research, was to perform association studies between pig genes considered as candidate for production traits and meat and carcass quality in different pig breeds.

Moreover, the procedures and the results of a protein study on pig muscle are presented. This part of the research has been conducted at Meat Laboratory, in Iowa State University (USA), during the my PhD studies six months stage.

In particular the specific aims of the present research were:

1. To perform association mapping of carcass and meat quality traits in the Italian Large White (ILW) population using multiple gene-tagged single nucleotide polymorphisms (SNPs) spread over the chromosome 2 region harboring QTLs for carcass and meat quality traits;

2. To search genes responsible for QTL effect on pig meat pH, single nucleotide polymorphisms (SNPs) detected in the transcribed sequences of coding genes located on three QTL regions (QTLRs) of SSC1 (60-80 cM), SSC2 (55-66 cM) and SSC3 (42-60 cM), were utilized to perform an association analysis with meat pH values;

3. To investigate the contribution of *PLIN1* and *PLIN2* genes to a wider range of performance, carcass, and meat quality traits in pigs and to confirm whether *PLIN1* and *PLIN2* genotype variants exert a differential effect on lean growth and IMF content;

4. To analyze the effect of normal and fast chilling of pork carcasses, on pork loin protein profile by proteomic comparison samples that were subjected to deep chilling temperature (1.4 °C) compared to conventional temperature (12.8 °C).

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Chapter 1

Association mapping of quantitative trait loci for carcass and meat quality traits at central part of chromosome 2 in Italian Large White pigs

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Abstract

Association mapping of the central part of porcine chromosome 2 harboring QTLs for carcass and meat quality traits was performed with 17 gene-tagged SNPs located between 44.0 and 77.5 Mb on a physical map (Sscrofa10.2) in Italian Large White pigs with records of estimated breeding values for average daily gain, back fat thickness, lean cuts, ham weight, feed conversion ratio, pH_1, pH_u, CIE *L**, CIE *a**, CIE *b** and drip loss. We mapped a significant QTL for fat deposition (adjusted $P = 0.0081$) and pH_1 (adjusted $P = 0.0972$) to *MYOD1* at position 44.4 Mb and a QTL for growth and meatiness (adjusted $P = 0.0238 – 0.0601$) to *UBL5* at position 68.9 Mb. These results from association mapping are much more accurate than are those from linkage mapping and facilitate further search for position candidate genes and causative mutations needed for application of markers through marker assisted selection.

Keywords:
pig; association; chromosome 2; carcass quality; meat quality

1. Introduction

Carcass and meat quality traits are amenable to marker assisted selection (MAS) as the majority of such traits can be measured post mortem. Earlier studies of quantitative trait loci (QTLs) for carcass and meat quality based on linkage mapping in experimental F_2 crosses with high level of linkage disequilibrium (LD) and lack of recombination usually have been able to map QTLs in intervals of 20–40 centimorgans (cM) (Georges, 2007). Fine mapping of QTLs that leads to identifying the mutations underlying phenotypic variation is necessary for use in selection programs.

Association or LD mapping that uses correlation between QTL alleles and marker alleles in the whole population is likely to be a more effective tool than linkage studies for examining complex traits because it can have greater statistical power to detect several genes of small effect. LD may occur if a marker allele and QTL allele were on the same chromosome in an ancestor of the current population and, due to chance effects and finite population size, that the chromosome segment is now common in the population. Since recombination will separate the marker and QTL alleles unless they are tightly linked, LD is expected and more often observed between genes that are tightly linked (Goddard, 2003). Consequently, LD mapping can only detect a QTL in the vicinity of a marker. If a QTL is close, however, it can map its position much more accurately than can linkage mapping because it uses all recombination events that have occurred since the common ancestor.

Generally, the amount of LD between a marker and a QTL useful for association mapping is assumed to be $r^2 \geq 0.3$ (Jungerius *et al*., 2005, Du *et al*., 2007). Association mapping enables precise QTL mapping in commercially exploited populations when the mapping is based on a sufficient number of markers in a specific region.

In addition to the imprinted *IGF2*-intron3-G3072A substitution with a major effect on body composition that maps to the proximal tip of pig chromosome 2 - SSC2 (Van Laere et *al*., 2003) other QTLs for fat deposition, growth (de Koning *et al*., 1999; Lee *et al*., 2003; Rattink *et al*., 2000; Thomsen *et al*., 2004; Stearns *et al*., 2005; Liu *et al*., 2008, Tortereau *et al*., 2010; Geldermann *et al*., 2010 and Qiu *et al*., 2010) and meat quality traits such as pH_u (Qu *et al*., 2002; Lee *et al*., 2003; Su *et al*., 2004; Rohrer *et al*., 2006; Liu *et al*., 2007; Heuven *et al*., 2009), pH_1 (Čepica *et al*., 2012), water holding capacity and meat color (Malek *et al*., 2001), drip loss (Li *et al*., 2010a) and intramuscular fat (Stearns *et al*., 2005; Čepica *et al*., 2012) have been mapped at the central part of porcine chromosome 2. The QTLs for carcass and meat quality traits and 95% confidence intervals are located in the chromosome region 55.0–77.9 cM (PigQTLdb, http://www.animalgenome.org/cgi-bin/QTLdb/SS/index).

The aim of this work was to perform association mapping of carcass and meat quality traits in the Italian Large White (ILW) population using multiple gene-tagged single nucleotide polymorphisms (SNPs) spread over the chromosome 2 region harboring QTLs for carcass and meat quality traits.

2. Material and methods

2.1. Animals

Two groups of sib-tested ILW pigs were used. Three siblings of the same litter (2 females and 1 castrated male) were performance-tested at the Central Test Station of the National Association of Pig Breeders (Italy) and slaughtered for genetic evaluation of a boar from the same litter. Animals were slaughtered at average live weight of 155 kg at about 8 months of age.

Group 1 used for selective genotyping approach comprised 200 pigs (138 females and 62 castrated males) and consisted of at least two-generation-unrelated performance-tested animals with highest and lowest estimated breeding values (EBVs) for average daily gain (ADG; high tail for ADG EBVs [N = 100] and low tail for ADG EBVs [N = 100]). These animals were selected from 3591 sib-tested pigs slaughtered between the years 1996 and 2007 (Table S1).

Group 2 comprised a random sample of 633 animals (422 females and 211 castrated males) encompassing two subgroups, of which subgroup 2A consisted of 277 animals (184 females and 93 castrated males) slaughtered in 2003 (6 different slaughter batches) and subgroup 2B consisted of 356 animals (238 females and 118 castrated males) slaughtered in 2008 (11 different slaughter batches). Subgroups 2A and 2B were analyzed as independent groups, as they were separated by 5 years of selection. The characteristics of subgroups 2A and 2B are presented in Table 1**.**

Animals of both populations had EBVs for average daily gain, measured in grams, from 30 to 155 kg of live weight with *quasi ad libitum* feeding (ADG); feed conversion ratio calculated as feed intake/weight gain from 30 to 155 kg (FCR); back fat thickness measured in mm and recorded post mortem at the *m. gluteus medius* (BFT); ham weight measured in kg (HW); and weight of lean cuts in kg including weight of neck, loin and HW (LC). EBVs for the traits reported above were calculated and provided by the National Association of pig breeders as described by Russo *et al.* (2000, 2008) using a BLUP multiple-trait animal model (Henderson & Quaas, 1976). Briefly, models were different for each trait and included fixed effects of batch in test, sex, age at beginning of test, age of sow, weight at slaughter, age at slaughter, and inbreeding coefficient as well as the random effects of litter, individual permanent environment, and animal. Pigs' genetic merit for the considered traits was calculated taking into account the additive relationship matrix. EBVs were expressed as differences from the genetic mean value for the considered trait in the year 1993. In addition, meat

quality traits such as pH_1 (measured about 1 h post mortem), pH_u (measured 24 h post mortem), CIE L*a*b* color (1976 CIE L*a*b* Colour Space, CIELAB; http://www.cie.co.at/index.php/index.php?i_ca_id=485), drip loss (DRIP; Grau & Hamm, 1957; Hoffmann *et al.*, 1982), and glycolytic potential (GP; Monin *et al*., 1987; Nanni Costa *et al*., 2009) were measured in *m. semimembranosus*.

2.2. SNPs and their genotyping

To optimize the number of genotyped animals, we used selective genotyping as a preliminary approach followed by sequential sampling. Selective genotyping involves phenotyping a large population of individuals, but the actual genotyping involves only those individuals whose phenotypes deviate substantially from the mean (Van Gestel *et al*., 2000). The genotyped SNPs were either retrieved from the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/snp_batchSearch.cgi?org=9823&type=SNP) and literature or obtained by comparative sequencing of the gene-tagged polymerase chain reaction (PCR) products prepared on DNA from 12 animals (4 Czech Large White, 4 Czech Landrace, and 4 Duroc). All 17 SNPs were gene-tagged. Of these, 14 were located in introns, 2 in exons, and 1 in the 3′ UTR. The SNPs used for genotyping, including reference sequences, PCR primers, PCR conditions, restriction enzymes used for PCR-restriction fragment length polymorphism, and literature references, are listed in Table 2. Pairwise measures of LD (r^2) were calculated using the Haploview software package accessible at www.broad.mit.edu/mpg/haploview/ (Barrett *et al*., 2005).

Group 1 was genotyped first and only for SNPs significantly associated with records for EBVs in the Group 1 subset. These SNPs and those with minor allele frequency (MAF > 0.05) were considered for genotyping in subgroups 2A and 2B. Further genotyping was then performed depending on the results of selective genotyping in Group 1 and sequential sampling in subgroups 2A and 2B.

2.3. Statistical analyses

Associations between genotypes and EBVs (obtained by the National Association of pig breeders) were assessed using the general linear model (GLM) procedure of SAS, release 9.2 (SAS Institute Inc., Cary, NC). The model included the fixed effects of genotype and sex (Group 1) or genotype, sex and day of slaughter (Group 2, subgroups 2A and 2B). The number of slaughter days was not available for Group 1.

The model for Group 2, subgroup 2A is as shown below:

$$EBV = \mu + SNP_i + Sex_j + e_{ij},$$

where EBV stands for estimated breeding value for ADG, BFT, LC, HW and FCR, respectively; μ = overall mean; SNP = fixed effect of each genotype (i = 1...3) (each gene was considered separately); Sex = fixed effect of sex (j = 1, 2); e = residual error.

The model for Group 2, subgroup 2B is as shown below:

$$EBV / Phenotype = \mu + SNP_i + Sex_j + Day_k + e_{ijk},$$

where EBV stands for estimated breeding value for ADG, BFT, LC, HW and FCR, respectively; Phenotype stands for pH_1, pHu, CIE L*, CIE a*, CIE b*, DRIP and GP, respectively; μ = overall mean; SNP = fixed effect of each genotype (i = 1...3) (each gene was considered separately); Sex = fixed effect of sex (j = 1, 2); Day = fixed effect of day of slaughter (k = 1...6 for subgroup A, 1...11 for subgroup B, and 1...17 for Group 2); e = residual error.

The MIXED procedure of SAS, including the sire and day of slaughter into the model as random effects and sex and genotype as fixed effects, was used to evaluate the association between the markers and those of the measured phenotypes showing a significant effect on the GLM analysis, as follows:

$$Phenotype = \mu + SNP_i + Sex_j + Day_k + Sire_l + e_{ijkl},$$

where Phenotype = pH_1, pH_u; μ = overall mean; SNP = fixed effect of each genotype (i = 1...3) (each gene was considered separately); Sex = fixed effect of sex (j = 1, 2); Day = random effect of day of slaughter (k = 1…6 for subgroup 2A, 1…11 for subgroup 2B and 1…17 for Group 2); Sire = random effect of the sire (l = 1…80 for subgroup 2A, 1…66 for subgroup 2B and 1…146 for Group 2; 1 sire was common to both subgroups); e = residual error.

In each of the proposed models, the effect of each SNP was considered separately. Nominal *P* values for association with the EBVs were adjusted for multiple testing (Benjamini & Hochberg, 1995, 2000) for each SNP using the MULTTEST procedure of SAS. The significance threshold for the adjusted *P* values was set to $P < 0.10$.

3. Results and discussion

3.1. Results of selective genotyping and sequential sampling in Group 1

Genotyping started in Group 1 with 17 gene-tagged SNPs located on pig chromosome 2 between 43.9 and 77.6 Mb, with spacing 1.98 Mb, corresponding to the interval 59.5–70.7 cM on the linkage map, with spacing 0.66 cM (Fig. 1). Genotype and allele frequencies of the genotyped SNPs are given in Table S2**.** SNPs within the *EIF4G2*, *WEE1*, *SYDE1*, *CARM1*, *ANGPTL4*, *RETN*, *INSR*, *CFD2* and *POLR2E* genes in Group 1 either were monomorphic or had MAF < 0.05 and therefore were not used for association analyses. The results of association analyses performed in Group 1 are summarized in Table S3. Of the segregated SNPs, highly significant (nominal $P < 0.01$) associations were detected for *LDHA* (ADG, BFT, LC, HW and FCR), *UBL5* (ADG, LC, HW and FCR), *LDLR* (ADG, HW and FCR) and *CNN1* (ADG, HW and FCR).

SNPs within *COPB1*, *FBN3* and *PIP5K1C* were excluded from further genotyping, while genotyping of the SNP within *MYOD1* was continued on the basis of sequential sampling of a subset of 2B samples. Further genotyping was not performed for those markers that clearly showed no significant effect (Weller, 2001). For economic optimization in detecting and locating QTLs, the results of selective genotyping in Group 1 and sequential genotyping in Group 2 were taken into consideration (Weller, 2001). If the sample of individuals recorded for the quantitative trait is large, the power per individual genotyped can be increased by selective genotyping of those individuals having the highest and lowest trait values. However, although the sample regression method may be used to estimate parameters with selective genotyping, the estimates will be biased upward relative to when using full data (Lander & Botstein, 1989, Darvasi & Soller, 1992; Henshall & Goddard, 1999). Moreover, if there are several quantitative traits of interest, it is necessary to genotype a different sample for each trait. Furthermore, if selective genotyping is applied to a single trait but other correlated traits are also analyzed, then QTL effects associated with the correlated traits will be biased, even if all individuals with phenotypes are included into the analysis (Weller, 2001). The extreme phenotypes in Group 1 were selected only according to EBV for ADG and used for EBVs for all analyzed traits. Therefore, our results concerning EBVs for BFT, LC, HW and FCT should be taken only as an auxiliary criterion in deciding about further genotyping.

3.2. LD mapping in subgroups 2A and 2B

Selective genotyping and sequential sampling reduced to five the number of SNPs genotyped in subgroups 2A and 2B. Genotyped gene-tagged SNPs, observed genotype numbers, allele frequencies, and agreement with Hardy–Weinberg equilibrium in subgroups 2A and 2B of ILW are given in Table 3**.** Genotyped SNPs were neutral mutations situated in introns or 3' UTR, except for

the non-synonymous substitution c.1653C>T within exon 13 of the *LDLR* gene. All SNPs were in agreement with Hardy–Weinberg equilibrium except for the SNP within the *LDLR* gene. Of those markers genotyped in this study, the SNPs within *LDHA* and *MYOD1* exhibited noticeable pairwise LD ($r^2 = 0.252$), thus implying that both SNPs could be associated with the same QTL, albeit with different *P*. The extent of pairwise LD between other neighboring SNPs was negligible ($r^2 = 0.014 – 0.021$).

The results of association analyses performed in subgroups 2A and 2B of randomly sampled animals are given in Table 4. At nominal $P < 0.05$, the traits were associated with LDHA (BFT, FCR), MYOD1 (BFT, LC, FCR, pH_1), UBL5 (HW, ADG, LC), LDLR (BFT, FCR) and CNN1 (DRIP, FCR). Associations of LDHA (BFT), MYOD1 (BFT) and UBL5 (ADG, HW, LC) remained significant after correction for multiple testing (adjusted $P < 0.10$).

On average, markers more tightly linked to a QTL show higher *P* values than do less tightly linked markers, thus implying that *P* values would be useful in establishing the map position of the QTL (Goddard, 1991). Hill & Weir (1988) had concluded that if a high *P* value is found in a large population, the marker and QTL must be closely linked.

For BFT, the most significant association was detected with the SNP within MYOD1 in subgroup 2B. Because the significance of association with LDHA in subgroup 2A showed a much lower P, the causative mutation should be closer to MYOD1 than to LDHA. MYOD1 is located approximately at position 60.0 cM on the USDA USMARC 2 linkage map. An additional, weaker signal for BFT was with the LDLR gene located approximately at position 63.0 cM on the USDA USMARC 2 linkage map.

Growth and meatiness were associated with the SNP within the *UBL5* gene located at position 68.9 Mb, corresponding to 62.1 cM on the USDA USMARC 2 linkage map (Čepica *et al.*, 2012). ADG, HW and LC were associated with *UBL5* in subgroup 2A, as was HW in subgroup 2B. In both subgroups, BFT and HW were associated with *MYOD1* and *UBL5*, respectively, but with the allele effects reversed.

At nominal level, meat quality traits pH_1 and DRIP were associated with *MYOD1* at positions 44.4 Mb (around 60.0 cM) and *CNN1* at position 70.5 Mb, respectively. The *CNN1* gene is located at about 63.0 cM on the USDA USMARC 2 linkage map, as the gene is situated on the physical map between *UBL5* and *RETN* mapped at positions 62.1 cM and 64.0 cM, respectively (Čepica *et al.*, 2012). After adjustment for multiple testing, these associations were not significant. When subgroups 2A and 2B were merged, however, pH_1 was significantly associated with *MYOD1* ($P = 0.0081$; adjusted $P = 0.0972$).

Here we confirm that some QTLs in the central part of *SSC2* affecting carcass composition and meat quality traits found earlier, mainly in the Meishan-derived F_2 experimental populations, segregate also in the highly selected ILW. The QTL for BFT was previously mapped between 37.6 and 64.1 cM (de Koning *et al*., 1999; Lee *et al*., 2003; Rattink *et al*., 2000; Stearns *et al*., 2005; Liu *et al*., 2008; Tortereau *et al*., 2010; Geldermann *et al*., 2010). Recently, Tortereau *et al*. (2011) confirmed the existence of a QTL for fatness at positions 62 and 66–71 cM using marker-assisted backcrossing design. A QTL at the *MYOD1* position was previously documented by linkage mapping in the Hohenheim Meishan-derived three-generation F2 populations (Geldermann *et al*., 2010). The existence of an additional QTL for fat accretion at the position of *LDLR* is supported by our previous finding that in a commercial Landrace x Chinese–European synthetic population the *RETN* gene (71.9 Mb, 64.0 cM) was associated with intramuscular fat (Čepica *et al*., 2012).

A QTL for growth and meatiness associated with *UBL5* (62.1 cM) was linkage mapped at 61.0 cM by Thomsen *et al*. (2004). QTLs for pH_1 in this chromosome region had been previously located at position 66.0 cM (Duan *et al*., 2008) by linkage mapping and at position 62.1 cM (Čepica *et al*., 2012) by association mapping in a commercial Landrace x Chinese–European synthetic population. DRIP has been linkage mapped to positions 74.8 and 59.0 cM (Malek *et al*., 2001; Li *et al*., 2010a).

3.3. Discussion on genes having positive association with investigated traits

The SNPs significantly associated with traits in subgroups 2A and 2B are mostly neutral mutations within the genes. Some of these genes, however, could be regarded as candidate genes for the studied traits, as suggested by their biological roles.

LDHA (*lactate dehydrogenase A*), found predominantly in striated muscle tissue, catalyzes the conversion of L-lactate and NAD to pyruvate and NADH in the final step of anaerobic glycolysis and therefore can potentially affect the pH of meat. Otto *et al*. (2007) found that the *LDHA* gene was associated with loin pH_1 and pHu. Using selective genotyping, Fontanesi *et al*. (2012) found *LDHA* associated with growth in ILW. The selective genotyping performed in Group 1 of ILW confirmed this result, but association analyses performed in a random sample of ILW (subgroups 2A and 2B) failed to corroborate this association. These contrasting or not always concordant results could indicate that this gene is more likely a marker than the causative gene itself.

The *MYOD1* (*myogenic differentiation 1*) gene encodes a nuclear protein that belongs to the basic helix-loop-helix family of transcription factors and the subfamily of myogenic factors that, in addition to *MYOD1*, also includes the genes *MYF5* (*myogenic factor 5*), *MYOG* (*myogenin*) and *MYF6* (*myogenic factor 6*). *MYOD1* regulates muscle cell differentiation by inducing *MYOG* cycle

arrest, a prerequisite for myogenic initiation. Recently, Lee *et al*. (2012) conducted an association study of three SNPs within the porcine *MYOD1* gene in a population of Yorkshire and Berkshire pigs. They found that SNPs were associated in joint population with several muscle fiber characteristics, loin eye area and lightness but not with BFT and pH_1. Analyses of individual populations revealed associations with BFT in both breeds and association with pH_1 in Berkshire. Association of *MYOD1* with pH in Large White pigs also was detected (Han *et al*., 2012). Further research is required to determine whether the *MYOD1* gene itself or one or more genes in the vicinity cause phenotypic variation in fat accretion and pH_1.

The *LDLR* (*low density lipoprotein receptor*) is a cell surface receptor that plays an important role in cholesterol homeostasis. In humans, mutations within *LDLR* that affect its function are spread fairly evenly through the gene and almost every single amino acid substitution that has been found has a deleterious effect. Mutations in this gene cause the autosomal dominant disorder familial hypercholesterolemia (for review see Soutar & Naumova 2007, Go & Mani 2012). Genetic defects in *LDLR* causing hypercholesterolemia have been described in Watanabe-heritable hyperlipidemic rabbits and rhesus monkeys (Watanabe *et al*., 1985; Scanu *et al*., 1988). Knockout (Ldlr -/-) mice have elevated plasma cholesterol levels but similar plasma triglyceride concentration when compared with their wild-type litter mates (Ishibashi *et al*., 1993), and a high-fat diet resulted in increased total cholesterol and glucose along with complications of obesity in these mice (Ma *et al*., 2012). Several non-synonymous polymorphisms have been identified in the porcine *LDLR* gene (Hasler-Rapacz *et al*., 1998; Grunwald *et al*., 1999; Pena *et al*., 2009), but their frequencies in commercial populations were low. Porcine non-synonymous mutations causing amino acid substitutions 84 Arg>Cys (Hasler-Rapacz *et al*., 1998) and 94 Arg>Cys (Grunwald *et al*., 1999) were associated with spontaneous hypercholesterolemia. The *LDLR* gene can be assumed to be a positional candidate for fat deposition in pigs.

UBL5 (*ubiquitin-like 5*) is a gene associated with ubiquitin and related proteins (collectively called ubiquitin-like modifiers, or UBLs) that covalently modify proteins and thus often critically alter substrate activity by influencing metabolic stability, binding behavior or localization (Hochstrasser, 2009). *UBL5* is involved in feeding behavior and development of obesity and type 2 diabetes in the Israeli sand rat, *Psammomys obesus* (Collier *et al*., 2000; Walder *et al*., 2002). In humans, genetic variants within the *UBL5* gene have been reported to be associated with obesity, triglyceride and cholesterol metabolism, and insulin levels (Jowett *et al*., 2004; Bozaoglu *et al*., 2006) but not with early onset obesity in children (Sentinelli *et al*., 2008). In a previous association study performed in a commercial Landrace x Chinese–European synthetic population, the *UBL5* gene was found to be associated with pH_1 (Čepica *et al*., 2012). Further research in other populations

is needed to determine whether variation in the *UBL5* gene can affect feed consumption and energy metabolism in pigs as well as growth and meatiness in pigs.

The *CNN1* (*calponin 1*) gene encodes a basic 34-kD protein (calponin, CaP) that is specifically expressed in smooth muscle and binds calmodulin, actin, and tropomyosin. Calponin 1 is able to inhibit the ATPase activity of myosin and is thought to play a role in smooth muscle contraction. Moreover, CaP was shown to function as a signal transducing and integrating molecule that controls actin-based cellular processes by regulating the stability of activity. *CNN1* was found to be upregulated in an oxidative muscle (*m. soleus*) compared to a fast twitch glycolytic muscle (*m. longissimus thoracis et lumborum*) in Meishan gilts (Li *et al.*, 2010b). The possible impact of the *CNN1* gene variation on pork quality should be the subject of further investigation.

3.4. Accuracy of association mapping and extent of LD in pigs

Association mapping uses the nonrandom association of alleles, called LD, between genotyped markers and an unknown mutation underlying phenotypic variation at a QTL. For this purpose, r^2 is used as a measure of LD. This measure is inversely related to the distance between a marker and QTL and directly proportional to the detected portion of QTL effect. Hence, for markers distant from the causative mutation with small r^2 values there is little power to detect association at the marker locus (Kruglyak, 1999; Pritchard & Przeworski, 2001).

The extent and amount of LD in the studied population are of primary importance for the accuracy of association mapping (Goldstein & Veale, 2001; Morton, 2005). Effective population size (N_e) is a factor affecting LD in farm animal species (Goddard, 1991; Amaral *et al.*, 2008; Uimari & Tapio, 2011; Badke *et al.*, 2012). The LD between a marker and QTL is inversely related to N_ec, where c is the recombination distance between the genes (Sved, 1971). Therefore, LD is found between genes further apart in small effective populations than in large effective populations (Pritchard & Przeworski, 2001; Hayes *et al.*, 2003). Generally, in Chinese breeds with higher N_e LD is mostly organized in haplotype blocks of up to 10 kb, while in European breeds with lower N_e LD haplotype blocks may be up to 400 kb in size (Amaral *et al.*, 2008). Recently, Badke *et al.* (2012) has estimated LD in US populations using PorcineSNP60 Genotyping BeadChip. They found that for markers 0.5 cM apart, average r^2 values were between 0.21 and 0.19, and for markers with average LD at r^2 of 0.25 and 0.28 the markers were 0.239 and 0.246 Mb apart in Yorkshire and Landrace breeds, respectively. While to date data about LD in ILW are not available, it can be assumed that LD is similar to that of other related Western commercial breeds including US Yorkshire and Landrace. This implies that for significant association the distance between markers and causative mutations should be shorter than 0.3 Mb. Analogous to this, traits significantly associated with genotyped

markers should be located up to 0.3 Mb from the associated marker. Such mapping of QTLs is much more accurate compared to that obtained by linkage mapping (Georges, 2007), and it facilitates the search for position candidate genes and causative mutations underlying particular QTL and subsequent marker assisted selection.

4. Conclusions

We performed association mapping of the central part of the porcine chromosome 2 region harboring QTLs for carcass composition and meat quality traits with 17 gene-tagged SNPs located between 43.99 and 77.49 Mb on the physical map (corresponding to 59.5 and 70.7 cM) in Italian Large White pigs using selective genotyping and sequential sampling approaches. We mapped a significant QTL (adjusted $P < 0.1$) for fat deposition and pH_1 to *MYOD1* SNP at position 44.4 Mb and a QTL for growth and meatiness to the *UBL5* SNP at position 68.9 Mb. At nominal level $P < 0.05$, DRIP was associated with *CNN1* at position 70.5 Mb and the second QTL for fat deposition with *LDLR* at position 70.1 Mb. Association mapping of QTL is much more accurate than linkage mapping and facilitates the search for position candidate genes and causative mutations needed for application of markers through marker assisted selection.

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Table 1

Mean breeding values for ADG, BFT, LC, HW and FCR; phenotypic values for pH_1, pH_U, CIE L*, CIE a*, CIE b*, DRIP, GP and CW; age at slaughter; and their standard deviations (SD) of subgroups 2A and 2B of Italian Large White pigs used for association studies.

	Subgroup 2A			Subgroup 2B		
Trait	N	Mean	SD	N	Mean	SD
ADG (EBV)	276	35.17	29.06	350	41.50	19.83
BFT (EBV)	276	-2.24	3.80	350	-2.31	2.85
LC (EBV)	276	2.03	1.91	350	3.36	1.62
HW (EBV)	276	0.55	0.59	350	0.64	0.43
FCR (EBV)	276	-0.14	0.16	350	-016	0.10
pH_1	275	5.94	0.24	347	6.21	0.27
pH_U	274	5.67	0.21	313	5.77	0.23
CIE L*	-	-	-	349	40.04	4.98
CIE a*	-	-	-	349	7.75	2.56
CIE b*	-	-	-	349	3.79	0.98
DRIP	-	-	-	350	69.54	19.33
GP	275	103.44	23.06	-	-	-
CW	276	120.05	9.57	350	114.58	8.41
AGE	275	239.56	7.92	350	238.21	7.76

EBV – estimated breeding value, ADG – average daily gain, BFT – back fat thickness, LC – weight of lean cuts, HW – hams weight, FCR – feed conversion ratio.

Traits: pH_1 – meat pH measured at about 1 h post mortem in *m. semimembranosus,* pHu – meat pH measured at 24 h post mortem in *m. semimembranosus,* color parameters according to 1976 CIE L*a*b* Color Space, DRIP – drip loss, GP – glycolytic potential, CW – carcass weight, AGE – age at slaughter.

Table 2. Genotyped SNPs.

Gene	Reference sequence	Position Mb	SNP	Localization	Primers	Allele	T_a (°C)	$MgCl_2$ mmol	Restriction enzyme	PCR bp	Reference
LDHA	FJ865398	43.9	c.795 A>G	Intron 6	F: gttgctggtgtctccctgaag R: ctgtccaccacctgtttgtga	1 - A 2 - *G*	55	1.5	*Ssi*I	95	Qiu *et al*. (2010)
MYOD1	U12574	44.4	g.1264C>A	Intron 1	F: ggcggagagcactacagc R: cgggttgggaaggaagag	1 - C 2 - A	62	1.5	*Dde*I	1008	Knoll *at al.* 1997
COPB1	FJ865397	48.0	c.3096C>T	3' UTR	F: gggcttactggactccaacat R: tggtcttgatacatgtgtgaaaca	1 - C 2 - T	55	1.5	*Rsa*I	205	Qiu *et al*. (2010)
EIF4G2	ENSSSCG00000013405	52.0	g.11687C>T	Intron 22	F: ctctctatgctcttcaggtg R: cagtgttgctattcagttttct	1 - T 2 - C	60	1.5	*Tsp*RI	1710	This study
WEE1	BV726831	53.2	93C>T	Exon 4	F: tgccattaagcgatca R: tgaaactcccaatttactgt	1 - T 2 - C	51	1.5	*Hind*II	290	gi:23131721
SYDE1	BV726641.1	62.0	g.236A>G	Intron 6	F: gcactgaaggcacacg R: agaatttgggcttgaacc	1 - G 2 - A	57	1.5	*Xba*I	402	gi:23130298
UBL5	AM950288	68.9	g.566G>A	Intron 4	F: ctcttcctcgttctatctcc R: cccctcccatctctcacagc	1 - G 2 - A	57	2.0	*Bsp*TI	1010	Čepica *et al.* (2012) gi:275523695

CARM1	BV726637.1	70.0	g.72A>G	Intron 16	F: tgtggccggtgagcaagg R: tgcagaaggcccagtgcg	1 - A 2 - G	65	1.5	*Aci*I	208	gi:23130236
LDLR	AF065990	70.1	c.1653C>T	Exon 13	F: ggcttcatgtactggctgattg R:gcttggagtcgacccagtaaag	1 - T 2 - *C*	58	1.5	*Hin*1I	≈ 500	Pena *et al.* (2009)
CNN1	ENSSSCT00000014869	70.5	g.6956C>T	Intron 5	F: tgcgggtgtgcccacttg R:gttgctgcccatctgtagg	1 - C 2 - T	57	1.5*	*Bse NI*	1188	This study
ANGPTL4	ENSSSCG00000013599	71.2	g.4136A>G	Intron 3	F: ctttttttgctttttcggtattt R: tctgggggctttttctgac	1 - A 2 - G	56	1.5	*Dde*I	465	This study
FBN3	BV726636.1	71.5	g.235T>C	Intron 21	F: ctacagctgcgccaaca R: ggaggccaccatatgctt	1 - T 2 - C	57	1.5	*Cfr*13I	382	gi:23129941
RETN	AM157180	71.8	g.1190G>A	Intron 3	F: ccccagccccagcccagat R: gcccccaaaggcaggagcag	1 - A 2 - G	61	2.5	*Btg*I	296	This study
INSR	AM950289	72.2	g.589T>C	Intron 12	F: gaaggtgggttggctcatcat R: gctgtggctctggcgtagg	1 - T 2 - C	58	1.5	*Hpa*II	566	Čepica *et al.* (2012) gi:275523697
PIP5K1C	BV726632.1	75.6	g.256T >C	Intron 7	F: ccgccctggtcaagacgc R: cggggtgggggttgtgg	1 - T 2 - C	57	1.5	*Ade*I	424	gi:23132002
CFD2	AM950287	77.5	g.306C>T	Intron 3	F: gcagtgggtgctgagtgca	1 - T	58	1.5	*Bse*NI	561	Čepica *et al.*

					R: atggtgccatcgtggtatgt	2 - C					(2012)
											gi:275523698
POLR2E	BV726629.1	77.6	g:374T>A	Intron 3	F: gggcgctgatcgtggtgc	1 - G	68	1.5	*Bsr*BI	472	gi:23131774
					R: cgccccacccagaccctt	2 - A					

T_a – annealing temperature.

Table 3

Genotyped gene-tagged SNPs, observed genotype numbers, allele frequencies, and agreement with Hardy–Weinberg equilibrium in subgroups 2A and 2B of Italian Large White pigs.

Gene	Subgroup	N	Observed genotypes			Allele frequencies		χ^2(*)
			11	12	22	1	2	
LDHA	2A	352	140	165	47	0.632	0.368	0.022
	2B	125	74	46	5	0.776	0.224	0.428
MYOD1	2A	351	105	166	81	0.536	0.467	0.967
	2B	277	57	139	81	0.457	0.543	0.035
UBL5	2A	347	196	123	28	0.742	0.258	1.901
	2B	275	140	122	13	0.731	0.269	4.492
LDLR	2A	351	8	30	313	0.066	0.934	**32.033**
	2B	273	3	24	246	0.055	0.945	**6.432**
CNN1	2A	333	153	158	22	0.697	0.303	5.013
	2B	277	135	121	21	0.706	0.294	0.743

(*) Genes not in Hardy–Weinberg equilibrium are in bold.

Table 4

Significant associations between the studied gene-tagged SNPs and estimated breeding values for carcass traits and phenotypic values for meat quality traits (nominal $P < 0.05$) in subgroups 2A and 2B of Italian Large White pigs. Traits with adjusted $P < 0.10$ are given in bold. For each haplotype, least squares means ± standard errors (LSmeans ± SE) are given.

Gene	Subgroup	Trait	*F* test			R^2	LSmeans ± SE			Mode of inheritance
			F value	Nominal *P* value	Adjusted *P* value		Genotype 11	Genotype 12	Genotype 22	(§)
LDHA	2A	BFT	**4.95**	**0.0086****	**0.0774^{+}**	0.10	-1.47 **±** 0.54^{1,2} N = 74	-2.80 ± 0.69^{1} N = 46	5.54 **±** 1.58^{2} N = 5	A* (-2.04)
	2B	FCR	3.69	0.0261*	0.2871	0.10	-0.18 ± 0.01^{a} N = 137	-0.15 ± 0.01^{a} N = 162	-0.15 ±0.02 N = 47	NS
MYOD1	2A	*BFT*	*3.95*	*0.0204**	*0.1632*	*0.05*	*-3.13 ± 0.51^{a}* N = 57	*-2.49 ± 0.34^{1}* N = 138	*-1.38 ± 0.43^{1,a}* N = 81	*A** (0.88)*
		FCR	3.06	0.0487*	0.1948	0.03	-0.11 ± 0.02^{1} N = 57	-0.17 ± 0.01^{1} N = 138	-0.13 ± 0.02 N = 81	D*
MYOD1	2B	*BFT*	***7.14***	***0.0009******	***0.0081^{++}***	*0.07*	***-1.74 ± 0.28^{A}*** N = 104	***-2.18 ± 0.23^{a}*** N = 165	***-3.34 ± 0.33^{A,a}*** N = 81	*A*** (-0.80)*

		LC	3.17	0.0431*	0.1293	0.10	3.13 ± 0.16[1]	3.37 ± 0.13	3.74 ± 0.02[1]	A* (0.31)
							N = 104	N = 165	N = 81	
		pH_1	3.49	0.0317*	0.1293	0.09	6.15 ± 0.03[a]	6.24 ± 0.02[a]	6.21 ± 0.03	NS
							N = 102	N = 164	N = 80	
UBL5	2A	ADG	**4.85**	**0.0085****	**0.0238**$^{++}$	0.06	**39.17 ± 2.52[a]**	**32.57 ± 2.72[1]**	**15.24 ± 8.05[1,a]**	A** (-11.97)
							N = 140	N = 122	N = 13	
		LC	**3.14**	**0.0451**	**0.0601**$^{+}$	0.04	**2.29 ± 0.17[1]**	**1.87 ± 0.18**	**1.11 ± 0.54[1]**	A* (-0.59)
							N = 140	N = 122	N = 13	
		HW	***4.51***	***0.0119****	***0.0238***$^{++}$	*0.06*	***0.64 ± 0.05[1,2]***	***0.47 ± 0.05[1]***	***0.25 ± 0.16[2]***	*A* (-0.19)*
							N = 140	N = 122	N = 13	
	2B	*HW*	***5.07***	***0.0068****	***0.0544***$^{+}$	*0.04*	***0.58 ± 0.03[2]***	***0.64 ± 0.04[1]***	***0.86 ± 0.08[1,2]***	*A** (0.14)*
							N = 196	N = 123	N = 28	
LDLR	2A									
		BFT	5.43	0.0206	0.1648	0.04	-	-0.65 ± 0.75[§]	-2.45 ± 0.26[1]	Not estimated
								N = 27	N = 245	
	2B									
		FCR	4.98	0.0263	0.2893	0.09	-	-0.13 ± 0.02[§]	-0.17 ± 0.01[1]	Not estimated
								N = 37	N = 308	
CNN1	2B	FCR	3.48	0.0319*	0.1755	0.10	-0.15 ± 0.01[1]	-0.17 ± 0.01[1]	-0.19 ± 0.02	NS

						N = 150	N = 157	N = 21	
DRIP	3.86	0.0221*	0.1755	0.10	67.29 ± 1.60[a]	68.20 ± 1.621	79.57 ± 4.16[1,a]	A** (6.14)	
						N = 150	N = 157	N = 21	

EBV – estimated breeding value, BFT – EBV for back fat thickness, FCR – EBV for feed conversion ratio, LC – EBV for weight of lean cuts, ADG – EBV for average daily gain, HW – EBV for hams weight, pH_1 – pH measured at about 1 h post mortem, DRIP – drip loss.

R^2 – the proportion of variability that is accounted for by the factors of variation included by the statistical model.
* nominal $P < 0.05$, ** nominal $P < 0.01$, *** nominal $P < 0.001$.
$^{+}$ adjusted $P < 0.10$, $^{++}$ adjusted $P < 0.05$; adjusted P value = P value of the F test adjusted for multiple testing. Reverse allele effects in subgroups 2A and 2B for BFT and HW are given in italics.
From *t*-test, matching capital letters indicate differences significant at level $P < 0.001$; matching small letters indicate differences significant at level $P < 0.01$; matching numbers indicate differences significant at level $P < 0.05$. A – additive mode of inheritance, D – dominant mode of inheritance (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).
(§) The allele substitution effect is indicated in parenthesis.

Fig. 1. Physical and linkage map of the central part of porcine chromosome 2 harboring QTLs for carcass and meat quality traits. Microsatellite markers are in bold.

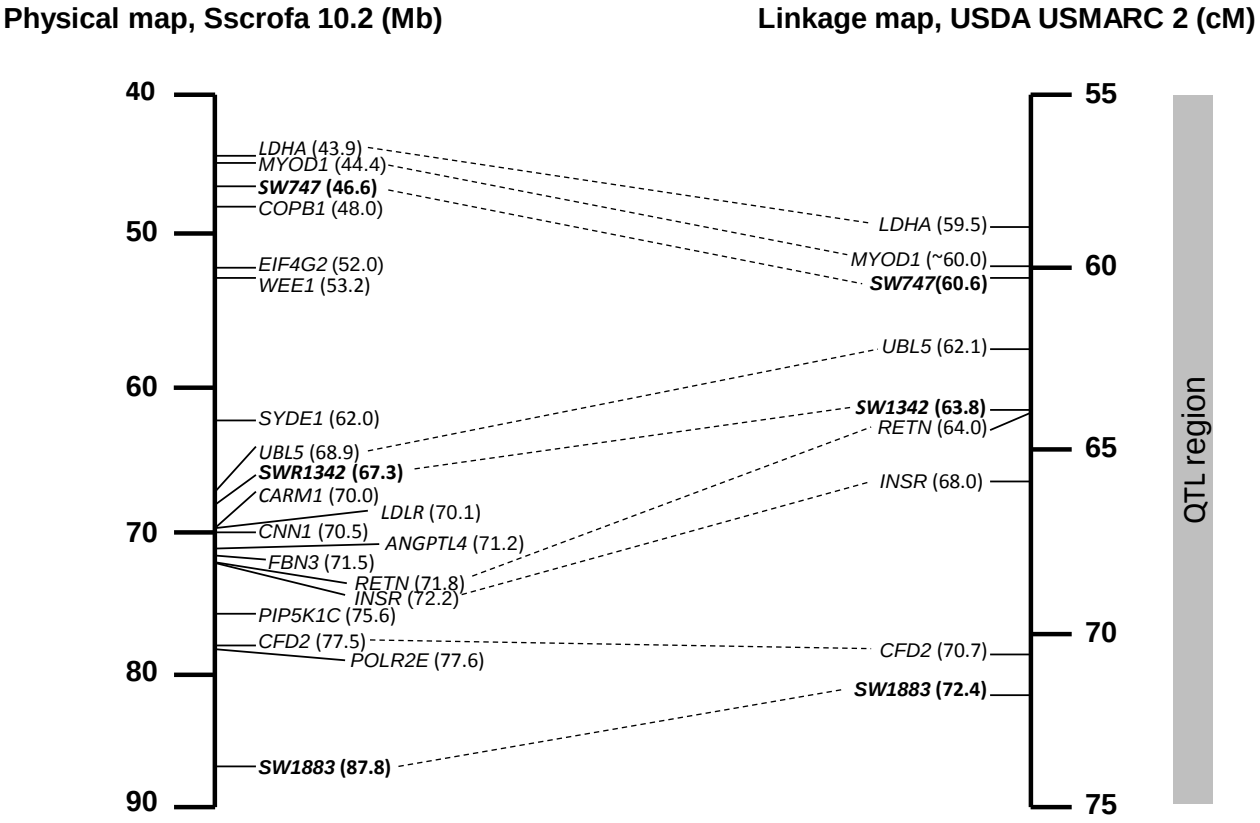

Table S1

Means of estimated breeding value (EBV) and age at slaughter and their standard deviations (SD) for the five traits studied in Group 1 of Italian Large White pigs that consisted of two divergent groups of performance-tested animals with extreme EBVs for ADG.

Group 1 (N)	Trait	Mean	SD	Minimum	Maximum
High tail for ADG (100)	ADG	105.42	9.85	94.00	133.00
	BFT	–6.95	2.64	–12.50	1.30
	LC	4.55	1.45	0.78	8.70
	HW	0.85	0.48	0.14	2.25
	FCR	–0.51	0.11	–0.84	–0.26
	AGE	234.34	10.58	205.00	258.00
Low tail for ADG (100)	ADG	–51.09	11.41	–104.00	–40.00
	BFT	–1.29	4.71	–14.30	9.20
	LC	–1.42	1.58	–4.40	2.77
	HW	–0.88	0.50	–2.39	0.22
	FCR	0.35	0.20	0.08	1.15
	AGE	253.44	8.33	227.00	274.00

ADG – average daily gain, BFT – back fat thickness, LC – weight of lean cuts, HW – ham weight, FCR – feed conversion ratio, AGE – age at slaughter.

Table S2

Genes with genotyped SNP, positions on physical map Sscrofa10.2, genotypes and allele frequencies in Group 1 of Italian Large White pigs (N = 200) consisting of two divergent groups of performance-tested animals with extreme EBVs for ADG.

Gene	Mb	Group (*)	N	Genotypes			Allele frequencies	
				11	12	22	1	2
LDHA	43.9	+	89	22	43	24	0.489	0.511
		–	91	52	35	4	0.764	0.236
MYOD1	44.4	+	54	18	26	10	0.574	0.426
		–	47	10	24	13	0.468	0.532
COPB1	48.0	+	79	20	38	21	0.494	0.506
		–	89	39	35	15	0.635	0.365
EIF4G2	52.0	+	13	13	0	0	**1.000**	**0.000**
		–	15	14	1	0	**0.967**	**0.033**
WEE1	53.2	+	19	19	0	0	**1.000**	**0.000**
		–	29	28	1	0	**0.983**	**0.017**
SYDE1	62.0	+	12	12	0	0	**1.000**	**0.000**
		–	20	20	0	0	**1.000**	**0.000**
UBL5	68.9	+	86	59	24	3	0.826	0.174
		–	90	30	40	20	0.556	0.444
CARM1	70.0	+	14	14	0	0	**1.000**	**0.000**
		–	29	25	4	0	0.931	0.069
LDLR	70.1	+	50	0	1	49	0.010	0.990
		–	50	0	8	42	0.080	0.920

CNN1	70.5	+	89	17	50	22	0.472	0.528
		–	92	43	42	7	0.696	0.304
ANGPTL4	71.2	+	10	10	0	0	**1.000**	**0.000**
		–	10	8	2	0	0.900	0.100
FBN3	71.5	+	20	8	12	0	0.700	0.300
		–	28	16	10	2	0.750	0.250
RETN	71.8	+	17	17	0	0	**1.000**	**0.000**
		–	10	9	1	0	**0.950**	**0.050**
INSR	72.2	+	19	19	0	0	**1.000**	**0.000**
		–	20	19	1	0	**0.975**	**0.025**
PIP5K1C	75.6	+	8	6	2	0	0.875	0.125
		–	19	6	13	0	0.658	0.342
CFD2	77.5	+	17	16	1	0	**0.971**	**0.029**
		–	28	26	2	0	**0.964**	**0.036**
POLR2E	77.6	+	18	18	0	0	**1.000**	**0.000**
		–	22	22	0	0	**1.000**	**0.000**

(*) + = high tail for ADG EBVs (N = 100), – = low tail for ADG EBVs (N = 100).

Genes with minor allele frequency ≤ 0.05 are in bold.

Table S3

Results of association analyses between gene-tagged SNPs and EBV for carcass and performance traits in Group 1 of Italian Large White pigs that consisted of two divergent groups of performance-tested animals with extreme EBVs for ADG.

Gene	M bp	N	ADG				BFT				LC				HW				FCR			
			P	R2	P Add	Add	*P*	R2	P Add	Add	*P*	R2	P Add	Add	*P*	R2	P Add	Add	*P*	R2	P Add	Add
LDHA	43.9	187	**<.0001**	0.16	<.0001	44.61	**0.0011**	0.07	0.0015	–1.588	**0.0001**	0.11	0.0001	1.42	**0.0034**	0.06	0.0016	0.34	**<.0001**	0.14	<.0001	–0.23
MYOD1	44.4	101	NS				NS				NS				NS				NS			
UBL5	68.9	179	**<.0001**	0.16	<.0001	–44.43	0.1100				**0.0008**	0.08	0.0005	–1.34	**0.0019**	0.07	0.0008	0.39	**<.0001**	0.15	<.0001	0.25
LDLR (*)	70.1	180	**0.0061**	0.04	–	–	0.6676				0.0340	0.09	–	–	**0.0058**	0.04	–	–	**0.0082**	0.04	–	–
CNN1	70.5	187	**<.0001**	0.12	<.0001	38.54	0.1986				0.0194	0.04	0.0198	0.85	**0.0031**	0.06	0.0009	0.37	**<.0001**	0.14	<.0001	–0.25

EBV – estimated breeding value, ADG – average daily gain, BFT – back fat thickness, LC – weight of lean cuts, HW – hams weight, FCR – feed conversion ratio.

R^2 – the proportion of variability that is accounted for by the factors of variation included by the statistical model, *P* Add – significance of additive effect, Add – allele substitution effect.
P < 0.01 are in bold.

(*) for *LDLR* one homozygote class was not detected, and for this reason the allele effect was not calculate

Chapter 2

SNPs detection in DHPS-WDR83 overlapping genes mapping on porcine chromosome 2 in a QTL regions for pig meat pH

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Abstract

Background

The pH is an important parameter influencing technological quality of pig meat, a trait affected by environmental and genetic factors. Several quantitative trait loci associated to meat pH are described on PigQTLdb but only two genes with influence on this parameter have been so far detected: Ryanodine receptor 1 and Protein kinase, AMP-activated, gamma 3 non-catalytic subunit. To search for genes influencing meat pH we analyzed genomic regions with quantitative effect on this trait in order to detect gene-tagged SNPs to utilize for an association study.

Results

The expressed sequences located within regions associated to pork pH mapping on porcine chromosomes 1, 2, 3, and selected after a bibliography survey, were searched *in silico* to find SNPs. 356 out of 617 detected SNPs were used to genotype Italian Large White pigs and to perform an association analysis with meat pH values recorded in semimembranosus muscle at about 1 hour (pH1) and 24 hours (pH24) post mortem.

The results of the analysis preformed using PLINK software showed that 5 markers mapping on chromosomes 1 and 3 were associated with pH1 and 10 markers mapping on chromosomes 1 and 2 were associated with pH24. After Bonferroni correction only one SNP mapping on chromosome 2 was confirmed to be associated to pH24. This polymorphism was located in the 3'UTR of two partly overlapping genes, Deoxyhypusine synthase (DHPS) and WD repeat domain 83 (WDR83), coded on opposite DNA strands. The overlapping of the 3'UTRs allows the co-regulation of the stability of both mRNAs by a cis-natural antisense transcript method of gene regulation. WDR83 has an important role in the modulation of a cascade of genes involved in cellular hypoxia defense by intensifying the glycolytic pathway to produce ATP.

Conclusions

Several DNA polymorphisms located in QTL regions in porcine chromosomes 1, 2, and 3 associated to pig meat pH have been analysed combining an *in silico* approach with SNP genotyping and association study with production traits. On chromosome 2 a SNP affecting meat pH was identified that was located in the common part of two partially overlapping genes (*DHPS* and *WDR83*) suggesting that glycolisis in muscle cells during post mortem phase and meat pH phenotypic variability could be influenced by molecular and biological processes involving one or both genes and the SNP detected in the overlapping 3' region. This finding, after validation can be applied to identify new biomarkers to be used to improve pig meat quality.

Keywords

Swine, meat pH, single nucleotide polymorphism, DHPS, WDR83, overlapping genes.

Background

Meat pH is an important parameter for the quality assessment of fresh and seasoned meat products (Russo and Nanni Costa, 1995). This trait is influenced by environmental factors mainly due to pre-slaughter events and it is also under genetic control. Phenotypic variation of meat pH is partially regulated by genes as indicated by Sellier (1998) who reported 0.16 as the heritability value for pH scored at about 1 hour *post mortem* (pH1) and 0.21 for pH recorded at 24 hours *post mortem* (pHu). Other researches showed that the heritability of pHu in Large White ranged from 0.29 to 0.45 (Nguyen *et al*., 2006; Rosendo *et al*., 2010). Up to now only two major genes related to pig meat pH have been identified: Ryanodine receptor 1 (*RYR1*) mapped on *Sus scrofa* chromosome (SSC) 6 (Fujii *et al*., 1991) and Protein kinase, AMP-activated, gamma 3 non-catalytic subunit, (*PRKAG3*), located on SSC13 (Milan *et al*., 2000). In addition to these evidences showing an effect of single genes, other researches reported significant Quantitative Trail Loci (QTL) for meat pH in several porcine chromosomes as showed in Pig QTL database (PigQTLdb http://www.animalgenome.org/cgi-bin/QTLdb/SS/index; Hu *et al*., 2007; Hu *et al*., 2010).

With the aim to identify genes responsible for QTL effect on pig meat pH, single nucleotide polymorphisms (SNPs) detected in the transcribed sequences of coding genes located on three QTL regions (QTLRs) of SSC1 (60-80 cM), SSC2 (55-66 cM) and SSC3 (42-60 cM), were utilized to perform an association analysis with meat pH values.

Results and discussion

SNPs detected in transcribed sequences located within the selected QTL regions

The comparison of the three selected chromosomal regions of porcine genomic sequence 10.2 (Groenen et al., 2012) with human genome showed synteny between the studied segments of SSC 1, 2, and 3 with regions of different human chromosomes. The QTLR located on SSC1 is homologous with part of human chromosomes 14, 15, and 18; the QTLR on SSC2 is correspondent to part of human chromosomes 11 and 19; and the QTLR mapped on SSC3 is aligned with part of human chromosomes 2, 9, and 16 (Table 1).

By multiple alignment of the sequences of the selected UniGene entries we localised 2409 clusters containing both mRNAs and ESTs sequences and, after filtering, we retained 1822 clusters (Table 1). Among them we detected 353 UniGene clusters containing SNPs. On the whole 617 SNPs were found and, after removing those separated by less than 80 nucleotides, the remaining ones were 379 that decreased to 356 after to have checked their suitability to be used on the GoldenGate system with a score >0.6 (designability rank = 1).

The position of the selected SNPs was precisely defined on porcine genome (version 10.2) allowing to align the location of the studied QTLR based on the linkage map to the physical map (Table 2). On SSC1 the examined region was 106.9-215.8 Mb, on SSC2 the considered segment was 32.7-77.9 Mb, and on SSC3 we analysed the region 18.7-62.6 Mb. By comparing the physical length of the three chromosome portions and the number of SNPs detected within each of them we observed that SNPs are not evenly distributed and the average interval between adjacent polymorphisms is always less than 1Mb. There are differences

among chromosomes with intervals ranging from 0.26 Mb on SSC2 to 0.77 Mb on SSC1. On the whole, the utilised approach for the detection of SNPs on transcribed sequences allowed to place approximately two to four markers for each cM assuming that 1 cM corresponds, on average, to a segment of 1.3 Mb. This correspondence was obtained by comparing the total nucleotide length of the porcine genome of 3,024,658,544 nt reported on ENSEMBL web site (http://www.ensembl.org/Sus_scrofa/Info/Annotation/#assembly) with the length of the linkage map reported by Roher *et al*. (1996) represented by 2286 centi Morgan. This marker density is higher than that utilized in QTL studies based on microsatellite markers found in literature with the possibility to define a more accurate mapping of the considered regions.

Identification of genes containing SNPs associated with meat pH

The SNPs detected by the *in silico* search were used to identify markers associated with meat pH in the three genomic regions studied. Results highlighted five markers significant at a nominal P-value <0.01 for pH1 (Table 3) and ten markers significant at the same nominal P-value for pHu (Table 4).

Out of the five markers associated with pH1 values, two SNPs detected on the same gene were mapped on SSC3 and three were identified on SSC1. Two of the SNPs located were originated by two different UniGene clusters but they correspond to the same gene. On the whole, the five SNPs associated to pH1 were detected in three genes that are listed here from the most significant to the less significant: 8E_018 and 8E018a (*KDM3A*), 2M_060 and 2M_059 (*EPB42* / *LOC100525673*), 2M_040 (*SPINT1*). For each gene the gene name, the chromosome localization and the position on the sequence are reported on Table 3. The markers associated with pHu values were mapped on SSC1 and SSC2 and the three most significant located on chromosome 2. The genes corresponding to the markers associated to pHu are (from the most significant to the least significant): 5E_003 (*DHPS* / *LOC100519413*), 5M_105 (Uncharacterized *LOC100513647*), 5M_011 (*FARSA* / *LOC100524304*), 2M_075 (*HERC1*), 5M_006 (*COL5A3*), 3M_020c and 3M_020 (*ACTR10* / *LOC100620619*), 5E_019 (*BRD2*), 5M_024 (*TRMT1*), 5M_032 (*MAN2B1* / LOC100518647). For each marker the gene name, the chromosome localization and the position on the sequence are reported on Table 4.

After Bonferroni correction for multiple testing only the SNP related to pHu that was found in the *DHPS* gene remained significant (P = 0.01937).

Using this marker we genotyped the Group 2 of pigs to analyse the additive effect of the SNP on the studied trait. The most frequent genotype was the homozygous TT (228 out of 311 tested pigs) while the frequency of the rarest C allele was 0.15 (Table 5). The TT pigs showed lower values of pHu than TT and TC animals and the difference with the other homozygous group (CC) was of 0.13 unit of pH (additive effect of 0.065 pHu unit, P<0.05). In view of the scarce number of CC genotypes detected (N=11) we performed an additional analysis considering together the TC and CC genotypes. This analysis confirmed the previous results showing a difference between the TT and the TC+CC pigs (data not showed).

Genomic characterization of the most significant SNP detected

By checking the UniGene cluster in which the SNP was detected, we found that the coded gene was Deoxyhypusine synthase-like (*DHPS*) mapped on a region of SSC2 homologous to human chromosome 19p13.2. Analysing the *Sus scrofa* 10.2 genomic sequence we found that the polymorphism was located on the ninth and last exon of the gene within the 3' untraslated region (3'UTR), nine nucleotides after the stop codon (Figure 1). The point mutation detected was located at nucleotide 66,686,842 of the current sequence of porcine chromosome 2 (g.66686842 T>C). This gene is a catalyzer of the first step of the reactions necessary to obtain Hypusine-eIF5A complex. This reaction is a posttranslational modification characterized by the transfer of the aminobutyl moiety of polyamine spermidine to one specific lysine residue of eIF5A precursor, to form an intermediate deoxyhypusine residue (Park *et al*., 2010). This intermediate product is hydroxylate in a second reaction by Deoxyhypusine hydroxylase/monooxygenase (*DOHH*) to obtain Hypusine [Nε-(4-amino-2-hydroxybutyl1)-lysine] that binds to the transcription factor eIF5A and together contributed to regulate cell growth and cell integrity (Jao and Chen, 2006; Wolff *et al.*, 2007).

Visualizing the genomic position of *DHPS* gene using NCBI MapViever we observed that its 3'end overlaps part of the 3'UTR of another gene, coded on the opposite chromosome strand, WD repeat domain-containing protein 83-like (*WDR83 – LOC100519823*). *WDR83*, called also *MORG1* (mitogen-activated protein kinase organizer 1) encodes a member of the WD-40 protein family and belongs to a modular scaffold system responsible of the regulation of extracellular signal-regulated kinase (ERK) pathway that has an important role in the modulation of various cellular processes, including gene expression, cells growth, cellular differentiation and apoptosis. *WDR83* interacts also with Egl nine homolog 3 gene (*EGLN3* also known as prolyl-hydroxylase domain-containing protein 3, *PHD3*): an increase in *WDR83* expression activate or stabilize the *EGLN3* mRNA level (Su *et al.*, 2012). The latter gene, *EGLN3*, in normoxic conditions hydroxylate the product of Hypoxia-inducible-factor 1 alpha subunit (basic helix-loop-helix transcription factor) gene (*HIF1A*) allowing the degradation of HIF1 alpha protein. On the contrary, at lower oxygen concentration *WDR83* expression decreases and *EGLN3* activity is reduced with the consequence of a higher stability of *HIF1A* mRNA allowing the activation of downstream metabolic processes essentials to reduce the effect of low oxygen level in tissues (Hopfer *et al*., 2006; Boulahbel *et al*., 2009) as angiogenesis, erythropoiesis, increased expression of glycolytic enzymes and glucose transporters to produce more energy (ATP) from anaerobic glycolysis.

The presence of two overlapping genes and the finding of a SNP on the shared part of their 3'UTRs may indicate a plausible connection between the roles of the two genes. In humans these genes are included in the group of genes for which was reported a bidirectional regulation of mRNA stability by the natural antisense transcript (NAT) method of regulation. In particular, *WDR83* and *DHPS* are an example of cis-NAT regulation i.e. the two transcripts are partially overlapping in their 3'UTRs, coded in opposite direction by the same DNA stretch (Su *et al.*, 2012). With this system of regulation the mRNA expression and proteins levels are regulated concordantly. The NAT method of regulation was identified in several mammalian genomes (Zhang *et al*., 2006; Su *et al*., 2010). NATs principal functions are to regulate the expression of sense transcripts, hybridize with them, and interfere with mRNA transcription or stability (Katayama *et al.*, 2005; Zhang *et al.*, 2006). Other roles proposed for

NATs are an involvement in DNA methylation, chromatin modification and mono allelic expression. In particular, *DHPS* and *WDR83* are two gene expressed in the same subcellular locations, overlapping in bidirectional relation so *DHPS* can regulate *WDR83* mRNA and protein expression level and vice versa. So, the overexpression of *DHPS* mRNA may result in a significant increase in *WDR83* mRNA too. Researches concerning these two genes are often related to cancer biology (Rossignol *et al*., 2002; Span *et al*., 2011; Su *et al*., 2012).

Little is known about the role of these two genes tissues like in skeletal muscle but a possible involvement may be related to the hypoxic conditions occurring in skeletal muscle due to exercise, stress or in *post-mortem* phase. We described above the role of WDR83 in regulating a gene cascade in normoxic / hypoxic conditions. Hypoxya is a condition that was reported to be present in several conditions: in cancers, when tumor cells growth rapidly their vascular supply become insufficient leading to hypoxia (Gourley *et al*., 2000; Griffioen *et al*., 2000); hypoxic condition occurs in ischemic cardiac myocytes (Graham *et al*., 2004) and in skeletal muscle under exercise (Wagner, 2001). Furthermore, muscle cells in apoptosis undergoes to relevant structural and functional changes involving all muscle metabolic pathways. The oxygen reduction and the energy deficit of *post mortem* phase will lead to hypoxic condition that is associated with acidosis due to the anaerobic glycolysis increase to produce ATP. The augmented glycolysis activity will cause a pH decrease for the lactic acid production until the exhaustion of ATP. The complex *DHPS*/*WDR83* is one of the factors modulating *EGLN3* and then *HIF1A* and the polymorphism detected on the common part of the 3'UTR of the two genes may activate this cascade with different efficiency between alleles to cause pH decline in the skeletal muscle cells during *post-mortem*. In order to validate this hypothesis further researches aimed to clarify and verify the link of the mutation found in the 3'UTR of *DHPS*/*WDR83* genes with meat pH are needed before to consider them as functional candidate genes and not only positional markers for the studied trait.

Conclusions

In the present work we studied QTLRs of 10-20 cM and detected some hundreds of SNPs that allowed a more refined analysis of these regions. In particular, a SNP found in the 3'UTR of *DHPS*/*WDR83* overlapping genes was associated with the ultimate pH of pig meat. The result confirms the localization of the QTL for this trait reported in literature and allowed to identify genes mapping on the QTL region of SSC2 for pork ultimate pH. If the association with meat pH of the detected marker will be confirmed it could represent a new biomarker useful to improve pig meat quality.

Methods

Animals, phenotypes and DNA extraction

For this study we sampled a pure-bred population of Italian Large White sib-tested pigs provided by the National Association of Pig Breeders (Associazione Nazionale Allevatori Suini, ANAS; http://www.anas.it). The animals were reared on the central ANAS Sib-Test station from about 30 kg live weight to about 155 kg live weight. The nutritive level utilised was *quasi ad libitum*, i.e. about 60% of pigs were able to ingest the entire supplied ration. For the

genetic evaluation of a boar, full sib triplets (two females and one castrated male) are performance tested. All pigs were slaughtered after electrical stunning in the same commercial abattoir during the year 2008 in 11 different days. Using the 356 SNPs detected in the QTLRs a first association analysis on 251 pigs (170 females and 81 castrated males, Group 1) was performed. The most significant marker was then tested using 251 animals of Group 1 and a larger group of 347 samples (231 females and 116 castrated males). We refer to this enlarged sample as Group 2. The sex distribution of the animals with a ratio females: castrated males approximately equal to 2 reflects the sex proportion characteristic of the Italian selection scheme described above.

Samples of *semimembranosus* muscle were collected at slaughterhouse from the right ham of the 347 animals and immediately frozen in liquid nitrogen. Genomic DNA was extracted from these samples using a standard protocol. Within about 1 hour *post mortem* the meat pH value (pH1) was recorded on the same muscle. Furthermore, at 24 hours *post mortem* the ultimate pH (pHu) was measured. The statistics of the recorded pH values are reported in Tables 6 and 7.

QTL selection, SNP detection and genotyping

We selected the QTL influencing porcine meat pH by searching in literature and by browsing the PigQTLdb. The most relevant QTL and the correspondent genomic positions were chosen according to these rules: a) significant effect described by different Authors and in different swine populations, b) a relevant part of the phenotypic variance explained by the QTL. Three QTLR were selected: SSC1 from 60 to 80 cM, SSC2 from 55 to 66 cM, SSC3 from 42 to 60 cM. A complete list of the literature utilised for the QTL selection is reported on Table 8. Map intervals were defined by searching for the position of the most significant markers reported in each original paper in the USDA-MARC linkage map (http://www.ncbi.nlm.nih.gov/projects/mapview/maps.cgi?TAXID=9823&MAPS=MARC) that includes all available microsatellite and DNA markers so far analysed. Furthermore, this map is implemented both in PigQTLdb and in NCBI map viewer (http://www.ncbi.nlm.nih.gov/projects/mapview/). In this way it was possible a comparison of the data contained in both websites. The alignment of porcine and human chromosomes was first performed using pig and human radiation hybrid maps using the tool available within PigQTLdb website, then the aligned regions were visualized using the NCBI map viewer. The obtained output was used to choose in each QTLR all expressed sequences (both mRNAs and expressed sequence tags, ESTs) located in the identified corresponding genomic regions that were grouped according to UniGene clusters (http://www.ncbi.nlm.nih.gov/unigene). The obtained clusters were filtered to retain only those represented by at least eight sequences, then putative SNPs were searched *in silico* by a multiple alignment of all members of each cluster using BLASTN (http://blast.ncbi.nlm.nih.gov/) with the algorithm MegaBLAST. We marked as putative SNP a mutation detected in at least three sequences to avoid inconsistencies due to sequencing errors and also to exclude SNPs with a rare allele. Moreover, the obtained multiple alignments were manually scored in order to detect those suitable to be analysed by the high throughput Illumina GoldenGate Genotyping Assay system (http://www.illumina.com/technology/goldengate_genotyping_assay.ilmn).When more than

one polymorphism was detected within each cluster we discarded those closer than 80 nucleotides each other because they were not suitable to design the probes to be used with this genotyping system. These SNPs were finally scored with the specific Illumina software to establish the SNP score of each sequence used to calculate the parameter indicated as designability rank. Genotyping of 251 samples of Group 1 was carried out by an outsource company.

Genotypes of the samples included in Group 2 were obtained by High Resolution Melting (HRM), that is an efficient technique to determine a genotype using the melting profile of small amplicons (Wittwer *et al*., 2003; Liew *et al*., 2004). For this aim, primers flanking the polymorphism were designed, to amplify a 92 bp fragment (For: 5'-TTATGCCCGAAAAGAACGAG-3', Rev: 5'- GGCTGGGTCTGCAAACTAAT -3'). Amplifications were performed with Rotor-Gene TM 6000 (Corbett Research, Mortlake, New South Wales, Australia), in a total volume of 20 µl containing 2 µl of 10X standard buffer, 3 mM MgCl2, 0.3 µM of each primer, 160 µM dNTP, 1 U EuroTaq polymerase, 1 U EvaGreen TM (Biotium Inc., Hayward, CA, USA) and 50–100 ng of template DNA. Cycling conditions were: initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 30 s, 56°C for 15 s and 72°C for 2 min, followed by a final extension step of 72°C for 2 min. Fluorescence was acquired at the end of each extension step to ensure that all reactions reached the plateau stage. After a holding step at 50°C for 60 s, a HRM analysis was performed heating the samples from 83 to 88°C, at a rate of 0.1°C each 4 s, with continuous fluorescence acquisition. The HRM data were analysed by Rotor-Gene TM 6000 software. Fluorescence vs. temperature plots were normalized by selecting linear regions before and after the melting transition. Genotypes were determined setting known genotypes samples as reference and using a reliability threshold of 0.90 for the genotype assignment.

Statistical analyses

The preliminary association study including the markers detected within the analysed QTLRs was performed with PLINK whole genome association analysis toolset (http://pngu.mgh.harvard.edu/~purcell/plink/; Purcell *et al*., 2007). The genotypes of animals belonging to Group 1 were filtered before the association analysis. All markers having a minor allele frequency below 0.01 (N=162) were discarded. Furthermore Hardy-Weinberg equilibrium was tested and four SNPs were discarded because not in equilibrium ($p<=0.01$). After filtering, 218 markers and all 251 individuals (Group 1) were retained. To correct for stratification of the considered population a clustering method based on an identical by state (IBS) approach included in PLINK was used. Furthermore, a stratified association analysis was performed using the Cochran-Mantel-Haenszel test implemented in PLINK. The significant markers were further assayed for multiple testing using the Bonferroni correction.

The most significant marker detected by PLINK was further analysed to validate the association between pH values and the genotypes scored on Group 2 of pigs using the MIXED procedure of SAS release 9.2 (SAS, Institute Inc., Cary, NC). The model included genotype of the analysed markers and sex as fixed effects and day of slaughter and sire as random effects:

$pHu = \mu + SNP_i + Sex_j + Day + Sire + \varepsilon_{ij}$

where: pHu = ultimate pH; μ = overall mean; SNP = fixed effect of each genotype (i = 1-3); Sex = fixed effect of sex (j = 1,2); Day = random effect of day of slaughter; Sire = random effect of the sire; ε = residual error.

List of abbreviations

3'UTR: 3' untraslated region

ANAS: Associazione Nazionale Allevatori Suini

EST: Expressed Sequence Tag

QTL: Quantitative Trait Locus

QTLR: Quantitative Trait Locus region

SNP: Single Nucleotide Polymorphism

SSC: *Sus scrofa* chromosome

Cited websites

ANAS: http://www.anas.it

BLASTN: http://blast.ncbi.nlm.nih.gov

ENSEMBL: http://www.ensembl.org/Sus_scrofa/Info/Annotation/#assembly

Illumina GoldenGate Genotyping Assay:
(http://www.illumina.com/technology/goldengate_genotyping_assay.ilmn

NCBI map viewer: http://www.ncbi.nlm.nih.gov/projects/mapview

PigQTLdb: http://www.animalgenome.org/cgi-bin/QTLdb/SS/index

PLINK: whole genome association analysis toolset
http://pngu.mgh.harvard.edu/~purcell/plink/

UniGene: http://www.ncbi.nlm.nih.gov/unigene

USDA-MARC linkage map:
http://www.ncbi.nlm.nih.gov/projects/mapview/maps.cgi?TAXID=9823&MAPS=MARC

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PZ performed the *in silico* and statistical analyses and drafted the manuscript; MB, LFdP and SB carried out the molecular genetic analysis; LB and MG contributed providing samples and

genetic indexes; VR and RD participated in the design of the study, coordinated the project and contributed both to draft and to revise the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Summary of the steps utilized to identify the genotyped SNPs.

Porcine chromosome	Homologous human chromosome	No of porcine UniGene clusters whitin QTLR	No of selected porcine UniGene clusters	No of porcine UniGene clusters containing SNPs	No of useful SNPs within QTLR
1	18	232	154	23	34
	15	224	157	41	53
	14	331	250	37	54
2	11	232	188	35	67
	19	563	444	83	104
3	16	425	340	61	35
	9	105	81	2	2
	2	297	208	71	30
Total		2409	1822	353	379

Table 2. Average SNPs distance within each of the three analyzed QTLRs.

Chromosome	QTLR (cM)	QTLR (Mb)	SNPs (No)	Average distance within each QTLR (Mb)
1	20 (60-80)	108.9 (106.9-215.8)	141	0.77
2	11 (55-66)	45.2 (32.7-77.9)	171	0.26
3	18 (42-60)	43.9 (18.7-62.6)	67	0.66

Table 3. Significant markers detected by association analysis with pH1 values using PLINK.

SNP (*)	Gene symbol	Gene name	Chromosome	Mb	P	
					UNADJ	BONF
8E_018	*KDM3A*	lysine-specific demethylase 3A	3	61.06	0.000837	0.1816
8E_018a			3	61.06	0.000837	0.1816
2M_060	*EPB42 / LOC100525673*	E3 ubiquitin-protein ligase UBR1-like	1	143.13	0.006212	1
2M_059a			1	143.13	0.00659	1
2M_040	*SPINT1*	serine peptidase inhibitor, Kunitz type 1	1	145.62	0.009615	1

(*) in this column the laboratory codes of the UniGene cluster selected are indicated

UNADJ = nominal p-values

BONF = p-values after Bonferroni correction

Table 4. Significant markers detected by association analysis with pH2 values using PLINK.

SNP (*)	Gene	Gene name	Chromosome	Mb	P	
					UNADJ	BONF
5E_003	*DHPS / LOC100519413*		2	66.69	8.926e-005	**0.01937**
5M_105	LOC100513647		2	65.38	0.001777	0.3857
5M_011	*FARSA/ LOC100524304*		2	66.28	0.002815	0.6109
2M_075	*HERC1*		1	119.56	0.004006	0.8693
5M_006	*COL5A3*		2	69.16	0.005301	1
3M_020c	*ACTR10 / LOC100620619*		1	208.21	0.007129	1
3M_020			1	208.21	0.008583	1
5E_019	*BRD2*		2	61.87	0.008863	1
5M_024	*TRMT1*		2	66.24	0.00914	1
5M_032	*MAN2B1 / LOC100518647*		2	66.72	0.009565	1

(*) in this column the laboratory codes of the UniGene cluster selected are indicated

UNADJ = nominal p-values

BONF = P values after Bonferroni correction

Table 5. Association analysis of 5E_003 (*DHPS*) SNP.

No	F	P	LSM ± SE (*)			Additive effect	P (Add.)	Dominance effect
			TT (No)	TC (No)	CC (No)			
311	4.00	0.012	5.749±0.024[a] (228)	5.822±0.032[b] (72)	5.879±0.067[b] (11)	0.065±0.033	0.047	NS

(*) Different superscript letters indicated differences in the estimated pHu values between pairs of genotype class significant at P<0.05.

LSM = Leas Square Means

SE = standard error

NS = not significant

Table 6. Statistic describing the muscle pH1 values measured in semimembranosus muscle recorded on Group 1 and in Group 2 of Italian Large White pigs.

	Group 1					Group 2				
	No (*)	Average	SD (**)	Min	Max	No (*)	Average	SD (**)	Min	Max
All	248	6.19	0.27	5.37	6.78	347	6.21	0.27	5.37	6.91
Females	167	6.19	0.29	5.37	6.78	231	6.21	0.28	5.37	6.91
Castrated males	81	6.20	0.22	5.72	6.69	116	6.21	0.23	5.72	6.81

(*) pH1 values are not available for all samples

(**) SD = standard deviation

Table 7. Statistic describing the muscle pHu values measured in semimembranosus muscle recorded on Group 1 and in Group 2 of Italian Large White pigs.

	Group 1					Group 2				
	No	Average	SD (**)	Min	Max	No (*)	Average	SD (**)	Min	Max
All	251	5.76	0.23	5.40	6.65	313	5.77	0.23	5.24	6.65
Females	170	5.73	0.21	5.40	6.47	209	5.73	0.21	5.24	6.47
Castrated males	81	5.84	0.24	5.50	6.65	104	5.84	0.25	5.43	6.65

(*) pHu values are not available for all samples

(**) SD = standard deviation

Table 8. List of the genetic crosses and the corresponding bibliographic reference utilized to identify the QTL regions used for this research.

Chromosome	Breeds	Reference
SSC1	Duroc x Berlin Miniature	Ponsuksili *et al*. (2005)
SSC1	Duroc x Pietrain	Liu *et al*. (2007)
SSC2	Meishan x Pietrain	Lee *et al*. (2003)
SSC2	Large White	De Koning *et al*. (2003)
SSC2	Duroc x Landrace	Rohrer *et al*. (2006)
SSC2	Duroc x Pietrain	Jennen *et al*. (2007)
SSC2	Duroc x Pietrain	Liu *et al*. (2007)
SSC2	White Duroc x Erhualian resource population	Duan *et al*. (2009)
SSC2	Commercial crossbred population	Heuven *et al*. (2009)
SSC3	Wild Boar x Pietrain	Beeckman *et al*. (2003)
SSC3	Iberian x Landrace	Ovilo *et al*. (2002)
SSC3	Duroc x Berlin Miniature	Wimmers *et al*. (2005)
SSC3	Duroc x Pietrain	Edwards *et al*. (2008)

Figures

Figure 1 – Exon-intron structure of *DHPS* gene and position of the detected SNP (g. 66,686,842T>C) in the 3' untraslated region present in exon 9. The stop codon and the polyadenylation signal are underlined.

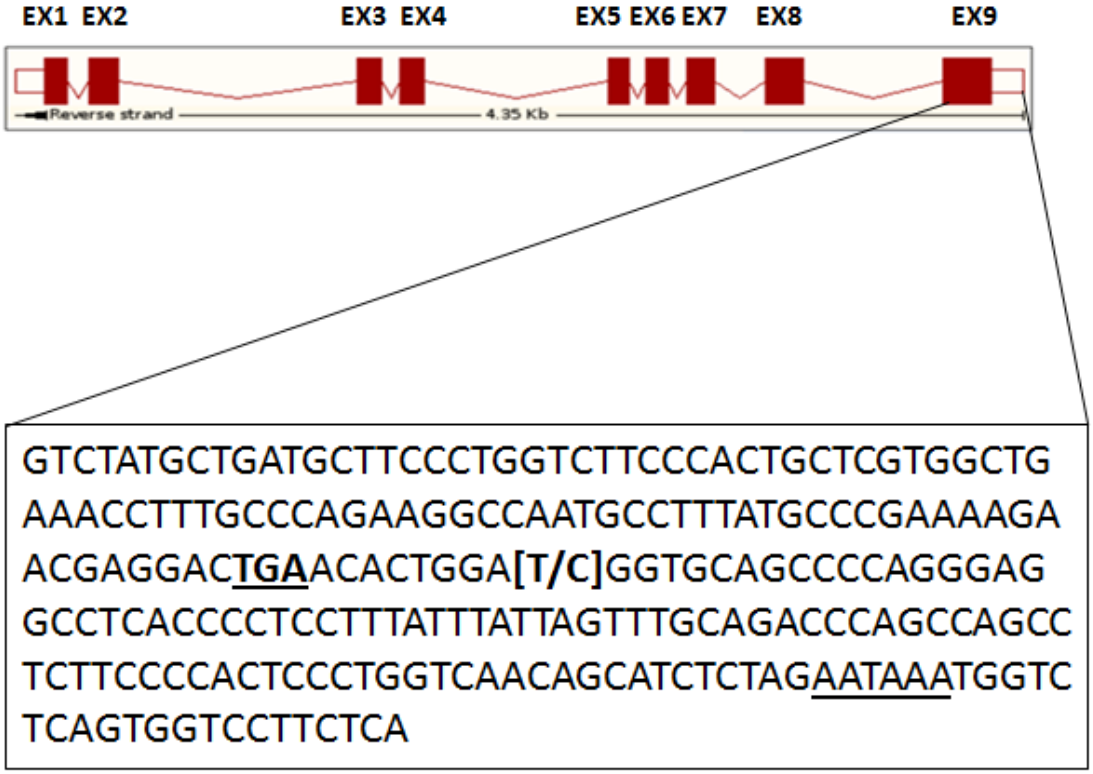
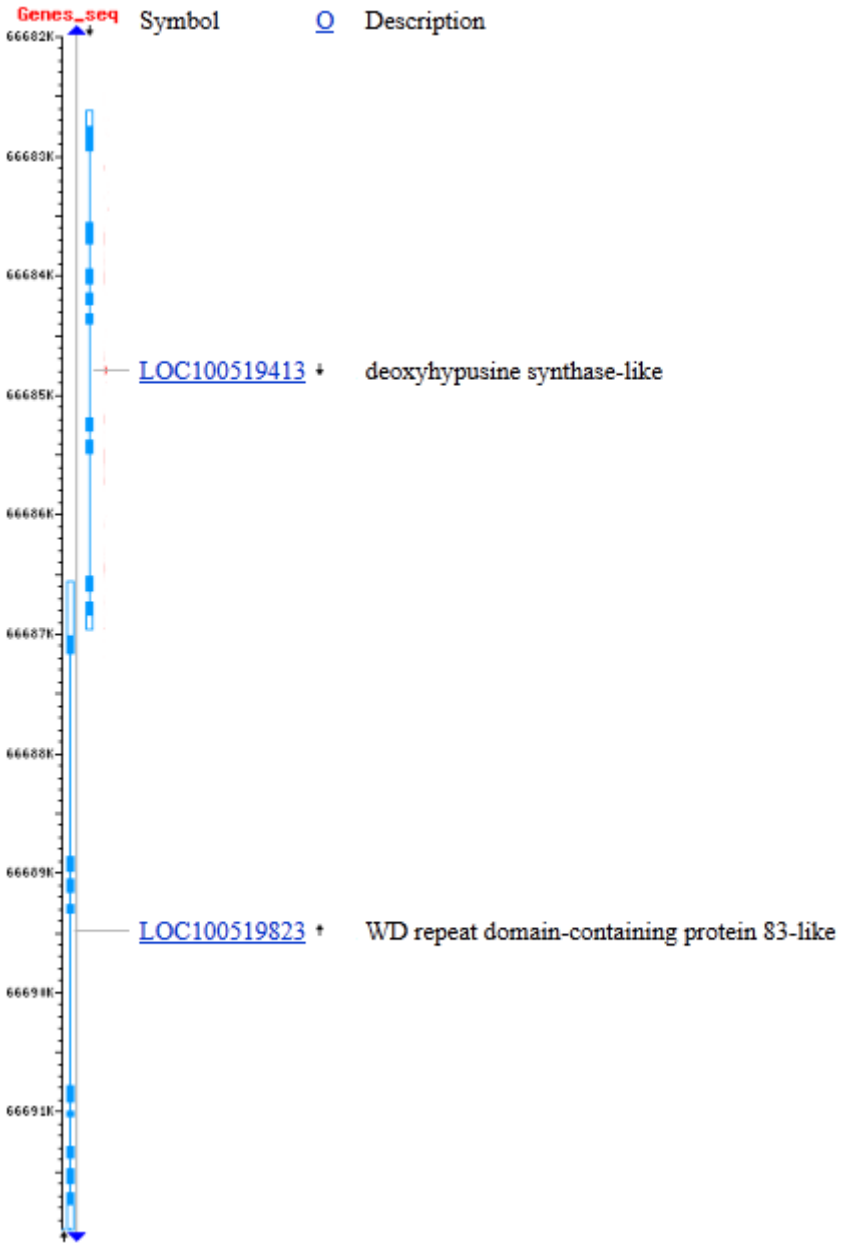
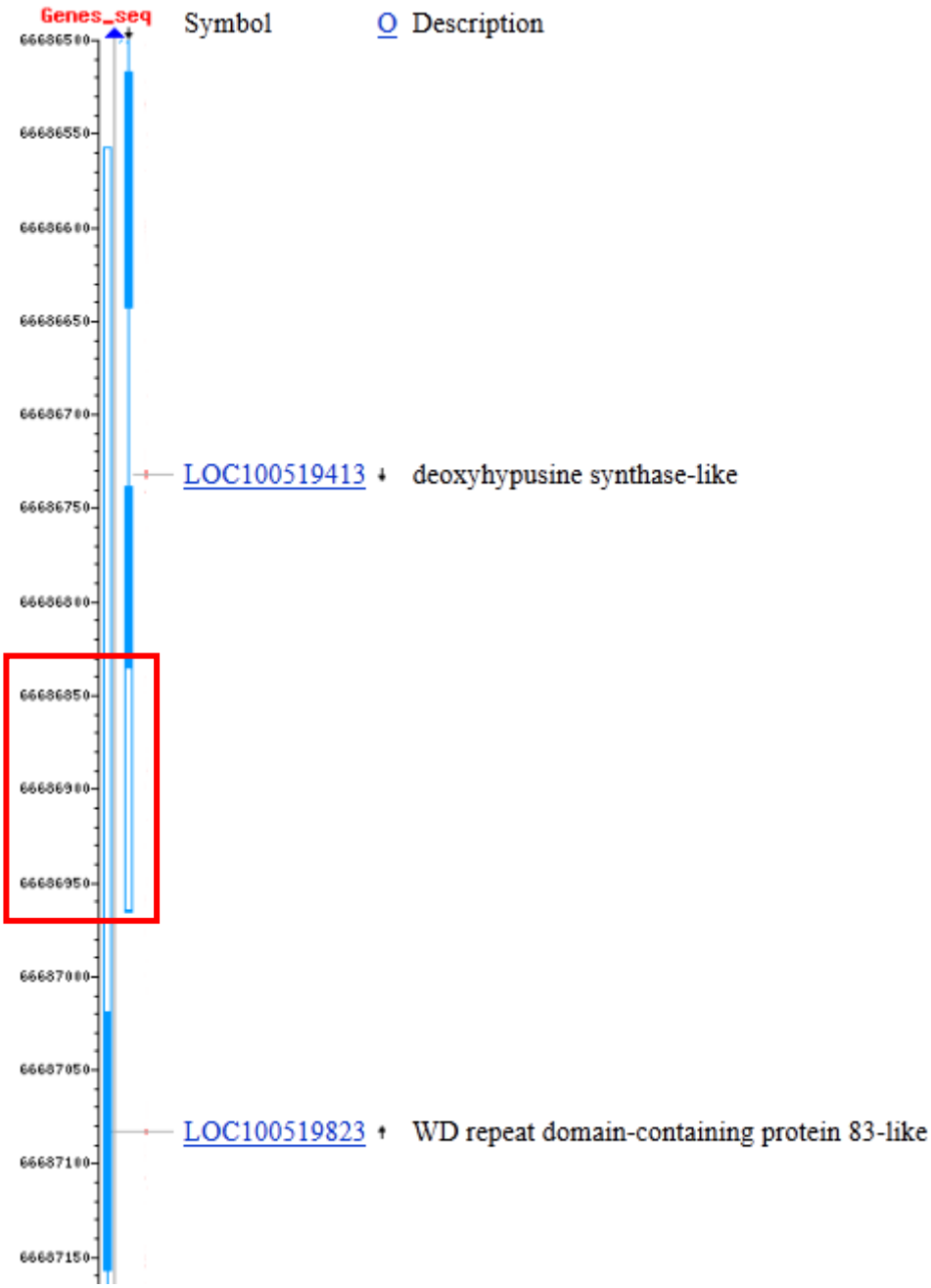


Figure 2 - Complexity of the region of *DHPS* and *WDR83* genes in pigs. The two genes are coded on opposite strands of porcine chromosome 2 at about 66.7Mb of the porcine genomic sequence 10.2.

A. Region of porcine chromosome 2 showing the two partially overlapping genes *DHPS* and *WDR83*.



B. Detail of the overlapping 3'UTRs of *DHPS* and *WDR83* genes. The 3' untraslated region of *DHPS* gene and the corresponding part of the 3' untraslated region of *WDR83* gene are included in the red box.



Chapter 3

Relationship between porcine perilipin genes polymorphisms and body weight and composition[1]

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Submited

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ABSTRACT

The perilipins (**PLIN**) belong to a family of structural proteins that coat the intracellular lipid droplets where they play a role regulating intracellular lipid storage and mobilization. Studies in mice and human have linked these proteins to BW, insulin resistance, and obesity. In the present study, *PLIN1* and *PLIN2* have been evaluated as candidate genes for growth, carcass, and meat quality traits in pigs. Two independent populations of Italian (n=429) and Spanish (n=268) Duroc pigs were used. Phenotypic data on the Spanish pigs were used to validate the results obtained in the Italian pigs, for which their estimated breeding values were available. All the 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*). No evidence of association with growth, carcass, and meat quality traits was found for the *PLIN1* polymorphism in the two Duroc populations. In contrast, the allele A at the *PLIN2* polymorphism was positively associated in both populations to ADG, particularly at earlier ages in Spanish Duroc, and lean weight, but not to lean percentage and intramuscular fat content. The additive effect of A for G at the *PLIN2* polymorphism in the Spanish Duroc for BW was 2.4 kg (at 120 d, $P<0.01$) and 1.9 kg (at 205 d, $P=0.02$). As a result, pigs carrying an additional copy of the allele A at the *g.98G>A PLIN2* polymorphism were longer (0.65 cm, $P<0.01$) and showed higher lean weight (0.88 kg, $P=0.04$). Thus, the *PLIN2* polymorphism can be a useful marker for lean growth. In particular, it may help to reduce the undesired negative correlated response on BW to selection for increased intramuscular fat content, a common scenario in some Duroc lines involved in the production of high quality pork products.

Key Words: body weight, composition, Duroc, fat, genetic markers, swine

INTRODUCTION

Growth rate and carcass lean content are crucial characteristics for economic viability of pork production. Selection emphasizing lean content has led to reduce some pork quality attributes, including intramuscular fat (**IMF**) content. The use of molecular markers may improve genetic progress in traits difficult and expensive to measure (Dekkers, 2004), but also to break down unfavorable genetic correlations between antagonistic traits, such as growth rate, carcass lean content and IMF content (Ros-Freixedes et al., 2012; 2013). In this regard, association studies with candidate genes affecting fat metabolism are of particular interest.

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

So far only two reports in pigs investigated the association of *PLIN1* and *PLIN2* SNPs with production traits. In the first report, two synonymous SNP of *PLIN1* showed suggestive associations with 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 the present 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 to confirm whether

PLIN1 and *PLIN2* genotype variants exert a differential effect on lean growth and IMF content.

MATERIALS AND METHODS

Animals, sample collection and traits

A panel of 20 unrelated pigs from three breeds was used for the SNP screening of *PLIN1* gene, including eight Italian Large White, four Italian Duroc and eight Italian Landrace pigs.

Two independent Duroc resource populations were used for the association analyses. The first was the Italian Duroc (**IDU**) managed by the Italian Association of Pig Breeders (www.anas.it), while the second was a commercial Spanish Duroc (**SDU**) line (Ros-Freixedes et al., 2012). In the IDU population, 139 pigs (95 females and 44 barrows, sampled over 11 slaughter days within the same year) and 429 pigs (292 females and 137 barrows, sampled over 119 slaughter days throughout 13 years) were used for the association analyses of *PLIN1* and *PLIN2* polymorphisms, respectively. All pigs were tested from 30 to 155 kg for genetic evaluation purposes at a Central Test Station, where they were fed a *quasi ad libitum* standard diet. For these pigs, the estimated breeding value for the following traits was available: ADG (calculated from 30 to 155 kg of live weight with a *quasi ad libitum* feeding level, expressed in grams), feed conversion ratio, backfat thickness at the level of the *gluteus medius* muscle measured at slaughter, lean cuts weight (expressed as the sum of neck and loin weight), ham weight, and visible intermuscular fat (a categorical trait that represents the probability to transfer to offspring the defect of excessive intermuscular fat, expressed in units of standard deviation). These values were provided by the Italian Association of

Pig Breeders after calculating them using a BLUP-multiple trait-animal model (Russo et al., 2000).

Additionally, a total of 268 barrows from the SDU line were taken for validation purposes. These pigs were sampled from 137 sires and 28 dams in three batches. They were performance-tested from 75 d to 210 d of age under commercial conditions following the protocol described in Ros-Freixedes et al. (2012). During the test period they had *ad libitum* access to commercial diets. The traits recorded have been described in detail before (Ros-Freixedes et al., 2012) and included, in live animals, BW at an average age of 120, 180, and 205 d, and the backfat and loin thickness, which were ultrasonically measured at the same ages. After slaughter at 210 days, the carcass weight and length, the carcass backfat and loin thickness, and the ham weight were measured, and the carcass lean percentage and lean weight estimated. After chilling for about 24 h at 2ºC, the pH was measured in the LM and in the *semimembranosus* muscles. Samples of at least 50 g of *gluteus medius* muscle and LM were taken, immediately vacuum packaged, and stored in deep freeze until required for IMF content and fatty acid determination (Bosch et al., 2009).

Single nucleotide polymorphism discovery and genotyping

Genomic DNA was isolated either from blood samples (IDU pigs) or from freeze-dried muscle samples (SDU) using standard protocols (Sambrook et al., 1989). 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 (http://www.ensembl.org) were compared for an *in silico* variability analysis. Seven primer pairs (**Supplementary 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 using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the four-capillary system ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences obtained were compared by multiple alignments, performed with MEGA software v4.0 (www.megasoftware.net/).

The genotyping of the *JN860199:g.173G>A PLIN1* SNP polymorphism was performed by PCR-restriction fragment length polymorphism assay. PCR products obtained with primer set "P2" (**Supplementary Table S1**) were digested with *Hin*1II (Fermentas, Vilnius, Lithuania) and the resulting products were resolved on polyacrylamide gels. For *PLIN2*, the *GU461317:g.98G>A* SNP, in the 3' UTR region of the gene, was genotyped by High Resolution Melting PCR in a Rotor-Gene™ 6000 (Corbett Research, Mortlake, New South Wales, Australia) following the protocol described in Davoli et al. (2011).

Association analysis

The *PLIN1 JN860199:g.173 G>A* SNP and the *PLIN2 GU461317:g.98G>A* SNP were tested for association using both IDU and SDU pigs. The phenotypic data obtained on SDU pigs were used to validate the prospective results obtained in IDU pigs for estimated breeding values. The effect of *PLIN* genotypes were estimated independently for each SNP and trait with a model including the genotype (the two homozygotes and the heterozygote), and the slaughter batch, either as a fixed (SDU) or random (IDU) effect. For IDU we included slaughter batch as random factor because of the large number of slaughter days. For SDU phenotypic data, the age at test was also included as a covariate. To test for additivity (a), the effect of the genotype was replaced by the covariates X_a which were coded as (1, 0, -1) for the most frequent homozygote,

the heterozygote, and the less frequent homozygote, respectively. The effect of the genotype was tested using the F-test and the differences between genotypes with the Tukey's test. The models were solved using SAS 9.2 (SAS Inst., Cary, NC, USA) statistical software.

RESULTS AND DISCUSSION

Polymorphisms and sequence variation of PLIN genes

The *in silico* analysis on publicly available genomic, EST and cDNA sequences 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 SNP in intronic regions. Seven genomic regions containing these putative SNP were subject to direct sequencing in the 20 animals from three Italian heavy pig breeds. A total of 2,437 bp of the pig *PLIN1* gene were screened, which covered 1,126 bp of the coding sequence, the complete 183-bp 5' UTR, and 1,128 bp of intronic regions and part of the promoter and 3' downstream genomic region, according to the annotation of the Ensembl entry [ENSSSCG00000001844]. The sequencing covered the positions of the putative SNP detected *in silico*, with the exception of the SNPs on exon 8, which was not analyzed due to the unsuccessful amplification of this region. Four SNP were detected by sequencing in the Italian heavy pig breeds (**Table 1**). The two intronic SNP were novel and the sequences were reported to GenBank [JN860199; SNP *g.173G>A* and *g.3484C>G*]. The two exonic SNP which were detected in our *in silico* analysis were both synonymous and had been reported before (GenBank: AM931171; SNP *g.4119A>G* and *g.7966T>C;* Vykoukalová et al., 2009). The four SNP were in complete linkage disequilibrium in the initial panel of 20 pigs. The intronic SNP

JN860199g.173G>A was selected for subsequent analyses because a restriction enzyme was available to analyse this mutation.

Allele frequencies and genotype counts of *PLIN1 JN860199:g.173G>A* and *PLIN2 GU461317:g.98G>A* SNPs are reported in **Table 2**. The alleles for *JN860199:g.173G>A* were evenly distributed in IDU (Minor Allele Frequency, MAF=0.13A) and SDU (MAF=0.30A). The *GU461317:g.98G>A* SNP in *PLIN2* segregate both in IDU (MAF: 0.37G) and SDU (MAF=0.43G).

Effect of PLIN genotypes

In IDU pigs, while the *PLIN1 g.173G>A* SNP was not associated to none of the production and carcass traits investigated (**Table 3),** the allele A at *PLIN2 g.98G>A* SNP showed a positive additive effect on ADG and lean cuts weight (**Table 4**). Both results were corroborated in SDU pigs. Thus, in SDU pigs, the *PLIN1 g.173G>A* SNP was not associated to neither on-farm performance-tested (**Table 5**) nor carcass and meat quality traits (**Table 6**). This finding, however, contrasts with the results by Vykoukalová et al. (2009), who found suggestive associations of the two exonic *PLIN1* SNP with ADG and backfat thickness in Large White pigs. Several studies in human and mouse have also correlated nucleotide variation at *PLIN1* gene to BW (Tansey et al., 2003; Qi et al., 2004; Corella et al., 2005; Ruiz et al., 2011).

The *PLIN2 g.98G>A* SNP association analysis on SDU pigs confirmed that the allele A positively affected BW and ADG (**Table 7)**. The substitution effect of A for G for BW was higher at 120 d (2.41 kg, $P<0.01$) than at 205 d (1.91 kg, $P=0.02$), thereby indicating that the beneficial effect of allele A on BW was due to increased growth at early stages. Thus, the effect of allele A for ADG was evident up to 120 d (19.31 g/d, $P<0.01$) but not thereafter, both from 120 to 180 d (-5.89 g/d, $P=0.45$) and from 180 to

205 d (-2.12 g/d, *P*=0.89). Consequently, the additive variance associated to the *PLIN2 g.98G>A* SNP, if estimated as $2pqa^2$ (Falconer and Mackay, 1996), with *p* and *q* being the allele frequencies (**Table 2)** and a the additive value (**Table 7**), is able to capture a greater proportion of the total additive variance of BW (Ros-Freixedes et al., 2013) at 120 d (9.3%) than at 205 d (6.0%). These results are consistent with those reported here and in Davoli et al. (2011) for estimated breeding values for ADG in IDU pigs (**Table 4**). 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 d (0.41 mm, *P*=0.04) vanishing at later ages. This result confirmed the results obtained in IDU pigs, where *PLIN2* genotypes did not affect backfat thickness at test (**Table 4**).

The beneficial effect of the allele A was also observed in carcass traits (**Table 8**). Thus, SDU pigs carrying an additional copy of the allele A displayed heavier (1.38 kg, *P*=0.04) and longer carcasses (0.65 cm, *P*<0.01), with no effect on carcass lean percentage (0.34 %, *P*=0.43). As a result, in line with the higher weight of lean cuts observed in IDU pigs (**Table 4**), pigs with the allele A showed higher lean growth (0.88 kg, *P*=0.04). The *PLIN2 g.98G>A* SNP reached to explain 3.1% of the additive variance of lean weight. However, it was not possible to detect the positive effect of allele A on ham weight as clearly as in IDU pigs (0.17 kg, *P*=0.08). No evidence was found in SDU pigs indicating that meat quality traits (pH and IMF) were additively affected by *PLIN2 g.98G>A* SNP (**Table 8**).

The results obtained here indicate that the allele A at the *PLIN2 g.98G>A* SNP has beneficial effects on early growth, thereby leading to correlated changes on BW at later ages and therefore in lean growth and in prime retail cuts. In agreement with our results, the genomic position of *PLIN2* on chromosome 1 co-localises with quantitative trait loci for ADG (Liu, 2007), BW at birth (Guo, 2008), and daily feed intake (Kim,

2000) (**Supplementary Table S2**). The five members of the *PLIN* family have been studied in depth in humans and model animals. Most reports have focussed on *PLIN1*, the main perilipin protein in mature adipocytes, particularly in relation to BW and obesity-related phenotypes (Smith and Ordovas, 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 both of our pig populations, 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 regulatory 5' or 3' 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. 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 can 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 BW to selection for IMF.

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Table 1. Single nucleotide polymorphisms (SNP) detected in the porcine *PLIN1* gene in this study

SNP[1]	Gene position [2]	Gene location	Amino acid change
JN860199 g.173G>A	691	Intron 1	-
JN860199 g.3484C>G	4,004	Intron 1	-
AM931171g.4119A>G	4,119	Exon 2	Synonymous[3]
AM931171g.7966T>C	7,966	Exon 5	Synonymous[3]

[1] GenBank accession number is indicated.

[2] Position from the start codon as referred to the entry [Ensembl:ENSSSCG00000001844; assembly Sscrofa10.2: chromosome 7; 601266014:60139897:-1].

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

Table 2. Number of pigs (N), allele frequency of the allele G (Freq), and genotype counts of single nucleotide polymorphisms at *PLIN1* and *PLIN2* genes in Italian and Spanish Duroc pigs

				Number of pigs		
SNP	Genetic type	N	Freq	GG	AG	AA
PLIN1 (JN860199:g.173G>A)	Italian Duroc	137	0.87	104	30	3
	Spanish Duroc	261	0.70	128	9	24
PLIN2 (GU461317:g.98G>A)	Italian Duroc	429	0.37	60	193	176
	Spanish Duroc	268	0.43	87	129	52

Table 3. Mean and standard deviation (SD) for the estimated breeding values for production and carcass traits and genotype differences (±SE) for PLIN1 JN860199:g.173G>A polymorphism in Italian Duroc pigs

				Genotype differences				Additive (a) effect	
Traits[1]	Mean	SD	P[2]	GG-AG	P[3]	GG-AA	P[3]	a	P[2]
Average daily gain, g/d	47.01	18.05	0.77	-1.93±3.74	0.86	5.05±10.60	0.88	-2.52±5.30	0.63
Feed conversion ratio, kg/kg	-0.19	0.11	0.55	0.03±0.02	0.52	0.01±0.07	0.98	-0.01±0.03	0.86
Backfat thickness, mm	-2.50	2.52	0.13	0.61±0.50	0.44	2.41±1.42	0.21	-1.21±0.71	0.09
Lean cuts weight, kg	3.23	1.32	0.79	-0.15±0.27	0.84	-0.35±0.77	0.89	0.17±0.38	0.65
Ham weight, kg	0.81	0.43	0.13	-0.16±0.09	0.16	-0.26±0.25	0.55	0.13±0.12	0.30
Visible intermuscular fat	-0.98	0.66	0.86	0.08±0.14	0.84	0.005±0.39	0.99	-0.00±0.20	0.99

[1] Average daily gain/ Feed conversion ratio from 30 to 155 kg; Visible intermuscular fat expressed in units of standard deviation.

[2] *P-values* associated to the genotype effect. Significance threshold set at 0.05.

[3] *P-value* associated to the difference between two given genotypes and to the additive and dominant effects. Significance threshold set at 0.05.

Table 4. Mean and standard deviation (SD) for the estimated breeding values for production and carcass traits and genotype differences (±SE) for *PLIN2 GU461317:g.98G>A* polymorphism in Italian Duroc pigs

				Genotype differences				Additive (a) effect	
Traits	Mean	SD	P[1]	AA-AG	P[2]	AA-GG	P[2]	a	P[2]
ADG, g/d	39.46	43.64	0.06	9.54±2.83	**<0.01**	8.91±4.29	0.10	-2.31±2.16	0.29
Feed conversion ratio, kg/kg	-0.17	0.23	**0.02**	-0.04±0.02	**0.02**	-0.05±0.02	0.11	0.02±0.01	**0.05**
Backfat thickness, mm	-2.79	3.09	0.30	-0.43±0.28	0.27	-0.35±0.42	0.68	0.18±0.21	0.40
Lean cuts weight, kg	2.84	2.36	**<0.01**	0.58±0.17	**<0.01**	0.66±0.26	**0.03**	-0.33±0.13	**0.01**
Ham weight, kg	0.88	0.90	**<0.01**	0.24±0.07	**<0.01**	0.21±0.10	0.10	-0.11±0.05	**0.04**
Visible intermuscular fat	-0.69	0.96	0.27	-0.13±0.09	0.30	0.16±0.13	0.46	-0.08±0.07	0.23

[1] Average daily gain/ Feed conversion ratio from 30 to 155 kg; Visible intermuscular fat expressed in units of standard deviation.

[2] *P-values* associated to the genotype effect. Significance threshold set at 0.05.

[3] *P-values* associated to the difference between two given genotypes and to the additive and dominant effects. Significance threshold set at 0.05.

Table 5. Mean and standard deviations (SD) for on-farm performance-tested traits at 205 d and genotype differences (±SE) for *JN860199:g.173G>A PLIN1* polymorphism in Spanish Duroc

				Genotype differences				Additive (a) effects	
Traits	Mean	SD	P[1]	GG-AG	P[2]	GG-AA	P[2]	a	P[2]
Body weight, kg	123.8	9.6	0.95	0.25±1.23	0.95	0.62±2.11	0.95	0.31±1.06	0.77
ADG, g/d	597	54	0.45	0.48 ± 0.63	0.73	3.14 ± 1.09	0.47	0.64± 0.54	0.24
Backfat thickness, mm	21.1	4.0	0.41	-0.15±0.51	0.96	-1.18±0.88	0.37	-0.59±0.44	0.18
Loin thickness, mm	48.4	4.3	0.20	0.28±0.54	0.86	-1.41±0.93	0.29	-0.70±0.46	0.13

[1] *P-values* associated to the genotype effect. Significance threshold set at 0.05.

[2] *P-values* associated to the difference between two given genotypes and to the additive and dominant effects. Significance threshold set at 0.05.

Table 6. Mean and standard deviation (SD) for carcass and meat quality traits at 210 d and genotype differences (±SE) for *JN860199:g.173G>A* *PLIN1* polymorphism in Spanish Duroc

				Genotype differences				Additive (a) effects	
Traits[1]	Mean	SD	P[2]	GG-AG	P[3]	GG-AA	P[3]	A	P[3]
Carcass weight, kg	94.0	7.5	0.95	0.30±0.97	0.95	0.01±1.66	1.00	0.01±0.83	0.99
Carcass backfat thickness, mm	21.9	3.7	0.63	-0.23±0.49	0.88	-0.78±0.84	0.62	-0.39±0.42	0.35
Carcass loin thickness, mm	46.6	6.9	0.74	0.44±0.91	0.88	1.13±1.58	0.75	0.56±0.79	0.47
Carcass lean percentage, %	44.6	5.0	0.61	0.34±0.65	0.86	1.07±1.12	0.61	0.54±0.56	0.34
Carcass length, cm	86.4	2.7	0.62	0.16±0.35	0.89	0.58±0.60	0.61	0.28±0.30	0.35
Carcass lean weight, kg	41.6	5.0	0.45	0.48± 0.63	0.73	1.27 ±1.09	0.47	0.64 ± 0.54	0.24
Ham weight, kg	12.3	1.1	0.65	-0.12±0.14	0.65	-0.12±0.25	0.88	-0.06±0.12	0.63
pH24 LM	5.7	0.2	0.49	-0.03±0.02	0.49	0.00±0.04	0.10	0.00±0.19	0.98
pH24 SM	5.7	0.2	0.27	-0.04±0.02	0.26	-0.03±0.04	0.69	-0.02±0.02	0.42
IMFGM, % DM	15.5	5.1	0.33	-0.94±0.64	0.30	-0.46±1.09	0.91	-0.23±0.55	0.67
IMFLM, % DM	10.4	3.1	0.89	-0.21±0.50	0.91	-0.29±0.88	0.94	-0.14±0.44	0.74

[1]pH24LM/ pH24SM: pH 24 h *post-mortem* in LM/ *semimembranosus* muscle; IMFGM/ IMFLM: intramuscular fat content in muscle *gluteus medius*/ LM.

[2] *P-values* associated to the genotype effect and to the difference between two given genotypes. Significance threshold set at 0.05.

[3] *P-values* associated to the difference between two given genotypes and to the additive and dominant effects. Significance threshold set at 0.05.

Table 7. Mean and standard deviation (SD) for on-farm performance-tested traits by age and genotype differences (±SE) for *GU461317:g.98G>A PLIN2* polymorphism in Spanish Duroc

				Genotype differences				Additive (a) effects	
Traits	Mean	SD	P[1]	AA-AG	P[2]	AA-GG	P[2]	a	P[2]
Body weight, kg									
120 d	62.15	9.72	**<0.01**	1.41±0.93	**0.01**	4.82±1.15	**<0.01**	2.41±0.57	**<0.01**
180 d	109.63	9.48	**0.03**	1.01±1.23	0.68	3.96±1.53	**0.02**	1.98±0.77	**0.01**
205 d	124.00	9.74	0.07	0.85±1.35	0.80	3.84±1.68	0.06	1.91±0.84	**0.02**
ADG, g/d									
0-120 d	510	80	**<0.01**	14.46 ± 7.74	0.15	38.64 ±9.75	**<0.01**	19.31± 4.87	**<0.01**
120-180 d	730	200	0.67	-9.68 ± 12.35	0.71	-11.79± 15.54	0.73	-5.89 ± 7.77	0.45
180-205 d	520	230	0.88	-12.40 ± 25.06	0.87	-4.22 ± 31.55	0.99	-2.12 ± 15.77	0.89
0-205 d	597	53	0.06	4.19 ± 6.44	0.79	18.78± 8.11	**0.05**	9.39 ± 4.05	**0.02**
Backfat thickness, mm									
120 d	11.46	3.07	0.10	0.11±0.33	0.94	0.83±0.41	0.11	0.41±0.21	**0.04**
180 d	18.28	3.71	0.62	-0.35±0.52	0.78	-0.68±0.64	0.54	-0.28±0.32	0.38
205 d	21.25	4.00	0.56	-0.57±0.56	0.57	-0.56±0.70	0.71	-0.28±0.35	0.56
Loin thickness, mm									
120 d	40.36	3.32	0.32	0.19±0.45	0.90	-0.69±0.57	0.45	-0.37±0.28	0.19
180 d	44.86	3.71	**0.04**	1.30±0.52	**0.03**	0.48±0.65	0.74	0.24±0.32	0.46
205 d	48.53	4.38	0.40	0.61±0.60	0.56	-0.22±0.75	0.95	-0.11±0.37	0.77

[1] *P-values* associated to the genotype effect. Significance threshold set at 0.05.

[2] *P-values* associated to the difference between two given genotypes and to the additive and dominant effects. Significance threshold set at 0.05.

Table 8. Mean and standard deviation (SD) for carcass and meat quality traits at 210 d and genotype differences (±SE) for *GU461317:g.98G>A PLIN2* polymorphism in Spanish Duroc

				Genotype differences				Additive (a) effects	
Traits[1]	Mean	SD	P[2]	AA-AG	P[3]	AA-GG	P[3]	a	P[3]
Carcass weight, kg	94.12	7.61	**0.05**	0.43±1.07	0.91	2.76±1.32	0.09	1.38±0.66	**0.04**
Carcass backfat thickness, mm	22.05	3.71	0.84	-0.26±0.55	0.88	-0.24±0.66	0.93	-0.20±0.33	0.55
Carcass loin thickness, mm	46.63	6.92	0.74	0.55±1.02	0.74	0.91±1.25	0.74	0.46±0.60	0.46
Carcass lean percentage, %	44.50	5.03	0.72	0.16±0.72	0.71	0.70±0.88	0.71	0.34±0.44	0.43
Carcass length, cm	86.47	2.78	**0.03**	0.36±0.39	**0.02**	1.31±0.48	**0.02**	0.65±0.24	**<0.01**
Carcass lean weight, kg	41.63	5.02	0.12	0.54± 0.69	0.71	1.75 ± 0.85	0.10	0.88 ± 0.43	**0.04**
Ham weight, kg	12.36	1.15	0.17	0.22±0.15	0.19	0.34±0.19	0.19	0.17±0.10	0.08
pH24 LM	5.74	0.25	0.40	-0.04±0.02	0.31	-0.02±0.03	0.72	-0.01±0.01	0.56
pH24 SM	5.76	0.29	0.14	-0.06±0.03	0.09	-0.03±0.04	0.72	-0.02±0.02	0.25
IMFGM, % DM	15.68	5.14	0.81	-0.17±0.71	0.97	-0.77±0.88	0.70	-0.28±0.44	0.52
IMFLM, % DM	10.57	3.09	**0.04**	-1.31±0.52	**0.04**	-0.63±0.67	0.62	0.27±0.34	0.42

[1] pH24LM/ pH24SM: pH 24 h *post-mortem* in LM/ *semimembranosus* muscle; IMFGM/ IMFLM: intramuscular fat content in muscle *gluteus medius*/ LM.

[2] *P-values* associated to the genotype effect and to the difference between two given genotypes. Significance threshold set at 0.05.

[3] *P-values* associated to the difference between two given genotypes and to the additive and dominant effects. Significance threshold set at 0.05.

Supplementary information

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

Primer	Sequence (5'-3')	Gene regions	Product size (bp)	Ta [1]
P1	F GTCAAATAACCATAGCAACCAAC R ATTCCCAGAAGACCCTAACC	partial promoter; exon 1; partial intron 1	253	61
P2	F AGGGAACTGATGGTGAGAGG 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 TGGTGGCCTCTTGGTAATTC	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

[1] Annealing temperature

Table S2. Quantitative trait loci (QTL) co-localizing with the porcine *PLIN2* mapping position[1]

QTL trait	QTL (cM)	Reference
PLIN2 (SSC1q2.3-2.7; 227.3 Mb on SSC assembly 10.2)		
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	100.8-118.5	Mohrmann et al. (2006)
Average daily gain	127.1-140.5	Evans et al. (2004)
Backfat thickness	80.0-110.5	Liu et al. (2007)
Body weight at birth	16.4-132	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 percentage	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 (http://www.animalgenome.org/cgi-bin/gbrowse/pig/), accessed on 06-09-2013.

Chapter 4

Proteomic to investigate conventional and deep chilling effects on pig meat quality traits

Chilling the meat as soon as possible after exsanguination is one way to remove heat from carcass and to guarantee a great impact on the quality, increasing shelf life and safety of meat, creating an environment that is unfavourable for microbial growth. Furthermore, lowering the temperature during post-mortem important meat technological parameters (i.e. pH value declines), which determines meat quality traits values in weight loss, tenderness, water holding capacity and colour can be modified. Therefore, the priority of chilling mechanisms is to remove heat from the carcass as quickly as possible after slaughter. The main effect of the application of chilling procedure on meat is that metabolic processes are slow down and the rate of pH decline is influenced by the low temperature. Therefore, the severity of the denaturation of myoglobin and other proteins is decreased and is showed the improvement of water-holding capacity and color quality values. Chilling temperature can also affect tenderness parameter. In early postmortem, the ability of the sarcoplasmic reticulum to sequester calcium, could be destabilized by very low muscle temperature, producing contraction and shortening in the muscle (Huff-Lonergan E. & Page J., 2001). Therefore in these steps, to obtain a high quality in meat product is necessary to find a balance in chill temperature applications.

The methods commonly used in commercial practices to decrease the meat temperature in pork, are chilling spray and rapid/accelerated chilling systems, or by hot –trimming to reduce the temperature in carcass or skinning of carcasses and handling practices of the pigs, prior to slaughter. The use of different accelerated chilling systems can be a valid method to prevent or to reduce unfavourable qualities effects, as the incidence of PSE (the opportunity to have the situation of high temperature and low pH was minimized) in pork and to support the extension of shelf life and the reduction of the evaporative weigh loss (Tomović V.M., *et al.*, 2008). The best system to chill carcasses would be the one that minimized the rate of pH decline and maximizes the rate of temperature decline without compromising pork quality (Huff-Lonergan E. & Page J., 2001).

Moreover, during the postmortem storage of meat, happened numerous changes in the myofibrillar and sarcoplasmic protein profile that may play a role in postmortem tenderization. For example in muscle tissue, posttranslational protein modifications can alter the proteins activity, by changing

their structural conformation and regulating the function of these proteins (Anderson M.J. *et al.* 2012).

Therefore, the application of proteomic approaches (in particular 2D-DIGE technique), to investigate and compare the proteins profile between different chilled muscle samples, can be useful.

The aim of this study was to use a proteomic approach to pork loin samples subjected to deep chilling compared to loin from the same pig that was conventionally chilled. Two-Dimensional Difference In Gel Electrophoresis (2D-DIGE), was use.

The identification and description of the proteins spots were performed by MALDI ToF and the results validated by 2D-Western Blot analysis.

2. Materials and methods:

2.1 Collection of samples

In this work carcasses of commercial USA pigs selected on the line on their weight (86-91 Kg) and percent of lean (55-58 %), were considered.

Samples from *Longissimus dorsi* (LD) muscle of two groups both of ten pigs, slaughtered in two different times, at the Iowa State University Meat Laboratory using normal approved humane slaughter procedures, were analyzed. The first group of animals was slaughtered in November 2012, the second group in February 2013, and these two groups will be called from now November and February group. Each sample was splitted in two parts (right and left side) and treated with different chill temperatures (conventional and deep, as indicated in Tab. 1). After four hours postmortem the temperatures measured in conventional chilled (CC) carcasses and in deep chilled carcasses (DC) were 12.83 °C (55.1 °F) and 1.5 °C (34.7 °F), respectively.

November 2012 group:

	Deep Chilling (DC)	Conventional Chilling (CC)
NUMBER	SIDE	SIDE
1	Left	Right
2	Right	Left
3	Left	Right
4	Right	Left
5	Right	Left
6	Left	Right
7	Left	Right
8	Right	Left
9	Left	Right
10	Right	Left

February 2013 group:

	Deep Chilling (DC)	Conventional Chilling (CC)
NUMBER	SIDE	SIDE
11	Right	Left
12	Left	Right
13	Right	Left
14	Left	Right
15	Left	Right
16	Right	Left
17	Right	Left
18	Left	Right
19	Left	Right
20	Right	Left

Tab.1: this table show how were been organized the samples in each group, utilized this study

Each LD sample (300 g) was homogenized and powdered in liquid nitrogen. Two separate extracts of sarcoplasmic proteins were prepared from each sample obtained (side left and side right).

2.2 Extraction of sarcoplasmic fraction

The highly soluble sarcoplasmic fraction was extracted from the homogenized and powdered sample (3 g) in 10 ml of cold sarcoplasmic extraction buffer (100mM Tris, 10mM EDTA, pH 8.3, modified from Carlin, Huff-Lonergan, Rowe & Lonergan 2006), a low ionic strength buffer designed to only solubilize sarcoplasmic proteins, using a Polytron PT 3100 (Lucerne, Switzerland). Samples were then centrifuged at 40,000 xg for 20 min at 4°C. The supernatant was filtered and the protein concentration of sarcoplasmic fraction determinated and adjusted to 10 mg/ml, using the method described by Lowry, Rosebrough, Farr and Randall (1951). SDS-PAGE analyse (using Hoefer SE260 gel formulation, with 12.5% acrylamide) was necessary to check the proteins concentration (mg/ml) and the molecular weight, to be sure that it was the same for each samples. The protein concentration was standardized to allow direct proteins comparison in the 2D-DIGE experiments.

2.3 Two-dimensional (2D) Difference In Gel Electrophoresis (DIGE) analysis

2D-DIGE is a powerful technology for proteins abundance studies because it can identify the protein profile of muscle, according to isoelectric focusing (IEF) in the first dimension and SDS PAGE in the second dimension (Marouga R. *et al*., 2005). Indeed, this technique allows the separation and detection of proteins that would appear as a single band in one dimensional PAGE analyses (Anderson M.J., *et al.*,2012). Two dimensional DIGE was used to determine differences in muscle proteins profile of conventional and deep chilled samples. The technique required prelabeling of the protein extracted using fluorescent cyanine dyes; a total of 50 µg of each individual sample was labeled with CyDyes 3 or 5 (GE Healthcare, Piscataway, NJ) according to the manufacturer's rules. CyDyes were alternated between conventional and deep chill side of each sample (as showed in the following scheme).

November 2012 group:

	Deep Chilling (DC)		Conventional Chilling (CC)	
NUMBER	SIDE	DYE	SIDE	DYE
1	Left	Cy3	Right	Cy5
2	Right	Cy5	Left	Cy3
3	Left	Cy3	Right	Cy5
4	Right	Cy5	Left	Cy3
5	Right	Cy3	Left	Cy5
6	Left	Cy5	Right	Cy3
7	Left	Cy5	Right	Cy3
8	Right	Cy3	Left	Cy5
9	Left	Cy3	Right	Cy5
10	Right	Cy5	Left	Cy3

February 2013 group:

	Deep Chilling (DC)		Conventional Chilling (CC)	
NUMBER	SIDE	DYE	SIDE	DYE
11	Right	Cy3	Left	Cy5
12	Left	Cy5	Right	Cy3
13	Right	Cy3	Left	Cy5
14	Left	Cy5	Right	Cy3
15	Left	Cy3	Right	Cy5
16	Right	Cy5	Left	Cy3
17	Right	Cy3	Left	Cy5
18	Left	Cy5	Right	Cy3
19	Left	Cy3	Right	Cy5
20	Right	Cy5	Left	Cy3

CyDye 2 was used to label the pooled standard sample. For each individual run, 15 µg of labeled protein (CyDye 3 or 5) from conventional and deep chill was used in addition to the pooled standard (CyDye 2) for a total of 45 µg of protein per gel. The labeled samples were then mixed, run and visualized using fluorescent imaging to enable detection of difference between protein abundance, in the two treatment. The protein mixture was added to individual wells in a reswelling tray and the labeled proteins were mixed and separated at the same time, on the same 2D gel, prepared using an immobilized pH gradient (IPG) strips (11 cm pH 4-7) (GE Healthcare, Piscataway, NJ). The different protein extracts labeled with different CyDye DIGE fluors can then be visualized separately by exciting the different dyes at their specific excitation wavelengths.

First dimension focusing was performed on an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare, Piscataway, NJ), for a total of 11,500 V h.

After isoelectric focusing, strips were equilibrated using two 15 min washes, first with equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and trace amounts of bromophenol blue) with 65 mM DTT and second with equilibration buffer with 135 mM iodoacetamide (IAA) (Rozanas & Loyland, 2008).

Equilibrated strips were loaded onto 12.5% SDS-,PAGE gels (acrylamine: N,N'-bis-methylene acrylamide 100:1, 0.1% SDS, 0.05% TEMED, 0.05% ammonium persulfate, and 0.5 M Tris-HCl, pH 8.8) using agarose as an overlay (Grubbs J.K., *et al.* 2013). The samples were run on a Ettan DALT SIX system (GE Healtcare , Piscataway, NJ) using 24 cm gels placing two 11 cm strips on each gel. Each gel was run in duplicate (see Run 1 and Run 2 in Tab.2A and 2B). Gels were imaged using an Ettan DIGE Imager (GE Healtcare, Piscataway, NJ). Images were processed and analyzed using DeCyder 2D software version 6.5 (GE Healthcare, Piscataway, NJ), using Student's paired *t*-test to determine differences in resolved protein relative abundance between conventional and deep chill treatments. A significant difference was determinated by a P-value of less than 0.15.

Sample number	Cy2	Cy3	Cy5	STRIP Run1	STRIP Run2
1LR	Pooled Standard	Deep Chill	Conventional Chill	44397	45549
2RL	Pooled Standard	Conventional Chill	Deep Chill	44398	45551
3LR	Pooled Standard	Deep Chill	Conventional Chill	44399	45552
4RL	Pooled Standard	Conventional Chill	Deep Chill	44400	45553
5RL	Pooled Standard	Deep Chill	Conventional Chill	44401	45554
6LR	Pooled Standard	Conventional Chill	Deep Chill	44402	45555
7LR	Pooled Standard	Conventional Chill	Deep Chill	44403	45556
8RL	Pooled Standard	Deep Chill	Conventional Chill	44404	45557
9LR	Pooled Standard	Deep Chill	Conventional Chill	44405	45558
10RL	Pooled Standard	Conventional Chill	Deep Chill	44406	45559

(Tab. 2A: November group experimental design using CyDye DIGE fluor labeling kit. Each sample was repeat two time)

Sample number	Cy2	Cy3	Cy5	STRIP Run1	STRIP Run2
11LR	Pooled Standard	Deep Chill	Conventional Chill	45560	42389
12RL	Pooled Standard	Conventional Chill	Deep Chill	45565	42390
13LR	Pooled Standard	Deep Chill	Conventional Chill	44407	42391
14RL	Pooled Standard	Conventional Chill	Deep Chill	44408	42392
15RL	Pooled Standard	Deep Chill	Conventional Chill	61532	42393
16LR	Pooled Standard	Conventional Chill	Deep Chill	61533	42394
17LR	Pooled Standard	Deep Chill	Conventional Chill	61534	42395
18RL	Pooled Standard	Conventional Chill	DeepChill	61535	42396
19LR	Pooled Standard	Deep Chill	Conventional Chill	61536	42621
20RL	Pooled Standard	Conventional Chill	Deep Chill	61537	42622

(Tab. 2B: February group experimental design using CyDye DIGE fluor labeling kit. Each sample was repeat two time)

The use of internal standard approach to derive statistical data, can help to eliminate the main technical errors of variation between gels. The DeCyder software consists of four modules:

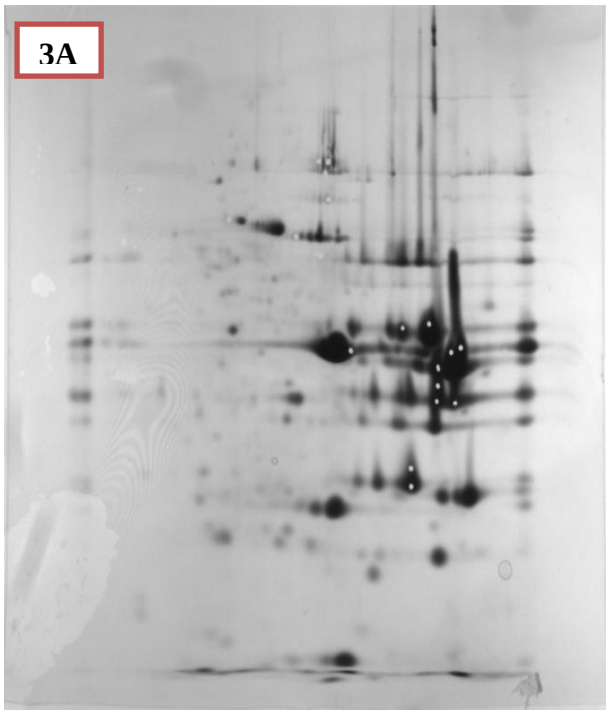
differential in-gel analysis (DIA), biological variation analysis (BVA), batch processor and XML toolbox. In this project was utilized DIA and BVA analysis tools.

From each gel, three scan images are generated: CyDye fluor Cy2 minimal dye for the pooled standard, Cy3 and Cy5 for experimental samples. The DIA module quantifies the spot volumes for each image and expresses these values as a ratio, comparing spot volumes on the sample image with corresponding spot volumes form the internal standard image, so the protein abundance for each spot in each sample is expressed as a ratio relative to the internal standard. This ratio can then be used for inter-gel protein abundance comparisons

When spot detection and quantitation of a single gel has been performed, data are transferred to the BVA module for inter-gel analysis. DeCyder BVA processes multiple gel images, performing matching of multiple images from different gels for comparison to provide statistical data on different protein abundance levels between multiple groups. This process enable comparison of protein abundance between samples on different gels, analysis of experimental designs with different degrees of complexity from a simple control/treated experiment through to a multi-condition experiment addressing factors such as dose and time, all performed in a single analysis (Marouga R., *et al*., 2005).

At first within-group analyses were performed, comparing samples belonging to the same slaughtering group. The protein spots in the same sample subjected at different treatment (e.g. sample 1 in conventional chilling vs sample 1 in deep chilling) were compared; moreover each sample was compared to all the others, irrespective of the treatment to which they were exposed (e.g. sample 1 in conventional chilling vs sample 2 in conventional chilling or sample 1 in conventional chilling vs sample 3 in deep chilling). Afterwards, were performed an inter-group analysis and the samples from the two different slaughter group, were compared.

Protein spots identified as being significantly different between conventional and deep chill (P value <0.15), were selected for identification (Fig. 3A and 3B). February group was discarded from next analysis because showed few significant spots. Unlabeled pooled protein references (750 µg) from each treatment in November group were resolved using 2D electrophoresis and stained with Colloidal Coomassie Blue Stain (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250) for a minimum of 24 h prior to destaining in water for a minimum of 24 h. Spots identified as different in the DeCyder analysis that were not the prominent on the Colloidal Coomassie stained gel, were not selected for identification.



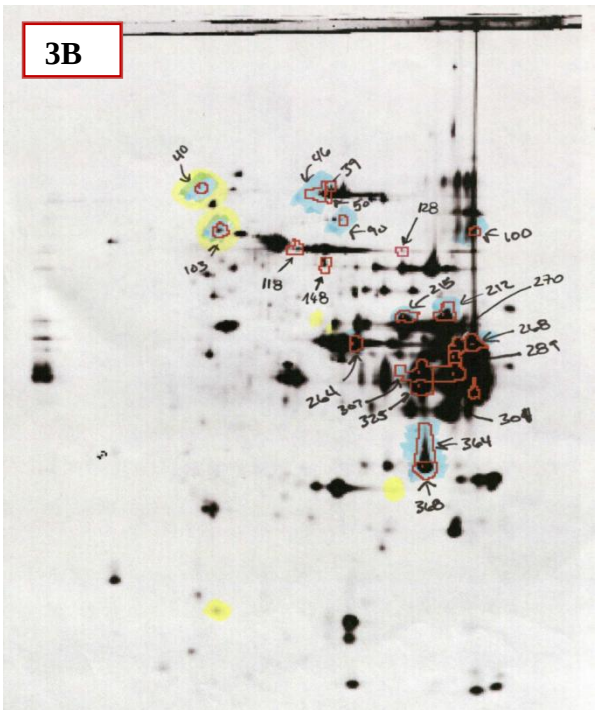


Fig. 3A represents the selected spots picked from 2D electrophoresis gel, on November group
Fig. 3B shows the numeration of selected spots, on November group

Selected spots from each gel were excised and sent to the Iowa State University Protein Facility for identification. In-gel trypsin digest using Genomics Solution ProGest (Chelmsford, MA) was performed. Peptides were dissolved in CHCA (5 mg/ml in 50% CH_3CN/0.1% TFA) and deposited to a MALDI target. MALDI Mass Spectrometry was performed using QSTAR XL. Quadrupole TOF mass spectrometer equipped with an o.MALDI ion source (AB7MDS Sciex, Toronto, Canada). Peak list was generated by Analyst QS Version 1.1 (AB/MDS Sciex, Toronto, Canada). Spectra were processed by MASCOT database search Version 2.2.07 (MatrixScience, London, UK). Search conditions included maximum one missed cleavage, fixed modification (carboxyamidomethyl cysteine), variable modification (oxidation of methionine), peptide mass tolerance of ± 100 ppm, and fragment mass tolerances of ± 1 Da. Identification was based on Mowse Score with a threshold of less than 0.05.

2.4 SDS-PAGE electrophoresis

To investigate the difference in abundance and molecular weight in Phosphoglucomutase 1 (PGM1), Fructose-biphosphate aldolase A iso 1 (ALDO-A) and GAL-3-Phosphate Dehydrogenase (GAPDH), one dimensional Western blot was performed.

At first unlabeled pooled protein references (40 µg) from each treatment and one reference sample, were separated by SDS-PAGE (using Hoefer 260 units, gel formulation; Hoefer Scientific Instruments). All samples were run on 12.5% acrylamide separating gels (10 cmx 10.5 cm; acrylamide: N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.05% N,N,N'N-tetramethylethylenediamine (TEMED), 0.05% ammonium persulfate [wt/vol], and0.5 M Tris-HCl, pH 8.8) for SDS-PAGE and Western blotting. A 5% stacking gel (acrylamide :N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.125% TEMED, 0.075% ammonium persulfate [wt/vol], and 0.125 M Tris-HCl, pH 6.8) was used on all of the acrylamide gels. The running buffer for all gels consisted of 25 mM Tris, 192 mM Glycine, 2 mM EDTA, and .1% [wt/vol] SDS (Anderson M.J., *et al.*,2012).

2.5 Transfer conditons

Proteins were transferred to polyvinylidene difluoide (PVDF) membranes (Millipore; Billerica, MA) using a TE22 Mighty Small Transphor electrophoresis unit (Hoefer Sceintific Instruments; Holliston, MA) at a constant voltage of 90 V for 1.5 h. Transfer buffer consisted of 25 mM Tris, 192 mM glycine, 2 mM EDTA and 15% [vol/vol] methanol. Temperature of the transfer buffer was

maintained at -5.0 °C using a refrigerated circulating water bath (Ecoline RE106;lauda Brinkmann, Westbury, NY).

2.6 Western blotting

Post transfer, all membranes were blocked in PBS-Tween [80 mM disodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% [vol/vol], polyoxyethlene sorbitan monolurate (Tween-20)] with 5% nonfat dry milk [wt/vol]. Membranes for the proteins to be analyzed were blocked for 1 h at room temperature (23 °C) and then were incubated with the appropriated primary antibody, diluited 1/20.000 in PBS-Tween and held overnight at 4°C (Antibodies sourced from Abcam, Cambridge, MA included: Anti-glyceraldehyde 3 phosphate dehydrogenase (AB8245) and anti-aldolase (ab169544). Anti-Phosphoglucosmutase (WH0005236M1) was sourced from Sigma-Aldrich, St. Louis, MO). All membranes were rinsed three times for 10 min with PBS-Tween to achieve a total rinse time of 30 min. Blots for the detection of Phosphoglucomutase 1 (PGM1), Fructose-biphosphate aldolase A iso 1 (ALDO-A) and GAL-3-Phosphate Dehydrogenase (GAPDH) were then incubate for 1 hour at room temperature (23°C) with the secondary antibody (secondary antibody from mouse, for PGM1 and GAPDH; secondary antibody from rabbit for ALDO-A), diluted 1/10.000 in PBS-Tween. Membranes were again rinsed three times for 10 min with PBS-Tween to achieve a total rinse time of 30 min before chemiluminescence detection (M.J. Anderson *et al.*,2012). Chemiluminescence detection was initiated using premixed reagents (ECL Plus kit; GE Healthcare, Piscataway, NJ) and detected using a 16-bit megapixel CCD camera (FluorChem 8800, Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corp.).

2.7 Statistical analysis

The intensity of each spot in 2D DIGE gel images was analyzed using the 3-dimensional spot density, calculated by the DeCyder 2D software v6.5 (GE Healtcare, Piscataway, NJ).

Student's paired *t*-test was utilized to determine differences in resolved protein relative abundance between deep and conventional chilling meat.

A significant difference was determined by a P value ≤ 0.15.

2.8 Two-Dimensional Western blotting for phosphoglucomutase 1

2D-Western Blot technique was used to identify Phosphoglucomutase 1 (PGM1) because by precedent studies (M.J. Anderson *et al.*, 2014) was known that PGM1 could be strongly associated with meat tenderness in beef.

Two – dimensional Western blotting gel for PGM1 were loaded with 100 µg of sarcoplasmic protein extracted from deep chilled samples. The first and the second dimensions were run under the same conditions using the same type of IPG strips (7 cm, pH 3 – 10) and performed on 12.5% SDS-PAGE gel. Gel transfer and Western blots for PGM1 was completed using the method described by Rowe, Maddock, Lonergan and Huff-Lonergan (2004). The primary antibody for PGM1 western blots was a monoclonal antibody against PGM1 (clone 3B8-H4, Sigma Aldrich, St. Louis, MO). Membrane was incubated in primary antibody at 4°C overnight. The secondary antibody membrane was rinsed three times for 10 min each in PBS-Tween then detected by chemiluminescence. Chemiluminescence detection was initiated using premixed reagents (ECL Plus kit; GE Healthcare, Piscataway, NJ) and detected using a 16-bit megapixel CCD camera (FluorChem 8800, Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Alpha Innotech Corp.).

2.9 Staining for phosphorylated and total proteins

One gel was prepared loading unlabeled deep chilled sample to localize phosphorylated proteins in the treatment and stained using Pro-Q Diamond stain (Invitrogen, Carlsbad, CA) method. This kind of stain lets to analyze the phosphorylation of a single protein and to obtain a totally vision of all phosphorylated proteins in the sample.

3. Result and discussion:

In the following Tabs. 4A-4B are reported the results of DeCyder DIA and BVA analyses, obtained from the comparison between deep and conventional chill treatment, in November and February groups.

In each table is possible to observe the result of Student's paired *t*-test, the abundance value (Average Ratio column) and the significance of this value (I-ANOVA column), in agreement with the P value established ($P \leq 0.15$). The negative or positive average-ratio value was calculated between deep chill / conventional chill and it indicates if the specific spot is more abundant in conventional (when negative value is obtained) or in deep chill treatment (when positive value is obtained).

In November group 22 spots different in abundance were selected, while in February group 7 were found. This means that 22 proteins in November group (Tab. 4A) and 7 in February group (Tab.4B), were different in abundance due to carcass chilling. February group hadn't enough spots useful for comparison, so this group was eliminated from next analyses.

Pos.	Master No.	Status	Appearance	T-test	Av. Ratio	1-ANOVA	Pick
1	268	Unconfirmed	45 (48)	0.0081	-1.17	0.0081	Pick
2	301	Unconfirmed	36 (48)	0.011	-1.44	0.011	Pick
3	39	Unconfirmed	30 (48)	0.020	1.37	0.020	Pick
4	215	Unconfirmed	30 (48)	0.026	-1.27	0.026	Pick
5	289	Unconfirmed	45 (48)	0.030	-1.14	0.030	Pick
6	307	Unconfirmed	30 (48)	0.031	-1.65	0.031	Pick
7	128	Unconfirmed	24 (48)	0.038	1.21	0.038	Pick
8	50	Unconfirmed	33 (48)	0.044	1.43	0.044	Pick
9	212	Unconfirmed	39 (48)	0.048	-1.17	0.048	Pick
10	100	Unconfirmed	30 (48)	0.055	-1.43	0.055	Pick
11	364	Unconfirmed	33 (48)	0.056	-1.12	0.056	Pick
12	325	Unconfirmed	45 (48)	0.076	-1.23	0.076	Pick
13	46	Unconfirmed	39 (48)	0.079	1.39	0.079	Pick
14	103	Unconfirmed	42 (48)	0.085	-1.13	0.085	Pick
15	90	Unconfirmed	27 (48)	0.096	-1.14	0.096	Pick
16	148	Unconfirmed	39 (48)	0.098	-1.11	0.098	Pick
17	332	Unconfirmed	39 (48)	0.13	-1.15	0.13	Pick
18	40	Unconfirmed	30 (48)	0.14	-1.13	0.14	Pick
19	264	Unconfirmed	48 (48)	0.14	1.09	0.14	Pick
20	270	Unconfirmed	33 (48)	0.14	-1.18	0.14	Pick
21	368	Unconfirmed	48 (48)	0.14	-1.08	0.14	Pick
22	118	Unconfirmed	42 (48)	0.15	1.14	0.15	Pick

Tab. 4A: November group protein table

Pos.	Master No.	Status	Appearance	T-test	Av. Ratio	1-ANOVA	Pick
1	390	Unconfirmed	45 (54)	0.026	-1.23	0.026	Pick
2	93	Unconfirmed	54 (54)	0.028	-1.12	0.028	Pick
3	92	Unconfirmed	51 (54)	0.069	-1.17	0.069	Pick
4	68	Unconfirmed	51 (54)	0.073	-1.23	0.073	Pick
5	357	Unconfirmed	51 (54)	0.076	-1.61	0.076	Pick
6	195	Unconfirmed	54 (54)	0.10	-1.14	0.10	Pick
7	219	Unconfirmed	45 (54)	0.14	1.06	0.14	Pick

Tab. 4B: February group protein table

After spots selection and MALDI-ToF analyses of November group, 18 spots that differed in abundance between the two treatments, were identified. In this table (Tab. 5) the identity and the abundance of each spot, selected and considerated in accordance with the P value ($P \leq 0.15$) indicated as significant, was reported. In the 2D-DIGE spotting table (Fig. 4) the identity numbers and protein identity names, were directly assigned.

GROUP	N spot	% Sequence	pI	Mol. Weigth (KDa)	Mowse Score	No. Peptides	PROTEIN ID	AKA	NCBI accession number	+ /-	P value
A	103	4%	5.11	68.912	129	3	Kelch repeat and BTB domain	KBTBD	gi|335302953	-1.13	0.085
A	118	13%	6.36	61.8	349	5	Phosphoglucamutax	PGM1	gi|116004023	1.14	0.15
A	148	6%	8.2	58.812	120	3	Dihydrolipoamide dehydrogenase prec	DLD	gi|181575	-1.11	0.098
A	212	11%	8.05	47.443	264	5	beta-enolase	ENO3	gi|113205498	-1.17	0.048
A	215	13%	8.05	47.443	319	3	beta-enolase	ENO3	gi|113205498	1.27	0.026
A	264	20%	6.44	43.205	424	6	creatin kinase M-type like	CKM	gi|126344225	1.09	0.14
A	268	17%	8.3	39.889	445	6	fructose-bisphosphate aldolase A iso 1	ALDO_A	gi|194219069	-1.17	0.0081
A	270	15%	8.45	39.925	333	4	fructose-bisphosphate aldolase A iso 1	ALDO_A	gi|194219069	-1.18	0.14
A	307	17%	8.51	36.041	363	6	GAL-3-phosphate dehydrogenase	GAPDH	gi|329744642	-1.65	0.031
A	325	15%	8.51	36.041	295	5	GAL-3-phosphate dehydrogenase	GAPDH	gi|329744642	-1.23	0.076
A	325X	12%	6.9	35.914	269	3	GAL-3-phosphate dehydrogenase	GAPDH	gi|329744642	-1.23	1.076
A	332	17%	8.51	36.041	403	7	GAL-3-phosphate dehydrogenase	GAPDH	gi|329744642	-1.15	0.13
A	364	23%	7.72	29.678	299	5	carbonic anhydrase 3	CA3	gi|56711366	-1.12	0.056
A	368	23%	7.72	29.678	304	5	carbonic anhydrase 3	CA3	gi|56711366	-1.08	0.14
A	39	10%	6.1	84.382	338	7	Muscle glycogen phosphorylase	PYGM	gi|190402253	1.37	0.02
A	46	1%	6.95	16.8152	67	2	Muscle glycogen phosphorylase	PYGM	gi|190402253	1.39	0.079
A	50	5%	6.1	84.382	188	4	Muscle glycogen phosphorylase	PYGM	gi|190402253	1.43	0.044
A	90	6%	6.93	78.971	207	4	serotransferrin	TF	gi|136192	-1.14	0.096

Tab. 5: Maldi - ToF analysis result of November Group that shows the identity of the significant spots. For each spot was been assigned the abundance value, found by 2D-DIGE analyses

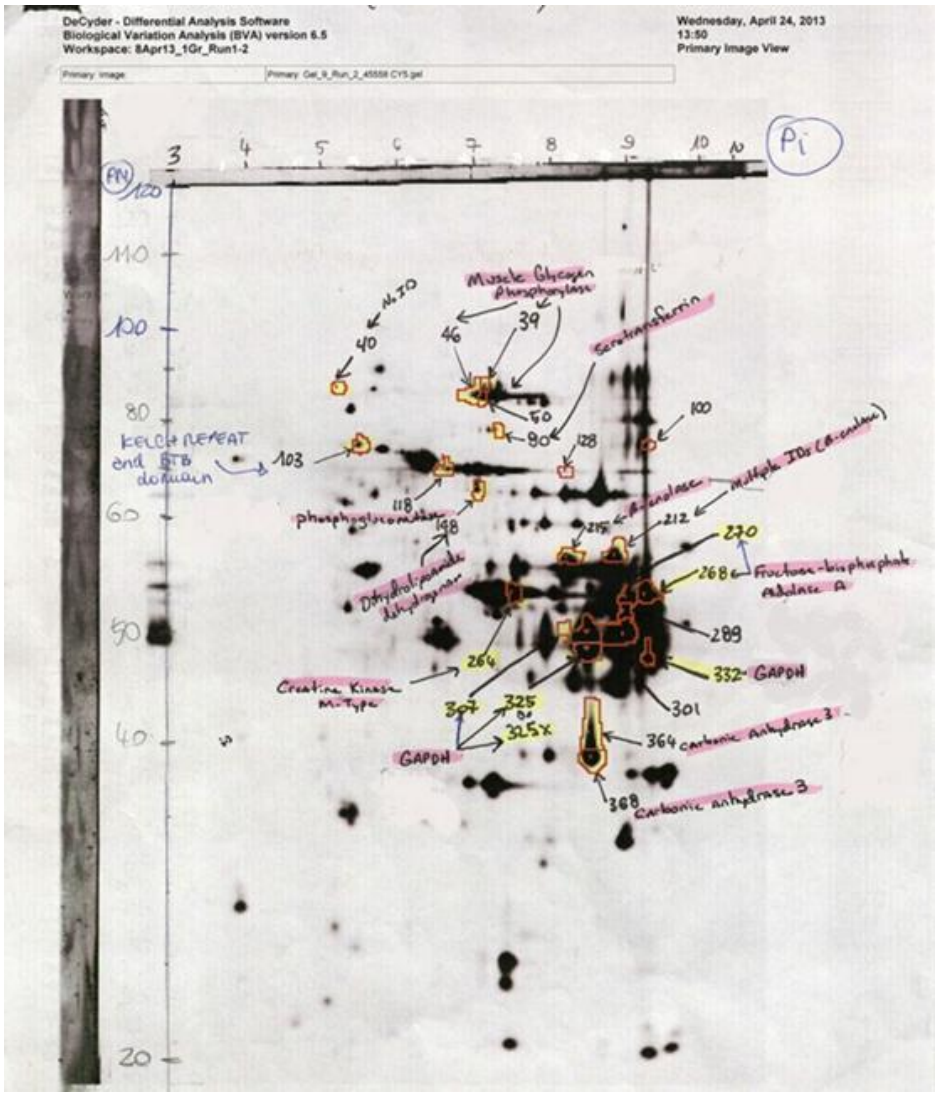


Fig. 4: 2D-DIGE "BVA" analysis of November Group. Each protein spot selected is marked by an identity number and protein name.

This table (Tab. 6) showed the results of the measures made after four hours post mortem on deep and conventional chill carcass samples.

Deep chill samples (at 1.5 °C) showed significant higher calpastatin and star probe values, than in conventional chill samples (at 12.83 °C). These results could be explained by muscle proteins activity, influenced by deep chill temperature.

Trait	Deep Chill	Conventional Chill	P-Value	SEM
Loin Weight (kg)	1.52	1.50	0.56	.04
Purge (%)	2.32	2.06	0.18	0.27
Cookloss (%)	25.67	22.98	0.25	1.56
Color	2.06	2.06		0.23
Marbling	2.56	2.69	0.45	0.18
L	50.46	50.14	0.49	0.621
a	13.62	13.59	0.91	0.26
b	3.38	3.28	0.47	0.127
Juiciness[a]	6.74	6.95	0.62	0.355
Tenderness[a]	6.40	7.21	0.166	0.381
Chewiness[a]	4.13	3.49	0.42	0.549
Flavor[a]	3.14	3.15	0.94	0.23
Off Flavor[a]	1.66	1.83	0.79	0.49
Star Probe[b]	7.10	6.29	0.0033	0.354
Calpastatin[c]	1.20	1.11	0.042	0.047
24 hr pH	5.79	5.7	0.0023	0.028

Tab.6: average values about the effects of chilling rate on meat quality and carcass traits.

[a] Measured on 15 cm line scale with 0 signifying a low degree of juiciness, tenderness, chewiness, pork flavor or off flavor and 15 signifying a high degree of juiciness, tenderness, chewiness, pork flavor or off flavor.

[b] Kilogram of force required to puncture and compress a cooked pork to 20% of its height.

[c] Units/gram of tissue measured the calpastatin activity.

SEM = Standard error of the mean

As this table showed, the meat quality average value is different between deep and conventional treatments, probably due to changes in proteins activities, during postmortem.

GAL-3-Phosphate Dehydrogenase (GAPDH), Fructose-biphosphate aldolase A iso 1 (ALDO-A) and Phosphoglucomutase 1 (PGM1) that appear as significant spots in 2D-DIGE analyses were further analyzed.

The position of these protein spots, on 2D-DIGE proteins map, let to affirm that these proteins were subjected to an horizontal translation, in agreement with them isoelectric point value (from pH3 to pH 10) (Fig 5).

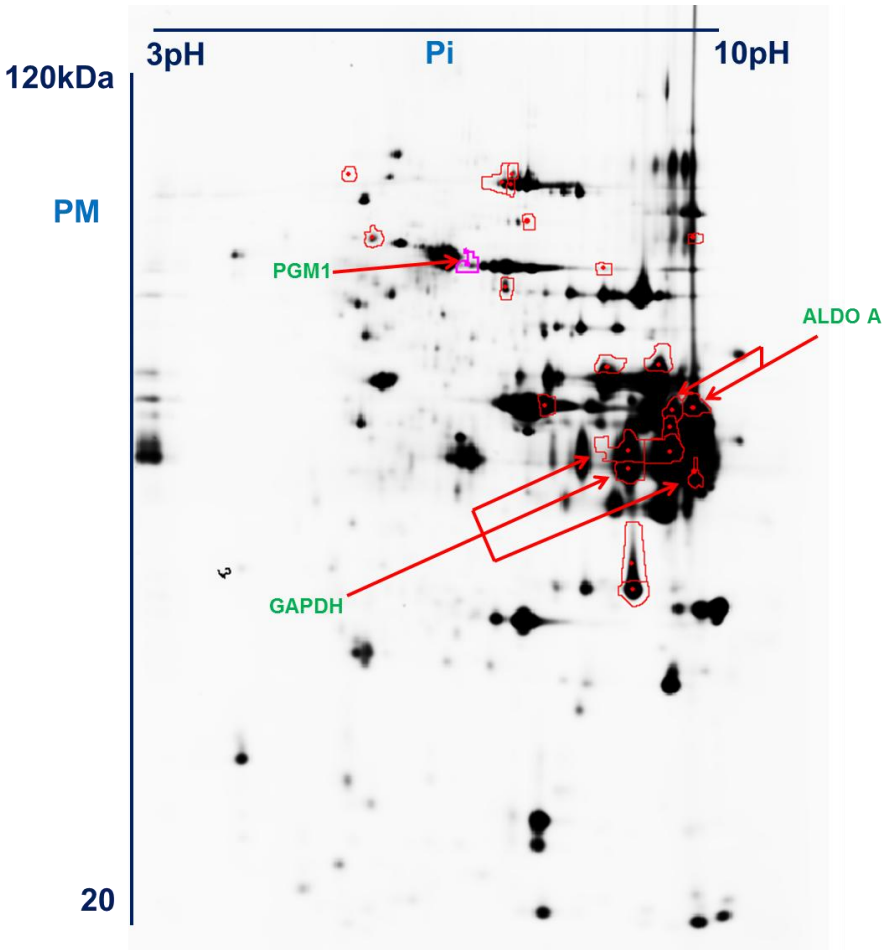


Fig. 5: DIGE analysis result from BVA analysis where are marked the principal studied spots: PGM1, ALDO –A, GAPDH

The analyses were focused *in primis* on Phosphoglucomutase 1 (PGM1) because by precedent studies of Anderson M.J., *et al.*, 2014 was reported that PGM1 could be strongly associated with meat tenderness characterization, in beef.

1D Western Blot analysis was conducted to investigate if the difference in abundance, observed between deep and conventional treatments in 2D-DIGE experiment, could be justified from difference in molecular weight or not.

Significant differences in GAPDH and ALDO-A molecular weight, using 1D Western Blots technique, were not observed (Fig. 6).

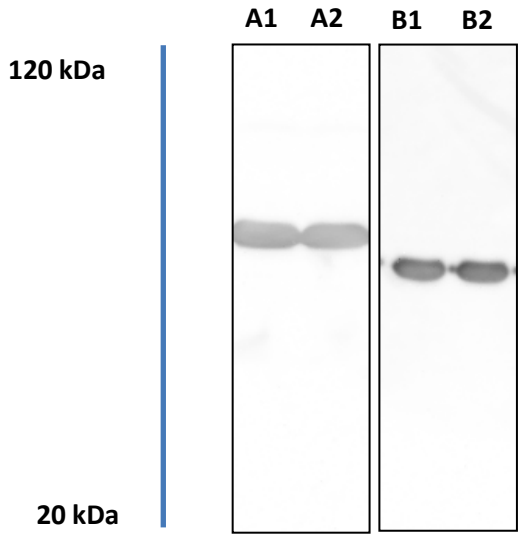


Fig. 6: one dimensional Western Blot in (A) ALDO-A, about 39 kDa: A1 deep and A2 conventional chill treatment; (B) GAPDH, about 36 kDa: B1 deep and B2 conventional chill treatment. Western blots were performed on 12.5% SDS-PAGE gels

In contrast to these result, PGM1 bands appeared more intense in deep chill than in conventional chill treatments(Fig. 7). The bands of PGM1 presented the same molecular weight but considering the reference sample intensity, the deep chill bands were more intense compared to conventional chill. This result could be due to the higher protein activity of PGM1 in deep treatment.

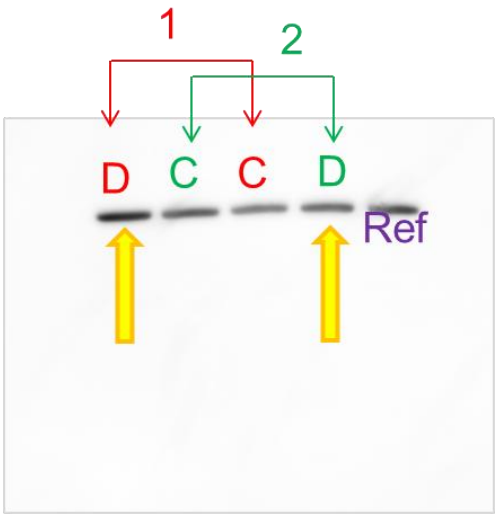


Fig. 7: PGM1 one dimensional Western Blot, comparing reference sample with two different samples (1;2) subjected to two different treatments. In deep chill treatment (D) PGM1 is more emphasize than in conventional chill (C)

Two-dimensional Western Blotting (2D-Western-blot) was performed, to validate if a different temperature treatment in loins during postmortem, could determine modification or different activity in the proteins. From Huang H., *et al* (2011) is known that in loins kept at 18 °C until three hours postmortem, PGM1 is phosphorylated and three distinct isoforms of the phosphorylated proteins are resolved. A monoclonal antibody against Phosphoglucomutase 1, was used in deep chill sample to verify and confirm the identity of this spot (Fig. 8). To obtain the best result, this analysis was repeated two times, and in the end, five spots, corresponding to five isoforms of PGM1, were visibly detected (Fig. 9).

Fig. 8: PGM1 2-D Western Blot overlay 8 min exposure, first picture

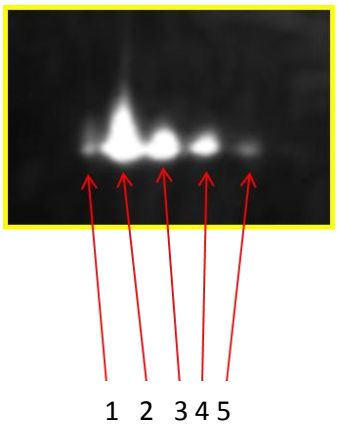


Fig. 8: PGM1 2-D Western Blot overlay 8 min exposure. This second picture was obtained after changing the focalization on the first picture. Here is possible to distinguish the five PGM1 isoforms in "beads-on-a-string" disposition

On light of the fact that PGM1 activity could depend from posttranslational phosphorylation and to confirm the result obtained from 2D-Western-blot analysis, the same gel utilizing Pro-Q Diamond (Invitrogen, Carlsbad, CA) stain, was prepared. This specific dye is utilized to display only the phosphorylated proteins spots, in the sample analyzed.

2D-DIGE was prepared without CyDyes fluorophores, utilizing the unlabeled deep chill sample. After the second dimension migration (by molecular weight), the gel was put in the Pro-Q stain, and rest in dark for some hours.

From literature is known that phosphorylated protein will migrate further towards the acidic portion of the gel compared to the same protein that is unphosphorylated. Additionally, a protein with multiple phosphorylated sites will migrate further toward the acidic portion of the gel during isoelectric focusing compared to the same protein with a single phosphorylated site (Anderson M. J., *et al.*,2014).

In November group was possible to confirm the presence of numerous phosphorylated proteins, among which PGM1 (Fig. 9A-9B).

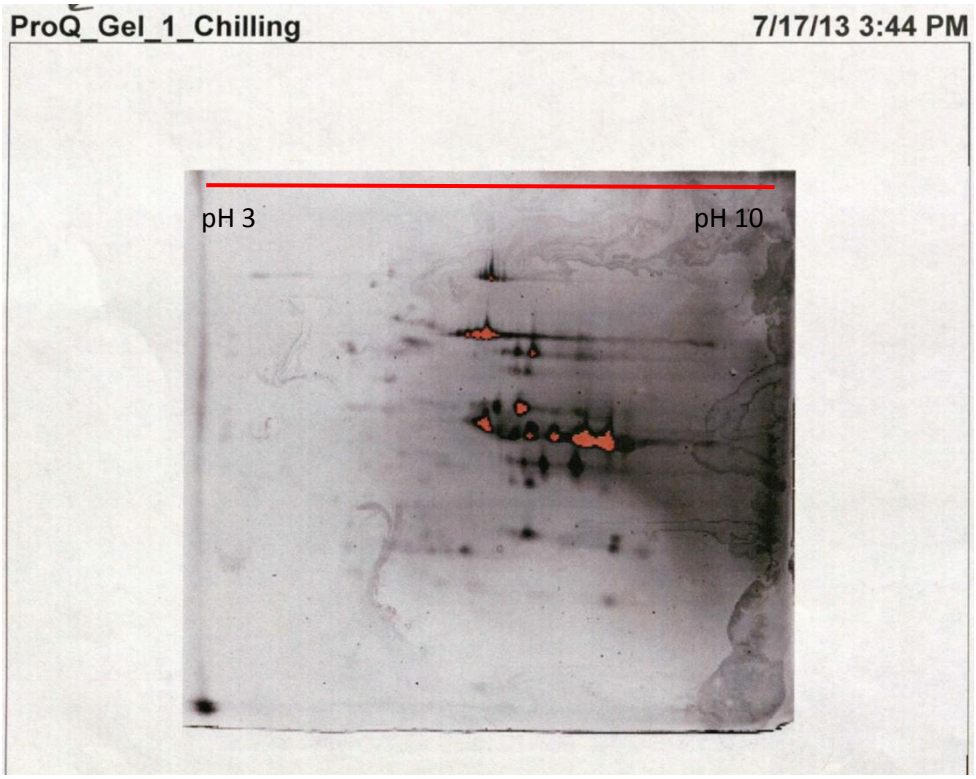


Fig.9A: ProQ Diamond (Invitrogen, Carlsbad, CA) technique to localize only the phosphorylated proteins from pH 3 to pH10. All the phosphorylated proteins in the sample, are showed

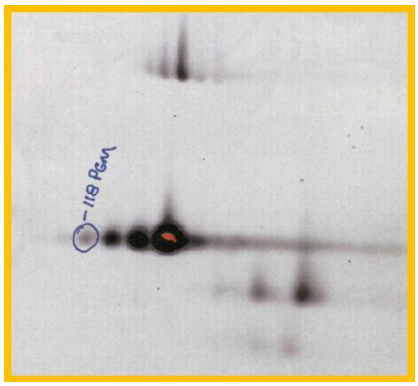


Fig. 9B: gel stained for total protein using ProQ Low exposure (Invitrogen, Carlsbad, CA). PGM1 spot was identified as protein subjected to phosphorylation

With the results obtained from this approach, it was possible to show that PGM1 protein is subjected to posttranslational modification by phosphorylation, in the muscle during postmortem.

4. Conclusions:

Rapid chilling of pork carcasses has the potential to influence pork quality (Shackelford et al. 2012).

In this study the difference in proteins abundance between samples subjected to deep and conventional chill temperatures, were displayed and verified.

Moreover, the identity of three selected protein spots (GAPDH, ALDO-A and PGM1) was validated and especially on PGM1 the final considerations were done.

The temperature can influence the protein activity, which can promote specific reactions, defining chemical, physical and technologic parameters in the muscle.

2D-DIGE analysis was performed to compare the divergent sample treatments (deep and conventional chill) and the results showed whole proteins characterized by significant ($P \leq 0.15$) different abundances in the two conditions. The abundance parameters vary probably because different chilling system can influence and change the proteins solubility and activity, in sarcoplasmic fraction, that includes the majority of enzymes and regulators in muscle. Therefore predominance of particular proteins in the sarcoplasmic fraction may be related to variations in tenderness and meat quality carcass traits.

Among all proteins identified, Phosphoglucomutase 1 (PGM1) resulted more interesting than others, because its abundance was higher in deep chill than conventional chill treatment, so its identity was validate by 2D-Western Blotting approach.

By 2D-Western Blot analyses, PGM1 was identify as a“beads-on-a-string”, composed from five spots, located at the same molecular weight, but different isoelectric point.

PGM1 is a glycometabolic enzymes that plays a pivotal role on the regulation of glycogen metabolism and glycolysis postmortem and its activity can be posttranslationally modified through phosphorylation, acetylation and methylation (Anderson M.J., *et al*. 2013).

From the reported results, it is possible to affirm that PGM1 was phosphorylated by deep chill temperature, that can alter its conformation and/or activity (including metabolism, transcription, signal transduction, etc.) and consequently the protein roles during postmortem and to influence meat quality properties.

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General conclusions:

In this thesis some factors influencing porcine meat and carcass qualitative traits have been analyzed focusing mainly on genomics.

The research technique mainly used was the candidate-gene approach, after selecting several polymorphisms in genes whose proteins were in same way associated with the studied traits. This procedure consists of genotyping and, later, performing a gene-trait association study, on samples from different breeds.

This method, applied in significant QTL regions for the studied traits allows to detect polymorphisms as possible markers of significant genes for the investigated traits, but considering the complexity of the biochemical reactions that happen in the muscle during the postmortem, the limitation of this method is the difficulty of accurately detecting the causative gene or genes. The phenotypic variability of quantitative traits, as the qualitative characteristics of meat and carcass, is based on the expression of several proteins, enzymes, hormones, etc... whose action regulates the activity of several metabolic pathways, so also several genes are involved to determine the phenotypic variability between animals .

Gene-candidate approach has been useful to detect new polymorphisms in candidate genes and to confirm more, which had already been pointed out in literature, significantly associated for the meat and carcass qualitative traits.

A new polymorphism has been detected on chromosome 2 (SSC2) *Calponin 1* (CNN1*)* to 70.5 Mb significantly associated to the studied traits and furthermore the results confirmed the effect already reported in literature for *Lactate dehydrogenase A* (LDHA) to 43.9 Mb, *Low density lipoprotein receptor* (LDLR*)* to 70.1 Mb, *Myogenic differentiation 1* (MYOD1*)* to 44.4 Mb e *Ubiquitin-like 5* (UBL5*)* to 68.9 Mb, in Italian Large White swine.

These results confirm the presence of significant QTLs regions on this chromosome and these QTLs could be useful to localize on the chromosome the causative genes responsible of the significant effect.

This approach has been also used to study the gene-trait association of *Perilipin 1* (*PLIN1*) mapping on chromosome 7 and *Perilipin 2* (*PLIN2*) mapping on chromosome 1, already reported on previous studies (Vykoukalová Z. *et al.,* 2009 and Davoli R. *et al.,* 2011).

Even in this case the results obtained from two pig populations of Duroc breed have shown significant associations with carcass traits.

An alternative method for the research of candidate genes has been utilized in the research for SNPs significant for the pH variation in pig meat after slaughtering, during the postmortem phase.

Using the database on pig genome (http://www.ncbi.nlm.nih.gov/genbank/) and PigQTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/SS/index, several SNPs on genes, mapping on chromosomes 1, 2 and 3, have been selected *in silico*, in regions described in literature as significantly associated to pH variations in pig meat.

This procedure has allowed to perform a selective search *in silico*, focalizing the research in specific regions of the genome (a few cM wide) where different genes are mapped that have been utilized to identify polymorphisms.

At a later stage, after the analysis of the genotype for the different polymorphisms in Italian Large White pig samples, has been possible to obtain one single SNP of *Deoxyhypusine synthase* (DHPS) to test on the population. Such approach has allowed to detect on SSC2 a polymorphism significant for the pH variation on meat in muscle samples collected at slaughterhouse from Italian Large White (ILW) pigs.

A further research on DHPS gene sequence and on the region where this gene maps in pig genome both, has allowed to highlight that DHPS maps in a region where another gene with opposite orientation on genome, is present. Indeed, DHPS sequence at 3' UTR level, is partially overlapping with 3' UTR *WD repeat domain 83* (WDR83) region.

These two genes, have the capability to influence and regulate each other's transcription, mutually inhibiting or favoring their activities.

Analyzing the overlapping genes function has been possible to know that these genes could be involved in the pH meat regulation, because the coded proteins could have a role in the muscle during anaerobic glycolysis after slaughtering, influencing the production of lactate.

On the whole the results obtained from the association studies utilizing different methodological approaches, have both confirmed the gene-trait relation for SNPs previously known and allowed to detect new markers associated to meat and carcass qualitative traits, to utilize after the validation of obtained results in the selection programs. The detected SNPs have likely to be considered positional candidates for the association with the studied traits and so, the search for the causative gene will have to be carried out starting from the results obtained in this work.

In this thesis both genomic approach and different methodological procedures more based on protein analysis were carried out, for a more in deep analysis and understanding of factors and processes developing in muscle cells after slaughtering and influencing qualitative meat traits.

These analyses evaluated the muscle proteins and protein composition variations, engendered by different carcass chilling temperature, which could be correlated to meat quality.

The results of a study carried out analyzing the protein composition in *Longissimus dorsi* (LD) samples, obtained from different pig carcasses, treated with different chilling temperatures during post mortem, have been considered.

These data indicate an effect of chilling treatment on carcass, related to the proteins of the muscle sarcoplasmic fraction, highlighting that the temperature reduction during carcasses chilling, can influence the quality of pig meat.

Using the electrophoretic method based on 2D-DIGE (2D- Difference In Gel Electrophoresis) and MALDI-ToF techniques it has been possible to identify and compare the different protein spots, found in the sarcoplasmic fraction of samples obtained from pig carcasses exposed to two different chilling temperatures. Moreover the abundances of the different proteins have been compared. Their protein activity hasn't been considered.

The attention has been focalized on Phosphoglucomutase 1 (PGM1) because recently some studies on beef steaks made by Anderson M.J. *et al.,* (2011), show that this protein can be involved in the determination of meat tenderness.

Using 2D-DIGE and a particular technique of staining proteins called Pro-Q, which show the phosphorylated proteins in the sample, it is possible to confirm that PGM1 in deep chill treatment compared to conventional chill, is modified. Indeed, several levels of phosphorylation characterize this protein in deep chill treatment.

Only after to have analyzed the activity of the protein PGM1, it will be possible to evaluate more precisely if the level of phosphorylation of this protein can have an effect on pig meat qualitative traits, as tenderness. Further studies are being carried out to this aim.

Environmental and genetic factors influence and concur to the definition of the characteristics determining qualitative traits of pig meat and carcass.

Although many studies have been already performed on this thematic, the full knowledge of biochemical and molecular factors of muscle, with effect on or regulating meat quality traits after slaughtering, is still quite sketchy.

In order to obtain a more complete frame and knowledge of these factors and their effects and how they act to regulate meat quality traits, it is important to plan studies and researches based on several approaches and strategies and to combine together all data.

Nowadays, the integration of different and innovative experimental approaches available (genomics, proteomics, transcriptomics…etc.) can allow to obtain a deeper and more complete knowledge of the biological and molecular processes developing in muscle tissue during the postmortem phase. Indeed in this critical postmortem phase both environmental and genetic factors

influence the conversion from muscle to meat and they can be responsible of phenotypic differences between individuals for meat and carcass quality.

Improving the level of knowledge on these aspects and with innovative and efficient experimental approaches it will be possible to use technology or genetic selection, to obtain fresh or seasoned meat products with excellent quality or with specific qualitative characteristics, to satisfy the requests both of the consumers and pig meat industry.