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IGF-I and its role in dairy animals:

investigations on Holstein Friesian cows and Sarda sheep

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Preface

Dairy sector is an essential component of the global food system, providing economic, nutritional and social benefits to a large proportion of the world's population. FAO defines milk as one of most relevant farming commodities worldwide, produced and consumed in all world's countries. The start point to obtain an efficient milk production is an adequate animal breeding and one of the most significant and influencing aspect is reproductive activity. Reproduction plays a pivotal role in dairy industry, since productive efficiency depends greatly on reproductive performances. To reach puberty at a proper age, maintain estrous cyclicity, achieve pregnancy and sustain it to term, as well uterine involution and follicular development postpartum are decisive features in dairy production. There are several hormones and metabolites, which act according to the physiological state of the organism in order to maintain normal homeostasis and regular reproduction function. Among these molecules there is a chemical mediator known as "Insulin-like growth factor-I protein" (also called IGF-I), encoded by the homonym gene, which plays a key role in various physiological processes such as in the establishment and maintenance of reproductive function. Moreover, several investigations show that IGF-I gene can exhibit singular nucleotide polymorphisms (SNPs) within its sequence, which can influence both reproductive activity and milk production in dairy animals.

This thesis is the result of two researches, carried out during three years of PhD School. Both investigations, although different, concern the same topic, that is the study of the IGF-I, as protein and as gene, and its effects on reproductive performances and milk production in two important dairy species, cattle and sheep. The aim of this work was to achieve scientific information that could be useful in dairy animal breeding, in consideration of the significant influence that IGF-I has, and the possibility to use it as marker to improve livestock performances. Indeed, genomic selection is recognized as an important tool that together with an adequate management can improve the production.

The "Study 1" has been realized during a period of up 6 months as a visiting student at the Royal Veterinary College, University of London, working with Professor Claire Wathes and her group, in the Department of Production and Population Health at the Hawkshead campus. The research carried through in the Host Institution was under an ongoing EU project "GplusE" (Genotype plus Environment). GplusE, taking in consideration the great distribution of dairy cattle in the World and in Europe, aims to contribute to the sustainability of dairy cow production systems through the optimal integration of genomic selection and novel management protocols based on the development and exploitation of genomic data and supporting novel phenotyping approaches. The overall objectives are improvement in productivity, efficiency, animal health, welfare and fertility in an environmentally sustainable way. Results from the project will also increase biological understanding of the mechanisms by which

genotype, environment and their interaction influence performance. One component of GplusE is to undertake a Genome Wide Association Study (GWAS) to link key physiological markers of cow health with genetic makeup. This will involve recruitment of between 10-15,000 Holstein cows from commercial dairy herds, of which about 1,000 have been sourced from the United Kindom (UK), and respectively 332 and 40 have been chosen to realize the present research.

The "Study 2" has been performed at the Department of Veterinary Medicine of the Sassari University, collaborating with my Tutor Doctor Maria Consuelo Mura and her team, within the section of "Endocrinology, animal husbandry and animal welfare". The research carried through was under an ongoing project "MIGLIOVINGESAR" supported by Regione Autonoma della Sardegna. This part of the research is focused on Sarda sheep, considering the great weight of this breed in Sardinia, and the importance of the milk sheep industry in this region. Moreover, it is important underline as productions of small ruminants are of great importance for many Mediterranean countries. These productions are the most valuable coming from the extensive and semi-intensive livestock systems typical of the Mediterranean pastoralism. These systems of livestock production often represent the only possible economic activities in inland areas and play a crucial role in maintaining both the vitality and the traditions of rural communities, as well as in preventing environmental issues (i.e., soil erosion, desertification, wildfire).

Current breeding programs, in dairy sheep, are based on quantitative

approach, and have achieved appreciable genetic gains for milk yield. However, further selection goals could be added, based on their importance in improving production traits. For this reason, the strengthening of research projects focused at finding causal mutation within genes affecting traits of economic importance is the most effective strategy for the future. The present study lies precisely in this perspective.

1. Introduction

1.1 The dairy sector

1.1.1 The world dairy sector

Dairy sector is considered a fundamental component of the worldwide food system, for the great production and consumption of milk and milk products. All over the world people cover approximately 13% of their protein requirement from milk, and about 40% of the world population consume milk daily (IFCN Dairy Report, 2008). Statistical analysis carried out by International Dairy Federation shows that the average level of consumption of milk and its products has increased consistently during the past decade and it was registered to be 110.7 kg pro capita in the 2014 (Figure 1) (FAO, IDF National Committees, 2016). Furthermore, this amount is projected to rise in the future, by 0.8% and 1.7% per year in developing countries, and between 0.5% and 1.1% in developed economies (FAO, The Global Dairy Sector: Facts, 2016).

The dairy sector represents a dynamic global industry, with constantly growing production trends, which are predicted to continue in the long-term. World milk production in 2010 reached 711 million tonnes (Blaskò, 2011) and it is forecast to grow by 1.4% to 831 million tonnes in 2017, with output set to expand in Asia and the Americas, stagnate in Europe and Africa, and decline in Oceania (FAO, Milk and milk products, Food outlook, 2017). Most of the global milk increase would originate in Asia, principally in India, but a

major production is also anticipated in Pakistan, Turkey, the Islamic Republic of Iran and Saudi Arabia. Instead, in China, output is expected to record a decline, as low domestic prices and competition from imported milk powder have weighed on profitability and led to a reduction in the national dairy herd.

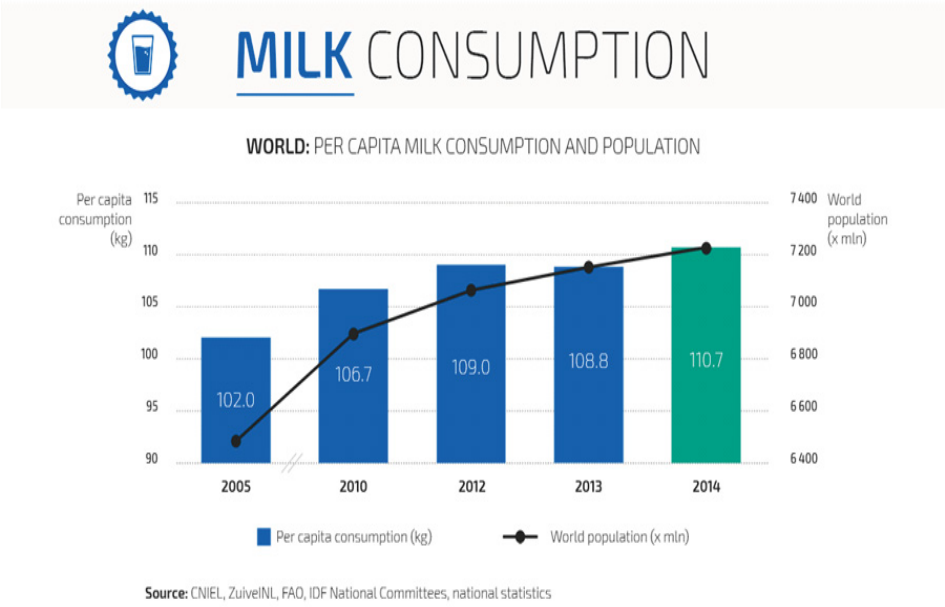


Figure 1. World milk consumption from 2005 to 2014 (FAO, IDF National Committees, 2016).

In Japan and the Republic of Korea, stable to lower milk production is anticipated due to the effects of herd reduction. In South America, recovery in milk production followed El Niño-associated extreme weather conditions, which caused overall milk production to fall by over 4% in the region in 2016. Anyway, from January to May 2017, weather conditions have been generally favourable in the main dairying areas of the central and northeastern part of

the country. Consequently, Brazil's milk production is expected to recover in 2017. Argentina and Uruguay both saw milk output fall by over 10% in 2016, mainly because of excessive rainfall and resultant flooding. In Mexico, continued modest growth in output is foreseen based on herd rebuilding and improvements in genetics and technology. In North America, output in the United States (US) is forecast to rise by 2%, continuing the expansion witnessed in recent years. Milk deliveries in Canada are set to grow by almost 4%, as quota limits for milk destined for processing were raised, due to increased domestic use of butterfat. In Africa, poor pasture conditions persist in large parts of Kenya, Somalia, Ethiopia and Tanzania, following inadequate precipitation during the October to December 2016 rainy season, affect milk production. Meantime, milk output in southern Africa may rise in a number of countries, as abundant seasonal rains have led to improvements in animal and pasture condition and alleviated some of the effects of the prolonged drought that had afflicted the sub Saharan region. In Oceania, milk production decreased in New Zealand, dropping by 1% and in Australia, milk production in the last two years is set to plummet by 8%, its lowest level in 21 years. Assuming normal weather conditions, dairy production is anticipated to recover somewhat in the next years, assisted by growth in domestic demand and improved international prices for milk products. Dairy production in the Russian Federation is predicted to fall by 0.5%. The dairy herd is expected to continue its decline and may fall by 3%, although this

would be largely compensated by productivity gains, as the movement towards increased large-scale production and reduced smallholder participation continues. In Europe, milk production is projected to have a low increase, by 0.4%. European Union (EU) milk output is still adapting to the 2015 removal of production quotas and the resulting intensification of exposure to international market forces. In the first part of 2016, a large rise in production and limited external demand caused milk prices in EU member countries to drop substantially, forcing many producers to cut output during the second part of the year (FAO, Milk and milk products, Food outlook 2017).

World milk production is almost entirely derived from cattle, buffaloes, goats, sheep and camels. Other less common milk animals are yaks, horses, reindeers and donkeys. The presence and importance of each species varies significantly among regions and countries. The key elements that determine the dairy species kept are feed, water and climate. Other factors that may influence the presence of a dairy species are market demand, dietary traditions and the socio-economic characteristics of individual households. Cattle are kept in a wide range of environments and represent the most important species for milk production (Figure 2), while buffaloes are bred especially in wet tropical regions. Goats allow milk production in regions with poor soils, sheep in semi-arid regions around the Mediterranean, camels in arid lands, yaks in high mountainous areas such as the Tibetan Plateau, whereas horses in the steppes of Central Asia. Reindeers are bred in the

indigenous region of Scandinavia and donkey milk is linked for its use since antiquity for cosmetic purposes as well as infant nutrition.

It is evident that in developed countries, almost all milk is produced by cattle (Figure 2), while about one-third of milk production in developing countries comes from other dairy species, as buffaloes, sheep, goats and camels, which make dairying possible in adverse environments that often cannot support any other type of agricultural production.

Figure 2. *(On the left) Milk production by species, it is evident how cows are the major producers of milk. (On the right) Cow's milk production per continents (FAO, IDF National Committees, 2016).*

Cattle produce 83% of world milk, followed by buffaloes with 13%, goats with 2% and sheep with 1%, while camels provide only 0.4%. The remaining share is produced by other dairy species such as equines and yaks.

Milk from dairy species other than cattle represents 39% of milk production in Asia, 26% in Africa, 3% in Europe and 0.3% in the Americas, while it is almost non-existent in Oceania. FAO declares that in 2016 were bred about 133 million dairy cattle; 28.5 million buffaloes; 41 and 19 million goats and sheep, respectively. Cows and especially Holstein dairy cattle dominate the milk production industry in developed countries; the reasons for their popularity are excelled production, greater income over feed costs and unequalled genetic merit. In developing countries farmers usually use indigenous animals, keeping them in herds of 2 or 3 heads. In industrialized economies, however, herds are often larger: the average dairy farms in the UK and the US manage 90 and 300 dairy cows, respectively. Nevertheless, farms with more than 100 cows represent less than 0.3% of all dairy farms globally (FAO, 2016).

In developed countries nearly all the milk produced on farms is delivered to dairies. The major processed products include: liquid milk, cheese, fermented products, processed skim milk for casein, butter, condensed milk, skim milk powder (SMP) and whole milk powder (WMP).

In regions that are more than self-sufficient in milk, such as the European Union, butter and SMP are residuals of total milk supplies. When all other milk requirements (mainly for higher value products such as cheese, fresh products and WMP) are satisfied, the remainder is processed into butter and SMP for storage. Production volumes are

therefore strongly linked to the supply and demand conditions of high-value dairy products. Thus, fluctuations in cheese production are a major determinant of butter production. In most developing countries, milk is produced by smallholders, and milk production contributes to household livelihoods, food security and nutrition. Some developing countries have a long tradition of milk production, and milk or its

products have an important role in the diet. Other countries have established significant dairy production only recently. Most of the former countries are located in the Mediterranean and Near East, the Indian subcontinent, the savannah regions of West Africa, the highlands of East Africa and parts of South and Central America. Countries without a long tradition of dairy production are also in Southeast Asia (including China) and tropical regions with high ambient temperatures and/or humidity.

1.1.2 The European Union dairy sector

The EU (Figure 3) produces nearly a quarter of the world's available milk. It is a major player in the world dairy market as the leading exporter of many dairy products, most principally cheeses. Milk production takes place in all EU Member States and represents a significant proportion of the value of EU agricultural output.

Figure 3. European Union-28 map (http://www.bbc.com).

The EU's main producers are Germany, France, the UK, Poland, the Netherlands and Italy, which together account for almost 70% of the EU production (Figure 4) [European statistic (EUROSTAT)]. Cow's milk is the most important dairy production and after milk quotas disappeared on 1 April 2015, in EU-28, production of cows' milk increased slightly (+0.8%), the price of farm milk fell and the number of dairy cows remained stable (+0.2%). Nevertheless, at national level, the dairy herd grew strongly in few countries (+9.9% in Ireland and +6.6% in the Netherlands), while it contracted in 18 Member States. Belgium, Denmark and the UK showed similar, but less

marked, growth. In many countries, the contractions in herd were compensated by an increase in productivity.

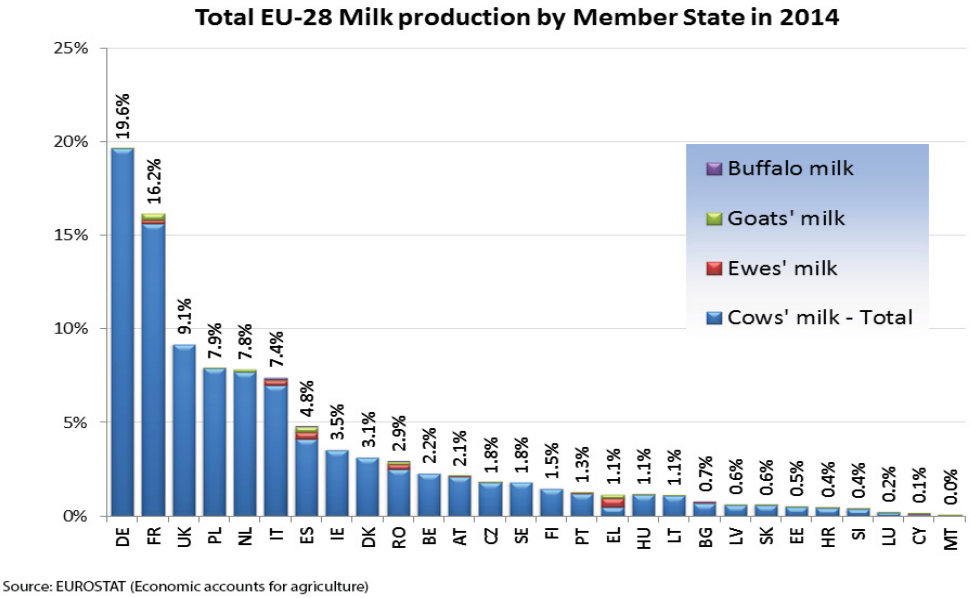
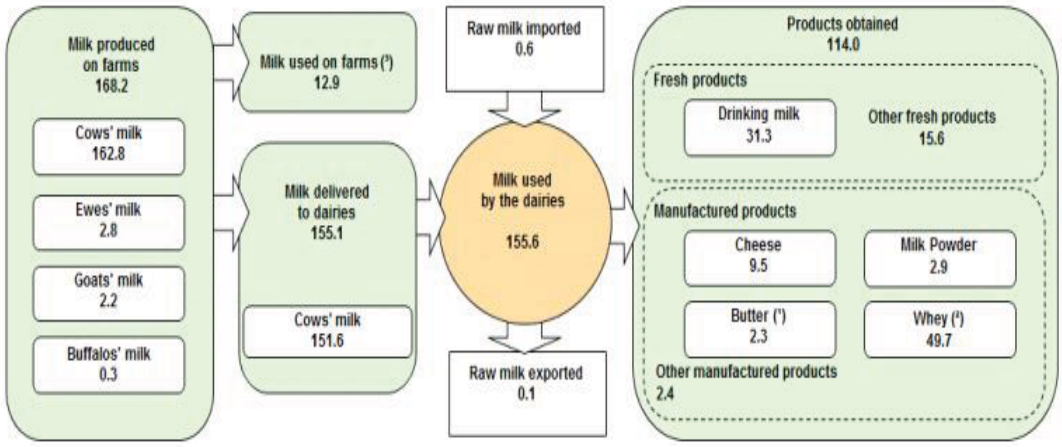


Figure 4. Total EU-28 milk production by Member State in 2014 (EUROSTAT).

The increase in cow milk productivity was the most spectacular in Greece, where the decrease by 18% in the dairy cow herd did not impact on milk production.

The apparent milk yield per dairy cow increased by 1.5% in EU-28 from 2014 to 2015, almost reaching 6.900 kg per dairy cow. In the Czech Republic, Spain, Hungary and Poland, the apparent milk yield grew by 320 to 420 kg per head, reflecting also development of the most productive farms and the ending of milking activity in the less productive ones, with various national balances. In 2015 in EU-28, the farms produced approximately 168.2 million tonnes of

milk. Production of cows' milk was 162.8 million tonnes (96.8% of all milk produced) while milk from ewes, goats and buffalos represented 5.3 million tonnes (3.1%). The main part of milk produced was delivered to the dairies (155.6 million tonnes) and the remaining amount (12.9 million tonnes) was used otherwise on the farms, i.e. processed, own-consumed, sold directly to consumers, or used as feed. The milk delivered to dairies is processed into a number of fresh products and manufactured products. It is used principally as drinking milk (31.3 milion tonnes) and other fresh products (15.6 milion tonnes). Between manufactured products there are cheese, butter, milk powder and whey (Figure 5) (EUROSTAT, Agriculture, forestry and fishery statistics, 2016 Edition). Over one fifth (21%) of all the cows' milk collected by EU-28 dairies in 2015 was produced in Germany, while slightly more than a sixth of the total (16.7%) in France. Spain (28.5%), France (21.2%), Greece (19.2%) and Italy (17.8%) collected more than 86% of the milk from sheep, goats and buffalo. Among them, Spain was the country that produced the highest quantity of nonbovine milk (1,004 thousand tonnes), which represented 14.8% of the total milk collected in Spain. Greece also produced about 678 thousand tonnes of milk from animals other than cows, representing 52.9% of the total milk locally collected.



(1) Includes other yellow fat dairy products; expressed in butter equivalent.
(2) In liquid whey equivalent.
(3) in whole milk equivalent.
(4) Inconsistency with the sum of milk by species are due especially to inconsistency in NL data.

Figure 5. EU Milk production and its destination in million tonnes (EUROSTAT, Agriculture, forestry and fishery statistics, 2016 Edition).

In 2015, 151.1 million tonnes (97.1%) of the milk available to the dairy sector was processed. Some 54.1 million tonnes of whole milk (36% of whole milk) and 14.4 million tonnes of skimmed milk were used to produce 9.5 million tonnes of cheese. The production of 2.3 million tonnes of butter and yellow products required 45.6 million tonnes of whole milk (30% of whole milk) and generated 42.3 million tonnes of skimmed milk. The skimmed milk, generated mainly during the fabrication of butter and cream, was used for processing into other dairy products. Some 16.4 million tonnes of whole milk and 14.8 tonnes of skimmed milk became drinking milk with a similar volume (31.3 million tonnes) (EUROSTAT, Agriculture, forestry and fishery statistics, 2016 Edition). In April 2017, 13,196 million litres of milk were delivered to

dairies in the EU-28, rising to surpass volumes delivered in April of last year by 0.4%. With a total of 600,000 dairy farms, 12,000 processing facilities and producing 15% of all EU revenue, the European dairy sector is a heavyweight in the European Union's agricultural economy. The EU dairy sector's second asset is its internal market of 508 million people, whose consumption is three time higher than the global average. Eighty-seven percent of Europe's milk is consumed by EU countries (European dairy association, 2016).

1.1.3 The Sardinian dairy sector

Sardinia, autonomous region of Italy (Figure 6), is for its extension the second largest island in the Mediterranean Sea, after Sicily. Its landscape consists of 13.6% mountain, 18.5% flat land and 67.9% hilly. Sheep farming in Sardinia is by far the most important agricultural activity, characterizing the island economically, socially, culturally and historically.

Figure 6. *Sardinia Island is the second largest island in the Mediterranean Sea (*http://d-maps.com).

The most important species bred is the Sarda sheep, with over 3,300,000 animals kept along the island for milk production [Istituto Nazionale di Statistica (Istat)]. Other species bred for dairy production are goats and cattle with respectively about 240,000 and 70,000 animals (Istat). Cow milk production is of minor importance in Sardinia, whereas this island is the main producer of sheep and goat milk in Italy. In Sardinian there are 12,669 sheep farms, with average about 230 animals per farm, especially in the provinces of Sassari and Nuoro; 7,852 cow farms, with average about 30 animals per farm, particularly in the zone of Arborea and 2,634 goat farms with approximately 100 animals per farm, above all near Cagliari and Nuoro (Sardegna Statistiche, 2013). Sardinia is the most important EU region for sheep milk production, with 3.5% of sheep of the EU total (EUROSTAT, 2012) and a milk production of about 330,000 tonnes per year (Sardegna Statistiche), which represents more than 12% of the total European production (EUROSTAT). More than half of Sardinian sheep milk production is delivered to cheese industry for "Pecorino Romano PDO" (Protected Designation of Origin, European quality label) production (Furesi et al., 2013). "Pecorino Romano PDO" (reg. CE 1107/1996), is one of the main Italian PDO products (ISMEA, 2012) and 95% of its production derives from Sardinian cheese factories (Idda et al., 2010). It is exported to Canada (60%), France, Germany and United Kingdom (Laore, 2008). The other two PDO cheeses are Pecorino Sardo and Fiore Sardo (reg. CE 1263/1996).

1.2 The dairy cattle

1. 2.1 The origin of domesticated cattle

The domestication of cattle, other livestock, and crop species, started 11,000 years ago and represent the beginning of development of agriculture, a turning point in human history, leading to extensive modifications of the diet, the behaviour, and the socioeconomic structure of many populations (Diamond, 2002). Migratory hunter-gatherer populations were transformed into sedentary farming societies, in which security and relative abundance of food permitted the population growth. Pastoral societies exploited the capacity of cattle, sheep, and goats to feed on grass or foliage, becoming the major source of milk, meat, and hides. Cattle accompanied human migrations, which led to the dispersal of domestic cattle in Asia, Africa, Europe, and the New World (Ajmone-Marsan et al., 2010).

The two principal taxonomic groups of domestic cattle are Bos taurus (taurine cattle) and Bos indicus (zebu cattle) (Figure 7).

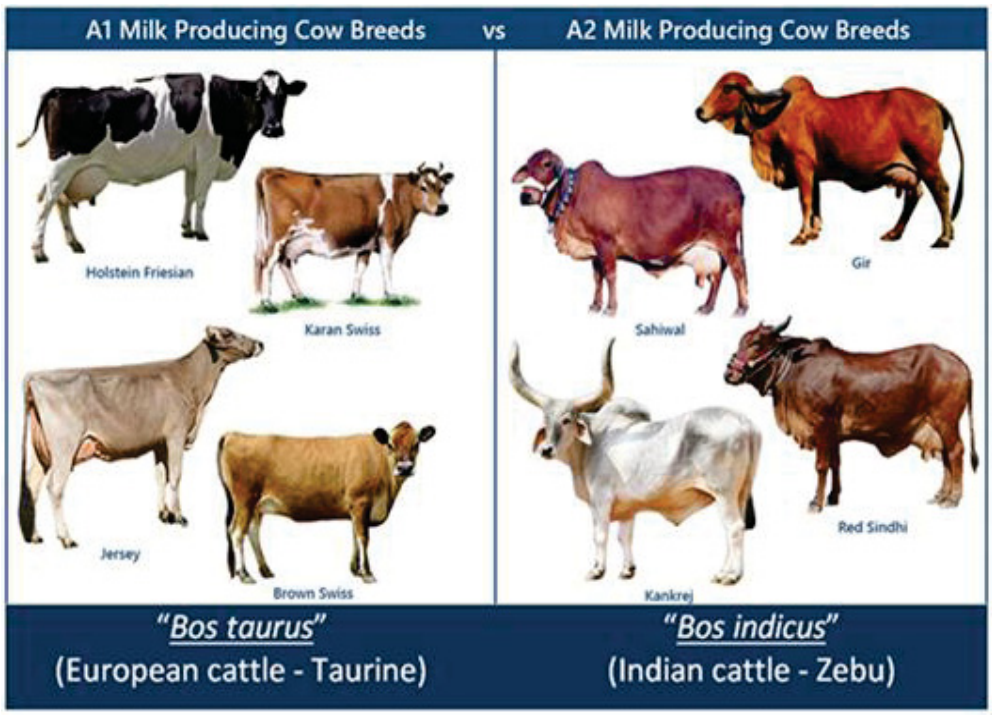


Figure 7. The most common current breed of Bos taurus and Bos indicus.

Bos indicus are distinguished from Bos taurus by the presence of a muscular-fatty hump, large and pendulous dewlap, skin anatomy and physiological characteristics that involve adaptation to arid conditions. Bos taurus are all humpless with a small dewlap and anatomy features for temperate climate zone (Ajmone-Marsan et al., 2010). Several studies (Loftus et al., 1994; Bradley et al., 1996 and MacHugh et al., 1997) showed that these two groups stem from independent domestication events in different geographical regions (Bollongino et al., 2007). The most recent genetic data suggest that maternal lineages of taurine cattle originated in the Fertile Crescent (Middle East) (Figure 8) with a possible contribution of South-

European wild cattle populations, while zebu cattle lineages originated from the Indus Valley (Ajmone-Marsan et al., 2010).

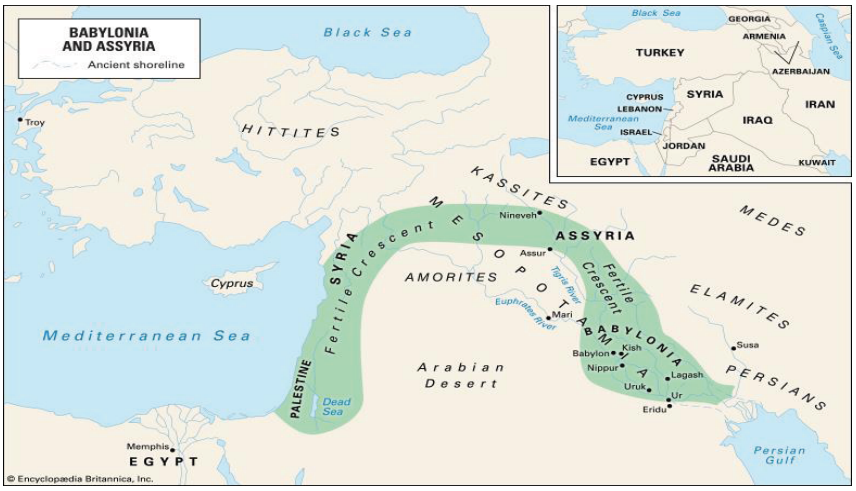


Figure 8. *The Fertile Crescent (Encyclopædia Britannica).*

Zebu cattle and their crossbreeds predominate in relatively arid regions such as the Indian subcontinent, the Near East, and most of Africa, whereas taurine cattle are native to North Africa, humid West Africa, and a vast swathe of Eurasia stretching from the Western European fringe to Japan (Bradley et al., 1998). These cattle have the same ancestor, a wild ox called auroch (*Bos primigenius*) (Figure 9), which, during the Pleistocene and Holocene, ranged from the Atlantic to the Pacific coasts and from the northern tundra to India and Africa (Zeuner, 1963).

Figure 9. A painting by Heinrich Harder showing an aurochs fighting off a wolf pack.

The auroch (*Bos primigenius*) was an important animal for humans, during prehistory when it was widely hunted, and in some areas also during historical periods (Wright, 2013). Based on bone finds, pictures and descriptions it is possible to obtain the characteristics of the aurochs (Van Vuure, 2012). They were giant beasts and such ferocious animals that Julius Caesar wrote about them while giving an account of the Black Forest in Germany : "They are but a little less than elephants in size, and are of the species, colour, and form of a bull. Their strength is very great, and also their speed. They spare neither man nor beast that they see. They cannot be brought to endure the sight of men, nor be tamed, even when taken young. The people, who take them in pitfalls, assiduously destroy them; and young

men harden themselves in this labour, and exercise themselves in this kind of chase; and those who have killed a great number – the horns

being publicly exhibited in evidence of the fact – obtain great honour" (Caesar, 65 B.C.). One of the intriguing aspects of the Holocene aurochs is its size. Many authors did their utmost to describe this size, especially the height at the withers. Based upon several methods of measuring bones, skeleton observation and sometimes a rich imagination, the reported withers height varied between 130-150 cm (the size of present-day cattle) and 200-220 cm (Guintard, 1999). Both aurochs bull and cow were relatively long-legged, especially when compared to modern cattle. Another conspicuous characteristic of the aurochs were its horn form. The aurochs cow had a small, hardly visible udder, like in other wild bovine species (van Vuure, 2012). Udder sizes as seen in dairy cattle nowadays are a typical domestication trait. The wild aurochs population of Europe has been extinct for 400 years (Bailey et al., 1996). Cow domestication from wild aurochs was among the most important innovations during the Neolithic agricultural revolution. Breeds domesticated in the Near East and introduced in Europe during the Neolithic diffusion probably intermixed, at least in some regions, with local wild animals and with African cattle introduced by maritime routes. Consequently, European breeds should represent a more diverse and important genetic resource than previously recognized, especially in the Southern regions (Beja-Pereira et al., 2006). Neolithic cattle were smaller than aurochs and

continued to decrease in size until the Middle Ages and cattle became adapted to environments, from green pastures to deserts

and developed a large variety of visible phenotypes (Ajmone-Marsan et al., 2010). Large horns for fighting became unnecessary and even undesirable in a farm environment, which led to the emergence of short-horned and even hornless (polled) cattle. Genetic characteristics in cattle have been manipulated during the history. The trivial feature of coat coloration and the horn size were a major preoccupation of the founders and improvers of modern breeds in the eighteenth century (Bradley et al., 1998). Observations have established that a drastic reduction in size occurs in bovines after only a few generations of domestication. However, even if domesticated cattle are normally significantly smaller than their wild ancestors, the morphological distinction between the two groups is often ambiguous. Some bones and teeth are of intermediate size and could therefore be either small aurochs or large cattle. During the last centuries, differentiation was emphasized by the development of hundreds of specialized breeds (Ajmone- Marsan et al., 2010).

1.2.2 *Bos taurus taurus*

Bos taurus taurus is a mammal belonging to order of *Artiodactyla*, family *Bovidae* and subfamily *Bovinae*. Most modern breeds of cattle are taurine cattle. Nowadays, there are several hundreds of breeds in the world,

but it is possible to classify them in few groups according to their main productive attitude: dairy cows, specialized to produce milk, beef cows, selected for meat, and cows with dual purpose, both milk and meat. Physical attributes of the dairy cows include long, thin neck, obvious spine, fine-boned legs, angular body, and truer rectangular shape (Figure 10).

Figure 10. Breeds of dairy cattle (Dairy Moos blog, photographed by Cybil Fisher).

Beef cattle are more muscular than dairy cattle, with a short, stout neck, thick back without any visible backbone, round leg bones and body (Figure 11). Approximately 480 cattle breeds are recognized today in Europe. This has resulted in their adaptation to different environments and selection processes, with the result of considerable variation in appearance and performance. Between the most important breed of dairy cows are collocated Holstein Friesian, Brown Swiss, Jersey, Guernsey and Ayrshire.

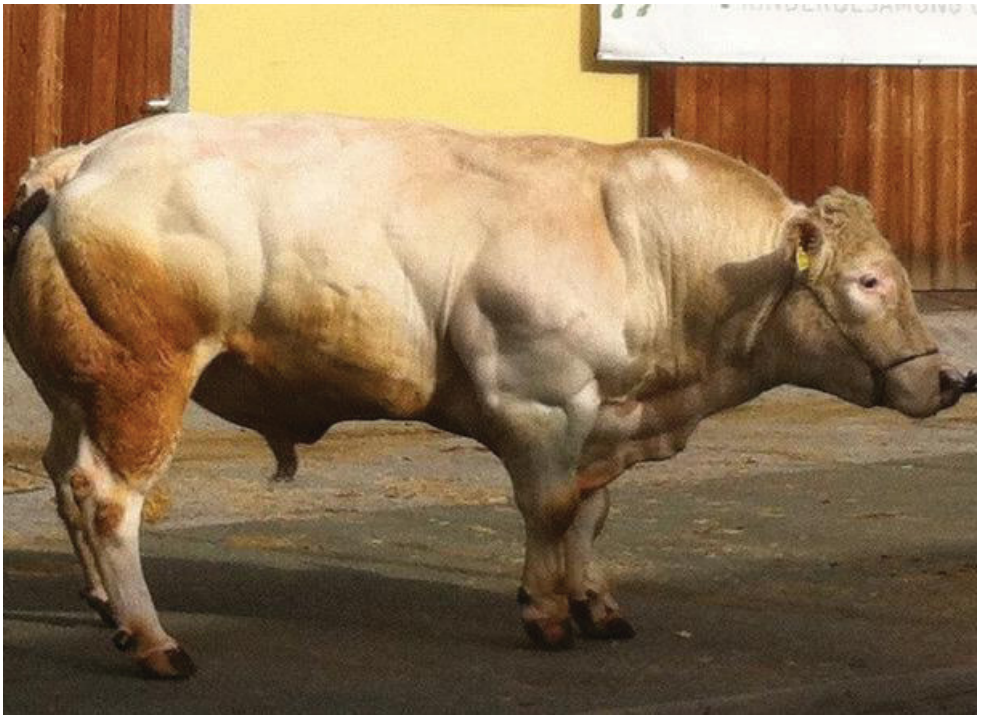


Figure 11. Blanc-Blu Belgian bull, a beef breed cattle (photographed by Maria Veronica Di Stefano).

1.2.3 The Holstein Friesian cattle

Holstein-Friesian is the most recognized breed of dairy cattle and it originated from Netherlands approximately 2,000 years ago. Two breeds of cattle, black animals from the Batavians (present day Germany) and white animals from the Friesians (present day Holland), were crossed to create a new breed of cattle, which led to a high milk-producing animal. Holstein-Friesians, is nowadays called more simply Holsteins. Friesian cattle still exist today, but are distinct from the Holsteins. After the World War II, the Holsteins was selected for a high yielding, large-framed, single-purpose dairy

cow with an exceptionally good udder. The modern Holsteins have distinctive black and white (Figure 12) or red and white markings (Figure 13).

Figure 12. Black and white Holstein Friesian.

Figure 13. Red and white Holstein Friesian.

The red and white colouring is a recessive gene that appears when both the dam and sire are carriers or exhibit the trait themselves. The Holstein cows are about 150-160 cm high at the withers, weighing up to 800-900 kg; bulls are 170-180 cm high, weighting 1,000-1,250 kg and sometimes up to 1,500 kg. A very precocious breed, it benefits from a rapid growth rate and heifers easily calve at two years of age. It is also endowed with an excellent functional morphology, that is, an udder adapted to mechanical milking, a body capacity that provides optimal valorisation of fodder, a slightly sloped rump for easy calving and feet and legs, which provide good locomotion. More than 19 million animals are registered in the Holsteins Association's herd book. The ancestry of most of these animals can be traced back to animals originally imported from the Netherlands. Holsteins can be found on every continent and in almost every country. Such convincing evidence of genetic superiority has created an active export market for Holsteins genetics.

Currently, live Holsteins females and males, including frozen embryos and semen are being exported to more than 50 countries and used extensively to improve foreign food supplies and dairy producer incomes. The Holsteins breed is known for high milk production (an average of 9-10 tonnes of milk for lactation), but its milk has less fat (3.9%) and protein (3.2%) compared to other less specialized breeds. Holsteins dairy cattle dominate the milk production industry; the reasons for their popularity are unexcelled

production, greater income over feed costs, unequalled genetic merit, and adaptability to a wide range of environmental conditions.

1.2.4 Reproductive activity in dairy cows

1.2.4.1 The estrous cycle

After the event of domestication, cattle were selected against seasonality, so domesticated cows are not sensitive to photoperiodicity and they can ovulate and conceive in every time of the year, with a polyestrous cycle (Hafez, 7th edition). However, photoperiodicity could influence the age of puberty in female cattle. Heifers born in autumn (short days) reach puberty earlier than those born in spring (long days) (Schillo et al., 1983).

Puberty starts with the first ovulation, the signs of estrus and normal luteal function. The average age at puberty is 10-12 months for dairy breeds and it is affected by both genetic and environmental factors, such as breed, nutrition, environment, management, bodyweight, skeletal growth and amount of body fat stores (Mialon et al., 2001; Hafez, 7th edition).

The estrous cycle normally occurs every 18-21 days after heifers reach puberty and it includes two phases: luteal and follicular. Follicular phase consists of proestrus and estrous, while luteal phase is divided in metaestrus and diestrus. In the follicular phase, during proestrus, the follicle stimulating hormone (FSH) trigs follicle development, and luteinizing hormone (LH) promotes further follicle growth and maturation of eggs. Ovarian follicular

growth is characterized by two or three consecutive follicular waves, where it is possible to recognize five steps: recruitment, selection, growth, dominance, and regression of follicle. Each wave involves the recruitment of a cohort of follicles and the selection of a dominant follicle. The dominant follicle continues to grow and mature to the preovulatory stage, while others undergo atresia. All follicles growing as a cohort contain specific receptors for FSH and depend on this gonadotropin in their growth. At this stage the growing follicles do not have a sufficient population of LH receptors to respond to a LH-like stimulation, for this reason this stage of follicular growth is sometimes called FSH-dependent. Selection of the dominant follicle is associated with a decrease in FSH, which is maintained at basal levels. The selected future dominant follicle acquires LH receptors, which allow it to continue growth in the environment of low FSH and increasing LH levels. Growing follicle produces high levels of estrogen that causes estrus and a surge release of LH (preovulatory LH peak) from pituitary gland, and provokes ovulation of dominant follicle. Ovulation occurs approximately 30 hours after the onset of estrous and after the behavioral signs of estrous have ceased. Proestrus starts after 17-20 days from the precedent cycle, while estrus is traditionally considered as day 0 of a new cycle. In late proestrus, the influence of estrogens on the reproductive tract and behaviour of the cow can be observed. Estrus is also called heat and lasts, on average, 4-24 hours. During estrus, the heifer is receptive to a bull and stands for mating (standing

heat). Behavioral signs of estrus are due to the influence of estrogens such as, restlessness, drop in milk production, standing to be mounted, presence of clear mucus, swelling and reddening of vulva. Luteal phase starts with metaestrus, after ovulation, when there is corpus luteum development, and progesterone levels in circulation begin to rise. The next phase is the diestrus, period of maximum corpus luteum size and function, and high levels of progesterone in circulation. At the end of diestrus, luteolysis of the corpus luteum begins. Metaestrus corresponds to 2^{nd}-4^{th} day of the cycle, and diestrus to 5^{th}-17^{th} day. Late in the estrous cycle, uterus produces prostaglandins (PG) which cause regression of corpus luteum. Progesterone regulates secretion pattern of LH pulses and hence, follicular development.

1.2.4.2 Dairy cows reproductive and postpartum management

Cows must calve to produce milk and the lactation cycle is the period between one calving and the next. In intensive dairying systems, heifers are reared with the intention of calving for the first time at 22-30 months of age, depending on the breed (Bittante, 2005, New Edition). The calf is normally taken from the cow shortly after calving and reared separately, usually receiving artificial milk substitute as a basic diet until weaning. Almost all the milk produced by the cow is thus available for sale (Ball and Peters, Third Edition). In an optimal situation, cows are expected to lactate for a period of 305 days, considering that they need a “dry period” of about

60 days before calving for regeneration of the udder tissue in preparation for the next lactation (Ball and Peters, Third Edition). In most intensive systems cows are inseminated between 45 and 60 days postpartum (De Rensis and Marconi, 1999), this "Voluntary Waiting Period" is important to ensure uterine regression and recovery of follicular activity. Moreover, it allows pregnancy by 80-85 days after calving, since gestation length is approximately 280–285 days and an optimal interval between calving is of 365 days. Anyway, also a calving interval up 410 days is considered acceptable (De Rensis and Marconi, 1999). The above, represents the ideal situation for an efficient reproductive management.

A very critical phase for reproductive cycle of dairy cattle is the early post-partum period. In this period, the reproductive function can be divided into recrudescence of ovarian follicular activity, ovulation, and formation of fully functional corpora luteal, which will maintain a pregnancy. Modern high-yielding dairy cows enter a state of negative energy balance (NEB) around calving when the energy demand for maintenance and lactation exceeds that of dietary energy intake with resulting extensive loss of body condition, which can be assessed by Body Condition Scoring (BCS).

Cows with excessive body tissue mobilization at this stage may take up to 20 weeks to regain a positive energy balance status (Taylor et al., 2004). One important link between NEB and fertility appears to be through the delaying of the first postpartum ovulation. Minimizing the interval to first ovulation

provides ample time for completion of multiple ovarian cycles prior to insemination, which in turn improves conception rate (Butler, 2000).

1.3 The dairy sheep

1.3.1 The origin of domesticated sheep

Small ruminants, sheep and goats, were among the first animals to be domesticated (Ryder, 1983) immediately after the dog, useful in defence and hunting (14 – 12,000 B.C.) perhaps because of their tameness, small size and ability to supply various products, attracting man's attention between 10,000 and 8,000 B.C. in the plains of the Fertile Crescent. Sheep were first reared for access to meat before human mediated specialization for wool and milk began 4,000–5,000 years ago (Kijas et al., 2012). It is important to note that not all animals can be domesticated and that the majority of the domesticated species share specific characteristics compatible with humans' necessities: they are sociable, breed readily in captivity, have a wide home range and a short flight distance (Clutton-Brock, 1999), features often found in herbivores as in the sheep. The effect of artificial selection is evident on the conscious decision of the herder in choosing a specific phenotype, either by controlling the mating in favour of the most desirable traits. This is the case with the absence or reduction of horns in sheep and goats. Whereas the wild counterparts (mouflon and bezoar) have large horns, the domesticated species either are polled (as for most sheep breed) or have smaller and twisted horns (as for the goat). This feature was advantageous for the man who would avoid managing animals with dangerous weapons.

Also, reduction in body size has been related to domestication and it is visible in almost all the early domesticated animals according to archeo-zoological findings (Zeder, 2008). Both body size and horn presence could also be a product of adaptation of wild animals to a novel anthropogenic domain (Barbato, 2016). The first region of importance, with the oldest human settlements in the Near East, is dated about 8,500 B.C. and located in the upper Euphrates valley in eastern Turkey, near the northern arc of the so-called Fertile Crescent (Peters et al., 1999). The Zagros region of modern day Iran and Iraq is also recognized as a primary Centre of sheep domestication (Zeder, 1999). There are a number of different theories regarding the origins of domestic sheep (*Ovis aries*). However, most sources agree that they could originate from feral animals as argali (*Ovis ammon*) (Figure 14), urial (*Ovis vignei*) (Figure 15) and mouflon (Figure 16). In reality, mouflon was the first to be domesticated for its meat and for major adaptation to nomad life, but after agriculture was introduced, men preferred sheep, which were tamer and smaller. There are two wild populations of mouflons still in existence, the Asiatic mouflon *(Ovis orientalis*), which is yet found in the mountains of Asia Minor and southern Iran and the European mouflon (*Ovis musimon*) of which the only existing members are on the islands of Sardinia, Corsica and Cyprus.

These two mouflons are closely related with the only difference being the redder coloration and different horn configuration of the Asiatic one.

Figure 14. *Argali (Ovis ammon).*

Figure 15. *Urial (Ovis vignei).*

Figure 16. *Sardinian muflon (Ovis musimon).*

A very useful approach to investigate the history of modern domestic animals is based on mitochondrial DNA analysis, which has allowed the identification of different maternal lineages in modern breeds, supposed to derive from distinct original wild populations domesticated independently (Hiendleder et al., 1998).

1.3.2 *Ovis aries*

Ovis aries is classified in the *Bovidae* family, subfamily *Caprinae*. According to molecular studies, the *Caprinae* subfamily diverged from *Bovidae* around 15-20 million years ago and, within *Bovidae*, the divergence between the genera *Capra* and *Ovis* dates to around 5-7 million years ago

(Bruford and Townsend, 2006). Wild and feral (domesticates that have returned to the wild state) sheep can be found across the majority of the northern hemisphere, and domesticated sheep are present almost all over the world (Barbato, 2016).

The genus *Ovis* is characterized by the presence of glands situated in a shallow depression in the lacrimal bone, the groin area, and between the two main toes of the foot. These glands secrete a clear semi-fluid substance that gives domestic *Ovis aries* their characteristic smell. Selection for economically important traits has produced domestic sheep with or without wool, horns and external ears. Coloration ranges from milky white to dark brown and black. It is possible to recognize over 200 distinct breeds of sheep. Classification can be made in many ways, including the degree of specialization for milk, meat or wool production. There are also sheep breeds with dual purpose, both milk and meat or meat and wool.

1.3.3 The Sarda sheep

This breed is indigenous to the island of Sardinia, where it has been kept since immemorial time (Chessa et al., 2009). From Sardinia, where it is the unique sheep breed raised, the Sarda sheep (Figure 17) spread across south (Abruzzo, Molise, Puglia, Campania and Basilicata), central (Toscana, Lazio, Umbria and Marche), north (Emilia and Liguria) Italian regions and also in other Mediterranean countries, as Tunisia. Not only it is considered to be

among the best Italian breeds for production of sheep's milk, but also it has been estimated that Sarda sheep produce 5% of the whole world ewe's milk.

Figure 17. A Sarda breed sheep flock (photographed by Maria Veronica Di Stefano).

As already mentioned, there are over 3 million Sarda sheep in the island and other 2 million in the rest of Italy. For its high adaptability it is suitable for both intensive and extensive or transhumant management (Carcangiu and Vacca, 2005). Anyway, in Sardinia it is carried out especially the extensive and semi-extensive management system. The Sarda is one of the autochthonous Italian dairy sheep breeds, such as Altamurana, Pinzirita, Comisana, Delle Langhe, Massese, Valle del Belice and Leccese, for which a

genealogical herdbook is kept by the Associazione Nazionale della Pastorizia (ASSONAPA), the Italian national association of sheep-breeders.

The herdbook for Sarda breed was established in 1928. Total numbers for Sarda sheep are estimated about 5,000,000, but in 2013 the number recorded in the herdbook was only 225,207 (ASSONAPA, 2013).

Sarda sheep (Figure 18 and 19) is a medium sized animal with weight between 42 and 59 kg and height between 63 and 71 cm, for females and males respectively.

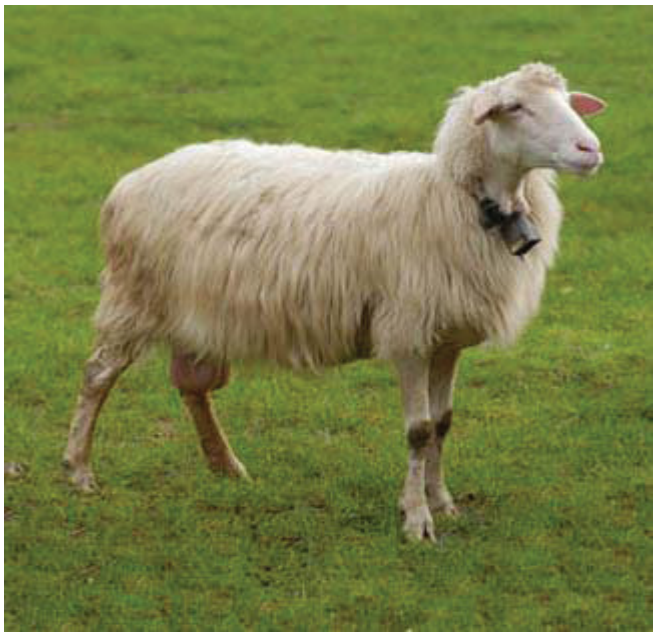


Figure 18. Sarda ewe.

Figure 19. Sarda rams.

The fleece is usually white in colour and long up to the forearms or near the hock. The skin is rose with possible marks black or brown marks on the face. Both ewe and ram are polled, but some males can have rudimental horns. Head is light, with straight and slightly Roman nose, the body is long with full abdomen; the udder is well developed, well attached, soft, spongy, elastic with teats well-proportioned and angled (ASSONAPA).

The main characteristic of the breed is milk productivity, with 250 litres of milk per year produced on average, and over 550 liters for the best producing animals. The prolificacy is between 1.1% and 1.5% respectively in extensive and intensive management (ARA). Wool quality is poor and mostly used as insulating material. Considering meat production, it consists principally in lambs fed only milk, and slaughtered at 4 weeks of age; it represents an IGP product (reg. CE 138/2001).

1.3.4 Reproduction in Sarda sheep

1.3.4.1 Reproductive activity in sheep

The sheep is a 'seasonally polyestrous' and "short-day breeder" animal, with breeding season that starts at the end of summer, when day length decreases (short days), since seasonality of estrus is controlled by the hours of light to which the animal is exposed. Day-length, detected by retina, is translated into a hormonal signal by secretion of melatonin from the pineal gland during the dark period, which stimulates the secretion of LH-Releasing Hormone (LHRH) from hypothalamus. The change in reproductive status is therefore controlled by modifications in the activity of the gonadotropic axis through variations in secretion of pulsatile LH, which in turn controls gonadal activity. In temperate areas of the world, ewes tend to enter a non-reproductive state during spring and most of summer, when day length increases. In tropical zones, where day length remains relatively constant ewes tend to remain sexually active throughout the year. The ewe lambs reach puberty by 6 to 9 months (Hafez, 7th edition) if they have gained a sufficient body weight. Recommended target weights are 50 to 70 percent of the adult weight. However, a ewe lamb should weigh at least 60 percent of her mature weight when is bred for the first time. After the onset of puberty, sheep are sexually active, and they have regular estrous cycles typically every 16-17 days during the breeding season. Estrus can last from 24 to 36 hours in sheep and ovulation normally occurs toward the end of this period. Precisely,

typical ovulation times for the ewe are about 24 to 27 hours from the beginning of estrous. The normal gestation period of ewes is approximately 149 days, ranging from 143 to 151 days (Hafez, 7th edition).

1.3.4.2 Reproductive management in Sarda sheep

Considering the natural cycle of ewes, in the past sheep breeding in Sardinia was during a mating season in autumn and a lambing season was in spring. This system, followed the natural cycle of Sarda ewes, which for its seasonal polyestrous have their highest sexual activity in autumn. Around the end of the XIX century, the increase for cheese demand induced a modification of the original breeding model. As a result, there was a modification of production system based on lambing season in autumn for adult sheep and late winter for primiparous, thanks to the progressive improvement of animal management. Milk production and cheese making are therefore concentrated during the winter – spring period, determining a break in the cheese factories' activities from the mid summer to the mid autumn (Piras et al., 2007).

1.4 The Insulin-like growth factor-I

Improvement of profitability in animal husbandry is an important goal that farmers try to obtain daily. The control of interaction of several factors, such as nutrition, management, genetic and endocrine system, represents a useful tool to increase animal production and reproductive success.

One of the most important endocrine system with an essential role in animal performances is the somatotropic axis, where the growth hormone (GH) and IGF-I are the major components, with important functions in growth and energy balance. Another fundamental endocrine element, which is crucial in animal husbandry is the hypothalamus-pituitary-gonadal (HPG) axis, which controls reproductive activity through several hormones, included IGF-I.

IGF-I, is a member of the insulin-like growth factor family, highly important in vertebrates at both the cellular and organismal level. The above consideration lay the foundation to understand why IGF-I represents one of the most significant signals that link somatic development to the reproductive system, since it is a regulator of both the somatotropic and the reproductive axis.

1.4.1 IGF system

The insulin-like growth factor (IGF) system is a complex of peptides, involved in pleiotropic actions. It works as mediator of several biological functions: at cellular level it intervenes in the synthesis of DNA, RNA, proteins, and in cell proliferation; inhibits apoptosis, participates in the activation of cell cycle genes (mediating mitogenic stimulation) (Mairet-Coello et al., 2009); increases the synthesis of lipids and the absorption of glucose (Etherton, 2004; De la Rosa Reyna et al., 2010). At organismal level, it exerts function in growth and metabolism during both fetal and post-natal life.

IGF system is composed of three ligands (IGF-I, IGF-II and insulin), their cell surface receptors [the IGF-I receptor (IGF-IR), the mannose 6-phosphate/IGF-II receptor (M6P/IGF-IIR), the insulin receptor (IR) and the hybrid IR/IGF-IR], six high affinity binding proteins (IGFBP-1 to 6) and their proteases (Figure 20) (Le Roith, 2003; Federici et al., 1997; Annunziata et al., 2011).

The knowledge about IGF-I and IGF-II have undergone an interesting evolution since their earliest classification. Both IGF-I and IGF-II were identified for the first time in 1957 (Salmon et al., 1957) and were designated as “sulphation factors” by their ability to stimulate sulphate incorporation into rat cartilage (Laron et al., 1971). Subsequently, both were described as the non-suppressible insulin-like activity (NSILA) of two soluble serum components (NSILA I and II) (Froesch et al., 1963).

In 1972, the labels sulphation factors and NSILA were replaced by the term "somatomedin", denoting a substance under control and mediating the effects of GH (Daughaday et al., 1972). In 1976, two active substances were isolated from human serum, which owing to their structural resemblance to proinsulin were renamed "insulin-like growth factor I and II" (Rinderknecht et al., 1976).

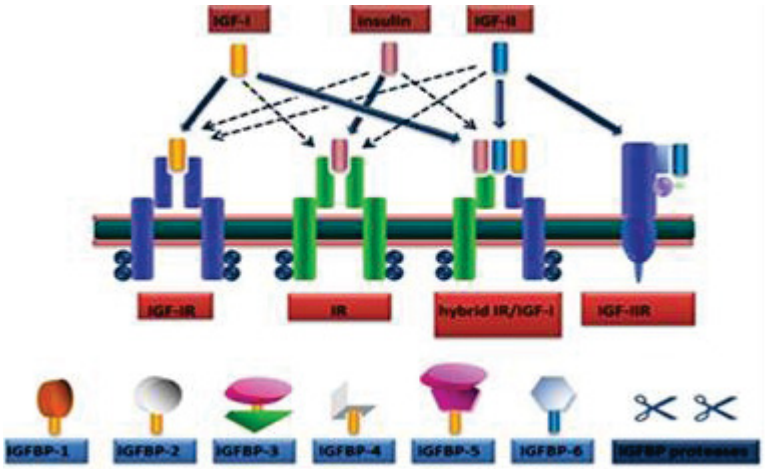


Figure 20. Insulin-like growth factor system. The IGF system comprises the ligands (IGF-I, insulin and IGF-II), their receptors (IGF-I receptor, IGF-IR; insulin receptor, IR; the hybrid IR/IGF-IR and the mannose 6-phosphate/IGF-II receptor, M6P/IGF-IIR), six IGF binding proteins (IGFBPs) and their proteases. Full arrows and dotted arrows indicate high and low affinity ligand binding, respectively (Annunziata et al., 2011).

IGF-I and IGF-II were termed in this way, also for the ability to interact with the insulin receptor and "because their effects on cell and tissue growth predominate over those on metabolic parameters" (Daughaday et al., 1987). The pronounced similarity of the primary structure of IGF-I with that of proinsulin indicates a common ancestor (Rinderknecht et al., 1978). Despite

the significant structural correspondence among IGFI, IGF-II and insulin, each ligand can result in unique signalling outcomes. For example, IGF-II is unable to compensate for the loss of IGF-I activity in patients with an IGF-I deficiency leading to severe growth and mental retardation (Chen et al., 2001; Walenkamp et al., 2005). Similarly, mice with a targeted disruption of the *IGF-I* gene are born at 60% birth weight compared to wild type litter mates (Liu et al., 1993).

1.4.2 IGF-I protein

IGF-I is a single-chain polypeptide that, like IGF-II, consists into four ''domains'': A, B, C and D (Denley et al., 2005). IGF-I has an A and B chain connected by disulphide bonds (Laron, 2001), a connecting or C-peptide region of 12 amino acids, that it has been shown to determine the high-affinity binding of IGF-I to the type I of IGF-I receptor (Bayne et al., 1989), and an eight-amino acid D-region peptide, which forms an extension of the carboxyl terminus (Brissenden et al., 1984). In comparison, proinsulin includes the A, B, and C domains, whereas mature insulin produced and secreted by the pancreas includes only the A and B domains (Le Roith, 2003), which have a 50% sequence similarity to the A and B chains of IGF-I protein (Figure 21) (Rinderknecht et al., 1978; Denley et al., 2005).

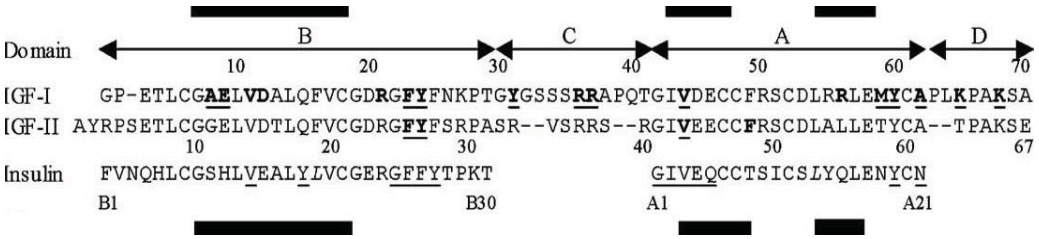


Figure 21. IGF-I, IGF-II and insulin. Sequence alignment highlighting domains and helices. IGF-I and IGF-II residues which have 5-fold (bold and underlined) or 2 (bold) lower IGF-1R binding affinity are shown. Insulin residues in the classic site 1 (underlined) or site 2 binding sites (italics) are also indicated (Denley et al., 2005).

Since its first characterization, it was evident that IGF-I protein displays high degree of homology with insulin, having 48% amino acid identity, identical disulfide bonding and similar tertiary structure. The structural similarity to insulin explains the ability of IGF-I to bind (with low affinity) to the insulin receptor (Laron, 2001). A second important difference is that specific amino acids at positions 3, 4, 15 and 16 within the IGF-I molecule, which are not present in insulin, confer binding to a family of six high affinity binding proteins (IGFBPs). Therefore, while most insulin circulates in a free form, more than 75% of IGF-I is confined to the vascular compartment as a 150 kDa ternary complex with the acid labile subunit (ALS) and IGFBPs (Clemmons, 1997; Firth et al., 2002).

The structures of IGF-I, IGF-II and insulin reveal three alpha helices that are the major secondary structural elements (Figure 22). Helix 1 (Gly8-Cys18 of IGF-I; Gly10-Val20 of IGF-II) is in the B domain whereas, helix 2 (Ile43-Cys47 of IGF-I; Glu44-Phe48 of IGF-II) and helix 3 (Leu54-Glu58 of IGF-

I; Ala54-Tyr59 of IGF-II) are both located in the A domain. Three disulphide bonds hold together the three-dimensional fold of the ligands (IGF-I residues: Cys6-Cys48, Cys18-Cys61, Cys47-Cys52) (Denley et al., 2005).

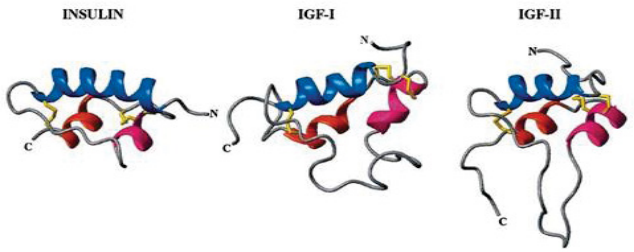


Figure 22. Ribbon structures of IGF-I, IGF-II and insulin showing the B domain helix 1 (blue), A domain helix 2 (pink) and A domain helix 3 (red) (Denley et al., 2005).

1.4.3 IGF-I receptor

IGFs actions are mediated by specific cell surface receptors termed the IGF-I receptor (IGF-IR), the mannose 6-phosphate/IGF-II receptor (M6P/IGF-IIR), the insulin receptor (IR) and the hybrid IR/IGF-IR.

IGF-I binds with high affinity to IGF-IR (Figure 23), which in turn initiates the physiological response to its ligand. Type I receptor is responsible for the mitogen effects of IGFs; while type II receptor interacts with IGF-II only and its main function is related to IGF-II internalization and degradation. The IGF-IR is also bound by IGF-II, although with six fold lower affinity than IGF-I, and by insulin, with one hundred fold lower affinity (Le Roith, 2003; Salmon et al., 1957).

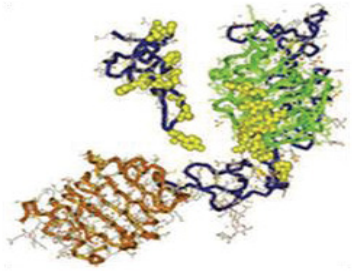


Figure 23. Structures of the IGF-I receptor and its ligand. Three-dimensional structure determined by X-ray crystallography. An extended bilobed structure comprises the two globular L-domains with a new type of right-handed helix fold that flanks the CR domain (Smith et al., 2010).

The IGF-IR exhibits high sequence and structural similarity with the insulin receptor (IR) (De Meyts and Whittaker, 2002). Given the significant structural similarity between IGFs and insulin, and their respective receptors, it is not surprising that these ligands can cross-activate the receptors when added at high concentrations in cell culture studies. The hybrid IR/IGF-IR have also been found, although their functional importance remains poorly understood (Taguchi and White, 2008; Duan et al., 2010).

The IGF-IR, member of a family of tyrosine kinase receptors, is a heterotetramer composed of two extracellular spanning α subunits and two transmembrane β subunits linked by disulfide bonds (Heldin et al., 1996). The α subunits have binding sites for IGF-I and are linked by disulphide bonds. The β subunits have a short extracellular domain, a transmembrane domain, and an intracellular domain. The intracellular part contains a tyrosine kinase

domain, which constitutes the signal transduction mechanism. Similar to the insulin receptor, the IGF-IR undergoes ligand induced autophosphorylation (Kato et al., 1994). The IGF-IR is localized in numerous tissues including muscle, ovary, pituitary, and brain. This wide distribution of the receptor underscores the multifaceted roles of IGF-I (Daftary et al., 2005; Le Roith et al., 2001; Bondy et al., 1990).

1.4.4 IGF Binding Proteins

In the plasma, 99% of IGFs are complexed to a family of binding proteins, called IGFBPs, which regulate the levels of IGFs by performing several functions, such as transporting the IGFs out of the vascular compartment in the circulation; localizing the IGFs to specific cell types; modulating both IGF binding to receptors and its growth promoting actions. (reviewd by Daftary et al., 2005; Hwa et al., 1999).

The IGFBPs are six binding proteins, which are present in the circulation as well as extravascular fluids, prolong the half-life of the IGFs and modulate their bioavailability and activity. They are well conserved among mammals and their affinity for IGF-I and IGF-II is in the same order of magnitude as that of IGFI-R. IGFBPs are themselves regulated by protease activity, which augment or decrease their bioavailability, adding a further layer of complexity to the IGF system. Moreover, some IGFBPs even display IGF-independent actions. IGFBP-3, for instance, has both growth-inhibiting

and growth-promoting effects at the cellular level, which can be either dependent or independent of IGF (Annunziata et al., 2011). Within a species, the IGFBPs share an overall protein sequence homology of 50%, whereas between species, up to 80% nucleotide sequence homology in corresponding IGFBP is observed. The IGFBPs prolong the half-life of circulating IGF and separate the activities of these hormones from those of insulin. Additionally, there is evidence that the IGFBPs may play a functional role not only in delivery, but also on local basis, ensure that IGF secreted by a cell are retained in the vicinity, and therefore exert their action on the same (autocrine) or adjacent (paracrine) cell population. In mature sheep and cattle the majority of circulating IGF are bound to IGFBP-3, as in humans (Conn and Melmed, 2nd Edition). Each IGFBP polypeptide chain, ranging in length from 216 to 289 amino acids, may be divided into three distinct domains of approximately equal size. The N- and C- terminal portions exhibit a high primary sequence identity across the six IGFBPs and contain spatially conserved cysteine residues that form intra-domain disulfide bonds. The central domain is the least conserved region and in some of the proteins contains post-translational modifications and proteolytic cleavage sites. Because of their sequence homology, it is assumed that IGFBPs share a common overall fold and have very similar IGF binding pockets. The N- and C- terminal domains are known to be involved in IGF binding (Clemmons et al., 1998). Proteolytic cleavage at the mid-region between the two domains of the proteins is

considered the predominant mechanism for IGF release from all IGFBPs, but several studies indicate that the resulting N- and C- terminal fragments still retain the ability to inhibit IGF activity (Sala et al., 2005).

1.4.5 Function of IGF-I protein

1.4.5.1 Role of IGF-I in several physiological systems

IGF-I is a protein that can influence cell function via endocrine, autocrine and paracrine signalling, with characteristics of both circulating hormone and tissue growth factor (Daftary et al., 2005). IGF-I protein is expressed by most tissues of the body and it is produced by both central and peripheral districts. Central IGF-I is synthesized throughout the brain and spinal cord, where it works as a potent neurotrophic factor for neurons and glial cells during development (de Pablo et al., 2005) together with peripheral IGF-I, which can cross the blood brain barrier, by a mechanism that is not entirely understood. The major source of peripheral IGF-I is the liver, where its production is induced by GH (Murphy et al., 1987), through the somatotropic axis. Secretion of GH hormone from pituitary gland is regulated by two hypothalamic peptides, growth hormone releasing hormone (GHRH) and somatostatin (SST), [called also somatotropin release–inhibiting factor (SRIF)], though other hypothalamic neuropeptides directly and indirectly participate in this process.

GHRH and SST induce a pulsatile secretion of GH following the alternation of the GHRH stimulation prevalence and somatostatin inhibitory prevalence.

When somatostatin is higher and prevents GH secretion, GHRH induces GH synthesis, which is produced and released when somatostatin decreases as a result of the feedback effect.

GH acts on specific cellular receptors inducing lipolysis, bone and protein synthesis, antagonizing insulin action. Body growth is generally recognized for this hormone but, from a biochemical point of view, it also affects the metabolism of proteins, carbohydrates and lipids; it also stimulates the proliferation of chondrocytes as well as the synthesis of RNA and DNA.

All the GH effects derive from its ability to interact with the GH-specific receptor, the GHR, a single chain protein of approximately 625 amino acids: this membrane receptor is widely distributed and belongs to the superfamily of the cytokines receptors and has structural similarities with the prolactin receptor, with the receptor for erythropoietin and with many interleukin receptors. Once GH is released, it circulates in the blood and acts on many organs to stimulate IGF-I production, which regulates, among other things, the effects on the growth of tissues. Although GH acts directly on adipocytes to increase lipolysis and hepatocytes to increase gluconeogenesis,

its anabolic effects and growth promoters are indirectly mediated by the induction of insulin-like growth factors (IGFs).

Initially, it was supposed that virtually all IGF-I protein originated in the liver and was transported by endocrine mechanisms to sites of action, but it is now recognized that IGF-I is also synthesized in other organs and tissues (Smith et al., 2010). Lung, thyroid, kidney, thymus, spleen, heart, muscle, stomach, gonads and adipose tissue, can produce IGF-I, which can act in endocrine, autocrine and paracrine way. In fact, a transgenic mouse with the *IGF-I* gene knocked out specifically in liver still has appreciable concentrations of circulating IGF-I resulting from extra hepatic synthesis (reviewed by Daftary et al., 2005).

The higher factors that regulate hepatic IGF-I biosynthesis are GH, insulin and nutritional status (Figure 24) (Oster et al., 1995; Le Roith, 2003).

Circulating IGF-I is the mediator of the anabolic and mitogenic activity of GH (Laron, 2001) and when growth hormone ties its receptor on the liver, called GH receptor 1A (GH1A) (Lucy, 2000), it generates IGF-I, which acts as an extension of the somatotropic axis by virtue of tonic pituitary stimulation of hepatic synthesis (Figure 25) (Maiter et al., 1988).

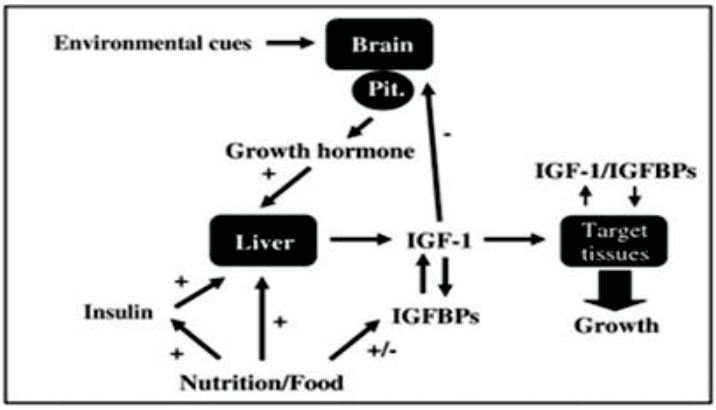


Figure 24. Illustration of the GH/IGF-I endocrine axis. Multiple hormonal and nutritional factors may stimulate (+) the production and/or modify (+/-) the activity of IGF-I. Negative feedback (-) by IGF-I inhibits growth hormone secretion by the pituitary (Cotè et al., 2007).

The consideration of interaction between IGF-I and nutritional status is very important, in consideration of the role that IGF-I have in metabolism. Indeed in a variety of species (including farm animals, laboratory animals and humans) higher concentrations of blood IGF-I are found in young, well-nourished and healthy individuals. Whereas, animals that are old, diseased, or malnourished have low blood IGF-I concentrations that reflect a compromised state of tissue, organ, and cell function (reviewed by Lucy, 2000).

In contrast to the levels of circulating (endocrine) IGF-I, which are determined primarily by GH stimulation, local expression of IGF-I protein in different tissues and its paracrine and autocrine actions can be relatively or completely independent of GH. Several studies reported that the block of GH signalling by deletion of GH receptors led to the expected profound

suppression of IGF-I message levels in the liver, while IGF-I expression in the kidneys was only partially suppressed and its expression in the heart and in the brain was not affected (Figure 25) (Bartke et al., 2013).

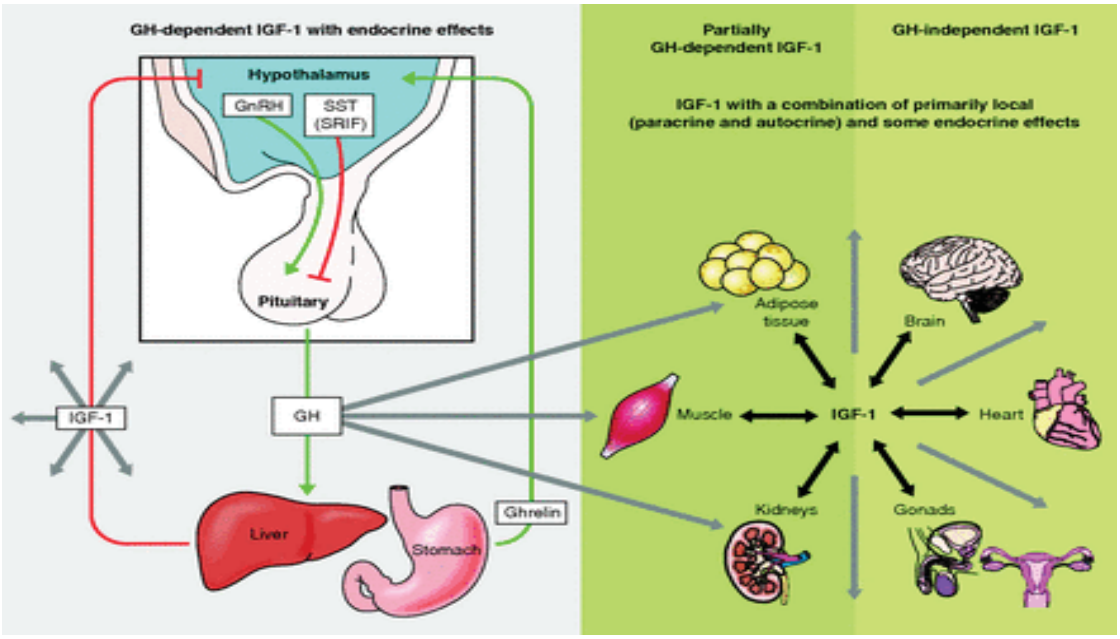


Figure 25. This diagram contrasts endocrine, primarily hepatic IGF-I that acts as a mediator of GH actions with partially or completely GH-independent IGF-I produced by other organs that could acts locally in paracrine or autocrine fashion (Bartke et al., 2013).

In extrahepatic tissues, *IGF-I* gene expression is regulated by several factors in addition to GH. Estrogen may play an important role in the local production of IGF-I in ovarian and uterine tissue (Murphy et al., 1987); angiotensin II controls IGF-I protein expression at the local level, by stimulating IGF-I production in the cardiovascular system (Brink et al., 1999); compensatory renal growth induces IGF-I mRNA expression (Mulroney et al.,

1992). Both prostaglandin E2 (PGE2) and parathyroid hormone (PTH) increase IGF-I mRNA levels in cultured osteoblasts, whereas GH has little effect on IGF-I protein expression in this system (Bichell et al., 1992). Estradiol also increases expression of IGF-I in osteoblasts. Thyroid-stimulating hormone (TSH) induces IGF-I mRNA expression in thyroid (Hofbauer et al., 1995; Le Roith, 2003).

The clear and important role of IGF-I in the somatotropic axis does not eliminate it as a candidate for the regulation of other neuroendocrine systems, particularly the reproductive axis. This neuroendocrine system, comprising the hypothalamic gonadotropin-releasing hormone (GnRH) neurons, pituitary gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)], and the gonads (ovary and testis), is responsible for the regulation of reproduction in all vertebrates (Gore, 2002). IGF-I could be consider a direct regulator of GnRH neurons, seeing as how it can regulate the hypothalamic-pituitary-gonadal axis via actions at the pituitary (Kanematsu et al., 1991) and gonadal levels. These findings, taken together with the role of IGF-I in the somatotropic axis, support IGF-I as a potential link between the reproductive and somatotropic neuroendocrine systems (Daftary et al., 2005).

1.4.5.2 IGF-I and reproductive system in female mammals

The role of IGF-I in prenatal and postnatal growth in mammals is well recognized; anyway, in vitro and in vivo observations indicate that IGF-I has a significant function also in the establishment and maintenance of reproductive activity (Daftary et al., 2005). In mammals, sexual maturation and reproductive function is critically dependent on the GnRH neurons. This decapeptide, into the hypophysial portal blood, triggers the production of gonadotropins, LH and FSH, which in turn stimulate the growth and release of sex steroids, as estrogen, progesterone and testosterone, by the gonads (Gore, 2002). Sex steroid hormones, produced and secreted into the circulation, act at their respective receptors that are located in many major cells and tissues. IGF-I is one of the many factors implicated in GnRH regulation and it is involved in the onset of reproductive activity at puberty and in the control of reproduction through adult life. Although the specific effects of IGF-I vary among species and sexes, and depend on hormonal status, IGF-I is usually considered to excite GnRH neurons and gonadotropins. The initiation of puberty involves the complex interaction of hypothalamic, pituitary, and gonadal hormones. During this process, there is an increase in the amplitude and frequency of GnRH pulses, which triggers a cascade of events including rises in the generation of FSH and LH pulses, followed by marked growth in gonadal sex steroidal output. Moreover, during puberty, serum GH release, including GH pulse amplitude, increases 2 to 3 fold from

the onset of puberty, and this is correlated with rises in IGF-I production (Suter et al., 2000) and serum estradiol concentrations. Thus, IGF-I influences the onset of puberty with the regulation of somatic growth, coordinated with the attainment of reproductive maturity.

In mammals, circulating levels of IGF-I change during key points of the reproductive life cycle. For instance, serum IGF-I levels increase during puberty in rodents, ruminants and primates, including humans, consistent with the possibility that the elevated levels of IGF-I may initiate or accelerate the onset of puberty (reviewed by Daftary et al., 2005). The effects of IGF-I can be exerted at all three levels of the hypothalamus-pituitary-gonadal axis. In the hypothalamus, IGF-I can arise from central or peripheral sources, acting in autocrine, paracrine, or endocrine manner. In the pituitary (Adam et al., 2000), IGF-I serves the dual process of down regulating pituitary somatotropin activity and influencing gonadotropic function, probably via endocrine actions (Daftary et al., 2005). At the ovary level, IGF-I can exerts local paracrine effects, because it is synthesized in ovarian tissues (Lucy, 2000), but also endocrine action, for the presence of IGF-I receptor (Vendola et al., 1999). The GH, synthesized by the pituitary, interacts with receptors located within the liver and other organs, as the ovary (corpus luteum and follicles), where GH can act through ovarian GH receptors (GH receptors 1B and 1C) to produce IGF-I. IGF-I can travel through the blood and affect ovarian function (endocrine IGF-I). The ovary produces IGF-I and IGF-II (ovarian IGF)

that can complement the IGF-I from endocrine sources (primarily liver). As opposed to other species, the ovarian IGF is not controlled by GH in the bovine. The "somatomedin hypothesis" states that the actions of IGF-I on the ovary are caused by both endocrine and ovarian IGF-I (Figure 26) (Lucy, 2000). In the ovary, IGF-I stimulates the proliferation of granulose and theca cells of the follicle and it inhibits follicular atresia. In addition, IGF-I stimulates the response of follicular cells to the hypothalamic gonadotropins (Hax et al., 2017).

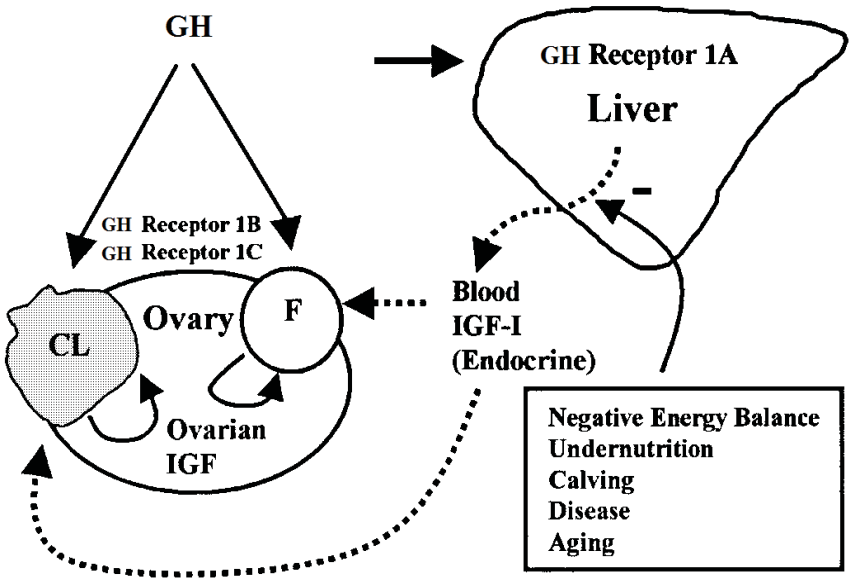


Figure 26. *GH and IGF-I action on the ovary (CL= corpus luteum; F= follicle) (Lucy, 2000).*

1.4.5.3 IGF-I and dairy cows

Several studies show that IGF-I concentration in blood is a heritable trait in cattle (Davis et al., 1997; Swali et al., 2006), in which endocrine IGF-I has been associated with important reproductive characteristic that are crucial for the dairy production. Age at first calving (Yilmaz et al., 2006; Brickell et al., 2007), conception rate to first service (Patton, 2007), twin ovulations (Echternkamp et al., 2004), and preimplantation embryo development (Velazquez et al., 2005) are highly connected with IGF-I concentration (Velazquez et al., 2008). Moreover, the metabolic hormone IGF-I is believed to be one of the main mediators of the effects of energy balance on the reproductive performance of the dairy cow after calving (Zulu et al., 2002), since circulating concentrations of IGF-I are associated to energy balance (Pushpakumara et al., 2003), follicular growth (Spicer et al., 1995; Gong, 2002) and resumption of ovarian cyclicity (Beam et al., 1999; Huszenicza et al., 2001; Nicolini et al., 2013). Relative to lactating cattle, prepartum animals have low blood concentrations of GH and high blood concentration of IGF-I (Cohick, 1998). At the time of calving and the initiation of lactation, blood GH concentrations increase and blood IGF-I concentrations decline. The increase in GH in postpartum cows may be secondary to the drop in IGF-I because IGF-I is the primary negative feedback inhibitor for GH secretion and animals with low IGF-I have greater blood GH

(Gluckman et al., 1987). The increase in blood GH and the decrease in blood IGF-I persist for several weeks after calving (Lucy, 2000).

The early post-partum period is considered a crucial phase in dairy cattle. It is characterized by prolonged negative energy balance (NEB) where feed intake is increasing, but energy balance (EB) remains negative due to the energetic costs of rising milk production. During this time the liver becomes refractory to GH (Vicini et al., 1991) and subsequently circulating IGF-I concentrations are dramatically reduced. The inability of GH to stimulate hepatic IGF-I production during periods of NEB is termed 'GH resistance' (Donaghy and Baxter, 1996) and has been documented in many species. The nutritional sensitivity of IGF-I is widely reported (Thissen et al., 1994) and severe NEB (SNEB) during early lactation attenuates gene transcription of *IGF-I* and also a number of *IGF*-related members in the liver, producing an endocrine environment of reduced IGF-I stability and bioavailability (Lucy, 2001; Fenwick et al., 2008). Transcripts for IGF-I and IGF-II have been detected in the oviduct of ruminants, primates and rodents (reported by Fenwick et al., 2008). Circulating IGF-I concentrations fall in the immediate postpartum period in dairy cows and may not return to pre-partum levels for more than 12 weeks in some cows (Taylor et al., 2004). The decline in hepatic IGF-I begins 2 weeks prior to parturition and is paralleled by a decline in plasma insulin. Changes in plasma GH over the same period are opposite to that of IGF-I and insulin (Bell et al., 2000). As the lactation progresses, the

blood concentrations of GH gradually decline, and the blood concentrations of IGF-I and insulin gradually increase (Lucy, 2000). The hypoinsulinemia of early lactation is part of a series of coordinated changes that occur around the time of parturition in support of lactation. Low plasma insulin levels reduce glucose uptake by insulin-responsive peripheral tissues (adipose and muscle) and facilitate greater uptake of glucose by the mammary gland (Bauman and Elliot, 1983), a tissue that is not insulin-responsive. Thus, it is not surprising that genetic advances for milk production have resulted in lower levels of circulating insulin in Holstein cows (Bonczek et al., 1988). As insulin is a key signal of metabolic status, it is hypothesized that the hypoinsulinemia associated with early lactation was responsible for the specific down-regulation of GHR 1A in liver and that this in turn was responsible for the uncoupling of the GH–IGF axis (Butler et al., 2003).

Several studies have established a correlation between blood IGF-I concentrations of postpartum cattle and reproductive function. Anestrus dairy cows had lower blood IGF-I compared with dairy cows that initiated estrous cyclicity earlier during the postpartum period (Thatcher et al., 1996). A similar relationship was reported for beef cattle; postpartum anestrus cows had lower IGF-I compared with cyclic cows (Roberts et al., 1997). In cattle, blood IGF-I was correlated with follicular fluid IGF-I because the majority of IGF-I in follicular fluid was derived from blood (Leeuwenberg et al., 1996). Therefore, endocrine IGF-I under GH control influences ovarian function

through its contribution to follicular fluid IGF-I. According to the somatomedin hypothesis, nutritionally induced changes in liver IGF-I secretion have a direct effect on the ovary through the endocrine actions of IGF-I (Lucy, 2000). Serum concentration of IGF-I also increases linearly until the moment of first postpartum ovulation and has a negative correlation with the duration of postpartum anestrous in beef cows (Roberts et al., 2005). Therefore, higher serum IGF-I is essential for an earlier return to postpartum cyclicity (Taylor et al., 2004). IGF-I and gonadotropins are synergistic for growth and differentiation of the follicle. Follicular growth and steroidogenesis in postpartum cattle, therefore, should be correlated with greater LH secretion as well as greater blood IGF-I concentrations. A positive correlation between LH pulsatility and ovarian follicular development has been established for postpartum cows. Likewise, a correlation between plasma estradiol during the first postpartum follicular wave and serum IGF-I was found. Postpartum ovarian function probably depends on both LH pulsatility and blood IGF-I concentrations, as both increase during the postpartum period and when nutrition is improved.

The bovine ovary can respond directly to GH because the GH receptor is found within ovarian cells. Therefore, the relatively high concentrations of GH found in postpartum cattle can potentially influence follicular growth, steroidogenesis, and oocyte health (Lucy, 2000).

1.4.5.4 IGF-I and dairy production

The somatotropic axis has a key role in the regulation of the metabolism and physiology of mammals. Polymorphisms in *IGF-I* genes have been linked to not only reproductive performance, but also other characteristics of economic interest, such as carcass traits, meat production and the synthesis and composition of milk (Siadkowska et al., 2006; Hax et al., 2017).

Although circulating concentrations of IGF-I may exert an endocrine effect on the mammary gland, evidence also exists for a paracrine or autocrine function of IGF-I in the mammary gland. The IGF-I mRNA, indeed, has been detected in mammary tissue of the pregnant (Hauser et al., 1990) and lactating bovine (Glimm et al., 1992; Sharma et al., 1994), suggesting that both local and hepatic production of IGF-I are important.

Some study shows that IGF-I is a potent mitogen for mammary epithelial cells and stimulated the synthesis of milk components. These findings led to the idea that IGF-I mediates the effects of GH on the mammary gland in a manner that is similar to its role in skeletal growth.

This is showed in researches where serum concentrations of IGF-I are increased in lactating cows that were treated with bovine somatotropin (bST) and IGF-I receptors are present in bovine mammary tissue. In addition, close arterial infusion of IGF-I into the mammary gland of goats increases milk yield.

Little evidence exists to support a direct galactopoietic effect of IGF-I in ruminants. However, IGF-I is a potent mitogen for mammary epithelial cells and it may also influence the inhibition of apoptosis of this cell type. Hauser et al. (1990) separated mammary tissue from pregnant heifers into fractions enriched in epithelium, stroma, and blood components and found IGF-I mRNA to be localized in the stromal component of the mammary gland. The IGF-I mRNA also has been identified in the stroma of normal and neoplastic human mammary tissue but not in the epithelia (Yee et al., 1989). Therefore, the stromal components of the mammary gland appear to be responsible for the local synthesis of IGF-I.

In light of the increased lactational persistency that is observed with long-term bST treatment, it seems logical that either increased proliferation or decreased apoptosis of mammary secretory epithelial cells must occur in addition to any changes in secretory activity to support the sustained increase in milk yield over time (Cohick, 1998).

1.4.5.5 IGF-I and immunity

Detection of IGF-I, IGF-IR mRNAs and their proteins in peripheral blood mononuclear cells suggests that IGF-I might serve some regulatory function in the immune system. Moreover, IGF-I production, action, and intracellular signalling can be influenced by multiple cytokines. Administration of GH and IGF-I promotes both B and T cell development.

Thus, there is reason to explore the potential for this endocrine pathway as a regulator of immunity.

IGF-I protein is also produced locally by many peripheral cell types under basal conditions and in response to inflammatory cues. In this case, IGF-I acts on peripheral tissues as an autocrine or paracrine factor resembling cytokines and other growth factors.

Several studies have demonstrated the importance of GH, IGF-I, and IGF-IR to many aspects of immune function. As an example, levels of IGF-I trend downward as a consequence of aging and chronic disease (Moldawer and Copeland, 1997; Grounds, 2002). Children diagnosed with one of several diseases associated with chronic illness exhibit alterations in growth and development attributable to GH/IGF-I dysfunction.

Immune system, IGF-I and IGF-II seem to modify several aspects of inflammation, at least in part by influencing the actions of cytokines and other small molecule mediators. These same mediators can in turn alter the abundance of IGF-I and modulate its actions on target tissues. Thus various components of the inflammatory machinery and the IGF-I pathway share a complex relationship that manifests in several ways. IGF-I can increase survival in rats treated with D-galactosamine and lipopolysaccharides (LPS), a strategy used to induce experimental acute hepatic failure (Hijikawa et al., 2008). When administered before the D-galactosamine and LPS, IGF-I prevented the biochemical stigmata of liver failure, such as elevations in

bilirubin and transaminases. Upon histologic examination, the growth factor seemed to decrease hepatic apoptosis and neutrophil infiltration. This effect seems to result from IGF-I blocking the elevation of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and neutrophil chemoattractant associated with D-galactosamine and LPS administration. In children with extensive thermal burns, the administration of IGF-I in combination with IGFBP3 as a continuous infusion, reduced serum levels of IL-1β, TNF-α, C-reactive protein (CRP) and complement C-3 (Jeschke et al., 2000). T cells display many different surface growth factor receptors, including IGF-IR (Schillaci et al., 1998). IGF-I, IGF-II, and insulin bind to the surface of T and B cells. Moreover, those studies demonstrated that IGF-I can induce T-cell proliferation and chemotaxis (Tapson et al., 1988). Both IGF-I and IGF-II play important roles in the development and function of T cells. However, both IGF-I and insulin can also suppress immune responses. IGF-I and insulin, although generally enhancing lymphocyte proliferation (Heulin et al., 1982; Schimpff et al., 1983; Walsh et al., 2002), can also block IL-2-dependent lymphocyte growth and function.

1.4.6 I*GF-I* gene and its polymorphisms in livestock

The ongoing evolution of the animal breeding has recently seen the inclusion of genomic selection, considering the great importance that different genes have on reproductive and productive traits. Recent advances

in genetics have had a significant impact on selective breeding programs. The phenotypic traits of animals are of interest to breeders and if these can be mapped onto particular genetic features, it makes easier for breeders to select animals with the required traits. Among genes involved in genetic selection, have particular relevance genes with function in growth as, the *IGF-I. (aggiungere referenza)*

IGF-I is a protein-coding gene that plays a key role in various physiological processes. A protein-coding gene consists of a promoter followed by the coding sequence for the protein, composed of exons, and then a terminator (Figure 27).

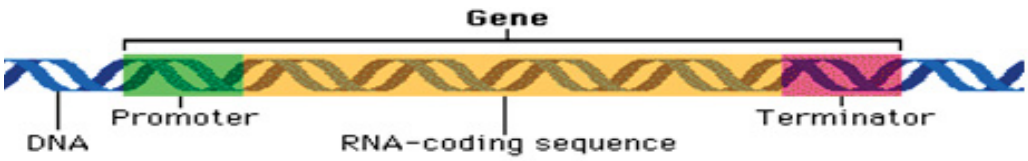


Figure 27. Protein-coding gene consists of a promoter followed by the coding sequence for the protein, composed of exons, and then a terminator (www.phschool.com).

The essential and multifactorial role of *IGF-I* in normal physiology and disease is potentially reflected in its complicated gene structure and by its complex patterns of regulation, such as its control by GH. *IGF-I* genes appear to be moderately conserved among vertebrates, but they have undergone substantial diversification during speciation (Rotwein, 2017). Indeed, the exons number is different between species, for example *IGF-I* gene in *Ovis aries* consists of 5 exons (GenBank N° NM_001009774), 7 exons in *Capra*

hircus (GenBank N° NM_001285697), 6 exons in *Bos taurus* (GenBank N° NM_0010778289) and 8 exons in *Sus scrofa* (GenBank N° NM_214256).

Anyway, although the length and genetic structure between species are variable, the expressed protein is enough conserved in vertebrates (Upton et al., 1998).

The *IGF-I* gene gives rise to a heterogeneous pool of mRNA transcripts, which is determined by several events or their combination, such as use of alternative transcription start sites located in leader exons (exons 1 and 2); alternative post-transcriptional exon splicing and use of different polyadenylation sites. These multiple IGF-I mRNAs transcripts encode different isoforms of IGF-I precursor peptide, which undergo post-translational cleavage to release the biologically active mature IGF-I (reviewd by Shavlakadze et al., 2005). For these reasons in bovine, as in other species, depending on the exon leader present, two transcripts are identified, class 1 (exon 1 as the leader) or class 2 (exon 2 as the leader). In class 1 transcripts, there are three transcription start sites, whereas there is one in class 2 (Wang et al., 2003).

A common assumption in animal breeding is that variation in quantitative traits, such as growth and lactation, is controlled by many genes. Usually, each of these genes has a small effect. However, the major gene model suggests that few genes may account for a relatively large proportion of genetic variation. Genes involved in the biology of a trait of interest are

candidates for association studies, and can be considered as "candidate genes". It was suggested that genes coding for hormones and factors of so-called somatotropic axis, including *GH*, its receptor *GHR*, transcription factor such as signal transducer and *activator of transcription 5 (STAT5)* (mediating actions of GH and prolactin), and *IGF-I* and *II*, fall into this category (Parmentier et al., 1999; Siadkowska et al., 2006).

It has been demonstrated that the polymorphisms in *IGF-I* gene are associated with productive and reproductive features. For this, in animal husbandry *IGF-I* is considered a strong candidate gene for productive and reproductive traits in several species, such as sheep (Yilmaz et al., 2005), cattle (Li et al., 2006; Kim, 2009) and chicken (Bian et al., 2008). Indeed, *IGF-I* has already been related with different reproductive traits in cattle, such as twin ovulations (Echternkamp et al., 2004), preimplantation embryo development (Velazquez et al., 2005) and conception rate to first service (Patton et al., 2007), and it is also connected to different productive marks, as body weight in several cattle breed (Ge et al., 1997, 2001).

In bovine, it was found a single nucleotide polymorphism (SNP) in the promoter region (IGF-I/SnaBI) that has been reported to be associated with production traits as weaning weight, weaning weight adjusted to 210 days and preweaning weight gain in several cattle breeds (Ge et al., 1997, 2001; De la Rosa et al., 2010). In chicken it was discovered that the coding region of the *IGF-I* gene was polymorphic, expressed differentially during the pre-hatch

and post-hatch periods, and haplogroups showed significant association with growth traits (Bhattacharya et al., 2015). *IGF-I* SNPs in five pig breeds (Berkshire, Duroc, Landrace, Yorkshire and Korea Native Pig) were associated with higher body weight (Niu et al., 2013). The polymorphisms of the *IGF-I* gene were linked also with cashmere yield, fibre diameter and body weight in cashmere goat (Wu-Jun et al., 2010). SNP in 5' regulatory region of ovine *IGF-I* gene influences litter size in Small Tail Han sheep (He et al., 2012).

All previous knowledge highlight as IGF-I has an important role in animal performances (both, as gene and protein). In consideration of its participation to several endocrine system, involved in growth, reproductive activity and milk production, it could be interesting to study how its concentration can change in different physiological and pathological phases of animal life. Moreover, the connection of its SNPs with productive traits could be a very useful within genetic selection program.

2. Aim

The axis composed by GHRH, GH, IGF-I and IGF-II and their associated binding proteins and receptors is a well-known mechanism (called somatotrophic axis) that regulates the metabolism and physiology of mammalian growth. Moreover, polymorphic sites located in genes involved in the mediation of growth are logical candidates for studies of possible association with livestock's production traits. Among the components of the somatotropic axis, growing interests are currently being focused on the function of the IGF-I, which is involved in controlling both reproductive and productive activity.

The overall aim of this thesis was to investigate the roles of IGF-I in dairy animals, precisely cows and sheep, in order to improve their production considering the importance of the both world and local dairy sector.

The choice of studying these two species was due to their importance in the dairy system:

- Holstein Friesian represents the most common breed of dairy cattle and its selection emphasized milk yield, which resulted also in increased demand for its reproductive efficiency.
- Sarda sheep is a native breed with high importance in milk production at national level.

Both these species have a great position in dairy sector and the obtaining of information about the function of IGF-I in their physiology and genetics could be useful to improve their production.

Milk producers can increase productivity and returns from dairying through selective breeding and control of reproduction. Reproductive efficiency (age of puberty, parturition intervals, conception rates) can be improved by using genotypes that are suitable to the production environment, and appropriate husbandry practices. Reproductive and productive performances of dairy animals are affected by such factors as the environment, nutrition, producers' socio-economic conditions, type of production system (intensive or extensive), dairy animals' adaptability and genetic traits.

Considering dairy cows, calving to conception interval is a crucial point for productive efficiency seeing as how a longer interval influences negatively the next milk production, with a lower return. After calving, cows go through a period of temporary infertility known as postpartum anestrus. It is also termed postpartum interval, which is the time from calving to the subsequent conception. Postpartum interval plays an important role in determining the number of days from calving date in one year to calving date the next year. To maintain a 365 days calving interval, a cow must have a postpartum interval of 80-85 days. Several factors can influence the length of the postpartum anestrous period, including uterine involution, short cycling and nutritional

status. The plasma IGF-I concentration in the periparturient period, could be used as an advantageous predictor for reproductive performance in this species, in view of numerous direct effects of IGF-I on ovarian cells, including stimulation of granulosa cell mitogenesis, progesterone secretion from luteal cells, and thecal cell androgen production. A bigger concentration of plasma IGF-I during the first 2 weeks of lactation has been associated with a greater conception rate to first service and an increased likelihood of a shorter interval to commencement of luteal activity. Consequently, cows with low IGF-I postpartum exhibit longer interval from calving to resumption of cyclicity. For these reasons it would be interesting to determine how the concentration of the plasma IGF-I changes in high-yielding Holstein-Friesian cows during early post-partum. Moreover, being post-partum a critical period for dairy cows, in which animals are more susceptible to disease, the evaluation of candidate immune genes expression, including chemokines, chemokine receptors, interleukins and other cytokines, and its relation with fertility, health and IGF-I concentration, can be useful to add important knowledge about the early post-partum in dairy cattle.

Despite the known differences among production systems in cattle and sheep, in sheep farming IGF-I is assuming increasingly importance for its involvement in several physiological processes and its role as mediator of many biological effects. Surely, its action in regulation of many hormones, which are

critical for reproductive system, such as the stimulation of the ovarian function, makes it a key factor of the entire production system, based on reproduction. IGF-I not only influences reproduction, but also can act directly on milk production, exerting an endocrine and paracrine-autocrine function in the mammary gland. *IGF-I* is considered a strong candidate gene and its polymorphisms can be associated with circulating IGF-I concentrations, growth, reproductive and productive traits such as meat, litter size, milk yield and composition, both in sheep and in cattle. Several mutations may influence the transcription of *IGF-I* gene and particularly SNPs located in 5'UTR region. Several studies show as nucleotide variations in this region could be responsible in different production and reproduction traits in sheep. In this way, it could be interesting to evaluate the presence and the variability of these polymorphisms in Sarda breed sheep and connected them with milk yield and reproductive performances, as litter size and parturition interval, assuming this latter as a crucial step to assure good reproductive performances. The study and analysis of *IGF-I* gene DNA, could be a valuable alternative approach to establish the allelic variants that are useful as markers to aid in selection. Especially in sheep, where the reports about this gene are still few, seems to be of interest to carry on researches targeted on possible genotype/phenotype correlations.

To summarize, the objectives of the present work were:

1) to determine how the concentration of IGF-I changes in high-yielding Holstein-Friesian cows during early post-partum, in relation to their parity, calving to conception interval, number of insemination needed to conception and positive or negative pregnancy diagnosis;

2) to evaluate the expression of candidate immune genes (*CCL5, IL8RB, CCR2, IL1A, IL1B, TNFa, SOD2, SELL*), 1-8 days after calving and its connection with IGF-I concentration and health status (interpreted as no recorded or recorded incidences of clinical mastitis in the previous lactation in multiparous cows with low IGF-I);

3) to detect possible polymorphisms within the *IGF-I* gene 5'UTR sequence in Sarda sheep in order to verify their influence on some crucial productive and reproductive traits, such as milk yield, distance in days from age at first lambing to conception, lambing to conception interval, interlambing period length and litter size.

3. Material and Methods

The present research was carried out in two different labs and time periods, through two investigations that are identified as Study 1 focused on Holstein Friesian dairy breed cows and Study 2, focusing on Sarda dairy breed sheep. The Study 1 was performed at the Royal Veterinary College, University of London and it investigated the relationship between plasma IGF-I concentration, reproduction activity and immune genes expression during early post-partum in Holstein Friesian cows. The Study 2 was conducted at the Department of Veterinary Medicine of the Sassari University and it was aimed to investigate, in Sarda sheep, possible association between *IGF-I* gene, fertility and milk yield.

3.1 Study 1

Investigation of relationship between IGF-I*, fertility and immune genes expression during early post-partum in Holstein Friesian cows*

3.1.1 Animals and blood samples

This study was realized at the Royal Veterinary College of the London University, using 332 lactating Holstein Friesian cows housed in "The Hill House dairy farm" (located at Lindfield, Haywards Heath, W Sussex, RH16 2QY, United Kingdom). In order to minimize variation caused by differences in management, cows came from the same farm, which housed a total number of 700 animals. At the time of the sampling, between August 2015 and March 2016, the cows involved in the study, did not show any clinical disease.

Due to limited pasture (especially during winter and autumn) available, cows were kept mostly indoors (litter bedded pens) and free stall cubical setup. Animals were fitted with pedometer for heat detection and they followed an AM-PM insemination regime. The AM/PM regime is a common rule for optimum conception rates in cattle: if a cow is in estrus in the morning (AM), it should be inseminated in the afternoon (PM) of the same day; likewise, if it is observed in estrus in the PM, then it should be inseminated the following morning.

Hill House was also fitted with a rotary milking parlour (DeLaval, UK) and individual milk meter for daily measurement. The chosen animals had a

mean milk daily production of 35±5 liters and a body condition score (BCS) of 2.5. They were fed twice daily with good quality hay, silage and concentrated grain according to their energy requirements and had free access to water and mineral block.

All animal procedures following institutional and national guidelines for the care and use of animals.

Individual 10ml blood samples were collected once at the same time, after the morning milking and feeding, into heparinized vacutainers and other 3ml into Tempus Blood RNA Tube (Figure 28), from the coccygeal vein and used respectively for plasma IGF-I measurement and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) of immune genes.

Figure 28. *Tempus Blood RNA Tubes.*

The sampling day routine was as follow: milked at 5 a.m., fed at 6 a.m. sampled at 8 a.m. The blood samples were taken between 0 and 27 days post-partum, and the 332 chosen cows were 98 primiparous and 234 multiparous

with a lactation number between 2 and 7 (and only one subject 9). For IGF-I assay, the plasma was separated by centrifugation and stored at -20°C. After IGF-I measurement, from the above specified animals, 40 cows were selected for qRT-PCR studies, based on the following criteria:

1) calving order (primiparous or multiparous);

2) IGF-I value measured in the first week after calving (Day 1- 8): ≤ 11ng/ml for "low IGF-I cows" ("L"), and ≥ 20ng/ml for "high IGF-I cows" ("H");

3) calving of a single live calf following a normal length gestation (279 days±3 in average).

The multiparous cows with low IGF-I were further subdivided into two subgroups: animals with a somatic cell count (SCC)< 70 in the previous lactation and no recorded incidences of clinical mastitis, and animals with a high SCC (>200) and/or at least one recorded incidence of clinical mastitis. Summarizing, five groups, each of 8 cows, were enrolled into the study:

1- PPL: primiparous with low IGF-I;
2- PPH: primiparous with high IGF-I;
3- MPLM: multiparous with low IGF-I and high SCC and/or mastitis in previous lactations;
4- MPLnoM: multiparous with low IGF-I and low SCC in previous lactation;
5- MPH: multiparous with high IGF-I.

For the qRT-PCR analysis, blood samples, taken into Tempus tubes

(Ambion™Applied Biosystems, USA), were stored at -80°C. The Tempus tubes contain 6ml of Stabilizing Reagent, which effectively lyses blood cells, inactivates cellular RNases and selectively precipitates RNA, whereas genomic DNA and proteins remain in solution. Using the Tempus tubes and associated extraction kit, it is possible to purify high quality RNA without sample pretreatments such as leukocyte isolation or selective red blood cell lysis.

Data about calving date, blood collection time (days post-partum) and parity were recorded for each animal. Further information on fertility and health were obtained from herd records.

3.1.2 IGF-I measurement

The IGF-I concentration of each sample was measured twice, using an enzyme-linked immunosorbent assay (ELISA) (DRG IGF-I 600 ELISA Kits, DRG Instruments GmbH, Marburg, Germany). All IGF-I assays included added IGF-II to block IGF binding proteins. The steps were as follows:

1) Acidification and neutralization of 50µl of samples, standards and controls in 1.5ml Reaction caps. Firstly, adding 50µl of 0.2 M HCL, mixing and incubating for 30 minutes; and finally, adding 10µl of Neutralization Buffer and mixing the solution. 2) Dispensation of 20µl of each acidified and neutralized samples, standards and controls into appropriate wells in the plate. 3) Dispensation of 100µl of Enzyme Conjugate with incorporated IGF-II (250ng/ml) into each well and thoroughly mix for 10 seconds. 4) Incubation for

120 minutes at room temperature. 5) Removal of the contents of the wells and rinse the wells 3 times with diluted Wash Solution (400µl for well). Strike the wells sharply on absorbent paper to remove residual droplets. 6) Dispensation of 150µl of Enzyme Complex into each well. 7) Incubation for 120 minutes at room temperature. 8) Removal of the contents of the wells and rinse the wells with diluted Wash Solution (400µl for well). Strike the wells sharply on absorbent paper to remove residual droplets. 9) Adding of 100µl of Substrate Solution to each well. 10) Incubation for 15 minutes at room temperature. 11) Block of the enzymatic reaction by adding 100µl of Stop Solution to each well. 12) Determination of the absorbance of each well at 450 +/- 10 nm with a microtiter plate reader within 10 minutes after adding the Stop Solution.

Results from each assay were recorded in Excel sheet, where the average absorbance values was calculated for each set of samples, standards and controls. Using a semi-logarithmic graph paper, a standard curve has been constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis. Using the mean absorbance value for each sample has been determined the corresponding concentration from the standard curve.

3.1.3 RNA extraction

RNA extraction was carried out using Tempus Spin RNA Isolation Kit (Ambion™Applied Biosystems, USA) according to the manufacturer's instructions. The RNA extraction procedure was as follows : 1) Defrost at room temperature blood samples previously collected in Tempus Isolation tubes (stored at -80°C). 2) Transfer 3ml of stabilized blood to 50ml conical tubes and dilute with 9ml 1X PBS (provided in the kit). 3) Vortex vigorously for at least 30 seconds. 4) Centrifuge at 4°C at 3,000x g for 30 minutes, then carefully discard supernatant. 5) Re-suspend the pellet in 500µl RNA re-suspension buffer. 5) Transfer re-suspended RNA into purification filter, then wash the column filter via RNA purification Wash Solution 1 (500µl) immediately followed by micro-centrifuge (16,000x g for 30 seconds). 6) Wash the column containing RNA with wash solution 2 (500µl) followed by micro-centrifuge (16,000x g for 30 seconds) twice. 7) Upon elution of RNA in Nucleic Acid Purification Elution Solution (90µl) samples were stored at (-20 °C or -80 °C for long-term storage).

Prior to storage, the quantity and quality (260/280, 260/230) of the RNA were verified using NanoDrop ND-1000 spectrophotometer (ThermoFischer, UK).

3.1.4 qRT-PCR

3.1.4.1 Primers design

Three housekeeping genes (based on previous studies carried out by Professor Wathes and her group at RVC): *Actin Beta* (*ACTB), glyceraldehyde-3-phosphate dehydrogenase (GADPH), receptor like protein 19 (RLP19*); and eight candidate genes involved in immunity: *C-C Motif Chemokine Ligand 5* (*CCL5), Interleukin 8 receptor beta (IL8RB), C-C Motif Chemokine Receptor 2 (CCR2), Interleukin 1 Alpha (IL1A), Interleukin 1 beta (IL1B), Tumor necrosis factor alpha (TNFa), Superoxide Dismutase 2(SOD2), Selectin L (SELL*), were selected, including chemokines, chemokine receptors, interleukins and other cytokines (Table 1). The Primer sequence for the studied genes and for the housekeeping genes (Table 2) was designed using a "Primer 3" web based program (http://frodo.wi.mit.edu/primer3) and DNA sequences from GenBank at NCBI (http://www.ncbi.nlm.nih.gov/Database/index.html). The primers were made by Eurofins MWG Operon (Ebersberg, Germany).

Table 1. *Immunity genes studied by qRT-PCR.*

Category	Gene	Gene info
Chemokines	*CCL5*	Functions as a chemoattractant for blood monocytes, memory T helper cells and eosinophils. It causes the release of histamine from basophils and activates eosinophils. This cytokine is one of the major HIV-suppressive factors produced by CD8+ cells. It functions as one of the natural ligands for the chemokine receptor chemokine (C-C motif) receptor 5 (CCR5).
Chemokine receptors	*IL8RB*	This receptor for IL8 mediates neutrophil migration to sites of inflammation.
Chemokine receptors	*CCR2*	This gene encodes two isoforms of a receptor for monocyte chemoattractant protein-1, a chemokine that specifically mediates monocyte chemotaxis.
Interleukins	*IL1A*	Pleiotropic cytokine involved in various immune responses, inflammatory processes, and hematopoiesis. This cytokine is produced by monocytes and macrophages as a pro-protein, which is proteolytically processed and released in response to cell injury, and thus induces apoptosis.
Interleukins	*IL1B*	This cytokine is produced by activated macrophages as a pro-protein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and it is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
Other cytokines	*TNFa*	Multifunctional pro-inflammatory cytokine mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.
Other immune mediators	*SOD2*	This gene is a member of the iron/manganese superoxide dismutase family. It encodes a mitochondrial protein that forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen.
Vascular Adhesion	*SELL*	A cell surface adhesion molecule that belongs to a family of adhesion/homing receptors. The encoded protein contains a C-type lectin-like domain, a calcium-binding epidermal growth factor-like domain, and two short complement-like repeats. The gene product is required for binding and subsequent rolling of leucocytes on endothelial cells, facilitating their migration into secondary lymphoid organs and inflammation sites.

Table 2. Oligonucleotide primer sequence information used for qRT-PCR assays.

Gene	Primer sequence (5'-3')	GenBank accession number	Product length (bp)	Annealing (°C)
GAPDH	Forward: GGTCACCAGGGCTGCTTTTA Reverse: TTCCCGTTCTCTGCCTTGAC	NM_001034034.2	147	61.4
RPL19	Forward: TCGATGCCGGAAAAACAC Reverse: ATTCTCATCCTCCTCATCCAG	NM_001040516	119	65
ACTB	Forward: GAAATCGTCCGTGACATCAA Reverse: AGGAAGGAAGGCTGGAAGAG	NM_173979.3	182	55
CCR2	Forward: GGGACAAATCGAAGCACAGC Reverse: AACAGCAGGTCAGAGATGGC	NM_001194959.1	162	62.8
IL8RB	Forward: GTGGGAGGGGTTTGAGGATG Reverse: AGCAAGAAGACCAGGGCATC	NM_174360.2	141	61.4
SELL	Forward: GGAGCCCAACAACAGGAAGA Reverse: GGGCTGTCTTTGCTTTGTGG	NM_174182.1	110	62.8
SOD2	Forward: AATCTGAGCCCTAACGGTGG Reverse: TATTGAAGCCGAGCCAACCC	NM_201527.2	151	61.4
TNFa	Forward: AGCACCAAAAGCATGATCCG Reverse: GGAAGGAGAAGAGGCTGAGG	NM_173966.3	112	65
CCL5	Forward: CTGCTGCTTTGCCTATATCTCC Reverse: ATGTACTCTCGCACCCACTTCT	NM_175827.2	159	60.2
IL1A	Forward: TGGATACCTCGGAAACCTCTAA Reverse: CTCTGGAAGCTGTAATGTGCTG	NM_174092.1	199	65
IL1B	Forward: ACGAGTTTCTGTGTGACGCA Reverse: TGCAGAACACCACTTCTCGG	NM_174093.1	147	60.2

3.1.4.2 DNase treatment of RNA

DNase treatment of RNA was carried out for each sample to eliminate potential genomic DNA contamination using a RQ1 RNase-Free DNase kit (Promega Corporation, Madison, WI, USA). A mix with 8µl of RNA (500ng/µl) in nuclease-free water, 1µl of RQ1 RNase-Free DNase 10X Reaction Buffer (containing 40mM Tris-HCl at pH 7.9, 10mM NaCl, 6mM $MgCl_2$ and 10mM $CaCl_2$) and 1µl of RQ1 RNase-Free DNase was prepared and incubated at 37°C for 30 minutes using G-Storm thermal cycler (G-Storm Ltd, Somerset, UK). After this step, 1µl of DNase Stop Solution was added to terminate the reaction. Finally, an incubation at 65°C for 10 minutes was performed to inactivate the DNase.

3.1.4.3 RT-PCR

The DNase treated RNA (stored at 4°C) was reverse transcribed into cDNA using random hexamer primers and a cDNA synthesis kit supplied by PCRBiosystems (qPCRBIO cDNA Synthesis Kit, PCR Biosystems Ltd, London). A Master Mix of reagents was prepared, to minimize potential variation due to pipetting, using 4µl of 5X cDNA Synthesis Mix [anchored oligo(dT), random hexamers, 15mM $MgCl_2$, 5mM dNTPs, enhancers and stabilizers], 1µl of 20XRTase, 5µl of PCR nuclease-free water for each sample. A quote of 10µl of the obtained mix was added to 11µl of RNA, then centrifuged and incubated at 42°C for 30 minutes and then at 85°C for 10 minutes to denature RTase. Before

store at 4°C, 1:5 time dilution was performed, with addition of 80µl of nuclease-free water for a total of 101µl of final volume.

3.1.4.4 Standards PCR analysis

A PCR was performed in order to both verify the specificity of each pair of primers for respective gene and amplify each gene to obtain qPCR standards.

PCR was carried out using Qiagen Multiplex PCR Kit (Qiagen) following the provided protocol. First, a Master Mix double size was prepared in two tubes for each gene, by mixing 20µl of 2X QIAGEN Multiplex PCR Master Mix (HotStarTaq DNA Polymerase, Multiplex PCR Buffer containing 6 mM $MgCl_2$, dNTP Mix), 4µl of Q solution 5X, 4µl of 10X primer mix (2µM each primer) and 2µl of Nuclease free water. Then 10µl of 5X diluted cDNA was added for a final volume of 40µl and briefly centrifuged before the passage in thermal cycler. Program cycler consisted in these steps: heat lid 105 °C, Taq activation at 95°C for 15 minutes, followed by 38 cycles of denaturation at 94°C for 30 seconds, annealing 57°C for 90 seconds, extension 72°C for 90 seconds, and a final extension at 72°C for 10 minutes.

The amplicons obtained from each gene were visualized by electrophoresis on a 2% agarose gel and then used to prepare the specific DNA standards in the next qPCR procedures.

3.1.4.5 Electrophoresis on agarose gel

Agarose gel was prepared mixing 1X TAE, 2% (w/v) agarose and SYBR Safe DNA Stain 10,000X (Invitrogen Carlbad, CA, USA). Each sample was loaded onto the gel after mixing 2µl of DNA gel Loading Dye 6X (Thermo Fisher Scientific, Massachusetts, USA) with 10µl of cDNA, and a DNA ladder 100bp (Thermo Fisher Scientific, Massachusetts, USA) was loaded as marker. After a run at a constant voltage of 80V for 40 minutes, gel was placed on UV light box (Syngene UV Gel Doc G: Box, Syngene, Cambridge, CB4 1TF, United Kingdom) for visualization.

3.1.4.6 DNA purification

The DNA amplified from cDNA used for standards in the qPCR assay was purified using a QIAquick PCR purification kit (Qiagen) following the protocol provided by the manufacturer. An amount of 70µl of PCR reaction was added to 350µl of Buffer PB (with high concentration of guanidine hydrochloride and isopropanol) using a QIAquick column placed in a collection tube. After a centrifuge for 30-60 seconds, the tube was discarded flow-through and the QIAquick column placed back in the same tube. An amount of 750µl of Buffer PE (confidential composition) was added to the column to wash the sample and after a centrifuge, the content of the tube was discarded flow-through,

and the QIAquick column placed back in the same tube. After removal residual wash buffer by centrifuge each QIAquick column was placed in a clean

1.5ml microcentrifuge tube. Elution of DNA was performed adding 50µl of Buffer EB (10 mM Tris-Cl, pH 8.5) and centrifuged for 1 minute.

Finally, quality and concentrations of cDNA purified was determined with the NanoDrop ND-1000 spectrophotometer.

3.1.4.7 qPCR procedures

Concentrations of the selected genes were quantified using qPCR. Annealing and amplicon-specific melting temperatures of the primers were determined using a gradient function of the qPCR machine (CFX96 Real Time System DNA, Bio-Rad Laboratories, Hercules, CA, USA). For each primer set was prepared a series of 8 identical reactions with 100µl of Absolute 2X qPCR SYBR Green Mix (PCR Biosystems Ltd, London), 8µl of forward and reverse primers (10µlM), 10µl of DNA standard (2ng/µl) and nuclease free water to a final volume of 200µl. Reactions and data acquisition were carried out in a CFX96 Real-Time System (Bio-Rad) with an initial Taq activation step at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 5 seconds, gradient annealing at 55-65°C for 30 seconds, extension at 63°C for 20 seconds and a final extension at 63°C for 5 minutes. The optimized annealing temperatures are shown in Table 2.

Eight DNA standards were prepared from 1×10^{1} to 1×10^{-6}ng/ml.

Standards and samples in the qPCR assay were performed in duplicate with the same final volume. Each well contained 10µl of 2X QPCR SYBR Green Mix, 0.8µl of forward and reverse primers (10µM), 3.4µl of nuclease free water

and 5µl of DNA standard or sample cDNA (Figure 29). Reactions and data acquisition were carried out in a CFX96 Real-Time System (Bio-Rad) with an initial Taq activation step at 95°C for 2 minutes followed by 38 cycles of denaturation at 95°C for 5 seconds, annealing for 30 seconds (the annealing temperatures are given in Table 2), extension at 63°C for 20 seconds and a final extension at 63°C for 5 minutes.

The results were analyzed using the CFX Manager Software package (Bio-Rad).

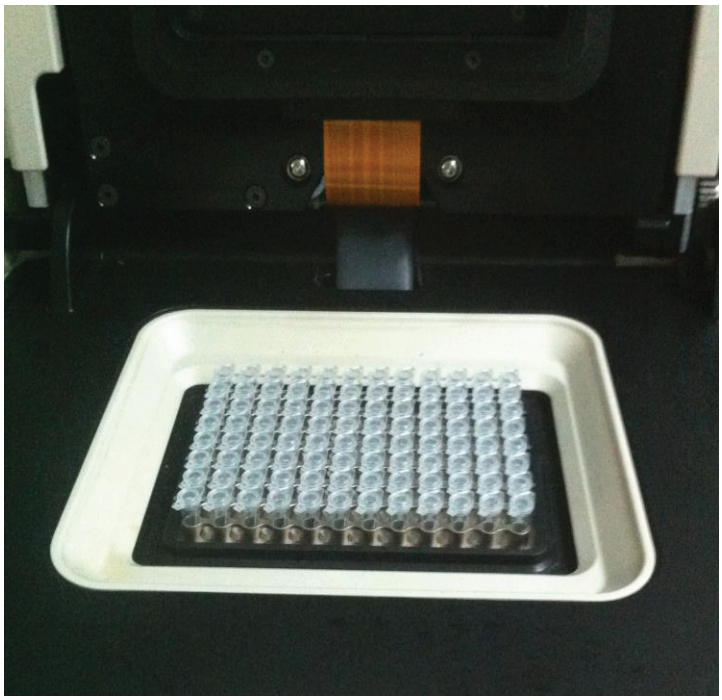


Figure 29. Microtubes with samples within the plate of CFX96 Real-Time System (Bio-Rad).

3.1.5 Statistical analysis

The values for plasma IGF-I were summarized as ng/ml and the values of gene expression generated by qPCR were normalized as femtogram per microgram (fg/µg) reverse-transcribed total RNA. They were expressed as mean ± standard deviation (SD). If the data were not normally distributed, a logarithmic conversion was carried out. Statistical analysis was carried out using R statistical software (Version 3.4.1).

The differences of plasma IGF-I concentration between primiparous and multiparous were identified using T-test. Whereas, differences between six groups, considering <5, 5-8, 9-12, 13-16, 17-21, >21 days after calving, were analyzed using one way analysis of variance (ANOVA). Considering high (≥20ng/µl) and low (≤11ng/µl) plasma IGF-I concentrations, 40 cows were divided in two groups, in order to obtain, using ANOVA, the connections between high or low IGF-I concentrations and respectively, calving to conception interval, number of insemination for a positive conception and pregnancy diagnosis. Statistical significance was considered at $P<0.05$. Where statistical significance was achieved in ANOVA, multiple comparisons using Tukey's HSD test were carried out to test the differences between each group.

Considering the 5 groups of cows (PPL, PPH, MPLM, MPLnoM, MPH) the relationship between the plasma IGF-I concentrations and each gene expression values was examined with Pearson correlation tests.

The relationship between the mean of gene expression in MLnoM and

MLM groups (only for *SELL* and *SOD2*) and health status (only for the multiparous cows with low IGF-I, interpreted as no recorded or recorded incidences of clinical mastitis in the previous lactation) was evaluated using T-test.

3.2 Study 2

Association between IGF-I gene, fertility and milk production in Sarda sheep

3.2.1 Animals and blood samples

For this part of the research, 214 adult and lactating Sarda ewes, in a good state of health and nutrition, were selected, from 4 farms, located in North Sardinia. In order to minimize the farm effect, farms were selected to be as uniforms as possible for size (approximately 800 animals for each farm), flock health, milk production (an average of 294±45 litres in 180 days for multiparous ewes; an average of 140±33 litres in 100 days for primiparous ewes), reproductive management and feeding conditions. During the day animals grazed on natural extensive pasture, were supplemented by 300g per head daily of commercial pellets (crude protein 20.4% and 12.5 MJ ME/kg DM), during milking and were penned at night, when they received hay (crude protein 11.1% and 7.2 MJ ME/kg DM) and water ad libitum. The ewes enrolled for this study were multiparous, aged 3-6 years in order to allow the evaluation of their reproductive and productive activity from their first lambing. The individual rumen bolus was recorded and each ewe was individually marked with a numbered collar in order to avoid identification mistakes.

An amount of 10ml of blood sample was collected from each animal from the jugular vein, using sterile vacuum tubes with K_2EDTA (10.8mg) as

anticoagulant. From each tubes, 4 aliquots of 200ml of whole blood were obtained and stored at -80°C until use. Data about lambing order, lambing date, mating date, number of newborn lambed, and milk were recorded for each animal and obtained from herd records by ASSONAPA.

Figure 30. Blood samples, tubes for aliquots and a P1000 pipette.

3.2.2 Genomic DNA extraction

DNA extraction was carried out from whole blood (from the 200µl aliquots preventively stored at -80°C) using a commercial kit (NucleoSpin® blood, Macherey–Nagel, Germany). The DNA extraction procedure consists in these steps: 1) Lyse blood sample. Firstly, pipetting 25µl of Proteinase K and up to 200µl of blood into 1.5 ml microcentrifuge tubes. Secondly, adding 200µL of

Buffer B3 to the samples and vortex the mixture vigorously for 30 seconds. Finally, incubating samples at 70°C for 30 minutes. 2) Adjust DNA binding conditions adding 210µl of ethanol (96–100%) to each sample and vortex. 3) For each preparation, take one NucleoSpin®Blood Column placed in a Collection Tube and load the sample. Centrifuge 1 minute at 11,000x g and discard Collection Tube with flow-through. 3) Wash silica membrane. For the first wash, add 500µl Buffer BW, centrifuge 2 minutes at 14,000x g and discard Collection Tube with flow-through. For the second wash, add 600µl Buffer B5, centrifuge 2 minutes at 14,000x g, discard flow-through and reuse Collection Tube. 4) Elute highly pure DNA, adding 50µl preheated Buffer BE (70°C), incubate at room temperature for 1 minute and centrifuge 2 minutes at 14,000x g. Repeat this step another time.

Prior to storage the amplicons at -20°C the quantity and quality of the DNA was evaluated by spectrophotometric reading (BioPhotometer Eppendorf, Hamburg, Germany). For the subsequent molecular analyzes the obtained DNA had a concentration > 50ng/µl, 260/280≥ 1.7 and 260/230≥ 2.

3.2.3 Amplification

One pair of primer was used to amplify the 5' untranslated region (UTR) according to He et al. (2012), considering the sequence X69472.1 Ovis aries gene for *insulin-like growth factor-I, exons W, 1 and 2* (GenBank). In mammals, exons 1 and 2 are differentially spliced to exon 3 producing alternate class 1

and class 2 transcripts. In sheep, there is evidence for a third leader sequence, which is spliced on to exon 3. This could indicate an additional exon or a differential splicing event from an extended exon, referred as exon 1W after Wong et al. (1989) (Scatà et al. 2010).

The primers were made by Thermo Fisher Scientific (Massachusetts, USA) and their sequence information are given in Table 3.

Figure 31. *Microtubes with samples for PCR within the plate of thermal cycle.*

Table 3. *Oligonucleotide sequence information used for IGF-I gene PCR assay in Sarda sheep.*

Gene	Primer sequence (5'-3')	GenBank accession number	Product length (bp)	Annealing (°C)
IGF-I 5' UTR	Forward: TGAGGGGAGCCAATTACAAAGC	X69472.1	294	55
	Reverse: CCGGGCATGAAGACACACACAT			

Polymerase chain reactions analysis was carried out in 25µl total volume containing 2.5µL of 10X PCR Buffer (supplied with Taq)[200 mM Tris HCl (pH 8.4), 500 mM KCl]; 0.75µl of 50 Mm $MgCl_2$ (supplied with Taq); 4µl of 1.25 mM dNTPs; 1µl of 10 µM of both Primers (Forward and Reverse), 0.1µl of Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA); DNA 100ng/µl and nucleasic free water until the total volume.

Program cycler using Mastercycle ep gradient S (Eppendorf AG, Hamburg, Germany) consists in the following steps: heating lid at 105°C, Taq activation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing 61°C for 30 seconds, extension 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

DNA products were visualized firstly for verifying the primers specificity using electrophoresis on a 1.5% (w/v) agarose gel and secondly to prepare amplicons for the following restriction fragment length polymorphism (RFLP) procedures.

3.2.4 Visualising nucleic acids on agarose gel

Agarose gel was prepared mixing 1X TAE with 1.5% (w/v) agarose. Each sample was loaded onto the gel after mixing 1µl of DNA gel Loading Dye 6X (Thermo Fisher Scientific, Massachusetts, USA) with 7µl of PCR product. In parallel a DNA ladder 100bp (Thermo Fisher Scientific, Massachusetts, USA) was loaded as marker. After a run at constant voltage of 100V for 30 minutes,

gel was colored with ethidium bromide for 20 minutes and then placed on UV Trans-illuminators (UVItec, Cambridge, UK) for visualization.

3.2.5 Restriction fragment length polymorphism analysis

RFLP analysis was performed using Thermo Scientific FastDigest BfoI restriction enzyme (Massachusetts, USA). BfoI is an isoschizomer, since it recognizes with other Restriction Endonucleases (Bsp143II, HaeII and BstH2I) the same sequence 5'-RGCGC^Y-3' and 3'-Y^CGCGR-5'.

A mix of 10µl of PCR product, 17µl of nuclease free water, 2µl of 10X FastDigest Green Buffer and 1µl of BfoI was incubated at 37°C for 5 minutes. RFLP products were visualized using a 1.5% agarose gel colored with ethidium bromide. An amount of 15µl of each sample was loaded, in parallel with a 100 bp DNA ladder onto the gel for a run at 80 V for 40 minutes. After coloration with ethidium bromide for 20 minutes, the fragments were analyzed using an UV Trans-illuminators (UVItec, Cambridge, UK).

3.2.6 Sequencing

After RFLP analysis, 42 PCR products in total based on the different found patterns were sequenced from both directions using a commercial service offered by BiofabResearch lab (Bio-Fab Research srl, Rome, Italy).

Sequencing was carried out using Applied Biosystems 3730 DNA Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), with Dye

Terminator 3.1 chemistry.

3.2.7 Statistical analysis

Allelic frequencies were determined by direct counting of the observed genotypes. R statistical software (Version 3.4.1) was used to analyse the associations between genotype and both milk production (in litres) and reproductive activities, measured as age of first lambing and conception (in days), distance of days from the parturition to the next birth, lambing to conception interval and litter size. The following linear model was used:

$$Y_{jk} = \mu + G_j + e_{jk}$$

Where Y_{jk} is the trait measured for each animal (distance of days from the second to the third birth or daily milk production), μ is the overall mean, G_j is the fixed effect of the genotype and e_{jk} is the random residual effect of each observation. When P was <0.05 it was considered statistically significant. In this case, multiple comparisons of the means were performed using Tukey's method (library *Agricolae*, R package version 1.2-3).

4. Results

Study 1

Enzyme-linked immunosorbent assay allowed to measure plasma IGF-I concentration from all the 332 analyzed cows, of which 98 were primiparous and 234 were multiparous. The mean ± SD of IGF-I was 22.2± 17ng/ml and 17.8± 10ng/ml, respectively in primiparous and multiparous cows. T-test showed that plasma IGF-I was significantly higher in the primiparous than in the multiparous animals (P= 0.004) (Figure 32).

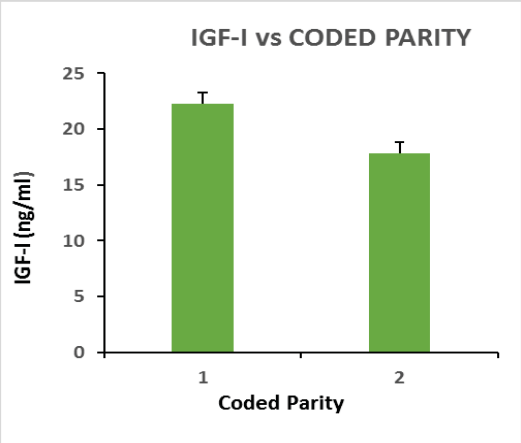


Figure 32. *Mean ± SD plasma IGF-I concentration in Holstein Friesian cows (n=332), in relation to the coded parity [1=primiparous (n=98); 2=multiparous (n=234)].*

To evaluate as plasma IGF-I concentration changes during the early post-partum (0-27 days), the cows were divided into 6 groups based on the

days after calving: <5, 5-8, 9-12, 13-16, 17-21, >21 (Table 4).

Table 4. Mean ± SD plasma IGF-I concentration in Holstein Friesian cows (n= 332) according to days post-partum (p.p.)

Days p.p.	<5	5 to 8	9 to 12	13 to 16	17 to 21	>21
Number of cows	113	77	73	31	31	7
IGF-I ng/ml± SD	16± 17	18± 7.5	21±8.5	22±9	25±14	13.6±2

ANOVA showed difference in the IGF-I concentration between these groups (P<0.006). Multiple comparison using Tukey's LSD illustrated that the IGF-I concentrations in the cows sampled <5, 5-8 and >21 days after calving were significantly lower than those sampled 9-12, 13-15 and 17-21 days after calving (Figure 33).

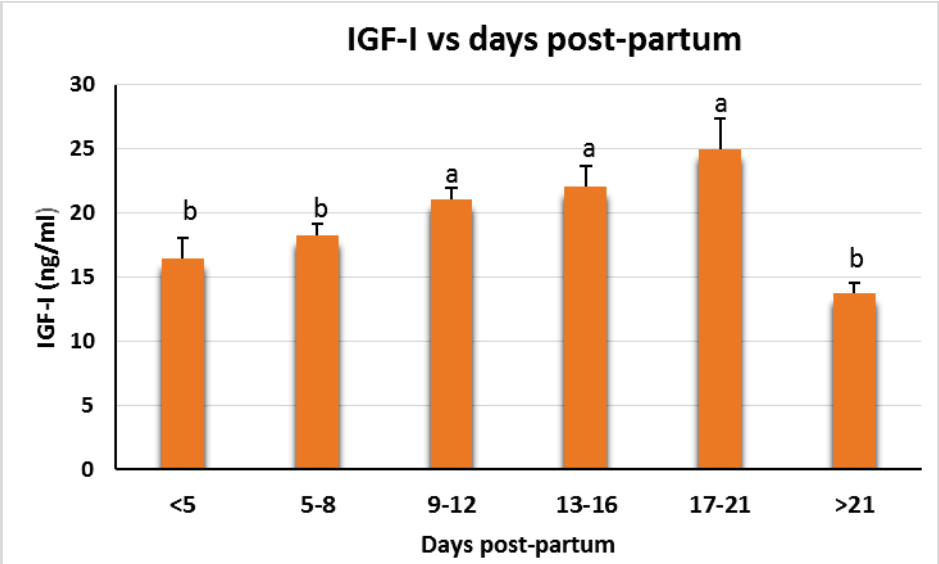


Figure 33. Mean ± SD plasma IGF-I concentration in Holstein Friesian cows (n=332) in relation to the days post-partum.

To evaluate how plasma IGF-I concentration changes during the interval in days (0-27) after calving, and according to parity, the cows were also considered divided in primiparous and multiparous groups (Table 5) (Figure 34).

Table 5. Mean ± SD plasma IGF-I concentration in Holstein Friesian cows according to days post-partum (p.p.) and parity (primiparous and multiparous).

Days p.p.	<5	5 to 8	9 to 12	13 to 16	17 to 21	>21
N° of primiparous	35	24	20	9	9	1
IGF-I ng/ml± SD	16±12	21±6	27±10	23±13	24±9	12
N° of multiparous	78	53	53	22	22	6
IGF-I ng/ml± SD	15±12	17±8	19±6	22±8	25±15	14±2

ANOVA showed that there were significant differences in IGF-I concentration between the multiparous' groups ($P<0.001$), whereas no differences were found between primiparous' groups ($P>0.05$) (Figure 34).

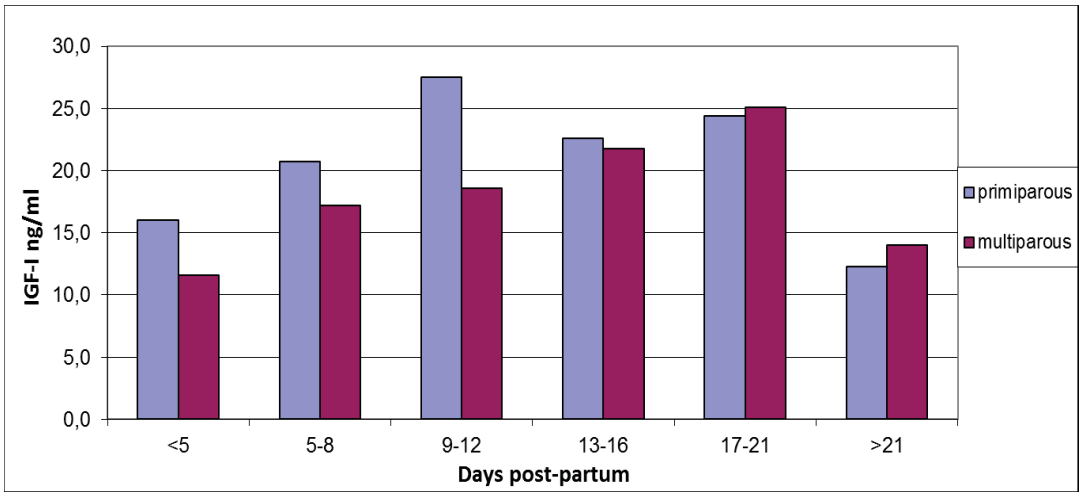


Figure 34. Mean ± SD plasma IGF-I concentration in Holstein Friesian cows (n=332) in relation to the days post-partum and parity (primiparous and multiparous).

After IGF-I measurement, from the 332 animals, 40 cows were selected for further data analysis according to the criteria specified in the Material and Methods. Subsequently, five animals were discarded, so that data on 35 animals were evaluated. They were divided into two groups based on the IGF-I concentration obtained in 1-8 days after calving: "H" for cows with high IGF-I (≥20ng/ml) and "L" for cows with low IGF-I (≤11ng/ml), in order to connect the IGF-I value with calving-conception interval (CCI), number of inseminations to conception and the pregnancy diagnosis (Table 6).

Table 6. Individual number of inseminations to conception (N.I.), calving-insemination interval, calving-conception interval (in days), based on the formed "H" and "L" group in lactating Holstein Friesian cows (n= 35).

Cow ID	Group	N.I.	C1I i	C2I i	C3I i	C4I i	C5I i	CCI	Pregnancy
AA04	H	1	61					61	yes
D016	H	1	93						no
D018	H	1	52					52	yes
D020	H	4	42	72	93	133		133	yes
FF15	H	2	64	83				83	yes
H047	H	2	79	99				99	yes
I023	H	2	68	115				115	yes
BB42	H	1	41					41	yes
BB48	H	1	79					79	yes
D009	H	4	80	122	205	233			no
H042	H	3	43	73	115				no
H044	H	2	65	91				91	yes
HH10	H	2	57	78				78	yes
HH12	H	1	106					106	yes
II30	H	3	55	81	144				no
D022	L	3	70	96	115			115	yes
DD36	L	1	84					84	yes
GG12	L	2	41	73				73	yes
HH06	L	1	66					66	yes
HH48	L	2	58	78				78	yes
II27	L	1	73					73	yes
AA06	L	3	60	103	145			145	yes
CC01	L	1	77						no
E004	L	3	61	85	132			132	yes
HH11	L	2	90	134					no
II26	L	3	61	97	119				no
J044	L	2	62	88				88	yes
BB46	L	1	136					136	yes
CC06	L	1	75					75	yes
D013	L	5	38	102	144	186	215		no
FF05	L	2	51	78				78	yes
FF25	L	5	73	83	104	127	148	148	yes
H038	L	1	88					88	yes
HH09	L	3	44	97	121			121	yes
J042	L	1	47						no

Cow ID = farm identification; Group= H for cows with high IGF-I (≥20ng/ml) and "L" for cows with low IGF-I (≤11ng/ml); N.I. = number of inseminations to conception; C1I i= calving-first insemination interval; C2I i= calving-second insemination interval; C3I i= calving-third insemination interval; C4I i= calving-fourth insemination interval; C5I i= calving-fifth insemination interval; CCI= calving-conception interval.

ANOVA showed no significant differences (P>0.05) between the percentage of cows with high or low IGF-I concentrations and the number of inseminations to conception (Figure 35).

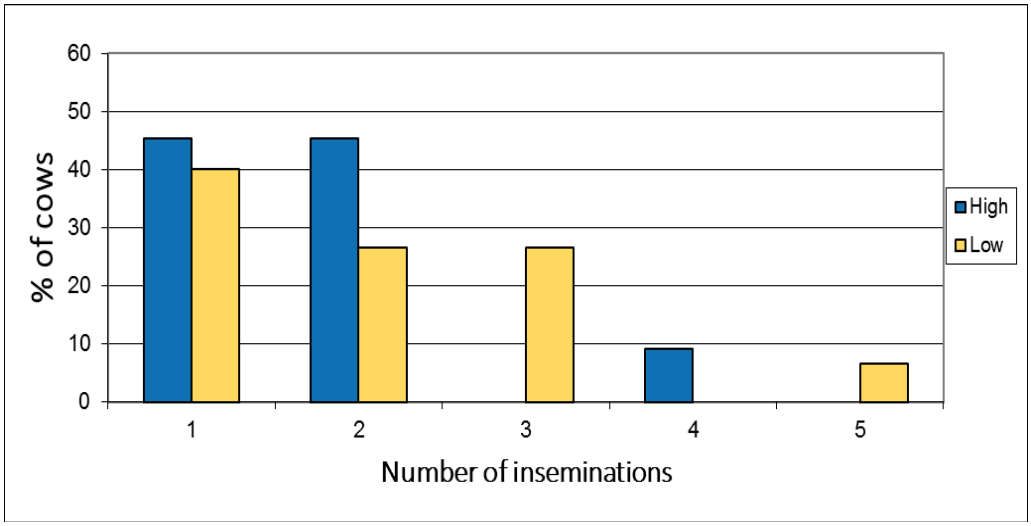


Figure 35. Percentage (%) of Holstein Friesian cows (n=35) with high (≥20ng/ml) or low (≤11ng/ml) IGF-I and number of insemination (1 to 5) to conception.

Moreover, ANOVA did not reveal significant differences (P>0.05) between the percentage of cows with a positive or a negative pregnancy diagnosis and high or low IGF-I concentrations (Figure 36). In addition, in consideration of calving to conception interval in days, statistical analysis did not show significant difference between the two groups of cows (P>0.05).

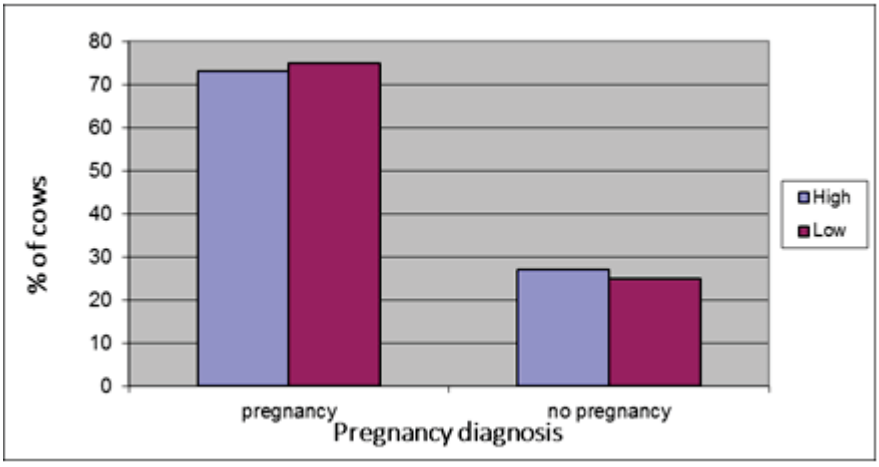


Figure 36. Percentage of Holstein Friesian cows (n=40) with high (≥20ng/ml) or low (≤11ng/ml) IGF-I and pregnancy diagnosis.

As well as being a period of altered energy balance and nutrient requirements, the post-partum in dairy cows is also characterized by a greater susceptibility to disease. The evaluation of the analyzed candidate immune gene expression produced the following results.

The PCR analysis allowed to detect a single band for each gene used for qPCR standards, confirming the specificity of each pair of primers and the amplification of each corresponding gene.

The qRT-PCR was performed for 3 housekeeping genes: *Actin Beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GADPH), receptor like protein 19 (RLP19);* and 8 genes involved in immune system*: C-C Motif Chemokine Ligand 5 (CCL5), Interleukin 8 receptor beta (IL8RB), C-C Motif Chemokine Receptor 2 (CCR2), Interleukin 1 Alpha (IL1A), Interleukin 1 beta (IL1B), Tumor necrosis factor alpha (TNFα), Superoxide Dismutase 2(SOD2) and Selectin L (SELL).* The expression of each gene, was obtained for 35 animals (although the animals selected were at first 40, but 5 samples were discarded, due to poor RNA yield or quality) divided into 5 groups: PPL (primiparous with low IGF-I), PPH (primiparous with high IGF-I), MPLM (multiparous with low IGF-I and high SCC and/or mastitis in previous lactations), MPLnoM (multiparous with low IGF-I and low SCC in previous lactation), MPH (multiparous with high IGF-I).

Since the expression of all housekeeping genes was not stable across the groups, their expression was not considered and an absolute quantification

rather than a relative method was used to evaluate the expression of the all eight immune genes analyzed.

The different expression of each gene within each of the 5 groups was evaluated using ANOVA, which showed difference of expression between groups for *SELL* (P=0.0368) and for *SOD2* (P=0.0249). Whereas, for other genes any significant difference (P>0.05) was found (Table 7).

Table 7. Individual IGF-I concentration (ng/ml), calving order (CO), and immune gene expression (fg/µg) based on the 5 formed groups in lactating Holstein Friesian (n= 35).

Cow ID	Group	IGF-I	CO	Immune genes expression (fg/µg)							
				TNFa	*CCR2*	*SELL*	*SOD2*	*IL8RB*	*CCL5*	*IL1B*	*IL1A*
AA04	PPH	27	1	354,6	2678	10840	24730	24730	780,4	4806	450,68
D016	PPH	26	1	219,2	439,6	1922	6620	6620	516	611	77,924
D018	PPH	75	1	157,8	3086	12300	29330	29330	1444,6	11666	363,52
D020	PPH	27	1	231,8	1160	4540	14428	14428	464	904,8	286,32
FF15	PPH	23	1	408,4	2608	11580	24420	24420	508,4	3859,6	305,62
H047	PPH	26	1	139,8	3444	14820	24860	24860	1371,6	4250	466,8
I023	PPH	24	1	314,6	2004	7580	36666	36666	760,8	3578,8	286,74
D022	PPL	10	1	68	404,4	1460	5370	4802	456,6	208,2	89,78
DD36	PPL	9	1	126,4	122,6	1500	4240,6	2958,4	300,04	111,12	72,7
GG12	PPL	9	1	222,6	1836	6800	21356	6082	294,58	1091	241,04
HH06	PPL	9	1	356,6	2786	11340	40140	15040	1313,8	5641,6	211,14
HH48	PPL	6	1	143,8	1292	5100	21826	8312	400,52	1987,2	1229,6
II27	PPL	11	1	292	91,6	264,4	346,86	5300	507,18	286,62	114,86
BB42	MPH	27	2	200,4	434	6460	22106	4219,8	426,94	600,2	117,914
BB48	MPH	21	3	212,2	1008	3148	16648	6780	427,46	3361,8	326,96
D009	MPH	23	9	608	3932	13140	22662	14546	718,2	3525,4	539,4
H042	MPH	25	3	171,2	1508	10660	22020	10872	721,8	5100	396,16
H044	MPH	27	3	420	1636	7120	42380	7250	392,56	2081,6	251,24
HH10	MPH	20	2	147,8	2442	11380	34166	13072	461,54	6620	366,06
HH12	MPH	21	2	149,2	3936	16900	54880	11856	611,4	3596,4	531,4
II30	MPH	19	3	207,2	53,6	404,8	137,94	2891,8	242,82	39,168	288,78
AA06	MPLM	9	4	197	1200	8260	35518	5492	404,02	920,4	126,76
CC01	MPLM	7	4	79,4	3440	12540	57800	8372	470,88	2867,6	701
E004	MPLM	8	6	120,2	2542	6760	17716	7374	427,2	3209,6	264,82
HH11	MPLM	7	2	144,6	3106	10820	58380	14064	380,9	3527,6	513,6
II26	MPLM	8	6	150,6	1790	7100	21674	9402	592	2750,8	185,14
J044	MPLM	10	2	110,2	3162	9520	33288	10234	447,4	4808	447,2
BB46	MPLnoM	9	3	396,6	3006	13240	53680	13032	835,2	2323,8	734
CC06	MPLnoM	5	3	313,8	1974	9260	33792	11448	499,8	4930	355,72
D013	MPLnoM	8	2	91,2	408,4	5660	18436	5728	409	652,6	162,54
FF05	MPLnoM	10	2	296,6	2386	13780	41254	21246	2343,6	11988	320,52
FF25	MPLnoM	7	3	234,8	2706	11140	61040	27784	713,4	8542	472,2
H038	MPLnoM	6	3	464	4068	16540	49020	16378	736,2	8986	493,2
HH09	MPLnoM	9	2	358	2930	8200	36362	6690	498,4	1864,4	545,6
J042	MPLnoM	10	3	173,8	3960	11540	16972	6550	485,82	1153,6	253,48

Cow ID = farm identification; Group identification: PPH = primiparous with high IGF-I, PPL= primiparous with low IGF-I, MPH= multiparous with high IGF-I, MPLM= multiparous with low IGF-I and high SCC and/or mastitis in previous lactations, MPLnoM= multiparous with low IGF-I and low SCC in previous lactation; CO= calving order.

Only for the multiparous cows with low IGF-I, it was evaluated the relation between *SELL* and *SOD2* genes expression and health status, interpreted as no recorded or recorded incidences of clinical mastitis in the previous lactation. Anyway, T-test did not show significance difference (P>0.05).

Study 2

Genomic analysis allowed to detect, in all the 214 tested samples, a single band of 294bp in length, corresponding to the *IGF-I* 5'UTR in Sarda breed sheep (Figure 37).

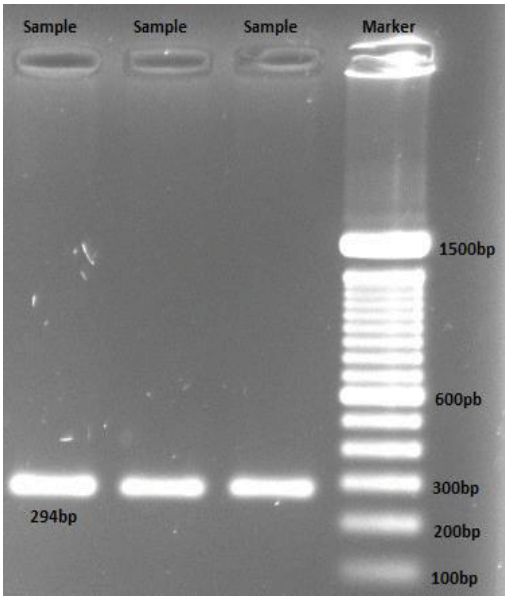


Figure 37. *Electrophoresis of PCR products in a 1.5% agarose gel in the Sarda sheep: lanes 1-3: amplicons of 294bp in length; lane 4: 100bp DNA marker.*

The obtained fragment corresponds to nucleotides 759-1052 of the GenBank locus X69472.1 (Figure 38).

>X69472.1 O.aries gene for insulin-like growth factor-I, exons W, 1 and 2
CAACCCCTTTCAAAACCAATCAGGTTTTCCTTTGAGGGCACCCACCTCTGCAACAGCTTCCTGGCCATGGCATAAAGACC
TAGACAAGAGTTTCAAGCAAACCTTTGTTTTTAGTCGAAGCATCAGGATCACTGTGTCCCCTGAGAATAAGAGGGAGAAA
TACAAGGTCCAGGCTACTCCCACCATTCCAGAAAACCATGCCCCAATAGACCCCAGGGAAAGTGGGATGTCTAACCTGGG
CTTTTGCAATCTTATTTCATAATCCACTTTCTTATCGCCTCCTTCACAAAACTGATGAGAAATTGGTACAAACTCTATCG
ACAAAAGATCACAACTTGATCCTCAATGGCAAAGGCAAGTATACATTATAAATAGCAAAACAGCTGGCTTGGACCATGTT
GCTGGCCACTCATCCAGCTGAGAGATTTGAATGACATCATAACCCTTGAGAGGGTATTGCTAGCCAGCTGGTGTTATTTA
GAATACACAAAAAGGGGGGAAAGAAAATGCACTCACATGCACACACACACAAATACACACACACACACAGGTTCAAGT
TATGCAGAAAAATATGAACAGTGGGAAAATCATTTGCCTCTCAGATGCCCTTCCCCTGGTGTGGGGTGGGGGTCGGGGTG
GGGGCCAAGCAGCAGAATAGAGGAAGGAAGAAAGAGATTCGATTTTATTTTTTCAGTTGGCTTTACAGCTCAGCAAAATC
TTTGCCCTGTCGTGGGCAAAAAGCATGAGACAGTGTCCTGAGGGGAGCCAATTACAAAGCTGCCTGCCCCTTTCCAGGTT
CTAGGAAATGAGATCATTCCCCTCACTTGGCAACCAGGACGAGGGGTCATCCCAGCGCCGTCTTCCAGTCTAGTTTACCC
CAGTCGTTTGAGGGTTAAAATCATAGAGTATGCTTGAGATGGTCTTTTTTTCATTTCTTGTTTTTTAAATTTTGTGTTGG
CTCTGGAATATAAAATTGCTCGCCCATCCTCCACGAATATTCCTTTCATACGGGTAAGGTGTATTAGCAGATGTGTGTGT
CTTCATGCCCGGTAGAAAGTTAATCAGAGGACAGCATCAGGATTTTAATGTCTGCTCCTCTTGTCACTAACACACATTCT
TTTAAGGGAAAAAAATGCTTCTGTGCTCTAGTTTTAAAATGCAAAGGTATGATGTTATTTGTCACCATGCCCAAAAAAGT
CCTTACTCGATAACTTTGCCAGAAGAGGGAGAGAGAGAAGGCAAGCGTTCCCCCAGCTGTTTCCTGTCTACAGTGTCT
GTGTTTTGTAGATAAATGTGAGGATTTTCTCTAAATCCCTCTTCTGTTTGCTAAATCTCACTGTCACTGCTAAATTCAGA
GCAGATAGAGCCTGCGCAATGGAATAAAGTCCTCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCCCTGGA
TTTCTTTTTGCCTCATTATTTCTGCTAACCAATTCATTTCCAGACTTTGCACTTCAGAAGCAATGGGAAAAATCAGCAGT
CTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCTTGAAGGTAAATATTTCTTACTCTTTGAAGTCATTGGGGAA
TTTTCTTTAAATTGTGTACTGCTTGCTTCTGCTTAGAAATGTTCTTCACTTTAGAATTTTCATTGTTTCGGCACTGGGAG
TTATTTATAAATTGCTGAATATGCAATTCTGTGGGATCTGAAAAAATAGCTCCGGGAGATAAATGCCTTTGCACAGATAT
CTGTATGAGTAAAAACTATTGCAAGGTACTTATGCTAAATCCTCCACTTCTTCAGGGCTTGAGTGGTGTCATTATAGAAG
ATTCCTTTAAATCCTGTCTATGGTTAAGGGCTATAGGGCATGGATATGAACTTTTGGATTTTTTTTGCAGGTGCAGATGT
TTTATTTTTAAGACCATGTTCATGTGTATGTAGGGCTGTGTGGTGTGTGTGTGTGCGTGTGTGTAACTGGCCAGGACTTT
TGATTACAAGACATGCCACATACAAAGAGTTACGTTTTAAATGATATTAAAGCTTTTAAATATGATCTTTGGAGCTAAGG
TCCCGGAACTCTCTGCACTTATGCCCAGAGAGTCAAAGTTAGAGTGAAGTTTCATTTGCTCTTCTGAAAAAGAACTCCTT
AAGAACTCCTGCTGACCTTGCATATTCGGATAATTTAAACAAATGCACACTGTATATGGAAAGCGGAAACTTTCTAGCAA
CTGCTATTCCAAGTTTTTTCTTTTGAAGAGGACTCTCAGGGGCGAAGTTTGGACTTGGGGTCTTGTGTTGTAAAACACGG
GTTTTGTATTTGGGATTGTAAAGTCTCTTTAGGTGAATTTGGCTAGCGCTGTCAATGCACTGACTTCGCCTTTCCAATAA
CTGGCCCTAGTTCAAGTTTCCATTCTCAGCAAAATTATATCTTTCAAGACTTGTATTTTTCCAATTTGCAAGCATTTTTA
AGCTGCTGTCACTGGCTCCCCGATGCAATTGCCTGTGGGCTCAATTCATGATCCGTCCCTACCGCTTAGTCCAACACTTA
TTTGCCGGCTTTCCAGCACTCCACCACTAGGACGTTTCTTAGTCAAGTCAGTGGCTTAGGAGTTAAGAATACATCCTTGT
CGGGCACCTGATCCTGCCGCCCTTGAGAAGCCAAGATGCACACTGCCTACCTTGCTTTTAAAAGAAATGAAGTCAGTATA
CACATATGCTATGTGGTCTGACAGCTGGGGATTTTGTATCCCTACTTAGAGGATCCTAAAAGGGGCTGTGTAGTGTTATC
TCTGCATTAATTACAAGTTTGGAAAACTCCAAATGAACTTTCCATGCTGTGTATGCTGAACTTTTCAGAAGTAGAGCTAG
CTAGCCATAAGTTGTTGCTTTTTCCTGTACTTGAAGCAGGAAGTGGTTTCAGGGAGCTACGTGGTTCTTTCAAATGTAAT
TCAATGAGTAAAGGTGTCTGCCAGGCAGAGCTCACAAGCTGATTGTACTGTGAGTCTCAAGATATTTCCAAGTGTTTGAG
TCAGAGGAAAGAGGGCACAGGGGAGGACTGGAGCTTCGGTCCCTGTCCAGGACGGCTACAATAGGCACACGATGGAAATC
AGTGGCTTGATTGGGAGGAAAAGATTGACTCAGATCCCAGCCGTGCAATTTGTTTATTGTCTGAATGGACAAAAGGCAGT
TTACCCAGGCTCGTAGCATACCTGCCTGGGTGTCCAAATGTAACTAGATGCTTTCACAAACCCCACCCACAAAGCAGCAC
ATGTTTTTAAGTCCTCAGTTTTCTATTCACATCAGTCTCATAATACCCACCCTGACCTGCTGTAAAAGATCTGGAACAAA
CAAAAATGGTTACACCTACAGTGAGTATTTTCTTATGACTATTGCCCTCAAATTTTGCTGGGCATTTTTATTATAACCCA
GACATCTGGAACCAATTGATATTCCATTTATTTAAGATAAAAAGAAAGGTTTTTAAAATTTTGGATTTGTGAATGATTTT
TGAGAAAGAGTGCTCTGAAATTTTTTTTTCTCGTCTCTTTCTAGTTCTTCTTTCATTTCTTTCTTTCAGATCTCTCCTTT
CTCTCTCTTCATTTCTTTTGTGTTTGGAAAAATAAAAGACCAAGGAAATGATGAACATATGGGGCCACTCATTTCAAACT
TTTAACTCCTAAGCAAGTTCTTCATTGTTTTCTTTTGGGGGAACATAATGTGATAAATTCTCTGGTTCTCTGTGGGTGTG
ATGGACTG

Figure 38. DNA sequence of the O.aries gene for insulin-like growth factor-I, exons W, 1 and 2 (GenBank accession number X69472.1) with evidenced amplified fragment corresponding to base 759-1052 (5'UTR).

The RFLP analysis, carried out using BfoI endonuclease, evidenced one polymorphic site in position 97 of the 294bp fragment, corresponding

to a C with a G substitution in position 855 of the entire sequence. Consequently, three genotypes were detected in the tested sheep: GG, GC and CC (Figure 39).

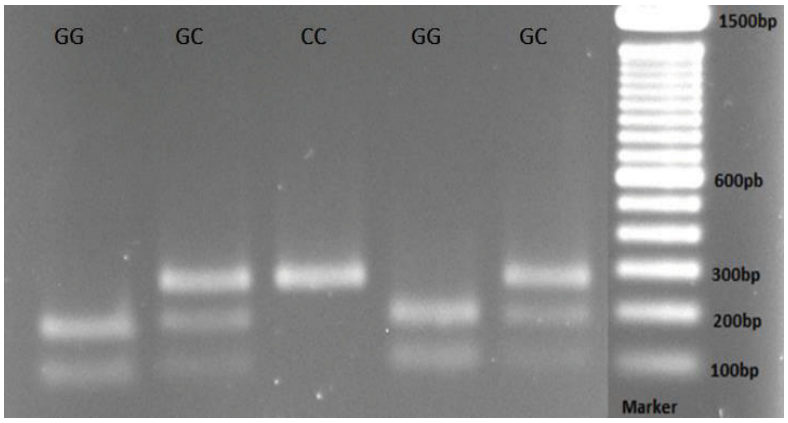


Figure 39. *Electrophoresis of digestion with BfoI in a 1.5% agarose gel in the Sarda sheep: lanes 1 and 4 GG genotype (bands of 196bp and 98bp); lanes 2 and 5 GC genotype (simultaneous presence of the 294bp band those of 196bp and 98bp); lane 3: CC genotype (a unique uncut band of 294bp); lane 6: 100bp DNA marker.*

Within the 214 Sarda ewes tested, 191 were found to be carriers of the GG homozygous genotype, 21 ewes were heterozygous GC and only 2 animals exhibit CC homozygous genotype. Accordingly, allele frequency was 94% for the G and 6% for the C allele.

By sequencing of the 5'UTR of the *IGF-I* gene it was possible to detect another nucleotide mutation: an A to a G substitution in position 99 of the amplified fragment, corresponding to nucleotide position 857 of the entire sequence (Figure 40).

This latter transversion, resulted in full linkage disequilibrium with the 855G>C, so that it was not considered as another SNP. In fact, there was

an exact correspondence between the G in position 855 and the G in position 857, and, conversely the presence of a C at position 855 resulted always in an A at position 857 as reported in Table 8 and in Figure 40. For this reason in the following section of the present thesis it will refer only to the genotype at position 855, implying also the corresponding one at position 857.

Table 8. *Allele and genotype frequencies at the 855 and 857 nucleotide position in the 5'UTR of the IGF-I gene in the Sarda sheep (n= 214).*

Genotype position		Genotype frequency	Allele position		Allele frequency
855	857		855	857	
GG	GG	0.89	G	G	0.94
GC	GA	0.10	C	A	0.06
CC	AA	0.01			

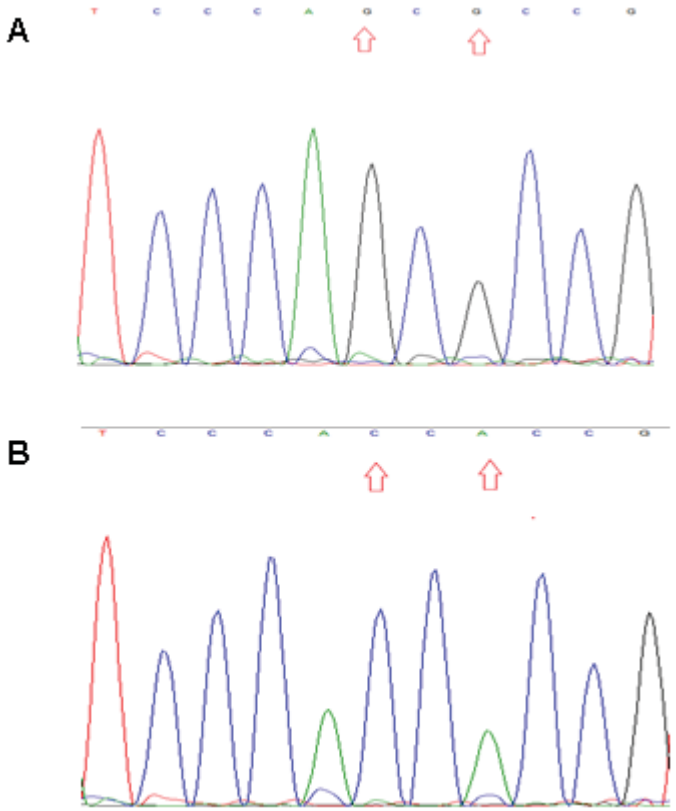


Figure 40. *Post-sequencing electropherograms showing the sequence comparison among the obtained genotypes in Sarda sheep IGF-I gene. Panel A= major homozygous genotype 855GG-857GG; Panel B= minor homozygous genotype 855CC-857AA.*

The association study between the found SNP and milk production traits showed a significant effect of the G allele. Indeed, ewes carrying GG genotype produced higher daily milk yield in all their lactation studied, with a significant P value ($P<0.05$) in their 1st, 2nd and 4th lactation, and an even bigger significance in their 3rd lactation ($P<0.01$) compared to those carrying GC or CC genotype (Table 9). Also in heterozygous genotype, G allele seems to be linked to a major daily milk production, although statistical analysis did not evidenced significant differences between GC and CC genotype.

Table 9. *Means ± SD of the individual daily milk production (in litres) in Sarda sheep (n=214), based on the different genotypes [GG (n=191), GC (n=21),CC (n=2)].*

Individual daily milk yiled (litres)	Genotype			P value
	GG(n=191)	GC(n=21)	CC(n=2)	
1st lactation	1.58±0.33	1.37±0.2	1.16±0.06	0.011
2nd lactation	1.80±0.4	1.54±0.34	1.50±0.09	0.021
3rd lactation	1.99±0.52	1.64±0.3	1.58±0.05	0.008
4th lactation	1.91±0.46	1.87±0.27	1.70*	0.048

*only one subject

Considering the importance of the reproductive traits in a dairy sheep breed, the age at first lambing, the lambing intervals and litter size were recorded to evaluate the possible association with genotype (Table 10). In addition, it was considered the age at first conception and the lambing to conception interval and these data were connected with the three genotypes (Table 11).

Statistical analysis revealed no significance in the comparison of the data considered and the three genotypes, although the allele G exhibited always a shorter interlambing interval and a shorter lambing to conception interval compared to C allele. Statistical analysis revealed no significance in the comparison of litter size and genotypes.

Table 10. *Mean ± SD of distance in days between interlambing intervals (the 1st lambing was considered as age in days at 1st lambing) based on the different genotypes [GG (n=191), GC (n=21), CC (n=2)] and litter size of Sarda sheep (n=214).*

Mean interlambing intervals (days)± SD	Genotype			P value	Litter size		
	GG(n=191)	GC(n=21)	CC(n=2)		GG	GC	CC
Birth/1st lambing	456±72	455±63	552±155	0.243	1.1	1.0	1.0
1st/2nd lambing	350±66	334±46	381±44	0.532	1.2	1.3	1.0
2nd/3rd lambing	335±33	335±24	383±13	0.135	1.3	1.2	1.0
3rd/4th lambing	354±53	357±30	385*	0.616	1.3	1.3	1.0

*only one subject

Table 11. *Mean ±SD of distance in days between lambing to conception intervals based on the different genotypes [GG (n=191), GC (n=21), CC (n=2)] of Sarda sheep (n=214).*

Mean intervals lambing to conception (days)± SD	Genotype			P value
	GG(n=191)	GC(n=21)	CC(n=2)	
Birth/1st conception	310±72	307±63	402±155	0.243
1st lambing/next conception	201±66	184±46	231±44	0.532
2nd lambing/next conception	185±33	185±33	236±13	0.135
3rd lambing/next conception	204±53	207±30	235*	0.616

*only one subject

5. Discussion

Study 1

The research carried out within the "Study 1" is based on measurement of plasma IGF-I concentration during the early post-partum in Holstein Friesian cattle, in consideration of the important role that this hormone plays in the resumption of reproductive activity.

The concentration of IGF-I does not fluctuate acutely either diurnally or with feeding activity (Ronge et al., 1988), and it therefore provides an indicator of nutritional status and energy balance. Moreover, there is a relationship between the plasma IGF-I concentration and reproductive performances in cows as described by several studies (Roberts et al., 1997; Taylor et al., 2004). Konigsson et al. (2008) found significantly higher IGF-I levels in the first two weeks after calving in cows with post-partum ovarian activity resumption, underling the important role of IGF-I as sensitive signal between metabolism and reproduction.

The early post-partum is crucial in Holstein cattle reproduction, since in dairy cows generally, the first wave of follicle growth begins a few days after calving (Wiltbank et al., 2011). Approximately 40–50% of the cows ovulate the

dominant follicle within 21 days after calving; another 30–40% ovulate follicles from subsequent follicular waves from 30 to 50 days after calving and

approximately 10–30% remain anovulatory by 50 days (reviewed by Galvao et al., 2010).

The relevance of IGF-I during this time is due to its action at hypothalamic, pituitary, and ovary level (Lucy, 2001), conditioning the resumption of reproductive activity after calving. Furthermore, IGF-I is highly linked with energy balance, considering its function in the somatotropic axis (Jones and Clemmons, 1995). For these reasons, IGF-I protein has been rated as a potential hormonal mediator of nutritional control of fertility (Zulu et al., 2002).

The "Study 1" permitted to assess the average levels of plasma IGF-I concentrations in primiparous and multiparous cows from 0 to 27 days after calving.

The results highlighted significantly higher ($P<0.01$) concentrations of IGF-I in primiparous than in multiparous cows (22.2±17ng/ml vs 17.8±10ng/ml, respectively). Parity is an important factor affecting metabolic, hormonal and reproductive parameters (reviewed by Konigsson et al., 2008). Considering IGF-I concentration, the diversity between primiparous and multiparous cows was reported by several studies (Kerr et al., 2001; Taylor et al., 2004; Wathes et al., 2007), and it could be associated with significantly lower milk production in the first calving cows, since the differing endocrine background in the less mature animals may limit partitioning of nutrients into milk. Dairy heifers are generally calved for the first time at about 24 months of age when they are not physically mature. Therefore, cows that approach their first calving are in a

different metabolic state compared to multiparous cows as they require nutrients for their own continued growth in addition to that of their developing calf.

Coffey et al. (2006) evidence as dairy cows continue to growth until the end of their third lactation, although growth rates decrease once animals reached about 450 days. At the start of their first lactation, the competing demands of the mammary gland are superimposed on the requirements for growth. IGF-I has growth promoting effects and it is the primary regulator of postnatal muscle hypertrophy, stimulating protein synthesis and inhibiting degradation (Etherton, 1982; Wathes et al., 2007).

Considering the above information, it is possible to affirm that the higher IGF-I concentration found in primiparous cows, were connected to their physiological process of growth and mammary gland development.

Taking into account that the sampling time covered a period from 0 to 27 days after calving, it was evaluated as, based on the following distance from calving date: <5, 5-8, 9-12, 13-16, 17-21, >21, plasma IGF-I concentrations were changing from calving till 27 days in lactation.

In early lactation, most dairy cows do not consume sufficient feed to satisfy their energy requirements for maintenance and milk yield, they therefore experience a period of negative energy balance during which they mobilize body reserves and lose body condition to maintain milk production (Beever et al., 2001). The tissue mobilization is driven in part by increases in the rate of secretion of GH, which acts on adipose tissue to stimulate lipolysis

(Dominci and Turyn, 2002). In this period, the liver becomes GH-resistant and the normal stimulatory action of GH on the IGF-I synthesis becomes uncoupled: while GH concentrations increase the IGF-I is low (Thissen et al., 1994; Lang and Frost, 2002).

Considering the above, in high-producing dairy cows, the plasma IGF-I concentration is low immediately after calving but afterwards it increases gradually as liver GH receptors are up-regulated and stimulate its production and release (Wathes et al., 2001; Taylor et al., 2003).

The results of this study indicate as IGF-I increases constantly from day 0 to 21, whereas there is an unexpected decrease after 21 days post-partum. The IGF-I concentration showed significantly lower values ($P<0.006$) in the cows sampled <5, 5-8 and 21-27 days after calving than those sampled 9-12, 13-15 and 17-21 days after calving.

This trend is confirmed also analyzing the IGF-I plasma concentration separately in the primiparous and in the multiparous cows (n= 98 and 234, respectively). The differences found among the multiparous groups resulted highly significant ($P<0.001$), whereas among primiparous groups, despite the trend resulted identical to that of the mature cows, there were no significant differences in the IGF-I concentrations. This last data might be due to the divergence in metabolic state and in nutrients requirements existing between young and mature cows, as stated above.

An upward trend of plasma IGF-I in the early postpartum period is desirable and also required for an early resumption of cyclicity in dairy cows

(Thatcher et al., 2006).

Therefore, the decrease in IGF-I concentration obtained >21 days after calving both in young and mature cows was surprising, as it is expected a constantly growing, as reported by other authors (Taylor et al., 2004).

This finding can be result of the lower number of these animals (they were only 7 animals, of which only one was in the primiparous group) and, it could be also due to other reasons such as age, genetics and/or differences in negative energy balance.

The importance of IGF-I concentration during the early post-partum has been examined by several researchers. Patton et al. (2007) described an association of IGF-I concentration in the first week after calving with conception rate to first service. The positive effect of IGF-I in the early postpartum period on the quality of the oocytes (Jorritsma et al., 2003) could be an explanation for improved fertility of cows with greater cumulative concentrations of IGF-I. Cows with high serum IGF-I concentrations resumed estrus cyclicity earlier and became pregnant faster than cows with a lower concentration of this hormone.

The mean concentration of IGF-I, observed in this study, is different from other experiments. In a study carried out by Falkenberg et al. (2008) IGF-I ranged from 57±18.9ng/ml within the first 12 hours post-partum to 74±19.9ng/ml at 40 days post-partum. This discrepancy might be due to various factors, such as differences in negative energy balance of the studied population that could result in divergence of IGF-I values; or a different

sampling time, since the concentration of IGF-I increases in the postpartum period so that a too early sampling, would result in lower IGF-I concentration. Another reason might lie in the methodology used for IGF-I quantification. In this respect, it is important to highlight that residual IGFBPs interfere with IGF-I measurements and may produce erroneously high values of IGF-I. This explains why conventional assays in which IGFBPs are not removed, result in incorrect IGF-I values. For this reason, in the experiment carried out in the present research IGF-II was added to block the IGFBPs and to obtain real IGF-I concentrations.

IGF-I, surely has numerous direct effects on ovarian cells, including stimulation of granulosa cell mitogenesis, progesterone secretion from luteal cells, and thecal cell androgen production (Spicer et al., 1994).

According to the IGF-I concentration, from the whole study population of 332 cows, 40 were selected and divided into "H" (cows with high IGF-I ≥20ng/ml in the first 8 days after calving) and "L" (cows with low IGF-I ≤11ng/ml in the first 8 days after calving) groups. Subsequently, five animals were discarded, so that 35 animals were evaluated. The aim was to verify correlation among IGF-I values during the 1st week and reproductive performance, such as calving to conception interval, number of insemination needed to conception and positive or negative pregnancy diagnosis.

Statistical analysis revealed no significant differences between "H" and "L" cows in the number of inseminations to obtain conception and in the calving to conception interval length. In addition, the percentage of cows with

a positive or a negative pregnancy diagnosis did not differ significantly between H and L groups.

These findings did not confirm precedent investigations. In fact, the plasma IGF-I in the periparturient period has been described as a useful predictor for reproductive performance in dairy cattle. Taylor et al. (2004), reported that a threshold of 25 ng/ml of IGF-I in the 7 days after calving might be suggested to distinguish between cows likely and un-likely to conceive to first service. Instead, in the present results the differences in IGF-I concentration had not a such weight in the ability to conceive at first service, or in the possibility to distinguish between cows able to get pregnant to later services and those that remained not pregnant despite services.

High IGF-I concentration in the considered periods had positive relationships to reproductive performance (Patton et al., 2007) and cows with low IGF-I postpartum had longer intervals from calving to resumption of cyclicity (Roberts et al., 1997; Beam and Butler, 1999). IGF-I has been demonstrated to influence ovarian functions such as follicular growth as well as maturation and the development of the embryo (Diskin et al., 2003; Thatcher et al., 1973). Concentration of IGF-I in plasma was also related to the recrudescence of corpus luteum activity (Thatcher et al., 1996). A greater plasma concentration of IGF-I during the first 2 weeks of lactation was associated with a greater conception rate to first service and an increased likelihood of a shorter interval from calving to commencement of luteal activity (Patton et al., 2007). Previous investigations have shown that both dairy and

beef cows with low IGF-I concentrations after calving take longer to resume estrous cyclicity (Roberts et al., 1997; Beam and Butler, 1999), probably because the dominant follicles that grow under conditions of low IGF-I fail to reach ovulatory size and produce sufficient estradiol to trigger ovulation. As a result, the cow goes through several waves of follicular development before it ovulates (Taylor et al., 2004).

Nonetheless, IGF-I has an importance role in reproductive activity, acting within the somatotropic and HPG axis, the results of the present thesis show that there are many other factors that contribute to regulate reproduction. For this reason, a predictive quality of different IGF-I measures in the postpartum period for reproductive performance in lactating dairy cows, was not confirm by the results obtained by this research.

Another important issue in the dairy cow farming is the control of factors that can make the animals more susceptible to diseases. In this respect, post-partum is a critical period that needs to be carefully monitored. Consequently, the different expression of some immune genes (chemokines, chemokine receptors, interleukins and other cytokines) has been evaluated in this study, in order to relate them with plasma IGF-I concentration in the 35 cows previously selected and improve the knowledge around the postpartum period in dairy cattle. Among the quantified immune genes, only *SELL* and *SOD2* showed a difference, among the five considered groups: primiparous with low IGF-I (PPL), primiparous with high IGF-I (PPH), multiparous with low IGF-I and high SCC and/or mastitis in previous lactations (MPLM), multiparous

with low IGF-I and low SCC in previous lactation (MPLnoM), multiparous with high IGF-I (MPH). This result could be due to the several factors that can influence immune gene expression, although IGF-I is considered serve some regulatory function in the immune system (Smith, 2010). Only for the multiparous cows with low IGF-I, it was evaluated the relation between *SELL* and *SOD2* genes expression and health status, interpreted as no recorded or recorded incidences of clinical mastitis during the previous lactation. These two genes are both involved in the immune response as immune mediators. The *SELL* gene, (also known as *CD62L*, or *Selectin L*) is a cell adhesion molecule (CAM), namely a protein located on the lymphocyte cell surface and involved in their binding with other cells. The *SOD2* gene belongs to the iron/manganese superoxide dismutase family, and encodes a mitochondrial protein that confers protection against cell death, contrasting oxidative stress by clearing mitochondrial reactive oxygen species (ROS). Dairy cows are under considerable metabolic stress in early lactation that together with negative energy balance, alter global gene expression. Cows are also under oxidative stress around calving, as indicated by an increase in reactive oxygen metabolites (Bernabucci et al., 2005). Higher mean values in the *SELL* and *SOD2* gene expression were found in the present thesis in MPLnoM cows compared to MPLM. This data confirm that animals with a lower expression of these genes (MPLM) are more susceptible to diseases.

Specifically the *SELL* gene acts as a “homing receptor” for lymphocytes to enter secondary lymphoid tissues via high endothelial venules. The

expression of this gene is critical to health status in animals, because it mediates the initial contact of circulating cells with the blood vessels (Weber et al., 2001). An adequate *SELL* gene expression by neutrophils is necessary for a successful innate immune response against bacterial infections. In cattle, neutrophil *SELL* expression decreases gradually for 2 weeks before calving and then still markedly decreases at calving. This decrement may contribute to increased susceptibility to mastitis or other diseases during early postpartum in cows (Burton and Kehrli, 1995).

During this period, dairy cows experience also energy deficiency. Researches carried out in pigs, proved that malnutrition alters the expression of MHC class I and II, consequently increasing susceptibility to infections, and prolonging their duration (McCracken et al., 1999; Zijlstra et al., 1999). In the present investigation, higher mean values in *SELL* gene expression levels were found in multiparous cows with low IGF-I concentration (<11ng/ml), and with low SCC and no mastitis diagnosis. Consequently, the generally high occurrence of infectious diseases around parturition in cows might be explained by decreased in *SELL* expression that, together with the NEB occurring in the same period, could make cows more susceptible to clinical mastitis (or other pathological conditions).

Certainly, all the above information increase the knowledge about the role of IGF-I in dairy cows during the early post-partum, in consideration of the great influence that this period has in reproductive activity.

Study 2

In the present study two single nucleotide variations were found in Sarda breed sheep, located in the 5' regulatory region of the *IGF-I* gene and named G855C and G857A. These variations were detected in the upstream regulatory region of the start codon of the *IGF-I* gene, considering the sequence *X69472.1 Ovis aries gene for insulin-like growth factor-I, exons W, 1 and 2* (GenBank). This exon, named 1W after Wong et al. (1989) research is considered an extra exon or a differential splicing event from an extended exon. Several studies showed that in mammals exons 1 and 2 of the IGF-I gene are differentially spliced to exon 3 and produce alternate class 1 and class 2 transcript (Wong et al., 1989; Ohlsen et al., 1993; Shen et al., 2005). In sheep, there is a third leader exon, which is spliced on to exon 3, so from these findings the evidence of the extra exon was born.

These two nucleotide variations resulted strongly related among them, so that they were not considered as two different SNPs: always the presence of a G at position 855 resulted in a G also at position 857, and, conversely the presence of a C at position 855 resulted always in an A at position 857. Thus, for greater convenience when referring to the genotype at position 855, is also mean to the corresponding one at position 857.

In this study, by the genotypic frequency count, was emerged a clear prevalence of the GG genotype at both the positions with 191 ewes carrying this genotype (89% of the total number). A number of 21 animals (10%)

were heterozygotes 855GC and 857GA, and only two subjects (1%) exhibited 855CC, and, consequently, 857AA genotypes. It could be important to specify that the two animals carrying the CC genotype came from the same farm. This distribution has strongly influenced the statistical evaluation, for the massive imbalance among the found alleles and the lack of data available to assess the productive features of homozygotes 855CC and 857AA.

A deficiency in 855C and 857A alleles was found also by Scatà et al. (2005), even if they registered a higher frequency than in the present research (12.5% vs 6%, respectively). The above authors studied 3 different Italian sheep breeds and found that these two alleles are in full linkage disequilibrium and contribute to worsen persistency of lactation, although their effect was no statistically significant just because of the low frequency in the studied population (Scatà et al., 2005).

The frequency of these two minor alleles seems to be completely inverted in the study by He et al. (2012) on some meat breed. These authors identified the same SNPs found in this study, but they detected a much higher frequency of the 855C and 857A alleles (from 65 to 97% in Hu, Small Tail Han and Texel sheep, and even 100% in Dorset). From these surprising results can be inferred that behind this inversion in the allele frequencies there is the productive attitude of the specific breeds. In the Small Tail Han, ewes carrying one or two G alleles at the above positions showed a

statistically higher litter size (P<0.05) than the homozygotes for C and A alleles, respectively (He et al., 2012).

Mutations in the 5' regulatory region of the *IGF-I* gene were found, also in other livestock species. Ge et al. (2001) reported that a polymorphism in the *IGF-I* regulatory region had a significant effect on meat traits in the Angus breed cattle. They hypothesized a direct action of the found mutation on gene transcription, and, subsequently on phenotypic traits (Ge et al., 2001).

Regulatory regions are sections in the genome that do not code for proteins, but instead control the expression of other district, that have coding function. Despite the lack of coding function, the regulatory regions play a crucial role in govern gene activity. Most of them exhibit binding sites for the transcription factors (TFs), proteins able to start DNA transcription. Other regulatory regions include binding sites for coactivators, coexpressor, or factors that regulate epigenetic modification of the DNA (Lengauer and Hartman, 2007).

Therefore, a SNP that occur in the regulatory region can have a great effect.

The two nucleotide substitutions (G855C and G857A) detected in the present research fall within the following sequence: ACGAGGGGTCATCCCA**G**C**G**CCGTCT. This sequence is recognized to be the core consensus binding sequence of the retinoid X receptor heterodimers (RXR).

A consensus binding sequence is a short sequence of nucleotides that are shared or similar among multiple distinct DNA sites and it is thought to play the same role in its different locations. In the specific case of this research, the consensus binding sequence of the retinoid X receptor heterodimers (RXR), above mentioned, is a member of the superfamily of nuclear factors that includes the vitamin D receptor (VDR). RXR and VDR form a heterodimeric complex and bind to the vitamin D responsive elements (VDREs) to activate or repress a great number of genes, involved in a variety of physiological functions.

Within human IGF binding proteins Matilainen et al. (2005) identified 15 VDRE, as candidate vitamin D response elements. These authors demonstrated that the activity of these elements *in vitro* was strongly associated to the presence of retinoid x receptor heterodimer. This means that, the *IGF-I* gene could have different functional activity in the different genotypes, as hypothesized also by Scatà and co-authors (2005).

In the present research, a significant effect of the GG genotype was found on milk yield in Sarda breed. The first four lactations of 214 Sarda ewes were included in this study and always the highest average values of milk yield was recorded in the animals carrying GG genotype both at position 855 and 857, with $P<0.05$ in the 1st, 2nd and 4th lactation, and even $P<0.01$ in the 3rd. This confirms the influence of *IGF-I* genotypes on production traits, probably due to the effect of SNPs in the transcription factor of the *IGF-I* gene. Despite statistical analysis showed no significant

differences between heterozygous and minor homozygous genotypes, however also the higher daily milk production recorded in the heterozygotes suggest that the positive effect is due to the G allele. It is not of secondary importance that the minor alleles were found in homozygosity only in 2 ewes. For its age and lambing order, in the record of the 4th lactation only one of them was included, so that it is easy to understand that a real statistical comparison could not be carried out.

Considering the importance of reproduction in dairy breeding, reproductive traits, such as the age at first lambing, the lambing intervals and litter size were recorded to evaluate as possible association with genotypes. In addition, it was considered the age at first conception and the lambing to conception intervals and these data were connected with the three genotypes. G alleles exhibited always a shorter interval in days from lambing to the next conception and a major litter size, despite no significant differences. As stated above, statistical analysis suffered for the lack of an adequate number of comparable data. On the other hand, the found unbalanced allele distribution and the almost complete absence of animals carrying minor homozygous alleles could be the results of the intense genetic selection aimed at increasing its milk production that has been conducted for nearly a century in this breed. Moreover, this consideration could also explain the full reversal in the allele distribution found in meat breed by He et al. (2012).

Despite further investigations on a higher study population would help to confirm the importance of the *IGF-I* gene on milk traits in a high production dairy breed, however this preliminary result give a valuable insight into its crucial role. In this research was found a genotype/phenotype association that it could become an important reference to be used in sheep breeding program.

6. Conclusions

This thesis aimed to investigate the role of IGF-I in dairy animals, in consideration of the significant influence that it has on productive and reproductive animal performances.

IGF-I has been chosen, for its implication in several physiological processes, including regulation of nutritional status, fertility and fetal development. It has an important role also on mammary gland growth and function, milk yield and composition, and immune system.

All these aspects are crucial in dairy animals and the

improvement of the knowledge about all these systems regulation it will lead to giant steps forward in the livestock's breeding.

In this thesis IGF-I was considered as protein in dairy cows and as gene in dairy sheep.

The present research studied a possible association between plasma IGF-I concentration after calving and reproductive performances in Holstein Friesian cows.

The average levels of plasma IGF-I in primiparous and multiparous cows was calculated, from 0 to 27 days post partum. The results highlighted higher values of IGF-I in primiparous than in multiparous animals confirming that parity and age influence IGF-I concentration, in view of its metabolic role.

Moreover, increasing trend in the IGF-I concentration was found from calving until the 21st day, as expected. Whereas, an unexpected decrease of IGF-I was observed from 21 to 27 days after calving. Probably, this result was

due to the low number of the cows sampled between 21 to 27 days post-partum. Furthermore, these animals could have shown lower IGF-I for several reasons such as age, genetics and differences in negative energy balance.

After measurement, IGF-I concentrations were connected with reproductive performances, such as calving to conception interval, number of insemination needed to conception, and positive or negative pregnancy diagnosis. Anyway, differences between the IGF-I measures were negligible, and cows with high IGF-I (≥20ng/ml) did not show any significant difference compared to cows with low IGF-I (≤11ng/ml).

Considering that the post-partum is a critical period during which cows are more susceptible to diseases, with repercussion on reproductive activity, the different expression of some immune gene has been evaluated.

In view of the action that IGF-I has on immune system, its concentration was related with expression of these immune genes, but any important information was found, even if the expression of 2 genes was significantly different between groups of cows with diverse IGF-I concentrations. This finding explains that although IGF-I is considered to serve some regulatory function, there are other several factors that can influence immunity system.

The results obtained in this study, provide further information for an association between IGF-I concentration after calving, reproductive performances and immune gene expression. Anyway, despite the role that IGF-I has on somatotropic and HPG axis, and on immunity system, any interesting

connection was observed in this study.

Considering the *IGF-I* as a potential candidate gene for reproductive and productive traits, its polymorphisms were investigated in dairy sheep.

In Sarda sheep, two nucleotide variations in the 5'UTR region of the *IGF-I* gene, were found. These variations (G855C and G857A) are located in the core sequence putative binding sites of transcriptional factor.

A genotype/phenotype association was detected, leading to a significant higher milk yield in the animals that carried the G alleles that also resulted the higher in frequency, compared to those carrying the other minor alleles. Moreover, a visible effect, despite not statistically significant, of the G alleles on the shortening of the lambing to conception interval and, consequently, of the interlambing period was found. Anyway, it is possible to infer that the low frequency of the minor alleles could influence the results of the statistical analysis on the considered reproductive performances.

About the poor frequency of the minor alleles, it is also hypothesized that the strong genetic selection aimed to improve milk yield conducted on the Sarda breed could have marginalized the less productive genotypes, strongly decreasing their number in the entire population.

It would be interesting to conduct further analysis comprising a higher number of animals to confirm the real frequency and effect of the found mutations. These variations are supposed to modify the functional activity of the IGF-I, as they seem to fall in a DNA region influencing several production performances in different species. Therefore, interesting results could be

obtained by the study of milk quality traits, such as fat ant protein yield, or lactation persistency in order to confirm the strength of the *IGF-I* as candidate gene in milk performance.

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