SCUOLA DI DOTTORATO IN
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SCIENZA, TECNOLOGIA E BIOTECNOLOGIE

DOTTORATO DI RICERCA
IN PRODUZIONI ANIMALI

# MAPPING QTL IN THE BROWN SWISS DAIRY CATTLE BREED FOR MILK QUALITY TRAITS

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# Table of Contents

# INTRODUCTION

The animal genetic improvement in livestock production adapts the animal genotype according to production requirements: such adaptation allows farmers and animal production sector as a whole (operators, manufacturers, industry) to provide food having the quality characteristics required by consumers and do it efficiently. In the last decade dairy cattle selection was aimed at optimization of qualitative and quantitative aspects of milk production of milk which caused, with the fast increase of production, a growing number of health problems (Kadarmideen and Coll., 2000; Kelm and Coll. 2000).

The marker-assisted approach to selection has been already applied in several species of interest. In cattle, for example, the German national selection of Holstein Friesian integrated classic progeny tests (Bennewitz and Coll. 2003) with information about genes and genetic markers (identified through research programmes co-funded by industry and public sector) to known the effect on production (1 and 2 series of gene markers). This has allowed to increase the accuracy of selection of young bulls used for progeny tests by adding to the estimated

genetic value only based on the pedigrees index (1/2 genetic value of the father + 1/2 the genetic value of the mother), information on actually available genes so far assessed only after the progeny tests through the estimation of expected breeding values (EBV). Nowadays the genomic selection became a reality in mostly dairy cattle population after Meuwissen and Goddard theorized it (2001) and Schaeffer (2006) shows the economic importance in selection programs.

The impossibility to collect phenotypic data on the entire population, fundamental basis for the statistical approach to genetic improvement, and the high cost of this procedure, made the genomic approach to selection desirable as it identifies genes responsible for the expression of traits of interest and then uses such of knowledge for the choice of improver individuals.

Customers are actually very interested on product quality, food safety, animal welfare and environmental impact of livestock herds and often ask for quality-enhanced products. Customers needs can be met only if all stakeholders of the milk production chain (operators involved in genetic improvement, animal breeders, milk processors,

industry and market) act in a coordinated and concordant way.

Selection for traits having a direct impact on the nutritional characteristics of the product and on animal health leads to a collaboration between farmers and processing industry: the ability to merge the different components of the milk production chain around common objectives allows to reduce the apparent gap between what is desirable by society and what is really feasible by farmers and industry.

**Mastitis** is a widespread disease, (Andreatta et al., 2009) common in the international realities of the herds of dairy cows (Feßler et al. 2010). It represents a major problem both in economic and health terms, as it causes serious losses in both fields: costs of drug treatment, possible residues in animal products, need to discard contaminated milk (both for the presence of pathogens and drugs), decreased of milk production, less efficient in the dairy products processing, etc. Many different approaches have been evaluated to reduce the incidence of mastitis in the herd, taking into account all possible different aspects involved in the development of this infection: etiology, pathogenesis, epidemiology,

individual susceptibility, hereditary, and so on. All the milk production chain, from Artificial Insemination centers, through farmers and finally to milk processing industry is interested and is involved in the achievement of the common goal of reducing the incidence of mastitis.

# MASTITIS

Mastitis is a mammary gland inflammatory process that can affect one or more quarters. The disease is the result of physiological immune response to a traumatic insult or an infection: the affected quarter activates its immune system to locally neutralize the pathogens and prepare the tissues for recovery (Vincenzoni et al. 1996).
Mastitis is the most common and expensive disease in dairy cattle throughout most of the world.
This process causes changes both in the mammary gland and in the produced milk, with different effects depending on the nature of mastitis.
The great variety of mastitis manifestations is mainly due to the interaction of three important factors: the animal, the environment, and the characteristics of the pathogens involved in the infection.

ANIMAL. Each individual can differentially be susceptible to mastitis, depending on its own genetic, physiological and anatomical characteristics. Among those:

- Age: several authors reported an increase in mastitis frequency with age (e.g. Schultz 1977, Dohoo et al. 1982, Benedixen et al. 1988). Even a more recent study (Abdel-Rady and Sayed

2009) confirm this findings, describing higher susceptibility to mastitis in 5-8 years old cow than those of 2-4 years;

- Breed: it has been reported that breeds originating in eastern France (Montbeliard and Abondance breeds) or central Europe (Simmental, Brown Swiss) have lower somatic cell count and low frequency of clinical mastitis than the Holstein breed (Rupp and Boichard 2003);
- Immune system: for different reasons such as stress or systemic diseases, the immune response can be inadequate to face the infection (Carroll and Forsberg, 2007);
- Lactation stage: it has been reported that clinical mastitis are more frequent during the first 90 days after calving (Zecconi and Ruffo, 1993);
- Teat conformation and integrity: the diffusion of milking machine able to recover a huge amount of milk from the mammary gland in the short time after the oxytocine shock (5 minutes), and the increasing need to reduce teat trauma, have lead to the selection of cows having large and short and teats. Such teats are less fit to resist against bacteria infection especially after the milking, when teat sphincters are dilated (Ballarini, 2004).

ENVIROMENT. Different aspects of the environmental factor can be considered as influencing the mastitis incidence.

- Cattle shed: proper conditions of light, humidity, temperature and air circulation are important to reduce the spread of infection by lowering the bacteria in the environment (Zecconi and Ruffo 1993); consequently also the season can influence the hygienic condition of the cattle shed especially considering the different sensibility of the bacteria to different temperatures.
- Feed supply: feed contaminated with undesirable substances can lead to mammary gland inflammation. Some kind of mycotoxins, for example, can facilitate the teat sphincter release, consequently promoting the penetration of microorganisms in the mammary gland (Ballarini, 2004). The lack of microelements and vitamins in the diet can cause a lowering of immune defenses. For example, deficiencies of selenium and vitamin E in the diet have been associated with an increased rate of new infection (Wattiaux, 2009). In fact, has been showed that low selenium and vitamin E are related to a depression of glutathione peroxidase

activity and a delay in leukocyte chemotaxis: both this conditions predispose to mastitis and lengthen the persistence of clinical symptoms (Zecconi and Ruffo 1993). Nevertheless, the integration with specific elements can help reducing the sensitivity to mastitis: zinc, for example, plays a key role in the stimulation of the immune response and in the production of keratin, lost from teat canal during each milking). For this reason, if correctly added in the diet, it can help to improve the defense barrier against the entrance of bacteria at the end of the milking operations (Dell'Orto and Savoini, 2005).

- Litter conditions: where the litter is used, its frequent replacement is one of the best management practices to avoid bacteria reproduction and spread. (Ballarini, 2004). Furthermore the choice of the litter can be an important if considering that some substances, such as the silica content in rice litter can be irritating for the udder.
- Milking machine: the opening and closing movements of the teat cup around the teat during the milking facilitate the penetration of eventually contaminated milk drops and debris in the teat itself. This phenomenon is

proportional to the entrance of the air in the milking unit that can be too energetic. To reduce the mastitis incidence, it is important that milking vacuum fluctuations and teat cup shape and integrity, are periodically monitored. During milking operation, infected cows should be kept separated from the others and should be milked as last.

BACTERIA: Although stress and physical injuries may cause inflammation of the mammary gland, infection by invading bacteria or other microorganisms (fungi, yeasts and possibly viruses) is the primary cause of mastitis. The main microorganisms causing mastitis in dairy cattle can be classify as follow:

- Contagious microorganisms: they usually cause subclinical mastitis. The infection can be transmitted to health cows via milking machine or operator hands or to health quarter(s) via infected teat(s) of the same udder. *Streptococcus Agalactiae* and *Staphylococcus Aureus* belong to this group.
- Environmental microorganisms: they usually cause acute clinical mastitis of short duration. The infection transmission occurs during the interval between the milking or during the

drying period because of the teat contact with the litter. In fact, in this group are included microorganisms commonly present in the ground, in the forage and in the litter such as *Sterptococcus Uberis*, *Streptococcus Dysagalactiae*, *Streptoccus Bovis*, *Streptococcus Faecalis* or microorganism of fecal origin such as *Escherichia Coli* and *Entorobacter spp*.

- Opportunist microorganisms: they are normally present on the udder and teat surface and are able to cause infection when the animal immune system is compromised. *Staphylococcus Hyicus*, *Staphylococcus epidermidis* and *Staphylococcus haemoliticus* belong to this group.

## Development of the disease

Infections begin when microorganisms penetrate the teat canal and multiply in the mammary gland.
The teat itself is the first line of defense against the penetration of bacteria into the udder. Normally, the sphincter muscle closes the teat canal tightly when the cow is not being milked (Wattiaux, 2009)
Invasion of the teat most often occurs during milking. Organisms present in the milk or at the teat end are propelled into the teat canal and cistern when there is admission of undesired air in the milking unit (slipping or squawking of the unit or

removal of teatcup without first shutting off the vacuum). After milking, the teat canal remains dilated for one to two hours; however, the canal of a damaged teat may remain partially open permanently. Organisms from the environment (manure, bedding, etc.) or those found on injured skin at the tip of the teat may easily invade an open or partially open canal.

Some bacteria may proceed into the udder by attaching and colonizing new tissue; others may move around via milk current produced by the cow's movement. Bacteria first damage the tissues lining the large milk-collecting ducts. The bacteria may encounter leukocytes (white blood cells) present naturally in small numbers in the milk. These cells are the cow's second line of defense because they can engulf and destroy bacteria. However, during this process, the leukocytes release substances that cause the movement of additional leukocytes from the blood into the milk. If bacteria are not entirely destroyed, they continue to multiply and begin to invade smaller ducts and alveolar areas (Figure 1A). Milk-secreting cells damaged by toxins and other irritants release substances that lead to increased permeability of blood vessels (Figure 1B). Additional leukocytes move to the site of infection. They enter the alveolar tissue in great numbers by

squeezing between the damaged milk secreting cells (Figure 1C). Fluids, minerals and clotting factors also leak into the affected area. Clotted milk may close ducts, and in effect, isolate the infected regions. Sometimes the microorganisms are eliminated rapidly and the infection is cleared. In this case, the clogged ducts are opened and milk composition and production return to normal in several days. However, as the infection persists and ducts remain clogged, the entrapped milk causes the secretory cells to revert to a resting (non-producing) state and the alveoli begin to shrink (Figure 1D) Substances released by leukocytes lead to the complete destruction of alveolar structures, which are replaced by connective and scar tissues (Figure 1E and F). The destruction of milk secretory tissue is, in effect, the cow's third line of defense to bring the infection under control. Thus as the disease progresses the number of somatic cells in the milk becomes elevated and associated with a (permanent) reduction in milk yield.

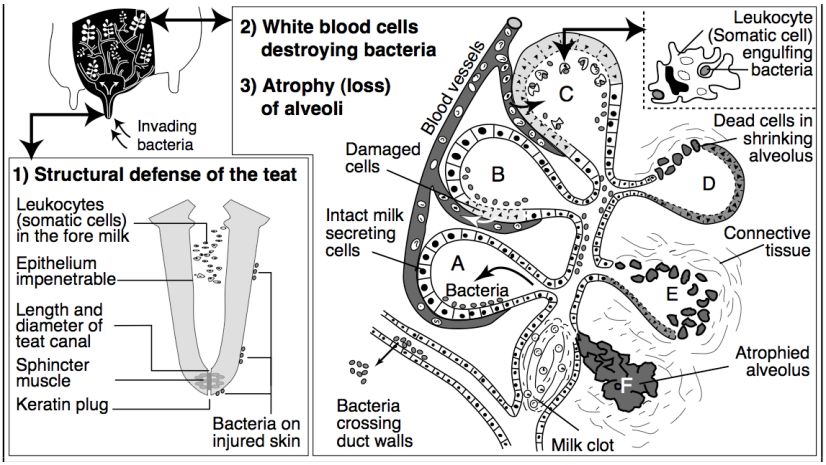


Figure 1:development of mastitis and cow's defense against infection (from Wattiaux, 2009)

## Mastitis classification

Mastitis can be classified both on the basis of etiology and clinical characteristics.

Classification based on clinical features includes:

1. ***Subclinical mastitis***. Subclinical mastitis is subtle and difficult to detect. The cow appears healthy, the udder does not show any signs of inflammation and the milk seems normal (Wattiaux, 2009). However, microorganisms and white blood cells (somatic cells) that fight infections are found in elevated numbers in the milk.

   A lot more milk is lost due to subclinical mastitis because the reduction in milk

production due to subclinical mastitis tends to persist for long periods of times and thus undermines the yield of infected cows. Nevertheless the vast majority of mastitis cases are subclinical: it has been reported that, on the average, for every clinical case, there are 20 to 40 subclinical cases (Wattiaux, 2009). For this reason the control of subclinical mastitis is more important than simply treating clinical cases because the cows that have subclinical mastitis are reservoirs of organisms that lead to infection of other cows and because most clinical cases start as subclinical; thus, controlling subclinical mastitis is the best way to reduce the clinical cases.

2. *Clinical mastitis*. In clinical mastitis the infected quarter often become swollen, sometimes painful to touch, and the milk is visibly altered by the presence of clots, flakes, or discolored serum and sometimes blood. In severe cases (acute mastitis), the cow shows signs of generalized reaction: fever, rapid pulse, loss of appetite and sharp decline in milk production.

   The loss of milk and income due to clinical mastitis are readily apparent-milk production drops sharply and milk from cows treated with

antibiotics must be discarded for three or four days.

3. *Chronic mastitis*. Chronic mastitis is characterized by persistent symptoms or symptoms coming from the alternatively occurrence of subclinical and clinical mastitis.

## Mastitis treatment

The mastitis treatment should be preferably carried out during the drying period: dry cow therapy, in fact, is the best way to cure chronic and subclinical mastitis that can rarely be treated successfully during lactation. The effective use of a long-term antibiotic infused in each quarter of the udder at the last milking of lactation reduces the incidence of new infections during the dry period.

The different classes of mastitis before mentioned require different treatment approach due to the diverse characteristics of the infection and related symptoms and effects.

1. *Subclinical mastitis.* High somatic cell counts in the milk indicate subclinical mastitis, but this should not be used as a criterion to treat cows with antibiotics because the rate of cure is generally very low. Cases of subclinical mastitis are better treated at the time of drying-off as antibiotic treatment during lactation is

ineffective for the most part. Conversely, treatment at drying off is the most effective way to cure existing subclinical mastitis. Intramammary infusion of slow-release antibiotics at the time of drying off (dry cow treatment) is an essential component of a mastitis control program on the farm. Dry cow therapy helps cure about 50% of the mastitis caused by *Staphylococcus Aureus* and 80% of environmental streptococci (*Streptococcus Uberis* and *Streptococcus Dysgalactiae*, etc.). One infected quarter treated and cured at drying off will produce about 90% of its potential during the next lactation. However, if a quarter remains infected or becomes infected during the dry period, that quarter will produce only 60 to 70% of its potential (Wattiaux, 2009).

2. ***Clinical mastitis***. Clinical mastitis should be treated timely and proper with an adequate therapy decided by a veterinarian and the affected cows handled accordingly to avoid the risk of spreading the disease. Prompt treatment of clinical mastitis limits the duration and possible spread of the disease. A veterinarian familiar with the history of the disease in the herd should prescribe the best therapeutic treatment.

When antibiotic treatment is recommended, it is critical to follow instructions, especially regarding the duration of treatment. Often treatments are discontinued too soon, preventing the antibiotics from reaching and destroying organisms in parts of the udder that are difficult to reach (the "deep-seated" infections) Only mastitis caused by *Streptococcus Agalactiae* can be treated successfully with antibiotics during lactation (more than 90% cure). However, when mastitis is caused by *Staphylococcus Aureus*, bacterial *coliforms* and many other organisms, the success rate of antibiotic treatment rarely exceeds 40 to 50% and sometimes is as low as 105 (Wattiaux, 2009). Acute mastitis such as that caused by *Coliform* bacteria endangers the cow's life. A veterinarian should be called immediately when the cow shows signs of generalized reaction to an udder infection (inability to stand, rapid pulse, fever, etc.). Milking the affected quarter every two to three hours helps to eliminate toxins.

3. ***Chronic mastitis.*** In case of chronically infected cows culling is suggested. Generally, this method is effective because

in most herds, only 6 to 8% of all cows account for 40 to 50% of all clinical mastitis.

## Mastitis control

To control mastitis problems in a herd, prevention of new infection is of much greater benefit than attempting to cure clinical cases. Even if the rate of new infection is reduced, existing infections that are treated can be cured with only limited success. The fight against mastitis is a long-term effort that must be persistent because it is impossible to completely prevent the transmission of bacteria or other organisms that cause the disease (Wattiaux, 2009). The prevention of mastitis can be achieved by following simple rules to be applied at different levels in the herd management and aimed at reducing the rate and duration of infection.

Milking machine should function and be operated properly: vacuum level in the milking unit should be between 275 and 300 mm of mercury and should fluctuate as little as possible. Fluctuations may be reduced considerably by avoiding squawking or slipping of the milking unit during milking, and shutting off the vacuum to the unit before teatcups are removed. The vacuum regulator should be kept clean and checked regularly for accuracy. Also, the

observation of the teats after milking can help disclosing defects in the vacuum system.
If milk is filtered, the presence of particles (soiled material) in the filter indicates insufficient cleaning of the teat during udder preparation or a lack of hygiene during attachment and removal of the milking unit.
Cleaning of the teats before milking and their disinfection after milking are key elements in preventing the mastitis. Teats should be cleaned and dried before milking using pre-dipping solutions and dried paper cloths; alternatively, cloth previously dipped in the washing solution could be used. It is important to take care of the use of one cloth for each animal to avoid contamination.
Researches (Bray and Shearer, 2009) indicates that the rate of new infection may be decreased by more than 50% when a suitable disinfectant is used to fully immerse or spray the teats after milking. Post-milking teat dipping is most effective against *Staphylococcus Aureus* and *Streptococcus Agalactiae*, the two most contagious mastitis-causing bacteria. Teat dipping does not affect existing infections. This explains why, in the short term, many farmers do not see the positive effects of teat dipping. Infected cows should be milked at the end of the milking operation, but to achieve a rapid decline in the level

of infection, it would be necessary to eliminate infected cows in the herd.
As already stated before, dry cow therapy should be performed to prevent mastitis. Advantages of this procedure are also the regeneration of damaged tissue before the start the following lactation and the decreased somatic cell count in milk. The treatment is carried out administering the drug via teat canal so that antibiotics can remain on site and the concentration of the drug can remain high for long periods without having an impact on milk production (Vincenzoni et al. 1996; Zecconi and Ruffo 1994, Intervet 2004). According to Srieys (2006) a systematic approach could be replaced with a targeted treatment adapted to different situations and after the identification of the epidemiological model prevailing in different farms; this solution would lead to a more conscious use of specific drugs and antibiotics and to a more accurate knowledge of the condition of the herd. Unfortunately, the complexity of this process leads to prefer a more traditional systematic approach.
Other simple management practices can help reducing the spread of mastitis as for example the use of comfortable and clean litter, for which a daily substitution is scheduled; the availability for each animal of sufficient room to lie down and stand up

(indicatively 8-10$m^2$ per animal); the availability and easily to access water (Labbè, 2004); a good nutrition to maintain the cow's ability to fight infections, the feeding immediately after milking so that they remain standing for at least one hour before they lie down (Wattiaux, 2009).

## Mastitis detection

Several approaches for mastitis detection and diagnosis have been proposed and compared (Viguier et al. 2009). Here below a list of the main approaches used for mastitis detection and their characteristics.

*Evaluation of the individual.* The evaluation of the general condition of the animal and the specific observation of the udder, allows an initial orientation of the diagnosis and permits to highlight possible systemic symptoms; the udder should be examined quarter by quarter after the milking (when it is empty) to be able to appreciate possible alterations such as edema, increase of temperature, malformations or atrophies. The accurate examination of the teats is also important to identify local alteration that can heavily compromise the overall udder condition. Signs of acute mastitis

include quarters that are swollen, warm and painful to the touch.

*Evaluation of first streams of milk.* This evaluation permits to detect abnormal milk that should be withheld. Abnormal milk may show discoloration (wateriness), flakes, or clots. Caution should be exercised during the removal of foremilk to avoid splashing of contaminated milk on the cow's limbs, tail, or udder. In addition, the operator should not collect the foremilk in the palm of the hand because of the risk of transferring bacteria from one quarter to another and from one cow to the other. In a stanchion barn, foremilk is typically drawn into a "strip cup" or plate. In a milking parlor, however, it may be drawn directly onto the floor and flushed away immediately after observation.
It is important to note that this evaluation is able to find out signs of possible clinical mastitis but not of subclinical mastitis, for which a laboratory test is needed.

*Californian Mastitis Test (CMT).* The Californian Mastitis test is a rapid test able to detect the presence of infection in each of the four quarter. To perform this test, milk of each quarter is mixed with an equal amount of a detergent solution, up to a

total of 4ml (2ml of milk and 2ml of detergent solution), and incubated at room temperature for 15-30 seconds. Milk of infected quarter(s) forms a gel and its consistency is visually evaluated. This reaction is related broadly to the number of somatic cells in the milk (specifically the neutrophils), and a positive reaction indicates mastitis. For a reliable result it is important to sample the milk during the milking, excluding the first streams. The CMT is used to: (i) to diagnose subclinical mastitis; (ii) to discriminate between infected and not infected quarters; (iii) to evaluate the pH of milk as the detergent reagent of CMT contains a purple pH indicator which become light violet if in contact with milk from healthy quarter (pH value between 6.5 and 6.7), dark violet if the milk comes from a quarter with acute infection (more alkaline pH) or greenish-yellow in case of colostrum (more acidic pH); (iv) to select individuals to be treat during the drying period; (v) to assess the general udder health; (vi) to assess the effectiveness of the prevention program implemented in the herd; (vii) to assess the impact of recent changes in management.
The accuracy of the CMT is founded on three principles:

1. leukocyte (white blood cells) numbers greatly increase when an injury or infection affects mammary tissue;
2. leukocytes, especially the polymorphonuclear ones, have large nuclei (DNA) compared to other cells or bacteria in milk;
3. leukocyte cell walls are mainly lipid (fat).

The CMT reagent dissolves or disrupts the outer cell wall and the nuclear cell wall of any leukocyte, which are primarily fat (detergent dissolves fat). DNA is now released from the nuclei and together form a stringy mass. As the number of leukocytes increase in a quarter, the amount of gel formation will increase linearly.
The CMT provides only an indication of somatic cell count, not an exact value. Furthermore, the results per quarter may not reflect the result from a composite sample taken for milk recording purpose.

*Evaluation of milk electrical conductivity.* Electrical conductivity measures the ability of a solution to conduct an electric current between two electrodes, and it is measured in milliSiemens (mS). When a cow is exposed to an intramammary infection, the electrical conductivity of the milk increases due to an increased concentration of Na+ and Cl caused by destruction of tight junctions and the active ion-

pumping system (Norberg, 2005). It has been reported that values of electrical conductivity for healthy, subclinically infected and clinically infected cows are 5.3mS, 5.75mS and 6.73mS respectively, but differences from these values have been reported for different lactations, parities and seasons (Norberg, 2005). For this reason the usefulness of this method to diagnose early mastitis is controversial because of the numerous factors that influence the milk conductivity.
Nevertheless many automatic-milking systems measures electrical conductivity on combined milk from all four quarters, consequently, automatic identification of the infected quarter is impossible.
Additionally, extremely different values of sensitivity and specificity for this test are reported (Ferouillet et al. 2006).

*Bacteriological test*. The bacteriological test enables the specific identification of microorganisms that cause mastitis. It is performed in subsequent steps including a first test consisting in the use of a general media (agar) used to grow bacteria, followed by more specific steps performed to reach a more detailed classification of the mastitis causative microorganismsin. In these steps differential or selective media are used to exclude some types of

bacteria or to reveals bacteria having specific metabolic capabilities. . The MacConkey's Agar, for example, grows only Gram-negative bacteria and contains a differential pH indicator that changes the color of bacteria that can ferment the sugar lactose. The mannitol salt medium, containing high sodium chloride (Nacl), is used to grow *Staphylococcus Aureus.*
Milk sampling for bacteriological test should be made avoiding possibilities of contamination. Sampling can be made for each separated quarter using different color tubes, or for the udder together. This last is less expensive but does not distinguish the infected quarter(s) and causes a dilution of the milk coming from infected quarter(s) with the use of milk from the healthy one(s). Sampling from each quarter is the most common procedure used for bacteriological test: even if it is quite expensive both in terms of time consuming and materials costs, it is the most reliable way to decide on the optimum antibiotic treatment for a particular cow. For this reason usually, the bacteriological test is performed on selected cows for which somatic cell counts of composite samples reveal a serious and persistent problem.
Cultures of bacteria in the milk may be useful to quantify bacteria and identify the organisms causing mastitis and high somatic cell counts. If bacterial

counts are elevated (>50,000 bacteria/ml), a culture may provide clues to the source(s) of contamination. Well-managed herds have bacterial counts less than 1,000 per ml.

## Somatic Cell Count (SCC)

The Somatic Cell Count (SCC) is evaluated applying a standard method and using specific instruments. Currently the Fossomatic™ instrument is the mainly used as it performs rapid reads with a low margin of error. The Fossomatic™ instruments count somatic cells by applying flowcytometry to recognise DNA from the cells. A mixture of milk and staining solution is surrounded by a sheath liquid and passed through a flow cell. In the flow cell, the stained somatic cells are exposed to light of a specific wavelength. The cells then emit fluorescent light pulses at a different wavelength; the pulses are counted and displayed. The design of the flow cell ensures that only one somatic cell is detected at a time. The evaluation of SCC can be performed both on bulk milk and on milk from each quarter and the SCC values are routinely registered by milk recording agencies.

Milk samples having a SCC lower than 100,000 cells/ml are considered to belong to healthy animals. It is important to consider that different

factors can contribute to SCC variations as for examples bovine age and breed, stage of lactation, season and various stresses. It has been reported (Harmon, 1994) that generally SCC increases with advancing age and stage of lactation. Additionally SCC is generally highest during the summer and lowest during winter (Harmon, 1994). Other studies (Rupp and Boichard, 2003) reported that breeds originating in eastern France (Montbeliarde and Abondance breeds) or central Europe (Simmental, Brown Swiss) have lower somatic cell count and low frequency of clinical mastitis than the Holstein breed.

Anyhow, the elevation of SCC is primarily a response to an insult to the mammary gland, and is modulated by inflammatory mediators. For this reason the major factor influencing SCC is infection status and the effects of all other factors are minor if the gland is uninfected (Harmon, 1994). Then SCC is definitely an effective way to monitor mastitis. SCC can be evaluated both for each quarter (see also CMT) and for bulk milk. When the milk of all cows in a herd is mixed, as in a bulk tank, the somatic cell count in a composite sample is a good indicator of the prevalence of mastitis in the herd alternatively it gives important information on single animal udder health status (Wattiaux, 2009).

Mastitis of environmental origin is often characterized by a considerable increase of SCC with a decrease to physiological values after some checking. Conversely, contagious mastitis has a constant rise in value over 300,000 cells/ml, value considered as a cut off also to suspect subclinical mastitis. A somatic cell count greater than 200,000 cells/ml indicates the presence of subclinical mastitis. Somatic cell counts under 400,000 cells/ml are typical of herds that have good management practices, but no particular emphasis on mastitis control. Herds with an effective mastitis control program consistently have counts below 100,000 cells/ml. In contrast, somatic cell counts greater than 500,000 cells/ml indicates that one third of the mammary glands are infected and the loss of milk due to subclinical mastitis is at the least 10%.

Mastitis causes an increase of the somatic cells normally present in milk and in particular of specific cell populations such as polymorphonuclear leukocytes, granulocytes, monocytes, macrophages, lymphocytes and mammary epithelial cells. A more negligible increase can be also observed for eosinophilic and basophilic leukocytes.

In a healthy mammary gland (i.e. SCC/ml < 100,000) macrophages and lymphocytes, neutrophils and epithelial cells are normally present. When an

inflammatory process occurs (i.e. SCC/ml >1,000,000) more than 98% of the somatic cells found in the milk come from the white blood cells (95% are neutrophils) that entered the milk in response to bacterial invasion of the udder (Wattiaux, 2009).

| Cells | % in healthy sample | % in mastitis sample |
|---|---|---|
| Epithelial cells | 20% | 0 - 7% |
| Lymphocytes | 5-10% | <10% |
| Macrophages | 50-60% | <10% |
| Leukocytes | 10-20% | >70% |

Table 1: proportion of different cells in healthy and mastitis milk samples

Somatic cell count is also used as a quality parameter for milk evaluation. High quality fresh milk must have a somatic cells count not exceeding 300,000 cells/ml. Nevertheless breeders selling to dairy industry milk with somatic cell count lower than 150,000 cells/ml, receive a premium. Therefore, many milk processors now offer premium payment programs to improve raw milk quality. These premium payment programs emphasize lowering the SCC of the milk. Also, other criteria in terms of

maximum SCC value, can be defined at national and international level for other dairy products: in Italy, for example, the DECREE 54/97 established that the maximum somatic cell value for heat treated milk must be 400,000cell/ml. Also in Europe, the 853/2004 CE Regulation reports that the rolling geometric average of raw milk somatic cell count, calculated over a period of three months, with at least one sampling per month, must not exceed the value limit of 400,000 cells/ml.

All these preliminary considerations lead to the understanding of impact and costs of mastitis for the entire milk-processing compound.

## Mastitis impact and costs

Mastitis is one of the most frequent pathologies in dairy cattle farms with a European average incidence of 25-40 cases every 100 cattle per year (Rupp and Boichard 2003, Gemmi, 2003). In many countries the frequency of clinical mastitis has increased over time (Rupp and Boichard 2003) having costs associated with cases of clinical mastitis reaching up to 250 Euro per cow per year (Wolfowa et al. 2006, Huijps et al. 2008, Viguier, 2009). Mastitis not only represents a serious public health problem because of its diffusion in the herds and the possible effects

of antibiotics residues present in the milk coming from treated cows, but is also a serious financial issue because of the losses along the overall milk production chain.

## Mastitis impact

In recent years, dairy product manufacturers have become very concerned about the impact of raw milk quality on finished dairy product quality. Milk quality has become a major concern because more time now elapses between milk production and dairy product consumption. At the same time, new milk-testing technology has made it more cost effective and easier to test milk for bacteria count, antibiotics, and somatic cell count (SCC) (Wattiaux, 2009).

Mastitis causes milk SCC to increase. During mastitis, the types of somatic cells present in the milk change to mostly white blood cells, which add many proteolytic and lipoytic enzymes to milk. Dairy product quality defects resulting from mastitis are due to enzymatic breakdown of milk protein and fat.

An enzyme called plasmin cause protein breakdown in milk produced by cows with clinical or subclinical mastitis. Plasmin is found commonly both in milk and in blood plasma and can cause extensive

damage to milk casein in the udder prior to milking. When milk is cooled, plasmin breaks down casein (the main milk protein) much more slowly. Unfortunately, plasmin is extremely heat stable. Therefore, pasteurization cannot inactivate it and plasmin will continue to damage milk protein during dairy product manufacture and storage.
As milk SCC increases gradually, other quality characteristics of dairy products will also change gradually.
In fluid milk, the rate of off-flavor development will increase. Rancid off-flavors due to increased lipase activity, bitter flavors due to proteolytic enzyme activity, and salty flavors due to a change in milk mineral balance will all gradually appear as SCC increases. In UHT shelf-stable milk, plasmin may cause milk to change from a liquid to a gel. Milk fat breakdown, on the other hand, tends to have an immediate impact on milk and dairy product flavor. Enzymes called lipases break down milk fat and release free fatty acids that produce off-flavors that are detectable at very low concentrations, especially in high fat products or dairy products with very mild flavors, such as butter or cream cheese.
The impact of the presence of casein breakdown products on flavor and functional characteristics of whey products that are used extensively as

ingredients in formulated foods is not known. Protein breakdown could also cause the body of other cultured products such as yogurt to be weak and result in undesirable separation of the yogurt into curd and whey in the package. The functional characteristics (foam stability, gel strength, heat stability, etc.) of the milk proteins in condensed and dried milk products can change as a result of protein breakdown and decrease the value of these products. Condensed and dried milk products and byproducts are used extensively as ingredients in other foods. Changes in protein functionality or the increased levels of heat-resistant protease (plasmin) may cause problems for food processors that use dried milk products as ingredients.

As already stated here before, plasmin damages milk casein by breaking the orginal large protein chains into smaller fragments. As a result, the milk casein does not curdle properly during cheesemaking, and some small casein fragments and an increased amount of milk fat are lost into the cheese whey (Rogers and Mitchell 1994). This causes low cheese yield. Cheeses made from high SCC milk (800,000 to 1,000,000 cells per ml) also have a higher incidence of unclean flavors and pasty textures (Wattiaux, 2009). It is very difficult to determine exactly how much a change in SCC will cause cheese

yield to change. There has been no formula a cheesemaker can use to predict decreasing cheese yield as the SCC increases in 100,000 cells per ml increments. This would be of importance especially for Countries, as for example Italy, for which the grater percentage (68%) of the total produced milk is used for to make cheese or other dairy products (Cassandro, 2003).

Many different studies have been conducted about the effect of milk SCC on cheese quality (e.g. Andreatta et al. 2009, Franceschi et al. 2009, Vianna et al. 2008, Mazal et al. 2007) but only few were aimed to determine the quantitative relationship between increasing milk SCC and cheese yield. Results from one of these studies (Barbano et al. 1991) reported that cheese yield efficiency was lower when milk SCC was high. The authors also reported that for milk coming from individual cows or small groups of cows all producing milk at the same SCC, cheese yield does not decrease linearly with increasing SCC. At a SCC of about 100,000 cells per ml, there is a sudden 1% decrease in cheese yield. As SCC increases from 100,000 to 1,300,000, cheese yield decreases by an additional 1-2% depending on time and temperature. Since milk handling and cheese making conditions used in this study were optimum, the observed changes in cheese yield are

probably conservative. It was concluded from this study that any increase in milk SCC above 100,000 cells/ml would have a negative impact on cheese yield efficiency for milk from individual cows.
Same results have been obtained in another study performed by Klei et al. (1998).
The change in cheese yield efficiency for commingled milk for a full herd would not show this same nonlinear trend. Herd milk will represent a weighted average of the milk characteristics from individual cows. If milk cooling and handling conditions after milking are very good, then the primary differences in cheese yield from bulk milks with different SCC will result from the damage to milk casein that occurred in the udder prior to milking. Thus, the cheese yield performance of the bulk tank milk from an individual farm will reflect the weighted average cheese yield performance of the milks of the individual cows in the herd plus the separate impact of the age of the milk at the time of cheese making. This will produce a linear decrease in cheese yield for herd milk with increasing milk SCC. Any negative impact of high psychrotrophic bacteria counts on cheese yield would be additional (National Mastitis Council web site http://www.nmconline.org/).

The impact of mastitis goes with the milk beyond the gate of the farm. Changes in milk composition (reduction in calcium, phosphorus, protein and fat, and increases in sodium and chlorine) reduce its quality. In addition, the antibiotic used in treating mastitis is an important industrial and public health concern. The presence of antibiotic residue in the milk interferes with the manufacturing process of many dairy products (cheese and other fermented products). Undesirable flavors reduce the value of dairy products and the presence of low levels of antibiotics may cause health problems to consumers (Wattiaux, 2009).
From a practical farm management viewpoint, prevention of mastitis is the key to high milk quality and high productivity per cow, especially because of the high costs related to mastitis cases.

## Mastitis costs

The extent of economic damage caused by this disease is mainly related to less production that represents the 70% of the total losses related to mastitis (Bailey t. 1996). Performing an accurate estimate of these losses is difficult given the complexity of the disease and the high number of sub-clinical mastitis whose symptoms are often evidence undetectable and difficult to measure

(Gemmi et al. 2003). Many different approaches have been proposed to estimate at best the cost related to mastitis, taking into account all the possible variables (e.g. Seegers et al. 2003, Wolfowa et al. 2006, Huijps et al. 2008, Sadeghi-Sefidmazgi et al. 2010). It is also important to note that only a small proportion (8%) of farmers estimate the economic losses of mastitis correctly: 20% of them overestimates these losses and 72% underestimated them (Huijps et al 2008).

New generation computer programs can offer opportunities to make more reliable estimates on the cost of this disease (Yalcin and Stott, 2000).

Factors influencing the cost of mastitis are numerous and can be grouped in direct costs and indirect costs as suggested by Gemmi et al. (2003).

Direct costs are related, for example, to the reduction of production, to the cost of milk dropped because altered by pathology or containing residues of antibiotics (during mastitis treatment), to the costs of medicines, veterinary and manpower for handling infected animals. Indirect costs are related to increased culling or death of animals and to the reduction in cheese yield.

Different causes of losses related to mastitis occur with different frequency and can affect the overall farm business in different way (Bailey. 1996): 70%

of the losses are related to lower milk production, 2% are related to cases of cows death and of early culling, 8% derive from the waste of milk during antibiotic treatments, 8% are related to the cost of medicines and veterinary.

**Direct costs**

As already stated before, the inflammatory process damaging the assessment of mammary gland, causes the reduction in milk production: it has been estimated that the reduction in milk secretion may vary from 2.4 liters/day, for mastitis caused by *Staphilococcus sp.,* to 4.6 liters/day for mastitis caused by *Streptococcus Agalatiae* (Zecconi and Ruffo, 1993).
Milk is discarded because of qualitative alterations both caused by mastitis itself and the presence of residues of pharmacological substances used for mastitis therapy (it is good to follow the guidelines of the manufacturers for the time of suspension).
In addition costs of veterinary intervention, costs of medications necessary for the resolution of mastitis and labor costs for the management of cattle who need therapy have to be considered among direct costs related to mastitis.

**Indirect costs**

Among the indirect cost related to mastitis, are the increasing of culling rate and the management of culled animals: considering the suspension time of medications, the moment to send animals to the slaughterhouse can be delayed, prolonging their stay in the farm, which leads to increased management costs without any gain. Also, cases of acute mastitis can lead to animal death, which causes in addition to financial losses related to the animals, additional costs for the carcasses removal.

The increased content of somatic cells in milk is one of the more important causes of indirect cost of mastitis. High SCC is directly linked to the decrease of milk production: if, for example, cells reach 200,000/ml the loss of milk can be equal to 1.8 q per lactation, or even 9 q for lactation with SCC 1,600,000 cells/ml (Daprà, 2006). Furthermore, SCC is used as milk quality parameter and to determine the milk payment to producer: high quality milk must have a content in somatic cells does not exceeding 300,000 cells/ml and premium is given for milk having less than 150 000 cells/ml.

Milk cellular and chemical composition changes lead to problems during milk processing, causing further indirect cost. Examples of these changes are:

- decrease in lactose from 5 to 20% due to damaged cells of the mammary gland secreting tissue;
- decrease in fat and caseins content for the same reason;
- alteration in coagulation time (Malacarne et al. 2006);
- high level of plasminogen, due increased transition from blood vessels through damaged secreting cells, with alteration of the curd;
- increased serum albumin and immunoglobulins (for the same reason of plasiminogen) which results in reduction of milk stability to heat treatment with difficulties during rocessing.
- Increased free fatty acids and lipase with higher possibility of rancidity of milk products. (Daprà, 2006).

# DNA STRUCTURE

DNA has a double-stranded DNA conformation (helix) whose backbone is made of sugar molecules of five carbon atoms (ribose) joined by bridges phosphoric esters. Each sugar molecule carries a nitrogenous base that can be a purine (adenine and guanine) or a pyrimidine (cytosine and adenine). Purines and pyrimidines are able to form hydrogen bonds between them and bind reversibly: complementarity between bases allows association of two strands of DNA to form a sort of "ladder" twisted spiral.

Possible pairs of bases are adenine-thymine and cytosine-guanine and then a date arrangement of bases along a strand of DNA must match on the opposite strand (anti-parallel) of the double helix. The determination of the double helix structure helps to explaining how the genetic information is encoded by the disposal of the nitrogenous bases along the DNA strand (nucleotide sequence) during meiosis and faithfully transmitted from one cell to the offspring, or how this information is translated into proteins.

Genomic DNA is organized into chromosomes that are present in different number in each species: in cattle, for example, 29 autosomes plus the two

sexual chromosomes are present. All chromosomes together constitute the specific individual karyotype. Chromosomes are chromatin complex structures whose shape changes during the meiosis. When meiosis is completed, the chromosomes have their definitive shape. In all "mature" chromosomes we can distinguish a centromere and 2 or more telomeres. Centromers and telomeres are the two regions that characterize each chromosome: the telomeres are the distal regions of a chromosome and serve to protect constantly the ends; the centromere, instead, which takes place during cells replication, is a sort of bottleneck that can be have a different position on different chromosomes giving them a unique and characteristic shape.

Within chromosomes DNA is organized into 3 different main structures, each having specific functions: (i) genes, that contain genetic information, (ii) regulatory sequences, DNA fragments precisely regulating the capabilities of each gene, and (iii) the so called "junk" DNA or DNA-junk, fragments of genetic material for which function has not been yet understood and that, according to some studies, might be simple evolutionary residues without a specific function. Eukaryotic genes are characterized by the presence of translated and transcribed sequences (exons)

alternating non-coding sequences (introns) whose function is unknown at the moment, even if recent studies suppose that also these sequences could be involved in specific genetic pathway (Brosius, 2009)

## DNA polymorphisms

Molecular analysis of Eukaryotic DNA sequence showed that it contains nucleotide sequences of variable length that can be divided in two different classes: unique sequences and repeated sequences.
Unique DNA sequences code for enzymes and structural protein. They are spread throughout the genome and represent 20 - 60% of the entire genome.
Repeated DNA sequences have different properties related to the number of repetitions.
Moderately repetitive DNA sequences include redundant genes, i.e. genes represented in many identical copies of the diploid genome. Genes coding for primary structure of introns and sequences having regulatory function are examples of redundant genes. Unlike highly repetitive DNA sequences, the moderately repetitive elements, are distributed throughout the genome, dispersed among the unique sequences and transcribed. They are never transferred to the cytoplasm, because of their activity on the adjustment of structural genes

transcription and selective involvement in gene expression during cellular differentiation.
Highly repetitive DNA sequences include very short DNA sequences that are repeated from 100 to 20 million times in tandem (each repetitive unit is linked by its terminal region with the beginning of the subsequent identical copy). According to their length repeated sequences are classified in satellites (thousand kilobases), midisatellites (few hundreds kilobases), minisatellites (from few hundreds to few kilobases) and microsatellites (dozens of bases). Microsatellites are typically neutral and co-dominant. They are used as molecular markers in genetics, for kinship, population and other studies. They can also be used to study gene duplication or deletion.

## Microsatellites markers

Microsatellites, also known as Simple Sequence Repeats (SSRs), or sometimes Short Tandem Repeats (STRs), include dinucleotides, trinucleotides and tetranucleotides repeated sequences of one to six base pairs of DNA that can be repeated 10 to 100 times (Turnpenny and Ellard, 2005).
Microsatellite markers (especially nCA repetitions) often present high levels of inter and intra specific

polymorphism, particularly when tandem repeats number is ten or greater (Queller et al, 1993).
More than 100 thousand microsatellite loci, at an average distance of 25-50 chilobases are present in haploid genome. Each sequence is characterized by microsatellite repeat pattern and flanking sequences that determine the uniqueness of the genome as markers.
As there are often many alleles present at a microsatellite locus, genotypes within pedigrees are often fully informative, in that the progenitor of a particular allele can often be identified. In this way, microsatellites are ideal for determining paternity, population genetic studies and recombination mapping. It is also the only molecular marker to provide clues about which alleles are more closely related (Goldstein et al. 1995).
Microsatellites owe their variability to an increased rate of mutation compared to other neutral regions of DNA. These high rates of mutation can be explained by frequent slipped strand mispairing (slippage) during DNA replication on a single DNA strand. Mutation may also occur during meiosis recombination (Blouin, 1996).
Interruption of microsatellites, perhaps due to mutation, can result in reduced polymorphism. However, this same mechanism can occasionally

lead to incorrect amplification of microsatellites; if slippage occurs early on during PCR, microsatellites of incorrect lengths can be amplified.

## Amplification of microsatellites

Microsatellites can be amplified by the polymerase chain reaction (PCR) process using the unique sequences of flanking regions as primers. The PCR activity was discovered by Kary Mullis in 1983 but is still one of the main pillars of the modern molecular biology. This process results in production of enough DNA to be visible on agarose or polyacrylamide gels; only small amounts of DNA are needed for amplification as the reaction creates an exponential increase in the replicated segment (Griffiths et al. 1996). In fact PCR is a cyclical process in which copies of genomic DNA are doubled at each cycle. During different PCR cycles DNA is repeatedly denatured at a high temperature to separate the double strand, then cooled to allow annealing of primers and the extension of nucleotide sequences through the microsatellite. This exponential amplification of DNA is based on the polymerization of new chains that use chains synthesized in the previous cycles as copy, so that in about twenty cycles it is possible to obtain almost a million copies of the original fragment.

## Role of microsatellites

Several researchers have suggested that microsatellites and other short sequence repeats can act as evolutionary tuning knobs (King 1997, Fondon and Gerner 2004, Verstrepen et al. 2005, Vinces et al. 2009). With proper means of expression, inherited length changes in repetitive DNA can act as digital genetic data, allowing for gradual changes in physical properties, with reduced risk of drastic mutations that might be lethal for the organism (King 1997).

Mechanisms for change

The most common cause of length changes in short sequence repeats is replication slippage, caused by mismatches between DNA strands while being replicated during meiosis (Tautz and Schlotterer, 1994). Typically, slippage in each microsatellite occurs about once per 1,000 generations. Slippage changes in repetitive DNA are orders of magnitude more common than point mutations in other parts of the genome (Jarne and Lagoda, 1996). Most slippage results in a change of just one repeat unit, and slippage rates vary for different repeat unit sizes, and within different species (Kruglyak et al. 1998).

Evolutionary changes from replication slippage also occur in simpler organisms. For example, microsatellite length changes are common within surface membrane proteins in yeast, providing rapid evolution in cell properties (Bowen and Wheals, 2006). Specifically, length changes in the FLO1 gene control the level of adhesion to substrates (Verstrepen 2005). Short sequence repeats also provide rapid evolutionary change to surface proteins in pathenogenic bacteria, perhaps so they can keep up with immunological changes in their hosts (Moxon et al. 1994). This is known as the Red Queen hypothesis (Van Valen 1973). Length changes in short sequence repeats in a fungus *(Neurospora crassa*) control the duration of its circadian clock cycles (Michael, 2008).
Short sequence repeats are distributed throughout the genome (King 1997). Presumably, their most probable means of expression will vary, depending on their location.

**In proteins**

In mammals, 20% to 40% of proteins contain repeating sequences of amino acids caused by short sequence repeats (Marcotte et al. 1998). Most of the short sequence repeats within protein-coding portions of the genome have a repeating unit of

three nucleotides, since that length will not cause frame-shift mutations (Sutherland and Rochards 1995). Each trinucleotide repeating sequence is transcribed into a repeating series of the same amino acid. In yeasts, for example, the most common repeated amino acids are glutamine, glutamic acid, asparagine, aspartic acid and serine. These repeating segments can affect the physical and chemical properties of proteins, with the potential for producing gradual and predictable changes in protein action (Hancock and Simon 2005). For example, length changes in tandemly repeating regions in the Runx2 gene lead to differences in facial length in domesticated dogs (*Canis familiaris*), with an association between longer sequence lengths and longer faces (Fondon and Garner 2004). This association also applies to a wider range of *Carnivora* species (Sears et al. 2007). Length changes in polyalanine tracts within the HoxA13 gene are linked to hand-foot-genital syndrome, a developmental disorder in humans (Utsch et al. 2002). Length changes in other triplet repeats are linked to more than 40 neurological diseases in humans (Pearson et al. 2005).

**For gene regulation**

Length changes of microsatellites within promoters and other cis-regulatory regions can also change gene expression quickly, between generations. The human genome contains many (>16,000) short sequence repeats in regulatory regions, which provide regulation on the expression of many genes (Rockman and Wray 2002). Length changes in bacterial SSRs can affect fimbriae formation in *Haemophilus influenza*, by altering promoter spacing (Moxon et al. 1994). Minisatellites are also linked to abundant variations in cis-regulatory control regions in the human genome (Rockman and Wray 2002). And microsatellites in control regions of the Vasopressin 1a receptor gene in voles influence their social behavior, and level of monogamy (Hammock and Young 2005).

**Within introns**

Microsatellites within introns also influence phenotype, through means that are not currently understood. For example, a GAA triplet expansion in the first intron of the X25 gene appears to interfere with transcription, and causes *Friedreich Ataxia* (Bidichandani et al. 1998). Tandem repeats in the first intron of the Asparagine synthetase gene are linked to acute lymphoblastic leukemia. A repeat

polymorphism in the fourth intron of the NOS3 gene is linked to hypertension in a Tunisian population (Jemaa et al. 2008).

**Within transposons**

Microsatellites are distributed throughout the genome (Richard et al. 2008). Almost 50% of the human genome is contained in various types of transposable elements (also called transposons, or jumping genes), and many of them contain repetitive DNA (Scherer, 2008). It is probable that short sequence repeats in those locations are also involved in the regulation of gene expression (Tomilin, 2008).

# SELECTION FOR MILK QUALITY TRAITS

## Genetic variability

The animal genetic improvement in livestock production adapts the animals genotype according to production requirements: such adaptation allows farmers and all the livestock production sector, operators, producers and industry, to provide food with the characteristics required by the consumer and to do it efficiently. The genomic approach to selection is becoming a reality that in the short to medium term will allow identifying the best animals for reproduction on the basis of their genome analysis. Given those circumstances, it is essential to know the genetic determination and the genes involved in character expression of economic interest.

The phenotype of an individual can be accurately described by a quantitative measure such as kg/year of product: such measurable characters are called quantitative traits.

When these characters are considered jointly for all the individuals in a population, they show a continuous distribution of values that have a

variability, part of which is due to the genetic component of every individual. The proportion of variability due to differences between genotypes and the total variability is called heritability.

## Quantitative Trait Loci (QTL)

The genetic theory of outbreeding populations, as dairy cattle, postulates the existence of a moderate number of genes for which it is possible to find variations (alleles) that differently influence the trait expression. The sum of the effects of these genes determines the observed genetic variability and each single gene contributing to this variation are called Quantitative Trait Gene (QTG). The group of the effects of all the genes that influence the trait determines, in part, the visible expression of the trait itself, so that it is not possible to establish a direct relationship between QTG and phenotype but it is possible to distinguish QTG based on their location on chromosomes (locus). For this reason the elements responsible for quantitative genetic variation of a trait are called Quantitative Trait Loci (QTL).

Molecular markers are DNA fragments whose chromosomal localization is known and that are easily and clearly identifiable using molecular genetic laboratory protocols. Usually these markers are not

genes and they do not have a direct effect on animal traits. Thanks to their size and distribution on the genome, molecular markers are used to track the transmission of genes responsible for variation of quantitative traits and to know the effect and location of a QTL on chromosomes, even if information about the genes responsible for the presence or the manifestation of a certain trait is not available.

The possibility of identifying QTL through genetic markers is an approach started about 30 years ago and the studies undertaken since then are many and have been performed on different traits, species and populations. Experimental designs and molecular techniques developed over the years have made the identification of QTL more efficient.

Recently an innovative experimental design, the "Selective DNA pooling" (Darvasi and Soller, 1994), has been proposed. It combines laboratory techniques and advanced statistical analysis (Lipkin et al., 1998; Mosig et al., 2001), to increase the efficiency of the experimental studies for QTL identification.

This experimental design has been used for the development of the work described in this thesis and is described in the "Materials and methods" section.

## Marker Assisted Selection (MAS)

The genetic improvement is the result of a process of reproduction and replacement of animals with progeny having the desired production characteristics. This process is slow and requires accurate choice of objectives that can give added value to breeders in the marketing of the product only after about 5 years from the choice of mate pairs.

Molecular genetics offers the opportunity to integrate today's selection schemes with information coming directly from animal genome and to implement the so-called Marker Assisted Selection (MAS). MAS is based on the principle of "DNA-level diagnosis" to identify individual's genetic superiority for a certain trait.

Selection within family can be carried out using two different MAS schemes: the "top down" and the "bottom up".

The top down scheme (Kashi et al., 1990) is based on research of loci of interest in the current population of the best bulls. To identify if these bulls are being segregated for a specific QTL, their sons in progeny test are grouped on the basis of marker haplotype received from the father. Segregation of the QTL can be identified when there is a significant difference in the average value

of EBV between groups of sons in progeny test. If there is a significant difference between groups of children, information on QTL for paternal grandfather is used for selection.
The bottom up scheme (Mackinnon and Georges, 1997) is based on the evaluation of bulls for QTL previously identified in interesting regions through the genotyping of the daughters born during sire progeny test. The daughters of bulls are grouped depending on the haplotype received from father and the difference in the productive deviation mean for the two groups of children is used to determine if the bull is heterozygous at the QTL. An heterozygous genotype at the QTL is assigned to the sire if, for example, the difference of milk production in the two groups of daughters having one of the two sire alleles at the marker or the different haplotypes, is larger than a specific value (Mackinnon and Georges, 1997). When a sire results heterozygous for QTL, only sons that received the best haplotype (Spelman and Garrick, 1998) are used in progeny test. Progeny tests are performed as usual but with the advantage of testing candidates bulls that were pre-selected for QTL and resulted genetically superior before performing the progeny test (Mackinnon and Georges, 1997).

An idea of the benefit of MAS may be given by comparison with DNA analysis techniques used in human. The diagnosis of genetic diseases can now be made through direct analysis of the genome of individuals, but in the past also studies on individual's relatives were necessary. Similarly the MAS will use an increasing number of genes that affect the genetic variation of traits of interest knowing the positive and negative effect on them. Thanks to the use of this information, it is possible to select sires and dams for reproduction based on the knowledge of their genotype, determined with molecular techniques. By combining genotypes and estimated breeding value the use of the progeny test could be reduced, making the selection more efficient thanks to shorter generation intervals and increased intensity and accuracy of the selection. For traits that are difficult to select, research and national and international industry have used MAS to select particular characteristics, such as those related to the diagnosis of hereditary diseases or the identification of subjects with undesired genes (e.g. the halothane gene in pig). Worldwide, MAS in dairy cattle begins to be applied in the context of selection programs (Dekkers, 2003) and various operators involved in genetic improvement are investing economic and organizational resources for

the integration, in a short time, of genomic information in selection schemes especially for economically important traits difficult and/or expensive to measure and having low genetic determination (susceptibility to mastitis, milk nutritional quality).

## Selection for resistance to mastitis and SCC

### General considerations

Direct selection for mastitis resistance has been considered inefficient because the heritability of mastitis is low. In addition, most countries do not widely record clinical mastitis incidences. Therefore, indirect measures of udder health, such as SCC, have been considered as an appealing alternative. The heritability of SCC is higher than that of clinical mastitis, and it reflects both subclinical and clinical mastitis, so that it can be used as an indicator of the presence of mastitis and susceptibility to this disease.

For this reason, many countries (especially Scandinavian countries) have chosen to select their breeding even for these characters and in almost all selection programs the decrease in the number of somatic cells in milk is inserted among the primary objectives.

In particular Denmark, Finland, Norway and Sweden have put in place an efficient system of registration of health traits data among whom are included also somatic cell counts and cases of mastitis.
The limits of this approach, which in Italy lead to failure registering mastitis data, are linked to the subjectivity of the diagnosis carried out in the farm and to the high costs of bacteriological tests. Somatic cell count is routinely recorded in most milk recording systems, and information on SCC is easily available on a large scale. It enables efficient and low cost monitoring of the pathology thanks also to the simplicity and reliability of the count.
Selection for low milk somatic cells is a valuable tool to reduce the incidence of mastitis but it will also improve the quality of milk and milk products, animal health (Cassel, 1994) and in general the economic production of the dairy sector.
The efficiency of SCC as a selection criterion for mastitis resistance depends on the genetic correlation between the 2 traits; a moderate to high correlation has been shown in a wide range of studies thus, in most cases when there are no records for clinical mastitis, improving udder health has widely been based on indirect selection for lower SCC. Additionally selection assisted by

markers and genes is highly promising for this character and has already been applied in some populations (Boichard et al. 2002).

## Selection against mastitis

During the last decades, the main breeding goal in dairy cattle was to improve milk yield using different conventional selection indices (Miglior et al., 2005). However, due to the unfavorable genetic correlation between milk yield and mastitis susceptibility, this selection leaded to an increased frequency of this disease (Rupp and Boichard 2003, Hinrichs et al., 2005).

The conventional selection methods based on EBV is not able, due to Mendelian segregation and independent assortment, to differentiate breeding values within progeny that share the same or similar pedigree (Griesbeck-Zilch et al., 2009).

Because of the limited progress in improving udder health by conventional selection procedures using solely indirect traits like milk SCC (Griesbeck-Zilch et al., 2009), there was an increasing demand for molecular marker information on mastitis susceptibility to be included in MAS schemes. Approaches to incorporate molecular information in the selection schemes have become feasible thanks to the detection of QTLs associated with this trait

and have been showed to be able to transcend the limitation of current selection methods.
Nevertheless some authors (Kehrli and Shuster. 1994 Schukken Y.H. and Coll. 1997) criticized the selection for lower SCC that are considered a key factor in response to the invasive pathogenic bacteria and are also present in milk of healthy cows. The same authors raised the possibility that selection for lower SCC can compromise the immune response against pathogens.
Thus, while selection against high SCC is supposed to reduce mastitis incidence, the dilemma is whether SCC should be decreased to the lowest possible level, or should not be lowered below a critical threshold.
Some studies have indicated that cows with very low SCC levels may be more susceptible to mastitis than cows with higher SCC (Kehrli and Shuster, 1994; Suriyasathaporn et al., 2000; Beaudeau et al., 2002). Studies based on experimental infection of cows have reported that animals having higher SCC before infection showed a lower severity of mastitis (Shuster et al., 1996; Schukken et al., 1998). This has been explained with the possibility of a higher number of leukocytes to be able to kills microbes by themselves before to initiate inflammatory response (Suriyasathaporn et al., 2000).

In contrast, results obtained by other researchers support selection for lower milk somatic cells as means to reduce the incidence of mastitis. Among these Nash et al. (2000) and Philipsson et al. (1995) have found that bulls whose daughters have a lowest counts of somatic cells, are the same who have daughters with lower frequency of mastitis. McDaniel (1993) found that one unit change in sire breeding value for SCC, corresponded to an increase of 36% in mastitis incidence.
Furthermore several studies report that low SCC does not increase the susceptibility of cows to clinical mastitis (Rupp and Boichard, 2000; Rupp et al., 2000; Boettcher et al., 2002).
Rupp and Boichard (2003) suggest that the best way to avoid undesirable consequences of selecting only on SCC would be to use clinical mastitis as an additional selection criterion for mastitis resistance, approach already applied in Denmark, Finland and Sweden (Heringstad et al., 2000). As already stated before, identification and registration of clinical mastitis is subjective and quite expensive and, for this reason, it is not routinely used by farmers.
It can be concluded that, since the largest component in variation of somatic cells content in milk between individuals is undoubtedly the presence of mammary infections, selection for low

milk somatic cell count will decrease the presence of mastitis and not the innate levels of cells in healthy mammary gland. Even if it is impossible to predict the role of the selection is difficult to expect a reduction of cells that could compromise the immune response.
Although the unfavorable genetic correlation between SCC and milk production, it has been shown (Koivula et al. 2005) that selection for lower SCC would not necessarily result in lower productivity. Also Nilsen et al. (2009) reached the same conclusion for protein yield. The authors demonstrated that the unfavorable genetic correlation between SCC and protein yield do not necessarily predispose animals selected for higher protein yield to increased incidence of mastitis.

## SCC correlation to mastitis and heritability

Selection for resistance to clinical mastitis based o reduced milk somatic cell is possible thanks to genetic correlation between the two characters, as shown in several studies (Nash et al. 2000; Rupp and Boichard, 1999; Shook, 1989; Schutz, 1994; Weller et al., 1992; Heringstad et al. 2006).
The positive genetic correlation between clinical mastitis and SCC was found to have a value of about 0.65/0.70 (then moderately high) in most of

the population examined. It has been also estimated that indirect selection on SCC is more effective than direct selection on mastitis (Heringstad et al., 2000). Moderate to high correlation with mastitis (0.65/0.80) has been also described for milk electrical conductivity (Norberg et al. 2004 and 2005), another tool used for mastitis detection. Limitation in the use of milk electrical conductivity as mastitis indicator is that it is not routinely recorded and could implicate additional cost for farmers using old milking machine not incorporating conductivity recorder. Nevertheless, variation in milk electrical conductivity not related to mastitis can present problems in diagnosis (Viguier et al. 2009).

Heritability for the somatic cell count was found to be higher than that for the susceptibility to mastitis. Heritability of susceptibility to mastitis has been estimated to varying within a range of 0.02 and 0.04 if estimated with a linear model (Emanuelson et al. 1998, Heringstad et al. 1999, Hansen et al. 2000) and from 0.06 to 0.12 when estimated with a threshold model (Simianer et al. 1991, Lund et al. 1999, Heringstad et al. 2003).

Heritability of SCC ranges from 0.04 to 0.12 (Emanuelson, 1988; Mrode and Swanson, 1996; Haile-Mariam et al., 2001). These heritabilities are

somewhat lower than heritability estimates for the level of electrical conductivity but of the same magnitude, highlighting that the indirect selection for the somatic cell count is easier to achieve and ensure a faster genetic progress compared with the direct selection against mastitis. This is also because most countries do not provide a rigorous registration of given "mastitis" (Rupp and Boichard, 2003). Always the same authors have suggested that somatic cell counts and mastitis can be an expression of a character involving common genes, despite the low correlation of phenotypes, from their estimated at 0.3.

## SCC correlation to productive traits

Different studies found positive genetic correlation between clinical mastitis and milk yield (Xiao-Lin et al. 2008, Carlè et al. 2004, Heringstad et al. 2005), confirming the genetic antagonism between production and udder health trait. Furthermore, this correlation indicates the involvement of common genetic factors or pathways in genetic expression and regulations of these two traits (Klungland et al. 2001, Sharma et al. 2006). From the viewpoint of genetic selection, the positive genetic correlations between mastitis and milk yield are unfavourable, because selection for higher production would be

associated with an increased susceptibility to clinical mastitis. It is known that there is an antagonistic genetic correlation between mastitis and milk production (Carlè et al. 2004, Heringstad et al. 2005) but knowledge is limited regarding how this association evolves in the course of lactation or among different lactations.

Koivula et al. (2005) found that genetic correlation between SCC and milk was positive in the first lactation, but negative, or near zero in the second lactation. This indicates that selection for lower SCC would not necessarily result in lower productivity, and at the same time SCC can be used as an indirect tool to select for mastitis resistance when clinical mastitis records are not available.

Genetic correlation between SCC and milk protein yield differs by parity and stage of lactation.

Samorè et al. (2008) showed that genetic correlations for lactation measures (305-d protein yield and lactation SCS) were positive in the first parity (0.31) and close to zero in the second (0.01) and third (0.09) parities. These results indicated that larger values of SCS were genetically associated with increased production. Furthermore in the same study it was estimated that the average overall correlation between SCS and protein yield was zero

or slightly positive in the first lactation and ranged from zero to negative in later lactations.
These correlations values demonstrate the dubiousness of applying a single genetic correlation measure between SCS and protein in setting selection strategies.
The evidence of a trend in additive correlations between SCS and protein yield should be taken into account when defining selection strategies. Usually, a single value of genetic correlation between SCS and protein yield is considered, ignoring the pattern of genetic correlations both with parities and within lactations. This would, consequently, influence the expectations of possible genetic progress.

The utilization of different additive genetic correlation values for various stages of lactation and parity would, therefore, be the only possible choice to give the correct emphasis to SCS and production yield into a selection program (Samorè et al. 2008).

# THE ITALIAN BROWN SWISS BREED

## Breed diffusion

The Italian Brown Swiss Breed, in the past also called Alpine Brown, originated in Switzerland and derives from *Bos Taurus brachycerus.*
Its peculiar rusticity, together with good production attitude, have leaded to the spread of the breed all over many European and American countries, with the differentiation of several genetic groups in relation to different environmental conditions.
In Italy, the introduction of the Alpine Brown Swiss Breed became in the 1850, trough the south Alps. Subsequently the diffusion of the breed reached the Pianura Padana, the center-southern Italy and the islands where it was often used for mating in substitution to autochthonous breeds.
After the 1940, thanks to the use of the artificial insemination, the Alpine Brown Swiss Breed have been mated with the American Brown Swiss Breed, having bigger size and higher dairy production attitude. The subsequent selection programmes have partially modified the characteristics of the breed in respect to the original alpine breed so that in the

1981 the name of the breed was converted from the original Alpine Brown Swiss Breed to the actual Brown Swiss Breed.
In the 1950, the Brown Swiss Breed was the most common dairy breed in Italy, counting 1.900.000 registered animals. In 1957 the Brown Swiss Breed National Breeder Association (ANARB) was established.
Actually the Brown Swiss Breed is the second dairy breed in Italy counting 750.000 animals bred in about 10.000 farms spread all over Italy mainly on mountain and hill.

## Breed description

The Italian Brown Swiss Dairy Cattle Breed has medium size, robust bone structure, low trunk with large pelvis and good muscularity. Its weight ranges from 550 to 700Kg.
The coat colour is uniform brown, darker in male and grey in calves.
The Brown Breed is present in Italy form century XVI. At the beginning it was a double attitude breed, but after years of selection, actually the main attitude for this breed is milk production.
The Italian Brown Swiss Breed production can reach 6000-9000kg of milk per lactation. The milk protein and fat content is 3.39% and 3.95%

respectively. An important characteristic of the Italian Brown Swiss Breed milk is the good cheese making attitude: in the Brown Swiss population, in fact allele A of the K-casein, having negative effects on curd formation, has low frequency in respect to other breeds (http://www.anarb.it/).
In order to support the particular attitude of the Italian Brown Swiss Breed milk to be processed for cheese making, ANARB promoted the "disolabruna®" registered mark used for the commercialization of typical cheeses produced using only milk coming from Brown Swiss Breed cows (http://www.disolabruna.it/).

## Breed selection

From 2006, the selection of the breed is based on the Total Economic Index re-examining the Total Economic Index adopted since 1998.
This index includes, together with productive and morphological traits also functional traits, as longevity, milkability and somatic cell count, introduced in the breed selection index in the 2005.
In the Italian Brown Swiss Breed aims of selection are not only production of animals having good size, weight, height and conformation, but also having good characteristics in terms of fertility, productivity and longevity.

For this reason the new Total Economic Index place 70% emphasis on production traits and 30% on functionality (Ghiroldi et. al 2005).
The quality, characteristics of the Italian Brown Swiss Breed milk, is the main selection goal of the breed. Because of this, the selection weight for protein content and percentage has been confirmed also in the new Total Economic Index as the most important traits (Ghiroldi et. al 2005).
Due to milk destination (mainly cheese production), K-casein has an extra value: +5% of protein yield for K-casein BB alleles, and 2,5% for K-casein AB alleles (Ghiroldi et. al 2005).

# OBJECTIVE

This research was carried out in the context of the international QuaLAT project developed at the Department of Veterinary Science and Technology for the Food Safety of the Veterinary Medicine Faculty in Milan and founded by the Regione Lombardia ("QTL detection for mastitis resistance and milk nutritional aspects in dairy cattle populations").
The scientific objective of the project was to identify chromosomal regions linked to genes (QTL) responsible for milk somatic cell counts (MSCC) and milk nutritional quality.

Although in literature numerous studies concerning the identification of QTL for productive and functional traits are reported, these have been mainly developed, both in Italy and abroad, on Holstein breed.
Results on QTL mapping are available in the Brown breed only for productive traits (milk kilograms and protein percentage), and have been obtained within the framework of a research project funded by the European Community (Bagnato et al. 2008).

No data are available in the Italian Brown Swiss breed about QTL mapping for milk somatic cell count or resistance to mastitis.

As for the other dairy cattle breed, also for the Italian Brown Swiss Breed, functional traits are of interest for selection purposes and among these, milk somatic cell count, considered as an indicator of susceptibility to mastitis and included in the official economic selection index of the breed.

Aim of this research, developed within 2 and half years of this PhD program jointly with international partners involved in the project, was to produce, for the Italian Brown Swiss dairy cattle breed, a moderate to high resolution map of QTL associated with milk somatic cell count.

Specifically, this thesis shows the progress of the project in the identification of SCC QTL after a genome scan performed with microsatellite markers and a fine mapping on 2 regions (BTA 1 and 8) identified as harvesting QTLs for MSCC in the Italian Brown Swiss Breed after the scan of the genome.

# MATHERIALS AND METHODS

## Experimental design

The research described in this thesis has been developed using a selective DNA pooling approach in a Daughter Design (Darvasi and Soller, 1994). The same experimental design has been used both to scan the genome and to fine map the 2 regions on BTA 1 and BTA 8 selected for high-resolution mapping.

The experimental design used in this research, is base on the principle of linkage disequilibrium that postulates that very close loci and their related alleles are transmitted together ("linked") to the progeny, and not independently as postulated by Mendel laws.

Consequently, if a marker and a QTL are very close one to each other, the recombination rate between them is very low and they are transmitted together, allowing, thanks to the marker, to follow the QTL from relatives to progeny.

## Daughter Design

The Daughter and the Grand Daughter Designs are widely used in dairy cattle populations to map QTL (Weller et al., 1990).
The QTL identification using genetic markers is based on the assumption that if markers and QTL are associated in the parents the progeny will receive together with the parental marker allele also the associated QTL allele. Consequently, if the progeny is grouped using the marker allele received by the heterozygous sire, the 2 possible different alleles at the QTL will create 2 progeny groups having different quantitative value. Consequently, a significant difference among the genetic values of the 2 groups indicates the presence of a QTL associated to the marker (Weller et al. 1990). Reversely grouping the individuals according to their phenotype may lead to significant difference between the two sire alleles, which may be linked to the QTL.
In dairy cattle, thanks to the possibility to have very large groups of half-sister daughters from a single sire, it is possible to verify the association between marker and QTL using a *t* test calculating the average difference of the genetic value of the daughters receiving the 2 possible alleles of all the markers from the heterozygous sire (Geldemann et

al. 1985). For small families the same procedure can be used combining data of different half-sister families to increase the test power.
If the sire is heterozygous at the markers but homozygous at the QTL, no difference in the daughter's genetic values is expected.
If the sire is homozygous at the markers, it is not possible to identify differences in the daughters that will receive all the same allele.
The identification of the marker-QTL linkage using a Daughter Design requires the usage of a very large number of samples to be effective (Darvasi et al. 1993, Kashi et al. 1990, Weller et al. 1990). This implicates high costs related to the genotyping, especially for the genome scan genotyping using a sufficient dense panel of markers, and has limited the used of this experimental design for cattle genetic improvement (Kashi et al. 1990), even if it very powerful.
Recent developments in the methodology of statistical analysis and laboratory procedures have made possible its application on a large scale, making the mapping of dairy cattle genome economically feasible.

## Selective DNA pooling

In addition to the recent developments of the laboratory and statistical procedures that make easier the application of the daughter design for QTL identification, the use of milk as a source of DNA and the introduction of the "Selective DNA Pooling" (Darvasi and Soller, 1994, Lipkin et al., 1998) experimental design mark a step forward in the QTL mapping experiments.

The use of milk samples as source of DNA makes possible the collection of a large number of milk samples (as requested by the Daughter Design) using the milk recording system for management purposes and genetic testing. Samples are sent to centralized laboratories for the testing procedure (including the somatic cell count) and virtually are available approximately once a month, at a very low cost and in few centralised milk labs.

The second, (Selective DNA pooling approach) is based on the theoretical demonstration that almost all the map information for a trait are linked to the allele frequency of the marker in the best and the worst 25% of the population phenotypic distribution for that trait (Darvasi and Soller, 1994) and the technical demonstration that microsatellite markers allele frequency in pools of DNA can be determined with great accuracy through genotyping

correction for "shadow bands" (Lipkin et al. 1998). In this way, for each trait, all map information usually obtained from a very large sample, is obtained at a very low cost, through the densitometric analysis of a small number of pools.

## Sampling

The experimental design adopted in this search, used the daughters family group that have been identified in 5 Italian Brown Swiss large families of at least 500 half sisters individuals.
For each of these 5 families, the sire and all the possible daughters have been sampled (Table 2).

| Sire | Number of samples |
|---|---|
| E | 2568 |
| B | 2254 |
| C | 1782 |
| G | 1255 |
| F | 947 |
| Total | 8806 |

Table 2: number of collected milk samples in each sire family

For sires both DNA and semen samples were available.

For the daughters milk was collected within the routine milk recording system and thanks to the collaboration of breeders associations. The sampling considered only the daughters born in Italy.
In order to prevent milk from degradation during shipment, 10ml tubes containing 50ul of a methylene blue conservative solution (Bronopol) have been prepared in the lab and then distributed to the regional/provincial breeder associations. Each tube was labelled with name of the bull, name of the daughter to be sampled, identification code of province and farm, and the barcode corresponding to the serial number of the subject to be sampled. This labelling procedure was adopted to facilitate samples identification, collection, sorting and storage.
The tubes were divided by farm to simplify the sampling activity performed by milk recording system controllers, and placed in plastic bags together with the list of all the samples to be collected in each farm and some indications on how perform the sampling.
Sampling procedures also indicated to report on the list of the animals the sampling date and the reason why a sample was not collected (e.g. dried, death or sold animals). The sampling date information was requested in order to be able to associate each

sample with the correct somatic cell count registration performed within a specific month.
Upon the arrival in the laboratory all filled and empty tubes were catalogued by reading barcode with optical scanner and divided for the subsequent storage at –20 °C.

## Data collection

In addition to milk and semen samples, it was necessary to acquire, from the breeders associations, the somatic cell counts data for each individual at sampling. These data were necessary for the application of the Selective DNA Pooling experimental design. Somatic cell count data were obtained from the breeders associations after the communication of sampling dates for each farm/individual. Data were then assigned to the corresponding individual merging the individual specific code.
Breeders associations also provided EBV for milk somatic cell count for each daughter, necessary to identify individuals according to the Selective DNA pooling experimental design.

## Pools constitution

For each family, the normal distribution of Estimated Breeding Value (EBV) for milk Somatic Cell Count (SCC) has been considered.
The EBV has been then corrected to account for maternal effect as described by Dolezal et al. (2003) and the resultant normal distribution of corrected EBV (cEBV) for SCC used as reference to identify samples for pools construction.
Two hundred samples in the high tail and 200 in the low tail have been selected for pools constitution. The 200 selected samples of each tail have been then randomly divided in 4 pools of 50 individuals/each.
In the formation of pools, the milk of each individual was included in the pools in different volume in relation to somatic cell counts to ensure that all individuals were equally represented within the pools i.e. in each pool each individual has the same number of cells and therefore the same amount of DNA.
Each subpools contained a total of 200,000 cells.
Milk samples for which the milk somatic cell count value was not available or for which it exact correspondence between somatic cell counts and date of sampling was not sure, were read again using

a Fossomatic ® instrument available at the Veterinary Medicine Faculty in Milan.
Pools formation requires precision and attention to avoid errors during construction and to limit contamination among pools. Each of the four subpools was divided in 5 different aliquots of 4,000 cells/each and stored in 10ml tubes at -20°C until DNA extraction. If during DNA extraction of an aliquot some problem occurred, the other aliquots were available without repeating pools constitution activity.

## DNA extraction

### DNA extraction from semen samples

DNA of bulls, if not already available, was extracted from semen samples using the ZR Genomic DNA™-Tissue MiniPrep commercial kit (http://www.zymoresearch.com). The procedure suggested by producer for biological liquid samples was applied to semen samples.
To ensure repeatability of the results and cross check among laboratories, another bull added to the 5 chosen for this research, has been sampled and DNA extracted from semen sample (control).

## DNA extraction from milk samples

As described by Lipkin et al (1993, 1998) cell lysate instead of purified DNA was obtained by pool samples to be used in the subsequent PCR. This procedure is very cheap and permits to avoid the use of toxic reagents.
The protocol envisages a series of cells washes performed in saline solution (NaCl 0.9%) followed by centrifugation at 2000rpm for 10 minutes. This step, performed directly in the 10ml tubes where the pools have been constituted, permits to wash cells from proteins, fat and other milk components and to obtain a clear white pellet of cells at the bottom of the tube. For each pools a different number of wash and centrifugation steps is needed depending on the milk samples used to construct pools. The advantage of using 10ml tubes during washing is the possibility to add more saline solution for each washing steps then limiting the number of steps needed to obtained a clear pellet.
Once the pellet is formed, cells are re-suspended in 50 ml of TE solution (Tris and EDTA) to obtain a final concentration of 200 cells/ml, transferred in 1,5 ml tubes and then incubated for 5 minutes at 50 °C, for 5 minutes at 100 °C and again for 5 minutes at 50 °C to break cells. The obtained cell lysate can be used directly for PCR or frozen until usage.

## PCR amplification

### Markers choice

A panel of 242 dinucleotide microsatellite markers was chosen (www.marc.usda.gov/genome/genome.html), to cover all the 29 bovine autosomes.
The characteristics of each marker (position, annealing temperature, amplified fragment size, etc.) have been acquired from online databases.
The dye for each forward primer was chosen considering each microsatellites fragment sizes and the possibility to perform multiplex PCR.
A total of 187 markers were used in the genome scan to genotype the 5 sires and the control.
After the genotyping of the sires, 3 microsatellites were excluded from subsequent analysis on the pools primers were not amplifying or were producing by products.
The remaining 184 markers have been used to genotype the pools.
After the genome scan and the identification of the regions to be mapped in high resolution, 55 additional markers in the interesting chromosomes have bee chosen for pools genotyping.

## PCR optimization

PCR amplification protocols have been optimized to obtain the best results both in terms of quality of the amplified fragment and possibility to reduce reactions (saving time and costs) using multiplexes.
Mg2+ concentration and annealing temperature, being the limitation factors for the success of the reaction, have been considered for the optimization of the PCR protocols and the multiplexes constitution. Markers amplifying at an equal or similar Mg2+ concentration and annealing temperature, have been considered for multiplexing according with the following criteria:

1. markers having similar fragment size but different dye so that, although the overlapping of size, they are distinguished by the dye colour;
2. markers having different size but the same dye colour so that although they cannot be distinguished by the dye colour, they are differentiated according to the size.

Between the two criteria, the second one was preferred as it allows a clearer distinction of the electrophoresis peaks.
Multiplex PCR were mainly used to amplified sires DNA, as it was purified and guaranteed good amplification results.

All the selected markers were used for the 5 sires DNA amplification.
Cell lysate from pools was amplified only for markers resulted heterozygous at the sire.
Cell lysate amplification required peculiar attention in limiting the formation of unspecific products, so, in some cases the PCR was repeated to verify the real sizes of the products and to distinguish possible by-products. Furthermore, in some cases the signal obtained during the electrophoresis run was very low and also in this case the PCR was performed again adding more DNA in the reaction to obtain higher and more readable peaks.
DNA of the control was amplified in all the PCR reaction and loaded on all the gel runs to verify the repeatability and the goodness of the results both in terms of amplification and electrophoresis.

## Electrophoresis run

### Acrylamide gels preparation

Electrophoresis of PCR products was carried out on polyacrylamid gels.
This kind of gel, differently from the agarose ones, permits to separate fragments that differ even of only one nucleotide.
Pre-mixed gels have been used to limit contact with acrylamide. The acrylamide mixture, also containing

polymerization factors, was passed between 2 glasses to obtained 0.1cm gel thickness and left at room temperature for 8-12 hours for polymerization.
After the gel polymerization, the glasses are fixed to the instrument in contact with the heater surface that during the run keep the gel at 50-60°C.

## Samples preparation and gel loading

Before loading DNA samples are added of formamide, methylene blue and standard.
The formamide solution is added to facilitate DNA denaturation at 95°C and then permits its run on the gel.
The methylene blue solution is added to the sample to make it heavy and visible and then easier to be loaded on the gel. The laser does not detect the methylene blue and the colour runs out after few minutes run so that it cannot be confused with the markers blue dye.
The standard (TAMRA350®, Applied Biosystems Foster City, CA) is added to each sample to permit the correct attribution of the fragments size. The used standard utilises a red dye to be discriminated among the other markers dye (yellow, blue and green).

The samples added with the aforementioned reagents are then denaturated at 95°C and loaded on the gel.

## Gel Run

During the run, single strand DNA fragments are separated according to their molecular weight. To keep DNA as a single strand during the run it is necessary to maintain the denaturation condition during the overall duration of the run. For this reason samples are denaturated before loading using formamide, and then keep at 50-60°C to maintain the denaturation.
The runs were performed on the ABI377XL automatic sequencer (Applied Biosystems Foster City, CA) reading dye markers using an infrared laser.

## Analysis

### Gel analysis

Each band on the gel is read by the laser and then converted into electrophoretic peak.
Before the conversion in to peaks, the gel bands must be analyzed to track lanes and optimize the standard position using the specific GenomeScan® software.

Then, using the Genotyper™ software, the aligned bands and the corrected standards are converted in to peak whose size height and area values are reported.
Size and height values are used for allele frequency estimation and shadow bands correction as described by Lipkin et al. (1998).

## Pools analysis

Thanks to the electrophoresis run on the acrylamide gel, alleles that differ in repetition numbers are solved as discrete bands. The analysis of the pools has been performed as described by Lipkin et al. (1998). Allelic frequency estimation in grouped DNA samples is based on a linear relationship between the initial number of allele copies in the pool and the band intensity of the final allele. This relation is determined both by the allele frequency in the group of individuals that made up the pool and by densitometry (Lipkin et al., 1998). However, this estimate is often biased by the presence of "shadow bands", that are PCR products artefacts derived from the repetition segment of genomic DNA and from deletion or insertion of one or more repeating patterns (Hauge and Litt, 1993; Litt, 1993, Murray et al., 1993). As a consequence, a band observed in the pool can be a collection of a main product together

with and a number shadow product generated from contiguous alleles on the same line (Lipkin et al., 1998). Lipkin et al. (1998) have shown that the relative intensity of a shadow band is a linear function of the repetition of the fragment from which it derives and of the number of insertion/deletion that made the fragment to differ in respect to the genomic fragment. This linear function has been calculated specifically for the macrosatellites markers used in this research and applied to adjust the intensity of the microsatellites bands from the overlapping shadow bands.

## Estimation of Sire-Marker Allele Frequencies in the Pools

Sire marker allele frequencies in the daughter pools were obtained by shadow band correction as described in Lipkin et al. (1998). This resulted in 16 pool frequencies for each sire-marker combination, as follows (Table 3):

| | | POOLS | | | |
|---|---|---|---|---|---|
| **TAIL** | **SIRE ALLELE** | *a* | *b* | *c* | *d* |
| **H** | **L** | A | C | E | G |
| | **S** | B | D | F | H |
| **L** | **L** | I | K | M | O |
| | **S** | J | L | N | P |

Table 3: Pool and sire allele frequency nomenclature

This table reads as follows, A is the frequency of the long sire allele in the high tail of pool *a* and B the frequency of the short sire allele in the high tail of pool *a* and so on. Sire by marker combinations having genotyping data for less than 2 pools per tail were excluded from subsequent analysis.

## Marker-QTL Linkage Tests

### Single Marker Single Sire Tests

Linkage between a marker and QTL is based on the rejection of the null hypothesis of equal sire marker allele frequencies in the high and low tail.

Briefly, significance of marker i for a single sire j, heterozygous at i, is determined by the single-sire test statistic (Darvasi and Soller, 1994):

$$Ztest_{ij} = \frac{Dtest_{ij}}{SD(Dnull)_{ij}},$$

where $Dtest_{ij}$ is the sire marker allele frequency difference between daughters from the high tail of the phenotypic distribution assigned to the high pool and daughters from the low tail of the phenotypic distribution assigned to the low pool calculated as follows:

$$Dtest_{ij} = \{[(A - I) - (B - J)) + ((C - K) - (D - L)] + \\ + [(E - M) - (F - N)) + ((G - O) - (H - P)]\}/8$$

$Dnull_{ij}$ is the average of the $i^{th}$ sire allele frequency differences at the $j^{th}$ marker between all possible couples of arbitrarily paired replicate pools within the high or low tail, respectively and $SD(Dnull_{ij})$ is calculated as the standard deviation of all $Dnull_{ij}$.

$$Dnull_{ij} = \left[ \frac{(A-C)-(B-D)}{2} + \frac{(A-E)-(B-F)}{2} + \frac{(A-G)-(B-H)}{2} + \right.$$
$$+ \frac{(C-E)-(D-F)}{2} + \frac{(C-G)-(D-H)}{2} + \frac{(E-G)-(F-H)}{2} +$$
$$+ \frac{(I-K)-(J-L)}{2} + \frac{(I-M)-(J-N)}{2} + \frac{(I-O)-(J-P)}{2} +$$
$$\left. + \frac{(K-M)-(L-N)}{2} + \frac{(K-O)-(N-P)}{2} + \frac{(M-O)-(N-P)}{2} \right] /12$$

The expectation of $Dnull_{ij}$ across all sire-marker combinations is zero as it is calculated within tails and therefore represents the null hypothesis of no linkage. Therefore the standard deviation (SD) of the $Dnull_{ij}$ values $SD(Dnull_{ij})$ is an empirical estimate of the standard error of $Dtest_{ij}$ under the null hypothesis. (Tal Stein et al., 2010).
$Znull_{ij}$, calculated as $Dnull_{ij}/SD(Dnull)_{ij}$ within tails distributes as N(0,1) and thus confirms the validity of the $SE(Dnull_{ij})$ used.
$Dtest_{ij}$ and $Dnull_{ij}$ can only be calculated for markers for which the sires were heterozygous. Under the normal approximation $ztest_{ij}$ is a standard normal variable and comparison-wise error rate (CWER) P-values for the individual sire-marker combinations were obtained accordingly.

## Single Marker Across Sire Tests

The test statistic for the j$^{th}$ marker across all n heterozygous sires was calculated as

$$TS_j = \sum_{i=1}^{n} Z_{ij}^2 \approx \chi_{(n)}^2,$$

according to (Weller et al. 1990), which distributes as $\chi_{(n)}^2$, with degrees of freedom equal to the number of informative sires (n) at the j$^{th}$ marker. CWER P-values were obtained accordingly.

## Multiple Markers Across Sire Tests

To overcome the influence of the number of informative sires at a given marker and to increase mapping accuracy, Multiple Marker Mapping (MMM) as described in (Dolezal et al. 2008) was used to identify marker-QTL linkage, which also allows calculation of test statistics at every cM along a chromosome based on a selection index analogy.
Briefly, given **t** a vector of observed across sire single marker test statistics calculated as

$$T_{ij} = Z_{ij}^2 \approx \chi_{(1)}^2$$

with $\mathbf{V}$ = var($\mathbf{t}$), the variance-covariance matrix of $\mathbf{t}$, and $\mathbf{b}$ the solution vector of $\mathbf{V}^{-1}$ $\mathbf{c}$, with $\mathbf{c}$ being a vector of covariances between predicted ($T_l$) and observed ($T_i$) test statistics, test statistics at each cM along the chromosome can be calculated as

$$\hat{T}_l = b't$$

For calculation of P-values, the degree of freedom at each cM position along the chromosome was calculated as the proportion of variance explained by the markers, namely,

$$df(MMM) = \frac{(b'Vb)}{t},$$

(Visscher et al., 1996) and summed across sires.
All statistical analyses were performed with SAS 9.2. (SAS Institute, Cary, NC)

## Setting Significance

A CWER cutoff of 0.02 was used, which corresponds roughly to a 10% proportion of false positives.

# RESULTS AND DISCUSSION

## Sires and pools genotyping

Among the 187 markers selected to scan the genome, 3 have been excluded from further analysis because of amplification problems in the genotyping of the sires.
Among the remaining 184 markers, 7 have been excluded because all the sires resulted homozygous at those loci and other 11 have been excluded because of resulting in by-products amplifying pools DNA.
These technical issues lead to 166 markers used for the association analysis in the pools. As the goal of the genome scan was to cover the entire genome with markers spacing on average 20 cM, other 30 markers have not been tested in the pools. These 30 have been chosen according to the following criteria:

1. markers for which less than 3 sires were heterozygous;
2. markers with similar position map respect to flanking ones.

In this way all the remaining 136 markers were successfully used in pools genotyping maintaining the average distance of 20cM one to each other as aimed in order to performed the genome scan.

After a single marker analysis performed as in Bagnato et al. (2008), 2 chromosomes were identified for high-resolution mapping and saturated with additional markers aiming at a 5 cM interval coverage in the selected QTL regions.
In total 55 additional markers have been identified on BTA1 and 8 to map at high resolution the two QTL regions identified with the single marker analysis.
Out of the 55 selected markers, 27 map on BTA 1 and 28 on BTA 8.
Because in genotyping sires, 6 markers on BTA 1 did not amplified, they were excluded from the pools genotyping leaving 21 microsatellites for the high resolution mapping on BTA1.
Three markers on BTA 8 were excluded for the same reason, leaving 25 markers to be used for subsequent analysis on pools.
After these markers preselection, both the chromosomes were fine mapped using markers spacing on average 5 cM one to each other.

Previous experience in similar mapping projects (Bagnato et al., 2008) suggested to identify a larger number of markers in respect to the goal (i.e. spacing on average 20cM in the genome scans or 2/5 cM in high resolution mappings), to account for

the markers not amplifying or in homozygosity in the population.

## Source of errors in genotyping

In this project the DNA obtained from pools is not purified as those extracted from semen samples and for this reason it could be more difficult to amplify.
Another source of genotyping results variability, are the markers themselves as microsatellites have proved to be versatile molecular markers, particularly for population analysis, but they are not without limitations.
PCR failure may result when particular loci fail to amplify, whereas others amplify more efficiently and may appear homozygous on a gel assay, when they are in reality heterozygous in the genome.
Point mutation in the primer annealing sites may lead to the occurrence of null alleles, where microsatellites fail to amplify in PCR assays (Jarne and Lagoda, 1996; Dakin and Avise, 2004). Null alleles can be attributed to several phenomena as for example sequence divergence in flanking regions that can lead to poor primer annealing, especially at the 3' end, where extension commences; or preferential amplification of particular size alleles due to the competitive nature of PCR that can lead

to heterozygous individuals being scored for homozygosity (partial null).
Among the 187 markers used in the genome scan about 7% had null alleles.
Null alleles complicate the interpretation of microsatellite allele frequencies and thus make estimates of relatedness faulty. Furthermore, stochastic effects of sampling that occurs during mating may change allele frequencies in a way that is very similar to the effect of null alleles; an excessive frequency of homozygotes causing deviations from Hardy-Weinberg equilibrium expectations. Since null alleles are a technical problem and sampling effects that occur during mating are a real biological property of a population, it is often very important to distinguish between them if an excess of homozygosity is observed.
Because of in the pools analysis using shadow band correction strategy, it is very important to correctly identify the sire allele contribution in the daughter, it was preferred to use more stringent criteria selecting markers than having biased results.

## QTL mapping

The QTL mapping was performed applying the MMM approach to the overall markers data set, including markers used to fine map BTA 1 and BTA

8. Results are showed in Figures 1 to 29 of the Appendix. Table 4 reported the 41 QTL regions identified in the 29 bovine autosomes and the QTL location in each region.

| | QTL region | | | QTL |
|---|---|---|---|---|
| **BTA** | **Number** | **Start cM** | **End cM** | **Position cM** |
| 1 | 1 | 14 | 27 | **17** |
| | 2 | **38** | **81** | 69 |
| | 3 | 87 | 95 | 89 |
| | 4 | 121 | 160 | **130** |
| 2 | 1 | 0 | 49 | 26 |
| | 2 | **51** | **111** | **80** |
| 3 | 1 | **37** | **116** | 68 |
| | 2 | 121 | 121 | 121 |
| 4 | 1 | **60** | **86** | 69 |
| | 2 | 88 | 104 | **100** |
| 5 | 1 | **27** | **77** | **58** |
| 6 | 1 | 65 | 69 | 67 |
| | 2 | **78** | **114** | 96 |
| 7 | 1 | **0** | **57** | **32** |
| | 2 | 60 | 75 | **67** |
| 8 | 1 | **0** | **51** | 31 |
| | 2 | **55** | **77** | **63** |
| | 3 | 101 | 118 | 113 |
| 9 | 1 | **0** | **40** | 29 |
| | 2 | **55** | **95** | 74 |

| | QTL region | | | QTL |
|---|---|---|---|---|
| **BTA** | **Number** | **Start cM** | **End cM** | **Position cM** |
| 10 | 1 | 94 | 105 | 104 |
| 11 | 1 | **60** | **108** | 82 |
| 12 | 1 | 0 | 16 | 10 |
| | 2 | **58** | **106** | 99 |
| 13 | 1 | **0** | **85** | **85** |
| 15 | 1 | **0** | **88** | **23** |
| 17 | 1 | **15** | **97** | 86 |
| 18 | 1 | **0** | **15** | 3 |
| | 2 | 17 | 18 | 17 |
| | 3 | **26** | **59** | 44 |
| 19 | 1 | **0** | **86** | **17** |
| 20 | 1 | **12** | **54** | 33 |
| 21 | 1 | **0** | **60** | 36 |
| 23 | 1 | 64 | 76 | **75** |
| 24 | 1 | **13** | **66** | **45** |
| 26 | 1 | **0** | **19** | 6 |
| | 2 | 34 | 50 | 43 |
| 28 | 1 | 0 | 25 | 15 |
| | 2 | 32 | 49 | **49** |
| 29 | 1 | **0** | **9** | 0 |
| | 2 | 28 | 45 | 40 |

Table 4: QTL regions identified within each chromosome (BTA); number of QTL regions per chromosome, QTL region start and end, QTL position.

As the MMM approach walks 1cM step estimating the QTL position cM by cM, start and end of each QTL region and the position of the corresponding QTL in each region are expressed accordingly.
The QTL regions and the respective QTLs are distributed all over the 29 *Bos Taurus* autosomes except on BTA 14, BTA 16, BTA 22, BTA 25 and BTA 27.
The grater number of QTLs has been identified on BTA1, with 4 different QTL regions.
Three QTL regions have been identified on BTA 8 and BTA 18.
Eleven chromosomes (BTA 2, 3, 4, 6, 7, 9, 12, 13, 26, 28, and 29) showed 2 QTLs, and the remaining ten chromosomes (BTA 5, 10, 11, 15, 17, 19, 20, 21, 23 and 24) showed only 1 QTL.

Table 4 also reports the results of the comparison performed between the results of this study and the QTLs previously mapped for mastitis and related traits (SCC and somatic cell score) and published in the databases considered for the comparison (see next section).
In bold are the QTL regions for which one or more QTLs from previous studies have been found also in this study.

## Comparison with literature results

Among the 3 on-line available Bovine QTL databases (www.animalgenome.org), only 2 are constantly updated and were used here to compare the QTL map obtained in this research with the map including all the QTL reported in literature from previous research efforts.
Aditionally the database of cattle candidate genes and genetic markers for mastitis and production traits created by Ogorevc et al. (2009) has been used to compare if the results of this research can be related to the effect of genes or of their expression patterns associated with mastitis traits and studied both in cattle and mouse species.

## QTL databases

The 2 bovine QTL databases considered for the comparison of the results are the *Bovine QTL viewer* (updated on February 12th 2011) and the *Cattle QTL db* (Release 13, December 2010), both available on line *via* the www.animalgenome.org web site.
The third available database was not considered because was last updated on April 2008.
In the *Bovine QTL viewer* database only 13 chromosomes are reported as carrying a total of 20

QTLs related to somatic cell score (BTA 2, 4, 5, 7, 9, 10, 13, 14, 18, 21, 22, 23 and 27). All the studies listed in this database have been performed on Holstein Breed except 1, reporting QTL identified in French Dairy Cattle Breeds.

The *Cattle QTL db* is the most complete database of QTL associated with mastitis or related traits as somatic cell count or somatic cell score. It reports more than 145 QTLs distributed all over the entire cattle genome and divided in QTL specifically associated with mastitis (33 QTLs), QTL associated with SCC (13) and those associated with somatic cell score (more than 100). This last trait is the most used as mastitis indicator in mapping QTL research and is obtained from SCC using the log transformation proposed by Ali and Shook (1980) to normalize the distribution of SCC:

$$SCS = \log_2(SCC/100) + 3$$

As in *The QTL Viewer* also in this database most of the researches have been performed on Holstein Breed, with exception of some researches carried out in the Finnish Ayrshire breed, in the Swedish Red and White populations, in the Danish Red, in the Normande and in the Montbeliarde breeds.

Most of the QTL in the databases are indicated with the exact position (higher significant marker). The criteria here followed to was to consider the QTL found in this research confirmed in previous studies if the published QTL position differs at most of 2 cM (taking into account the map upgrade).
Some of the previous mapped QTLs lie in the QTL region identified in this study, even if the exact QTL position doesn't match among different studies: this discrepancy could be due to the different marker association methods applied or to the different number of markers used for the association study. It has been indicated in the following paragraphs if an already published QTL lies in a QTL region identified in this research, but this kind of comparison should be more accurately deepened in a metha-analysis taking into account the experimental designs and in the association tests used by different authors.
QTL for which only the QTL region starts and ends were indicated in the databases (7 cases), have not been considered for comparison.

**Chromosome 1**

Among the 4 QTLs mapped on chromosome 1 in this research, the 2 located at 17cM and 130cM are confirmed by previous findings: the first was

mapped in the Hostein breed, the second in an unspecified breed.
QTL mapped at 69 cM and the QTL region identified between 87 and 95 cM and the corresponding QTL at 89 cM have not been mapped previously and may be loci segregating exclusively in the Brown population that has not been used in any previous mapping study.

**Chromosome 2**
The QTL mapped on BTA 2 at 80 cM is confirmed by previous studies performed on the Holstein Breed. Other 3 published QTLs lie in the QTL region here identified spanning from 51 to 111 cM.
According to the QTL databases considered the QTL region segregating between 0 and 49 and the corresponding QTL at 26 cM was not mapped before.

**Chromosome 3**
The 2 QTLs mapped on this BTA don't appear in any previous study included in the databases.
The QTL reported in literature for clinical mastitis at 102.95 cM lies in the QTL region identified in this study between 37 and 116 cM. As the trait considered for the published QTL is clinical

mastitis, this discrepancy could be related to the use, in this study, of somatic cell count.
Other 2 published QTLs in association with somatic cell score, lie in the QTL region beginning at 37cM and ending at 116 cM as identified in this research but at different locations. Even if not at same locations and with same traits the findings in this study identify the presence of QTLs on chromosome 3, confirming also for the Brown Swiss population that segregation for this mastitis resistance is occurring on BTA3.

**Chromosome 4**
The QTL mapped at 100 cM is confirmed by previous research.
Three QTLs previously mapped on BTA 4 lie in the QTL region spanning from 60 to 86 cM and 2 have are associated with clinical mastitis. QTL mapped at 69 cM was not previously reported.

**Chromosome 5**
Among the 10 published QTLs, only 1 associated with clinical mastitis map in the same location of the one identified in this study at 58 cM. Nevertheless among the other QTLs reported in literature, 6 lie in the QTL region here identified between 27 and 77 cM.

**Chromosome 6**

Two QTLs previously mapped are placed in the QTL region spanning from 78 to 114 cM.

No confirmation has been found for the QTL region identified between 65 and 69 cM and the related QTL at 67 cM. Indeed chromosome 6 has been deeply studied for the loci mapping productive traits as numerous studies report QTLs for protein traits.

**Chromosome 7**

The 2 QTLs mapped on this chromosome have been previously mapped in the Holstein Breed, and other QTLs have been localized in the QTL region starting at 0 cM and ending at 57 cM.

**Chromosome 8**

This chromosome has been fine mapped in this study because resulted particularly interesting in the Brown Swiss Breed as being a BTA carrying QTL associated with somatic cell count (data not showed).

Among the 3 QTLs identified, only 1 (63 cM) was previously reported in literature for the Ayrshire Breed, Swedish Red and White Breed and Holstein. It is interesting to note that the same QTL was

mapped in these breeds for SCC, as in this study for the Brown Swiss Breed. Furthermore, both the Ayrshire Breed and the Swedish Red and White breed are form Northern Europe and could have different characteristics in term of resistance to mastitis and low somatic cell count in respect to the Holstein breed.
Even if without any confirmation on the exact QTL position, other QTLs have been previously mapped in the QTL region spanning between 0 and 51 cM.
No confirmation has been found for the QTL region identified between 101 and 118 cM and the related QTL at 113 cM that appears to be unique for the Brown Swiss breed.

**Chromosome 9**
Although none of the 2 QTLs identified on this Chromosome was previously reported, many of the QTLs in databases are located within the 2 QTL regions respectively spanning from 0 to 40 cM and from 55 to 95 cM.

**Chromosome 10**
The QTL region identified between 94 and 105 cM and the associated QTL at 104 cM was not previously reported in other studies and seems to be characteristic of the Brown Swiss population.

**Chromosome 11**

Even if the QTL location here identified at 82 cM on chromosome 11 is not exactly reported previously, all the 5 QTLs reported in the Cattle QTL db as associated with somatic cell score, have been mapped with the QTL region, spanning from 60 to 108 cM as identified in this study.

**Chromosome 12**

Among the QTLs published in the 2 considered databases, 2 map at 83.55 and 102 cM respectively.
Even if the location reported is different from the one found here both these QTLs are located in the QTL region identified in this study between 58 and 106 cM. The other QTLs were not reported previously in other studies

**Chromosome 13**

The QTL indentified in this study on BTA 13, was not previously reported and seems to be characteristic of the Brown Swiss population.
The 3 QTLs reported in databases are located within QTL region spanning from 0 to 85 cM identified in this study.

**Chromosome 15**
The QTL here mapped at 23 cM in the Brown Breed was previously reported in the Holstein breed for clinical mastitis.

**Chromosome 17**
This study locates a QTL at 86 cM, position not previously reported. Nevertheless on BTA 17 literature report a QTL lying in the QTL region here located between 15 and 97 cM.

**Chromosome 18**
None of the 3 QTLs locations mapped on chromosome 18 was previously reported from the 2 QTL databases used for comparison. Four among the 9 QTLs previously identified on this BTA mapped in 2 QTL regions identified in this study and specifically the one spanning from 0 to 15 cM and the one spanning from 26 to 59 cM.

**Chromosome 19**
The QTL mapped at 17 cM on this chromosome has been previously mapped in the Holstein Breed. All the other 3 QTLs mapped in previous studies and reported in the QTL databases are located in

the QTL region identified in this study between 0 and 86 cM.

**Chromosome 20**

All the 3 QTLs reported in the QTL databases and previously mapped on BTA 20 are located in the QTL region identified in this study between 12 and 54 cM.

No other study reports the QTL at 33 cM that appears to be unique for the Brown Swiss breed.

**Chromosome 21**

Among the 6 QTLs previously mapped and published in the QTL databases, only 1 doesn't lie in the QTL region identified in this study and spanning from 0 to 60 cm.

The QTL identified in this chromosome at 36 cM has not been previously reported and seems to be characteristic of the Brown Swiss population.

**Chromosome 23**

The QTL found on this chromosome at 75 cM has been previously mapped in the Holstein Breed for somatic cell score and in an unspecified breed from Sweden for somatic cell count.

**Chromosome 24**

Two QTLs have been previously reported on this chromosome as associated with somatic cell score and both of them confirm the findings in this study. The first QTL was mapped in the Ayrshire Breed and lies in the QTL region identified by this research between 13 and 66 cM. The second QTL was previously mapped in the Holstein Breed in the same location of the one here mapped at 45 cM.

**Chromosome 26**

Among the QTLs identified in previous studies on this BTA, only 1 is located in the QTL region identified in this research and spanning between 0 and 19 cM.

QTL mapped at 6 cM and the QTL region identified between 34 and 50 cM and the corresponding QTL at 43 cM have not been mapped previously and may be loci segregating exclusively in the Brown population that has not been used in any previous mapping study.

**Chromosome 28**

The only one QTL previously identified in other studies as associated with somatic cell score, map in same location as in this study at 49 cM.

No other QTLs from previous researches has been found to be located in the QTL region here identified between 0 and 25 cM and the corresponding QTL at 15 cM.

**Chromosome 29**

One QTL reported on the databases and identified in the Ayrshire Breed as associated with somatic cell score, is located in the QTL region identified in this study between 0 and 9 cM.

QTL mapped at 0 cM and the QTL region identified between 28 and 45 cM and the corresponding QTL at 40 cM have not been mapped previously and may be loci segregating exclusively in the Brown population that has not been used in any previous mapping study.

## Genes and genes expression

In the Database of cattle candidate genes and genetic markers for milk production and mastitis (Ogorevc et al. 2009) a list of genes tested for association with mastitis traits is reported.

Among the 10 genes listed, 2 are located very close to 2 QTLs mapped in this study.

The TLR4 gene (*toll-like receptor 4*) is located on BTA 8 between 112,418,500 and 112,446,100 base pair (bp).

Using the pb/cM relation suggested Cockett et al. (1994), 1cM corresponds to 1,000,000 and so 112,418,500 is approximately equal to 112.42 cM.
In the same chromosome a QTL at 113 cM has been mapped within this study.
The TLR4, a receptor located on cell surface, recognizes a broad class of pathogen-associated molecular patterns activating innate and adaptive immune responses, and playing an important role in pathogen defense (Wang et al. 2007).
It mainly recognizes the molecular patterns presented by lipopolysaccharides that compose the Gram-negative bacteria cell wall and induces the over expression of the interleukin inflammatory factors, which participate in innate immune responses conferring disease resistance (Shizuo et al. 2001).
The QTL found in this study very close to the TLR4 gene, didn't find any correspondance in the QTL databases. Also, in a recent study performed on the Norwegian Red cattle breed (Opsal et al. 2008) and specifically aimed to test the association between markers in the TLR4 region and clinical mastitis, the authors found no significant association in this region for this particular breed.

Conversely, Sharma et al. (2006) found significant association of TLR4 polymorphisms with somatic cell score.

The NOD2 gene (*nuclotide-binding oligomerization domain 2*) is located on BTA 18 between 18,141,400 and 18,148,000 bp, corresponding approximately to 18.14 cM. On the same chromosome a QTL at 17 cM has been mapped in this study.

In order to be able to activate an efficient immune response against a wide variety of pathogens, the host must possess receptors that recognize conserved molecular patterns associated with different classes of pathogens. This recognition leads to the secretion of cytokines and chemokines, recruiting neutrophils and monocytes to the site of the infection where they contribute to the host inflammatory immune response.

The NOD2 is a cytosolic protein able to recognize pathogen associated molecular patterns and specifically a peptide that is commonly present in the cell wall of Gram-positive and Gram-negative bacteria (Pant et al. 2007).

As for the QTL mapped on BTA 8, also the one mapped on BTA 18 didn't find any confirmation in the QTL databases, although it is very close to the NOD2 gene.

Discrepancy between the results could be due to the different breeds used in these studies suggesting that TLR4 and NOD2 genes may influence mastitis resistance in some breeds and not in others. As in the Holstein cattle the linkage disequilibrium extends over large distances (Vallejo et al. 2003), it was supposed (Sharma et al. 2006) that the association found between markers in the TLR4 and somatic cell score may be due to linkage with other gene(s) that truly influences this trait.
It is therefore important to take into account the complexity of the mastitis disease and the numbers of factors (genetic, environmental, related to causative pathogens) that contribute to the immune response.

Genes with expression patterns associated with mastitis are also listed in the database of cattle candidate genes and genetic marker for mastitis.
In this study, we found 7 QTLs located very close to genes that are expressed during mastitis in different biological pathways.
One of these genes is the already mentioned TLR4, for which it has been showed, in studies performed on cattle (Goldammer et al. 2004) and mouse model (Zheng et al. 2006), that the severity of the infection is correlated with an increased abundance of

messages for this specific receptor directly involved in the inflammatory response.
In this same process (inflammatory response) is also involved the gene CCL7 (*chemokine (C-C motif ligand 7*) located at 15 cM on BTA 19 near the QTL mapped in this study at 17 cM. Expression of this gene has been reported to be significantly associated, in mouse model, not only with the inflammation response to mastitis but also with the chemotactic process (Zheng et al. 2006): this gene, in fact, together with others having similar activity, is induced during mammary gland infection and contributes to the influx of inflammatory cells (particularly neutrophils) into the udder.
In this study a QTL at 96 cM on BTA 6 has been mapped: this QTL is located very close to the TACC3 gene (*transforming acid coiled-coil containing protein 3,* 94cM) whose expression in mouse model has been showed to be associated with mastitis in the biological process of cell proliferation (Zheng et al. 2006) encoding protein kinases and trascription factors that are associated with the regulation of the cell cycle.
Another gene involved in two different biological processes activated during mastitis infection is the CEBPB (*CCAAT/enhancer binding protein (C/EBP), beta*), located at 83 cM on BTA 13, near the QTL

mapped in this study at 85 cM. This induced transcription factor is involved in the cytokine biosynthesis, the process having statistically the larger relative amounts of gene expression changes (Zheng et al. 2006) and in the apoptosis, necessary to maintain the efficiency of he immune system.
It has been observed in mouse model that this gene is responsible for the induction of the acute-phase proteins in the mammary gland through the pathway of 2 interleukins. The role of the acute-phase proteins is not clear, but it seems they are involved in the leukocyte attraction (Badolato et al. 1994).
The AHNAK gene (*AHNAK nucleoprotein*), involved in cell differentiation, belongs to the class of genes encoding for growth and differentiation factors. This gene is located approximately at 42 cM in BTA 29, close to the QTL mapped in this study at 40 cM. A study comparing mammary gland expression patterns of cows with and without clinical mastitis (Schwerin et al. 2003) showed that this gene has an expression patterns associated with mastitis.
All the above-mentioned genes encode for proteins activating specific and well-regulated chain reactions to counteract the infection. Other genes have been found to have, during mastitis, a repression function on several biological processes as the potassium

transport, the main pathway of carbohydrate metabolism or the fatty acid metabolism.
One of these genes is the OXCT1 (*3-oxoacid CoA transferase 1*), located approximately at 35 cM on BTA 20. On the same chromosome at 34 cM, a QTL associated with somatic cell count has been mapped in this study.

There is no available literature about mapping QTL for SCC, somatic cell score or mastitis in the Brown Swiss Breed: more than 60% of the QTL listed in 2 bovine QTL databases considered is mapped in the Holstein, 18% on unspecified breeds (or generally indicated as French or Swedish dairy cattle breed) and the remaining 22% on Breed from North Europe (Ayrshire, Danish Red, Swedish Red and White, Normande and Montbeliarde breeds).
For this reason it was not expected that the findings of this research completely confirm QTL mapped in previous studies. In fact, it has been reported that the breed can play an important role in resistance to mastitis and in particular breeds from central Europe (including the Brown Swiss breed) have shown to have lower somatic cell count and low frequency of clinical mastitis than the Holstein breed (Rupp and Boichard, 2003). This occurred also for productive traits mapped in the same breed

(Bagnato et al., 2008) where novel QTL were identified mapping in the Brown Swiss population

# CONCLUSIONS

Continuous monitoring of mastitis, and its careful management is essential for the health of dairy herd.

This can be achieved through the detection of disease at its early stages and, subsequently, the treatment of the mastitis infection.
Traditional and well-established tests for mastitis detection include SCC and culture-based methods. However, the development of novel analytical platforms incorporating enzymatic assays, immunoassays, biosensors and nucleic acid tests are progressively replacing the more conventional methods.
Also, with advances in proteomics and genomics, new biomarkers are being discovered, allowing the disease to be detected at earlier stages and more resistant animals to be selected.

Results of this study will integrate the knowledge available about markers associated with milk quality traits and their use in markers and genes assisted selection.
Selection of individuals using markers and genes information will increase the efficiency of the selection schemes and will permit to improve the

animal health and welfare and the dairy products quality both in term of food safety and nutritional aspects.

The emphasis dedicated to productive traits, if not balanced with functional traits (e.g. resistance to mastitis) in the selection indexes, should cause animal health decline: the possibility to select individuals resistance to the more common pathologies (e.g. mastitis), will also increase the animal welfare in the farms.
Animals having higher resistance to mastitis will do not need to be treat with antibiotics guaranteeing that milk products will not have residuals: this will also meet the requirements of the consumers that are now aware on health and safety food products and animals welfare.

The milk production sector is of prime importance for the Italian agronomic system. All the stakeholders involved in this sector (genetic improvement and selection industry, breeders associations, farmers, milk processing industry and consumers) will then benefit of the results of this study that integrate other researches performed in the same field and will make possible the improvement of dairy cattle selection to obtain a

more efficient and competitive milk production system.

The tools available for the genomic selection may provide a further evidence of the QTL mapped in this study. So far genomic information is available on sires and GWAS studies are based on the male population. The findings of this study are based on a female population thus an independent sample that can confirm the region possibly highlighted by dense marker (SNP) chips.

The research here described will proceed with studies on milk somatic cell count and other milk quality traits (e.g. milk fatty acids composition) using the Illumina Bovine SNP chips, array at medium (50K) and high (750K) density of SNPs.

# APPENDIX

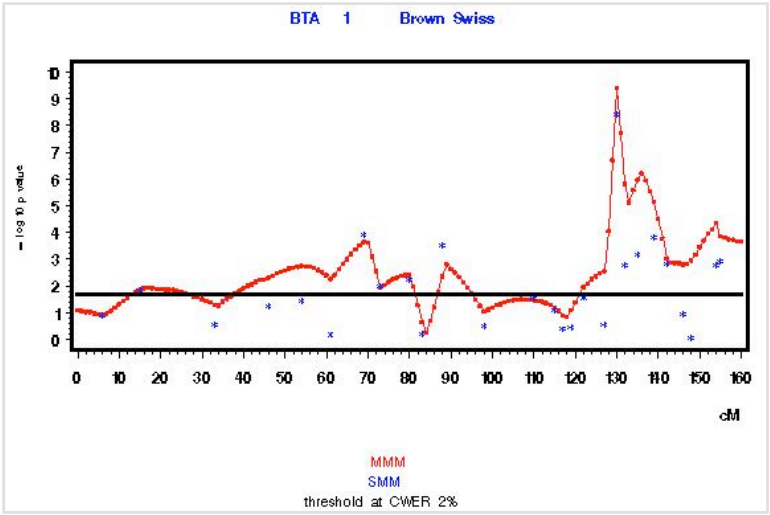


FIGURE 1: MMM results on BTA 1

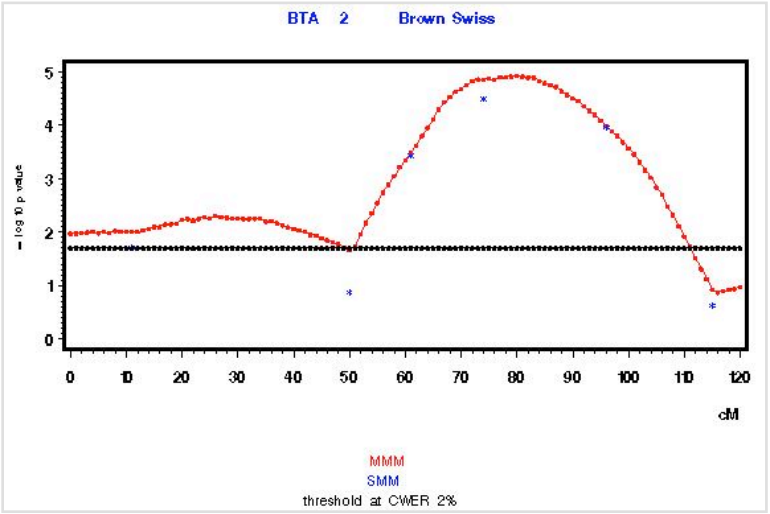


FIGURE 2: MMM results on BTA 2

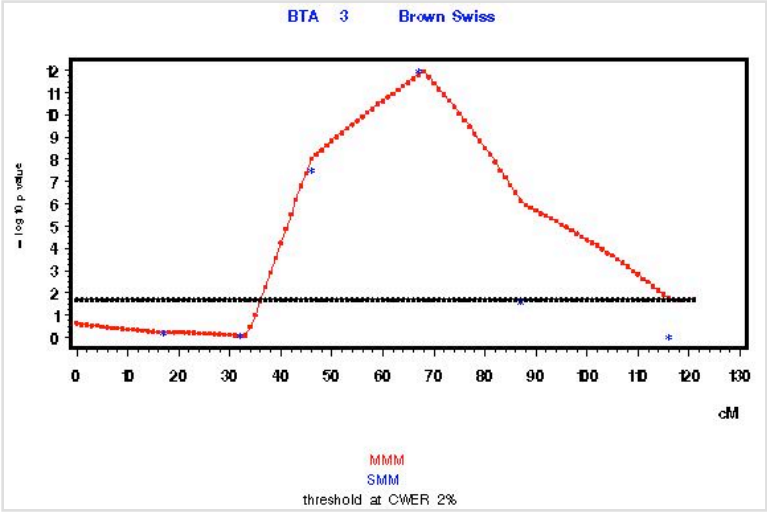


FIGURE 3: MMM results on BTA 3

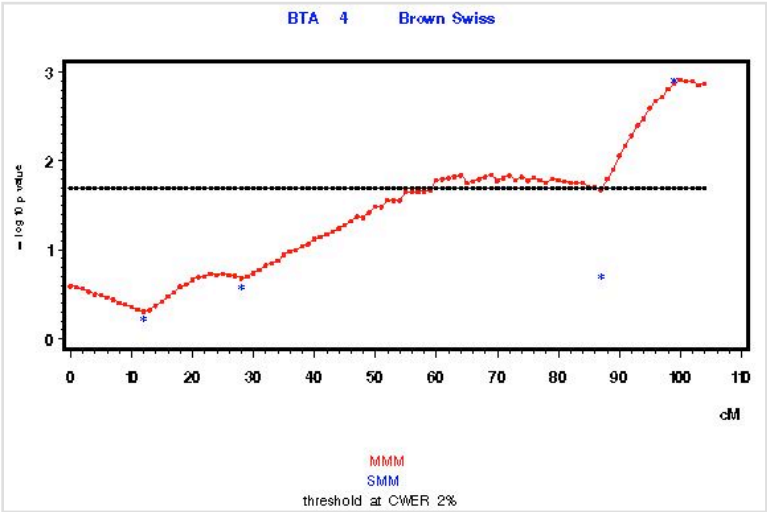


FIGURE 4: MMM results on BTA 4

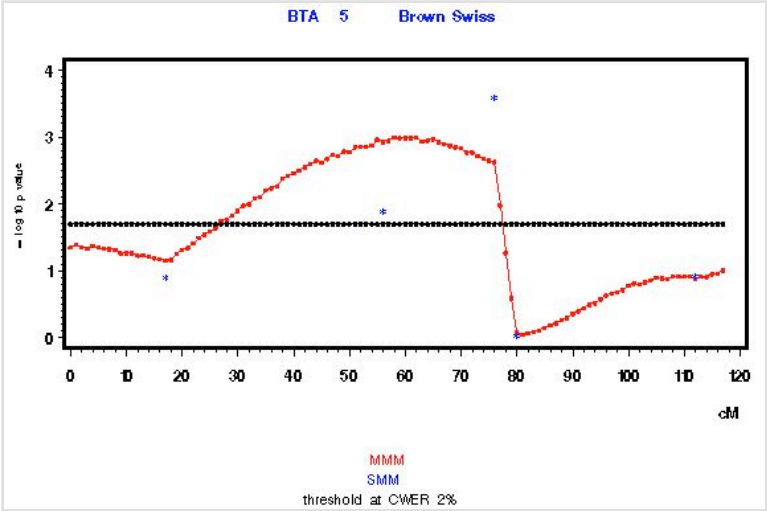


FIGURE 5: MMM results on BTA 5

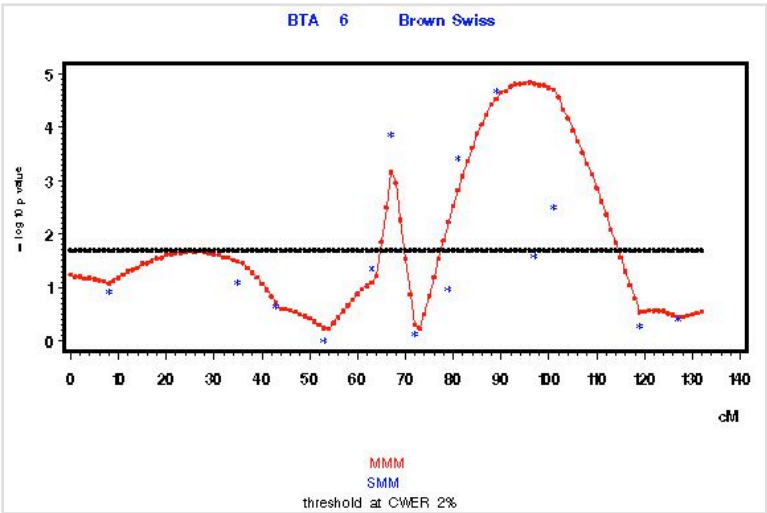


FIGURE 6: MMM results on BTA 6

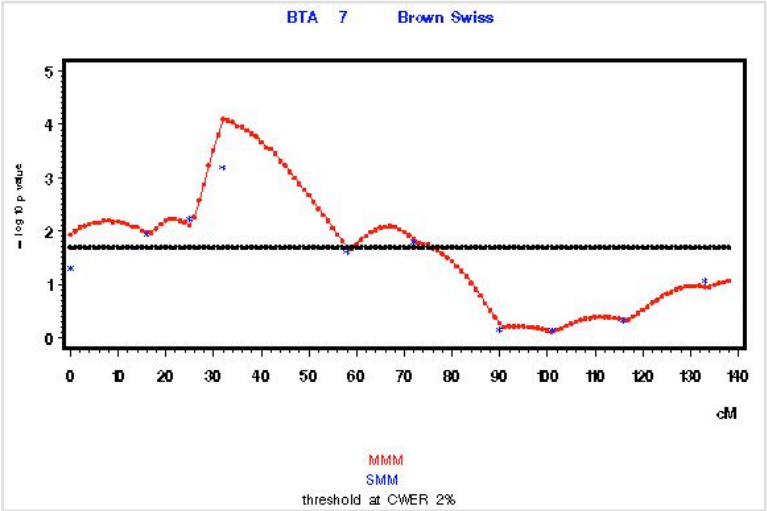


FIGURE 7: MMM results on BTA 7

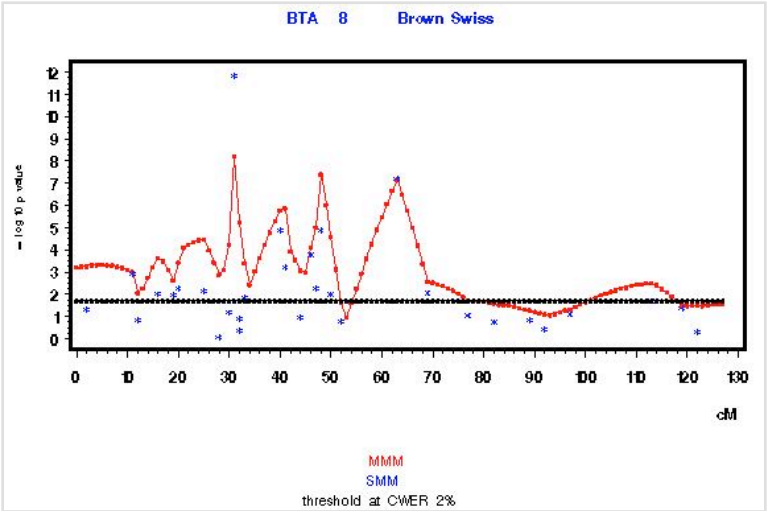


FIGURE 8: MMM results on BTA 8

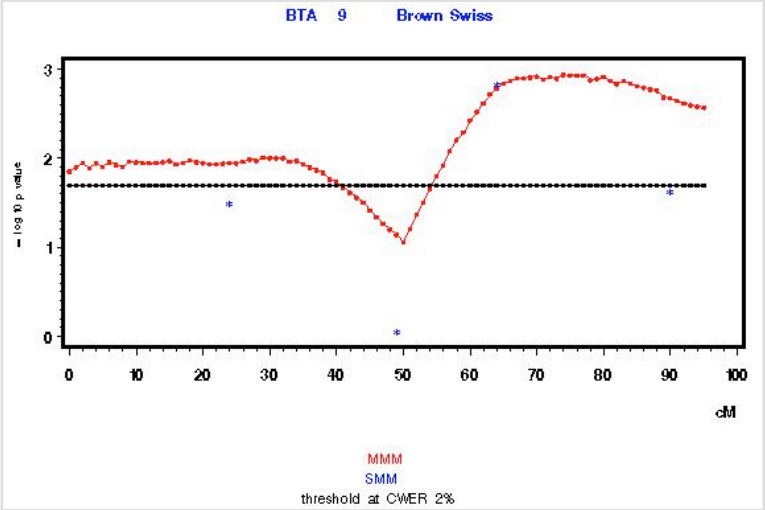


FIGURE 9: MMM results on BTA 9

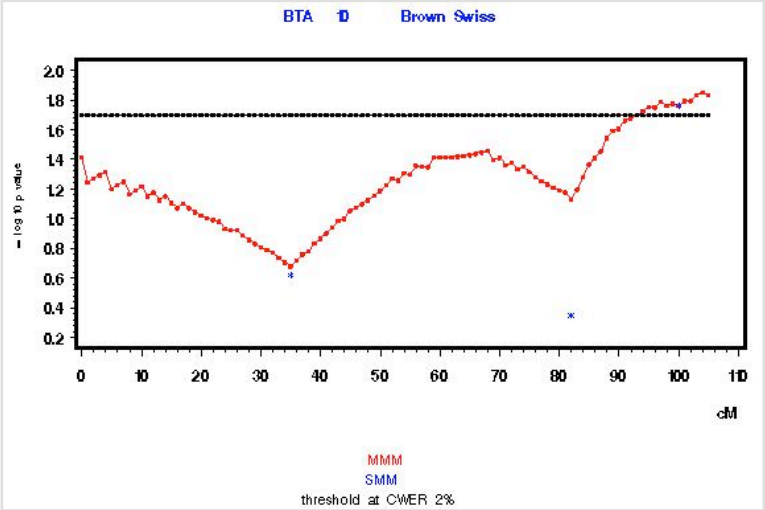


FIGURE 10: MMM results on BTA 10

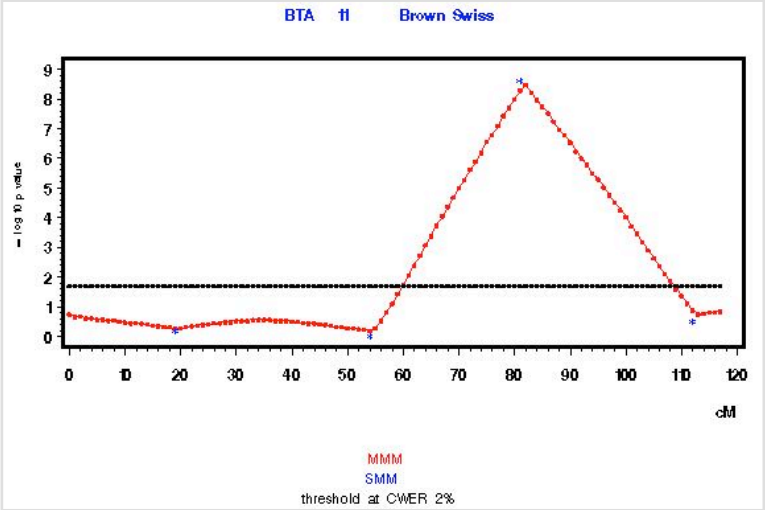


FIGURE 11: MMM results on BTA 11

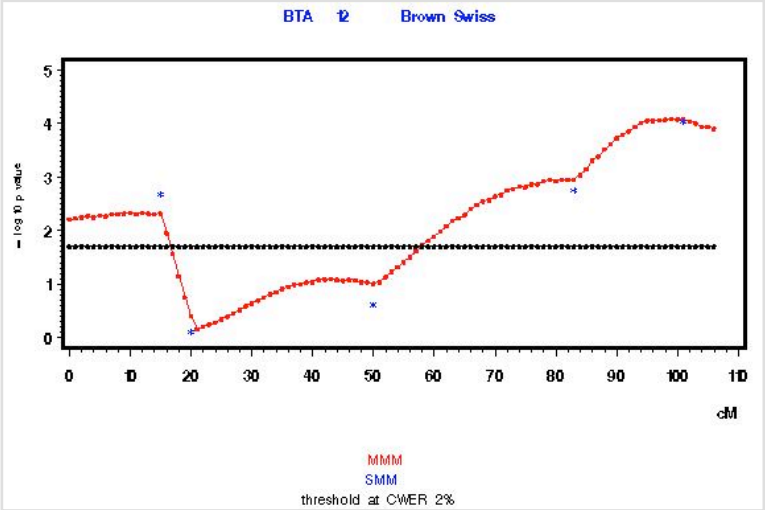


FIGURE 12: MMM results on BTA 12

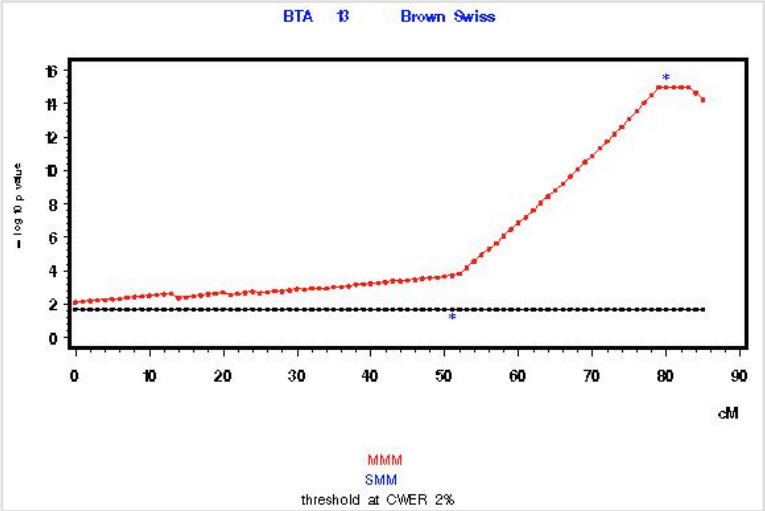


FIGURE 13: MMM results on BTA 13

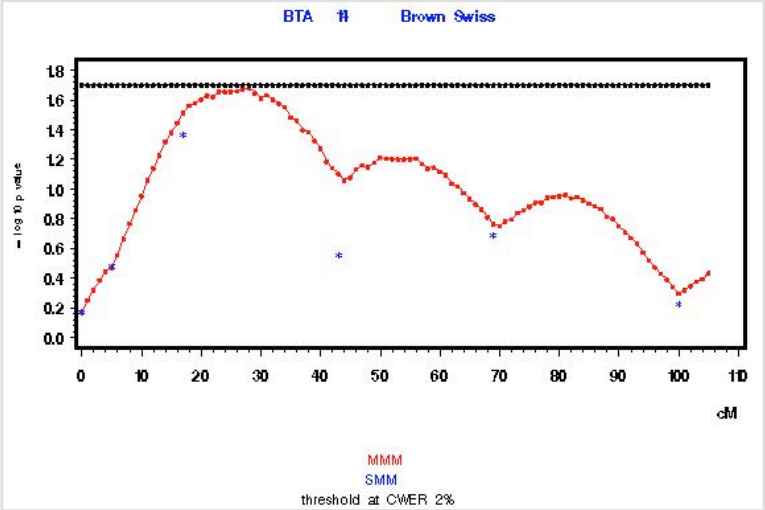


FIGURE 14: MMM results on BTA 14

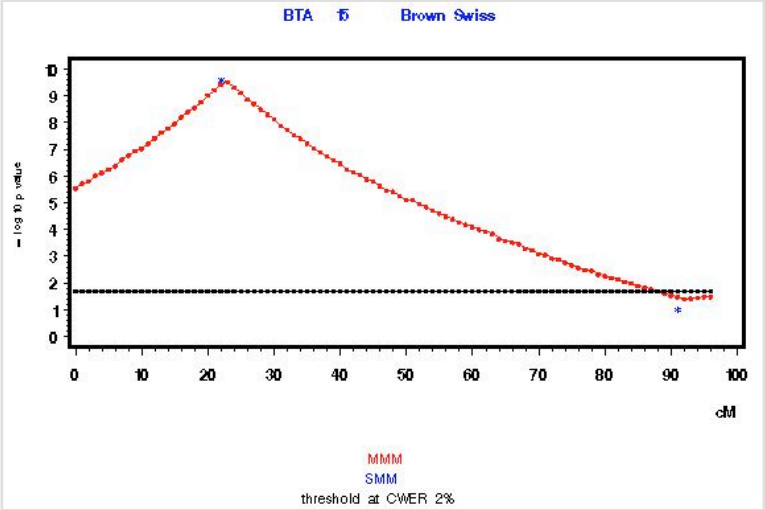


FIGURE 15: MMM results on BTA 15

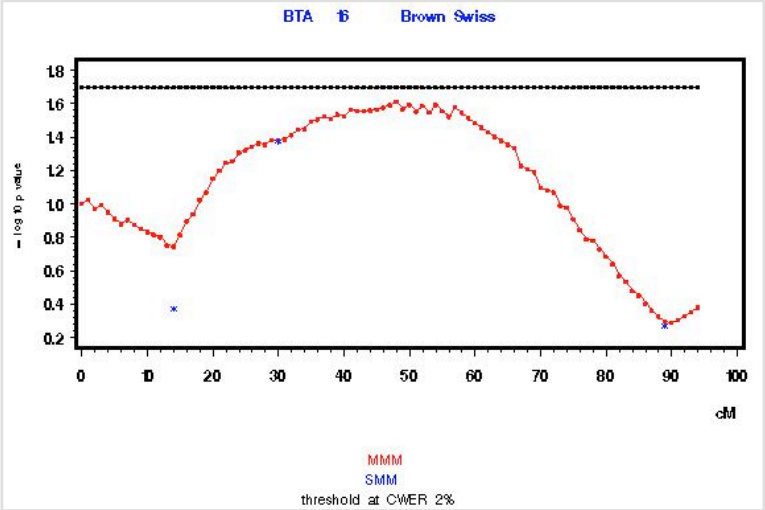


FIGURE 16: MMM results on BTA 16

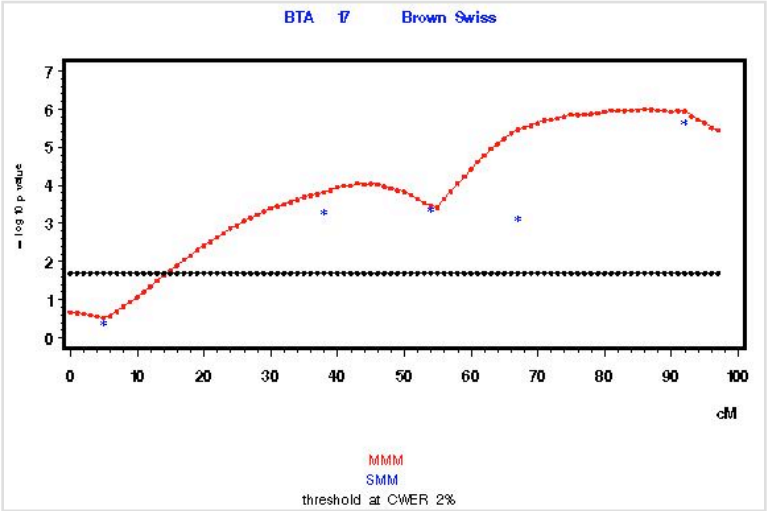


FIGURE 17: MMM results on BTA 17

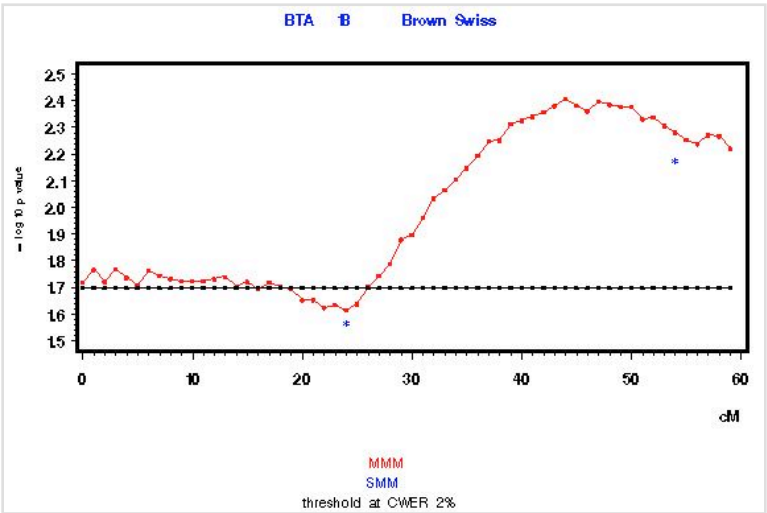


FIGURE 18: MMM results on BTA 18

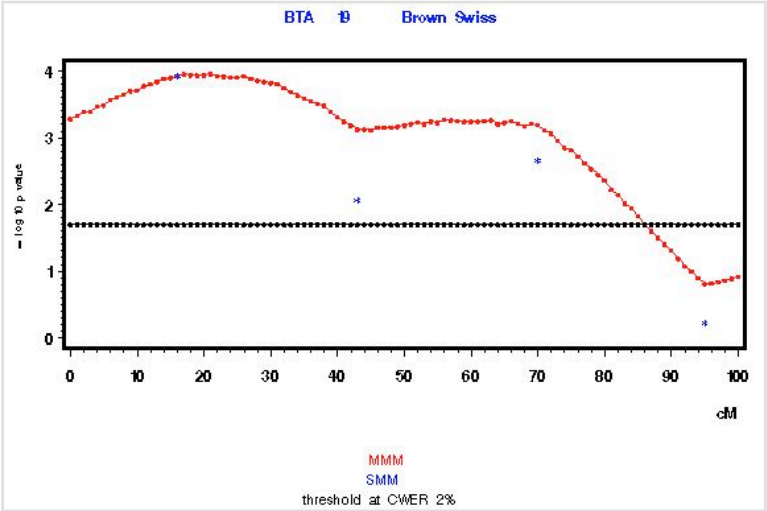


FIGURE 19: MMM results on BTA 19

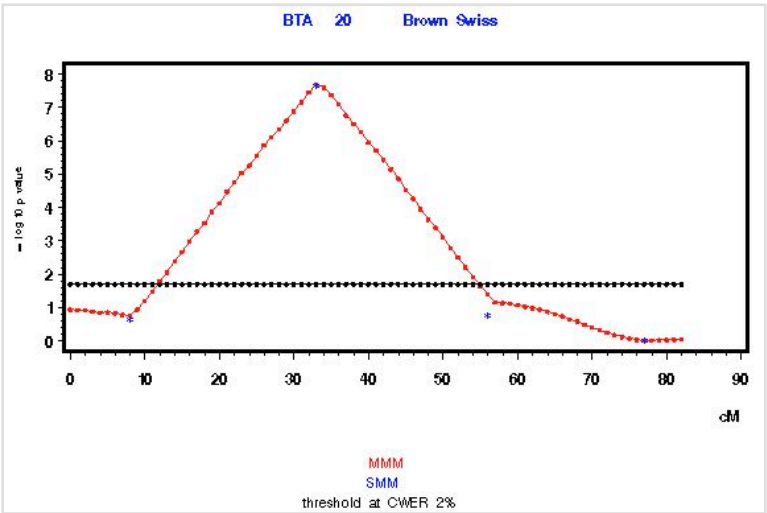


FIGURE 20: MMM results on BTA 20

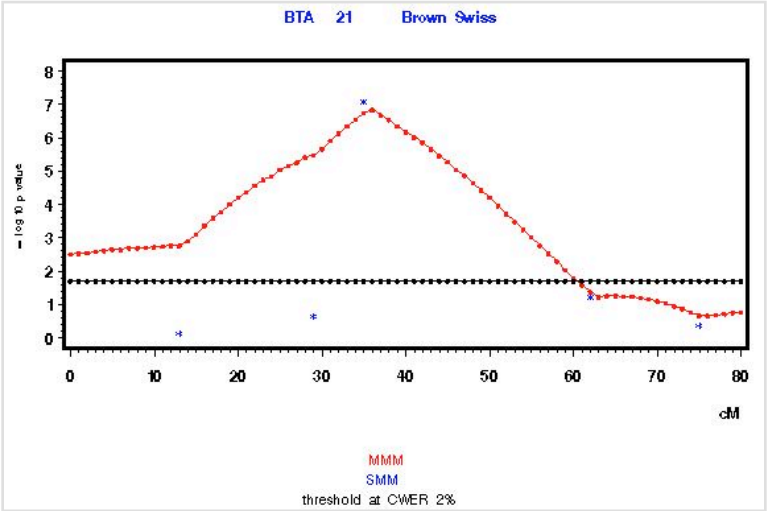


FIGURE 21: MMM results on BTA 21

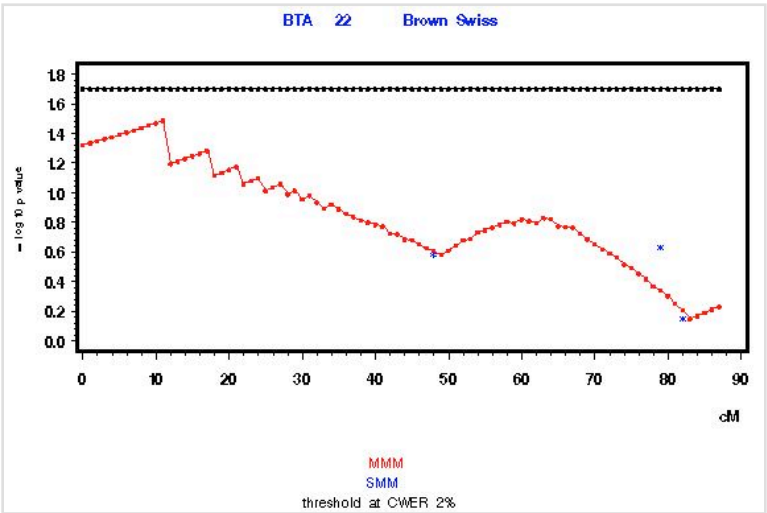


FIGURE 22: MMM results on BTA 22

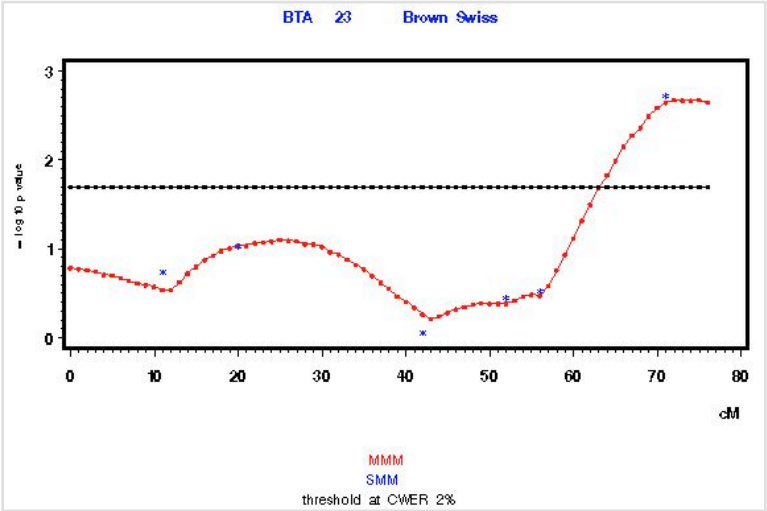


FIGURE 23: MMM results on BTA 23

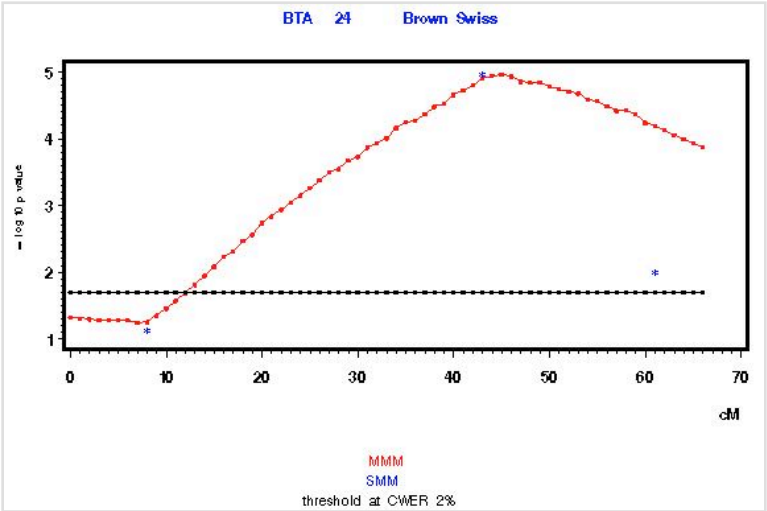


FIGURE 24: MMM results on BTA 24

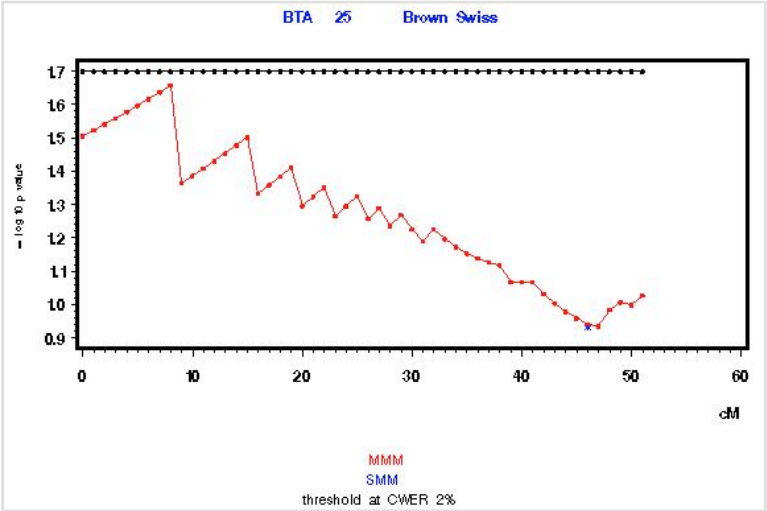


FIGURE 25: MMM results on BTA 25

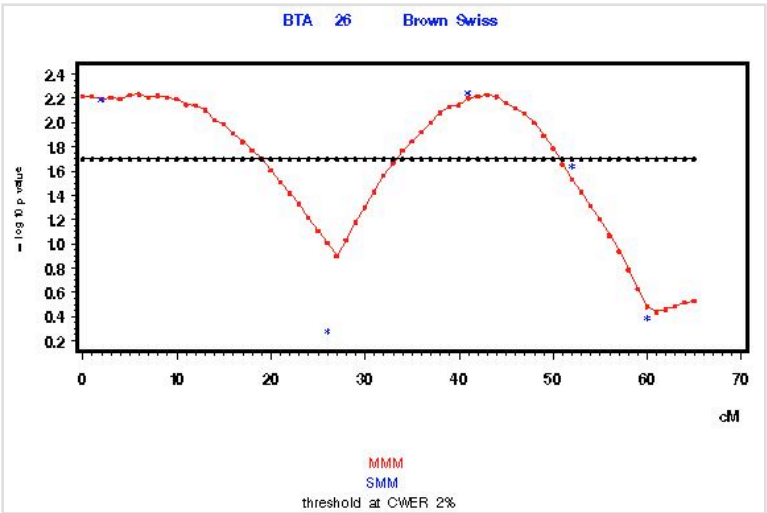


FIGURE 26: MMM results on BTA 26

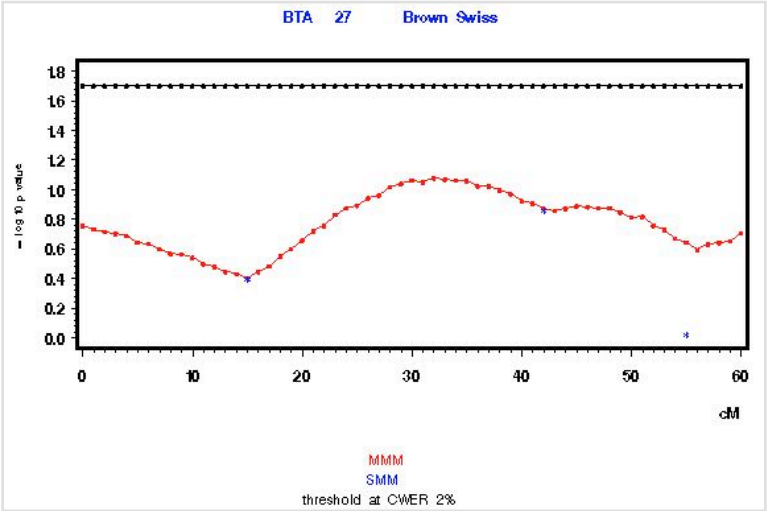


FIGURE 27: MMM results on BTA 27

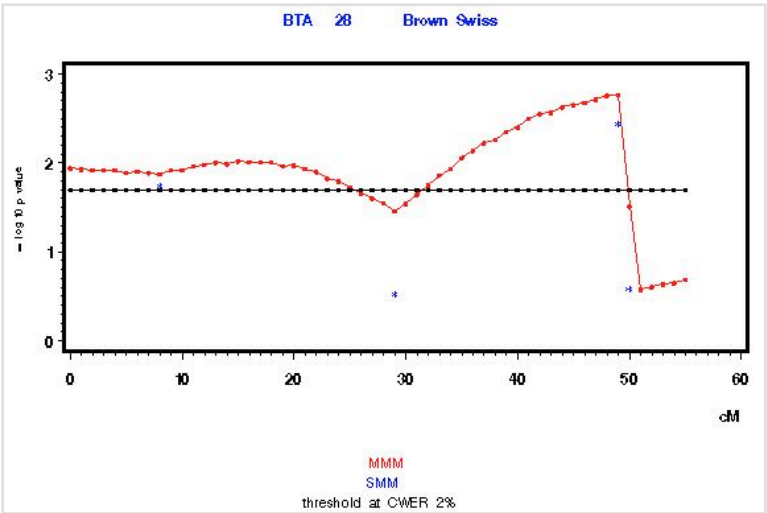


FIGURE 28: MMM results on BTA 28

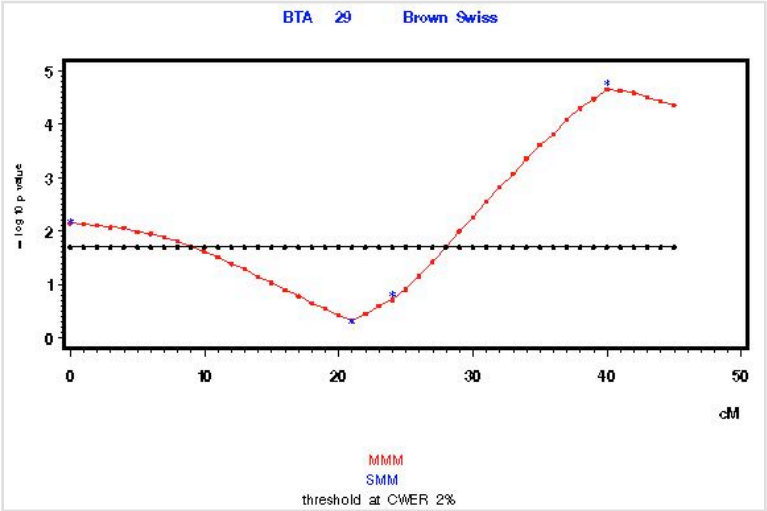


FIGURE 29: MMM results on BTA 29

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*" La perplessità è l'inizio della conoscenza."*
*(Kahlil Gibran)*

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