UNIVERSITA' DEGLI STUDI DI MILANO

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MORPHOMETRIC AND MOLECULAR STUDIES OF THE CORRELATION BETWEEN CHRONIC MASTITIS AND INFERTILITY IN DAIRY CATTLE

A THESIS

By
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Registration Nr. R 07928

Submitted to the Doctoral School in fulfilment of the requirements for the degree of
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(Chiar. mo Prof. **Fulvio GANDOLFI**)	(Chiar. mo Prof. **Fulvio GANDOLFI**)
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Faculty of Veterinary Medicine
UNIVERSITA' DEGLI STUDI DI MILANO

“Who hath created seven universes one above another: Thou seest not, in the creation of the All-merciful any incongruity, Return thy gaze, seest thou any rifts. Then Return thy gaze, again and again. Thy gaze, Comes back to thee dazzled, aweary”

Al-Quran,
(Chapter 67: Verse 3-4)

“This, in effect, is the faith of all scientists; the deeper we seek, the more is our wonder excited, the more is the dazzlement for our gaze”

To my Family, My Parents and
My Supervisor, With Love

INDEX

LIST OF ABBREVIATIONS

AI	Artificial insemination
APPs	Acute phase proteins
BHB	β Hydroxy-butyrate
BMP	Bone morphogenetic protein
CHSCC	Cumulative herd somatic cell count
CON	No clinical mastitis
CR	Conception rates
CRL	Crown-rump length
DCS	Deep cortical stroma
DF	Dominant Follicle
DFH	Days from calving to first heat
DFS	Days from calving to first service
DHI	Dairy herd improvement
DIM	Days in milk
DIPAV	Divertimento di Pathologia Animale, Igiene e Sanità Pubblica Vet.naria
DOPN	Calving to conception interval/ Days open
EGF	Epidermal growth factor
ESF	Early secondary follicle
FPAI	First postpartum artificial insemination
GC	Granulosa cell
GDF	Growth differentiation factor
GEA	GDF expressed area
GEF	GDF expressed follicles
GN	Gram negative
GP	Gram positive
IMI	Intra-mammary infection
IMM	Intra-mammary
KGF	Keratinocyte Growth Factor
LDH	Lactate dehydrogenase
LPCV	Lectin-positive capillary vessels
LSCC	Linear somatic cell count
MEC	Mammary epithelial cells
MG	Mammary gland
MG	Mastitis group
NEFA	Non-esterified fatty acids
NMC	National Mastitis Council
OSCT	Ovarian stromal connective tissue

OSE	Ovarian stromal epithelium
PMNs	Polymorphonuclear Neutrophils
RT	Room temperature
SCC	Somatic cell count
SCN	Somatic cell number
SPC	Services per conception
TGF	Transforming Growth Factor
TS	Total Score
VA	Vascular area
ZP	Zona pellucida

ABSTRACT

Dairy cow fertility has declined over the past decades even within the recent developments in functional genomics and genetic selections. There is overwhelming evidence that increasing genetic merit for milk yield considerably reduces fertility and the strength of negative associations between yield and fertility is obviously towards a rising trend. The deterioration of resistance or increased susceptibility to mastitis as well as reduced fertility in today's cow, are the examples of undesirable side-effects of the high emphasis on production traits in selection decision. Although, recent studies suggested an association between reproductive failure and mastitis in lactating dairy cows, our understanding of how mastitis affects reproduction is still limited. In this context, we investigated the effects of naturally occurring chronic mastitis on fertility particularly at the level of bovine ovarian stroma with matrix fibers, follicular growth factors, characteristic micro-architecture of stromal vasculature, as well as growth and development of follicles by macroscopic and microscopic morphological as well as immuno-detection based molecular analysis of the ovaries collected at slaughter.

Ovaries and milk samples were collected from 74 cows at slaughter. Milk samples from each quarter, were analyzed following National Mastitis Council procedures. Following macroscopic assessment, ovaries of 63 animals were scored as healthy, intermediate and pathological. Based on the presence of major pathogens and somatic cell count results, animals were sorted in 9 groups but only the two extremes were considered for further analysis: uninfected (n = 8) and affected by chronic mastitis (n = 9). Primordial, primary and secondary follicles were counted and scored on similar surface area of ovary sections for each animal (mean ± SD= 5.65 ± 0.25 cm^2). They were analyzed with Fisher's exact test and the association between health status and follicle number was estimated by odds ratios ± confidence limits. Stromal cells with matrix collagen fibres were visualized with Masson trichrome staining. Vasculature area in the ovarian cortex of healthy and mastitic animals was identified using *Bandeiraea simplicifolia*-I (BSL-I) Lectin. Results were quantified with the dedicated software MacBiophotonics image J, NIH, USA and subsequently analyzed with t-test for statistical significance. Follicles were further characterized by immunostaining with a GDF-9 specific antibody. GDF positive follicular area was measured as well as using a relative scale its fluorescence was semi-quantified.

Our results indicate no noticeable macroscopic alteration amongst various macroscopic parameters. Similarly, no ($P>0.05$) difference was observed between the numbers of primordial and primary follicles in healthy and affected animals. In contrast, the number of secondary follicles was significantly lower in sick animals (Odds ratio 10.50*; $p<0.05$), indicating a 10 times higher risk for a mastitis animal to have less than 2 secondary follicles per square centimeter. In the healthy and mastitic animals the stromal collagen were (1.61±0.90% vs. 6.046±1.85% $P<0.001$), indicating mastitis is linked with stromal alterations. Ovarian stromal vasculature represented the cortical area of 6.38±0.66 % in healthy vs. 4.24±0.37%; ($P<0.001$) in affected cows. Likewise, reduced GDF-9 expressed cortical area, healthy vs. sick (1.97±0.83% vs. 1.04±0.11%; $P<0.05$), with less immuno-fluorescence was observed in mastitic animals.

Our results show that chronic mastitis is associated with considerable alterations in follicle growth and differentiation with a decreased ability of primary follicles to develop into the secondary state in affected animals. This is accompanied by a significant increase of stromal collagen with reduced vasculature and the down regulation of the follicle differentiation factor GDF-9. All together, these findings substantiate the hypothesis that mastitis can reduce fertility by exerting a negative effect on ovarian function.

بِسْمِ اللهِ الرَّحْمٰنِ الرَّحِيْمِ

"In nome di Dio, il Compassionevole, il Misericordioso."

01. INTRODUCTION

A. MASTITIS

1.1 BRIEF OVERVIEW

The dairy industry worldwide has achieved many significant advances over the last fifty years, with knowledge-based genetic selection and optimized nutrition playing important roles in the delivery of increased milk yields of improved quality in most breeds. Obviously, the confrontations associated with achieving pregnancy in modern, high-producing dairy cows have received considerable attention from scientists, veterinarians, and farmers in recent years. Nevertheless, today's dairy cows tend to have lower fertility and greater likelihood of culling due to infertility than their counterparts from two or three decades ago. Infertility or sub-fertility is the varying degrees of aberrations from typical levels of reproductive performance. In fact, the poor reproductive performance is multifactorial and largely undeceived. However, mastitis has been implicated for one of its etiology and remains as a foremost challenge to the global dairy industry regardless of the extensive implementation of strategies.

Bovine mastitis, an inflammatory disease of the mammary gland, can have a broad spectrum of infectious or noninfectious etiology. The disease can occur in any lactating animals and even non-lactating animals can harbor this infection. Organisms as diverse as vast majority of the bacteria (Gram positive & negative), mycoplasma, yeasts and algae have been implicated to successfully establish the disease within the bovine mammary gland (Pyorala, 2003). *Escherichia coli, Staphylococcus aureus, Streptococcus dysagalactie, Streptococcus agalactiae* and *Streptococcus uberis* are the most common to cause the disease (Anon, 2001). Coagulase-negative staphylococci, *Streptococcus pyogenes*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Enterobacter spp*. are also often account for diagnoses (Table 1).

Classically, mastitis pathogens have been classified as either contagious or environmental (Blowey and Edmondson, 1995). In essence, the contagious pathogens can be considered as organisms adapted to survive within the host, in particular within the mammary gland. They are capable of establishing sub-clinical infections, which are typically manifest as an elevation in the somatic cell count of milk from the affected quarter; typically spread from cow to cow at or around the time of milking (Radostits *et al.,* 1994). In contrast, the environmental pathogens are best described as opportunistic

invaders of the mammary gland, not adapted to survive within the host; typically they invade, multiply, engender a host immune response and are rapidly eliminated. The major contagious pathogens comprise *S. aureus*, *Str. Dysgalactiae* and *Str. agalactiae*; the major environmental pathogens comprise the Enterobacteriacae (particularly *E. coli*), *Str. Uberis* and coagulase negative *Staphylococcus* species (Bradley, 2002).

Mastitis is prevalent on dairy farms all over the world and a major cause of less efficient milk production. Often cows have mastitis without obvious clinical symptoms (increased body temperature, depressed appetite, red, warm, lumpy or swollen mammary quarters): these subclinical infections also result in reduced milk production and elevated leukocyte counts in milk and constitutes a major source of economic loss for dairy farms due to unrealistic production potential (Hortet and Seegers, 1998; Huijps *et al.,* 2008). The disease affects milk quality directly through a change in technical and hygienic milk quality and indirectly through the intrinsic milk quality (Hogeveen and lankveld, 2002; Hogeveen, 2003). Apart from compromised milk production and altered milk composition and appearance, the disease is costly to the dairy industry through several other factors including, increased health care costs, death or premature culling of genetically superior animals, as a result limiting the choice of animals available to produce replacements and eventually limiting genetic progress (Seegers *et al.,* 2003; Holtsmark *et al*., 2008; Bell *et al*., 2010; Inchaisri *et al*., 2010).

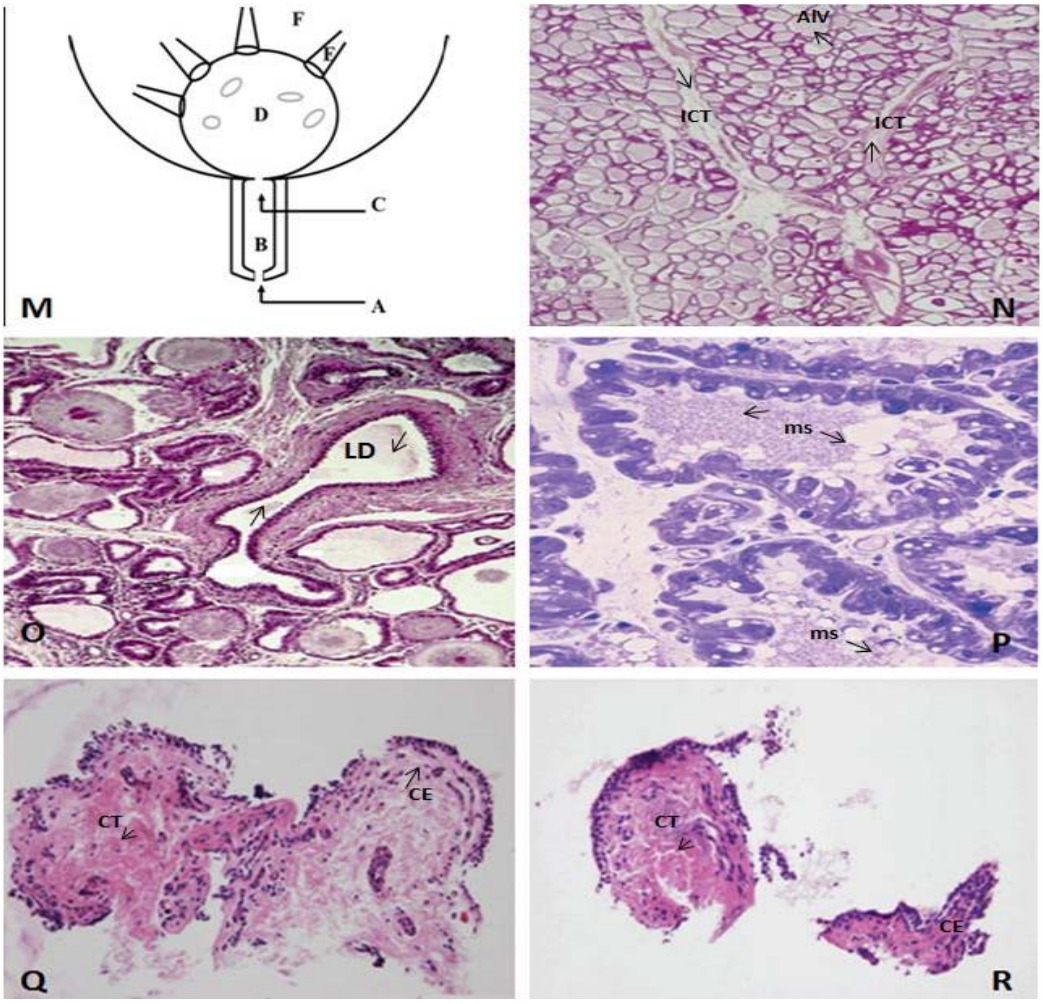

Figure 1. M) Schematic view of the anatomical structures of the lower part of the bovine mammary gland. A = teat canal, B = teat cistern, C = plica annularis mucosae (Furstenberg rosette), D = gland cistern (cistern lactiferous), E = large milk duct and F = alveolar parenchyma; where, N, O, P are the light microscopic images showing the morphology of mammary tissue with increasingly higher magnifications (original x 10, 20, 40) respectively; ALV=alveolus, ICT=interstitial connective tissues, MS=mammary secretions, LD= Lactiferous ducts. Q) Light microscopic images of teat cistern; R) Transition zone of teat and gland cistern; CT=connective tissue fibers and CE= double layered cuboidal epithelium (Vangroenweghe *et al.*, 2006; Liebich, 2006).

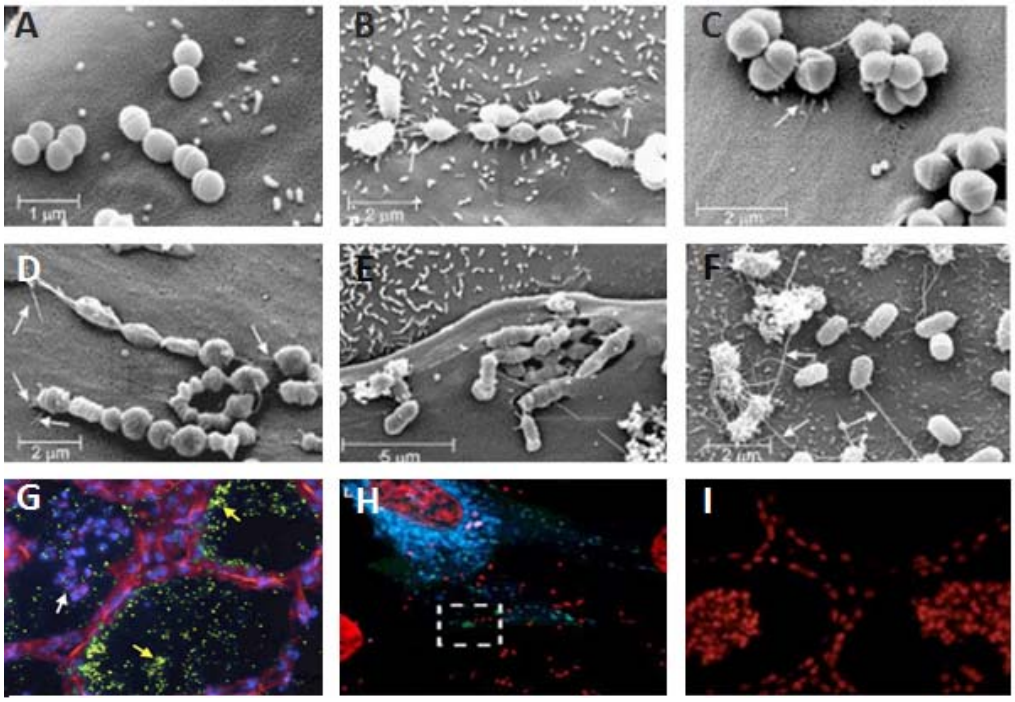

Figure 2. The interactions between the major mastitis pathogenic species and cultured bovine mammary gland cells (A-F): A) adhesion of *S. uberis* strain 7573 to cubic cells; B) adhesion of *S. uberis* strain 7572 to cubic cells; C) *S. aureus* cells adhered in grape-like clusters; D) adhesion of *S. dysgalactiae* to the elongated cells; E) *S. dysgalactiae* cells seemed to be internalized by the elongated cells; F) adhesion of *E. coli* (strain 7561) with the cubic cell type; thread-like structures connecting the bacterial cells, are indicated (arrows). Confocal micrographs: G & I) propidium iodide stained nuclei of mammary alveolar epithelium with invasion of *E. coli* ; H) invasion of persistent mastitis *E. coli* strain LT2649-M in the bovine mammary myoepithelial cells *in vitro* (Passey *et al.,* 2008; Lammers *et al.,* 2001).

Classification	Bacteria	Contagious or Environmental	Source	Spread
Staphylococcus spp.	*Staphylococcus aureus*	Contagious	Infected udders, hands of milkers	Milking time
	Coagulase negative *Staphylococcus* & *S. hyicus*	Neither	Skin flora & occasionally environment	Infect teat canal from skin sources
Streptococcus spp. and *Enterococcus* spp.	*Strep. agalactiae*	Contagious	Infected udders	Milking time
	Strep. dysgalactiae	Contagious and environmental	Infected udders and environment	Milking time & environmental contact
	Strep. uberis	Environmental	Environment - early dry period	New IMI during early dry period
	Environmental *Streptococcus* spp.& *Enterococcus* spp.	Environmental	Environment	Environmental contact
Gram negatives	*Escherichia coli*	Environmental	Bedding, manure, soil	Environmental contact
	Klebsiella spp.	Environmental	Organic bedding	Environmental contact
	Enterobacter spp.	Environmental	Bedding, manure, soil	Environmental contact
	Serratia spp.	Environmental	Soil and plants	Environmental contact
	Pseudomonas spp.	Environmental	Water & wet bedding	Environmental contact
	Proteus spp.	Environmental	Bedding, feed & water	Environmental contact
	Pasteurella spp.	Probably contagious	Upper respiratory tract of mammals and birds	Unknown – likely cow to cow
Other	*Mycoplama bovis*	Contagious	Respiratory system	Milking time & environmental contact
	Yeast & mold	Environmental	Soil, plants, water	Dirty infusions
	Corynebacterium bovis & other coryneforms	Contagious	Infected udders	Cow to cow
	Prototheca	Environmental	Soil, plants, water	Dirty infusions, infected udders
	Bacillus spp.	Environmental	Soil, water, air	Dirty infusions
	Arcanobacterium pyogenes	Contagious/Environmental	Teat injuries	Flies
		(IMI, intramammary infection)		

Table 1. Lists of major bacteria and other microbes causing mastitis, potential infection sources as well as mode of transmission in cow (Petersson and Currin, 2010).

1.2 DEVELOPMENT OF MASTITIS

Recent scientific developments, specifically in the areas of genomics and proteomics at host and pathogen level, provide exponentially growing opportunities for a deepening of our understanding of mastitis pathogenesis; and evidently, a wide-ranging comprehension of the pathogenicity could lead to potential therapeutic applications and the identification of biomarkers for the diagnosis and predicting the consequences of the mastitis episodes.

In brief, the teat canal is normally tightly closed by sphincter muscles, preventing the entry of pathogens. It is lined with keratin, a waxy material derived from stratified squamous epithelium that obstructs the migration of bacteria and contains antimicrobial agents, such as long-chain fatty acids, that assist in combating the infection. However, the efficiency of keratin is restricted (Craven, 1985; Capuco *et al*., 1992; Paulrud, 2005). Fluid accumulates within the mammary gland as parturition approaches, resulting in increased intramammary pressure (Paulrud, 2005) and mammary gland vulnerability caused by the dilation of the teat canal and leakage of mammary secretions (Sordillo and Stricher, 2002). Additionally, during milking, the keratin is flushed out and there is distention of the teat canal (Rainard and Riollet, 2006). The sphincter requires~2 h to return back to the contracted position (Capuco *et al*., 1992).

Once inside the teat, bacteria must also elude the cellular and humoral defense mechanisms of the udder (Sordillo and Stricher, 2002). If they are not eliminated, they start multiplying in the mammary gland (Figure 3). They liberate toxins and induce leukocytes and epithelial cells to release chemo attractants, including cytokines such as tumor necrosis factor-α (TNFα), interleukin (IL)-8, IL-1, eicosanoids (like prostaglandin F2α [PGF2α]), oxygen radicals and acute phase proteins (APPs) (e.g. haptoglobin [Hp], serum amyloid A [SAA]). This attracts circulating immune effector cells, mainly polymorphonuclear neutrophils (PMNs), to the site of infection (Giri, 1984; Paape *et al*., 2003; Sordillo and Daley, 1995; Zhao and Lacasse, 2008).

PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems. They contain intracellular granules that store bactericidal peptides, proteins, enzymes (such as myeloperoxidase) and neutral and acidic proteases (such as elastase, cathepsin G, cathepsin B and cathepsin D) (Owen and Campbell, 1999; Bank and Ansorge, 2001). The released oxidants and proteases destroy the bacteria and some of the epithelial cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-β-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH). Destruction of most of the PMNs takes place by apoptosis once

their task is fulfilled. Subsequently, macrophages engulf and ingest the remaining PMNs (Paape *et al*., 2002; Paape *et al*., 2003). The dead and sloughed off mammary epithelial cells, in addition to the dead leukocytes, are secreted into the milk, resulting in high milk SCCs.

If the infection persists, internal swelling within the mammary epithelium, not normally detectable by an external examination, can occur. The mammary gland alveoli become damaged and start losing anatomical integrity (Figure 3). The blood–milk barrier is breached, causing extracellular fluid components, such as chloride, sodium, hydrogen, potassium and hydroxide ions, to enter the gland and mix with the milk (Zhao and Lacasse, 2008).

When extensive damage to the blood–milk barrier has occurred, blood might be detected in the milk. This leads to visible changes on the udder, such as enhanced external swelling and reddening of the gland. Changes also occur in the milk, including increased conductivity, increased pH, raised water content and the presence of visible clots and flakes (Lee *et al*., 1980; Kitchen, 1981; Milner *et al*., 1996; Zhao and Lacasse, 2008). This marks the initial stage of clinical symptoms, and the most severe infections might ultimately result in the death of the animal.

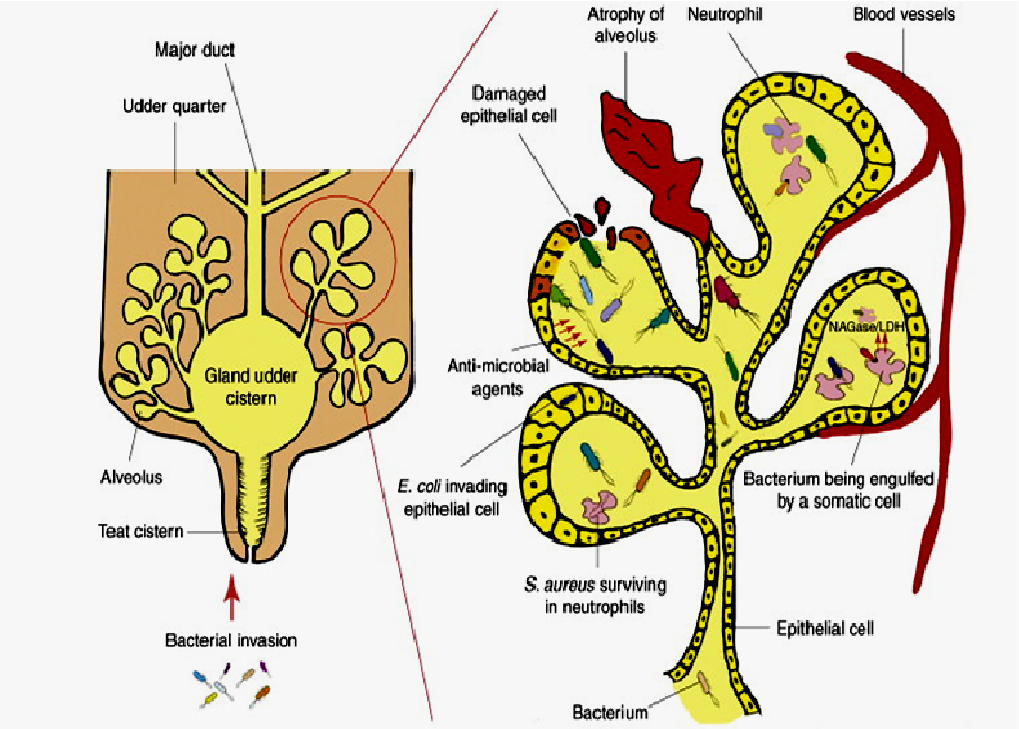

Figure 3. Schematic representation of mastitis development in an infected udder. Environmental and contagious microorganisms invade the udder through the teat cistern. They then multiply in the udder where they are attacked by neutrophils while damaging the epithelial cells lining the alveoli, with subsequent release of enzymes like NAGase and LDH. The epithelial cells also secrete anti-microbial compounds. Considerable tissue damage is observed once the immune effecter cells begin to combat the invading pathogens (Viguier *et al.*, 2009).

1.3 MAMMARY GLAND IMMUNITY AND MASTITIS

The availability of potent research tools in genomics, transcriptomics and proteomics makes possible the study of mammary gland immunity and mastitis pathogenesis in more detail and on a larger scale than it was previously. In general, immune system is characterized by its capacity to recognize and discriminate between foreign invading agents and molecules produced by the organism (Janeway and Medzhitov, 2002). The mammary gland performs a variety of immunological functions conferring protection;

even pre-partum antibodies secreted in colostrums are produced to protect the new born against infectious agents (Sordillo *et al.,* 1997). The mammary gland tissue is protected by two forms of immune defense mechanisms: innate immunity and adaptive or acquired immunity. Innate immunity has no memory, whereas the efficiency of adaptive immunity rests on memory. In fact, it is not easy to delineate immunity precisely because innate immunity is intricately enmeshed with adaptive immunity, and both systems share many effector mechanisms to provide protection against mastitis microorganisms (Sordillo *et al.,* 1997; Burvenich *et al.,* 2003; Rivas *et al.,* 2002). The efficiency of these immune responses to antimicrobial activity determines mammary gland susceptibility or resistance to infection. The boundary between local and mobilized innate defenses is drawn by inflammation, so that when resident defenses are not sufficient to contain or eradicate infection, systemic defenses are recruited to come to the rescue.

1.3.1 RESIDENT DEFENSE (TEAT CANAL BARRIER)

The teat canal is the first line of defense against mastitis since this is the route by which pathogens gain entrance to the mammary gland. This canal is sealed between milking, and during the dry period, by a keratin plug derived from the stratified squamous epithelial lining of the canal. Probably the major role of this waxy plug is to achieve a physical barrier preventing the penetration of bacteria. Keratin is able to bind and immobilize most strains of non-encapsulated mastitis-causing bacteria (Craven, 1985). Additionally, some components of the keratin such as long-chain fatty acids have microbicidal activity that assist in combating against mastitis causing bacteria. However, the efficiency of keratin is restricted (Craven, 1985; Capuco *et al*., 1992; Paulrud, 2005). The milking is a critical operation in relation to the barrier efficacy of the teat canal. Milk flushes out the keratin plug, and the teat canal is distended by the vacuum and the milk flux. The teat end contains sphincter muscles that maintain tight closure between milkings. After milking, two hours are required for the sphincter to contract and close the teat canal (Schultze and Bright, 1983).

Machine milking can have a profound influence on the integrity of the teat duct, by inducing mechanical and circulatory impairments in teat tissues (Zecconi *et al.,* 2000). Improper machine milking use or maintenance favors teat end erosion, and in the long term is likely to alter the functioning of the teat sphincter. Machine milking may also modify the immune defenses of the teat duct (Zecconi *et al.,* 2000). A healthy skin condition of the teat reduces the risk of contamination because it reduces the colonization of the teat skin by bacteria such as *Staphylococcus aureus*, which predisposes the cow to new intramammary infections. It was shown that in many cases

bacteria are present in the teat canal for weeks before causing intramammary infections, despite regular teat dipping (Preez, 1985). Long-lasting teat canal colonization shows that mastitis-causing pathogens have adapted to the teat duct milieu, and suggests that the proximal region of the teat duct may be in a position to play a special role in the immune defense of the mammary gland. It may in particular fulfill a role of sensing of and protection against invading bacteria, in relation to the presence of numerous intraepithelial leucocytes. As a result of the usual efficiency of the teat canal barrier, the intramammary lumen is an aseptic milieu. Important consequences for the immune innate defenses are likely to ensue. On the contrary to other epithelia such as the intestinal, buccal, or upper respiratory epithelia, the mammary epithelium is infrequently stimulated by bacterial components, and any bacterium must be taken as an intruder. Peculiarities of the immune equipment of the mammary gland, such as the sub- and intra-epithelial leucocytes, or the repertoire and distribution of sensor receptors on mammary epithelial cells (MEC) are likely to be conditioned by the aseptic character of normal milk. In this respect, the mammary gland resembles more the urinary system than the intestine (Rainard and Riollet, 2006).

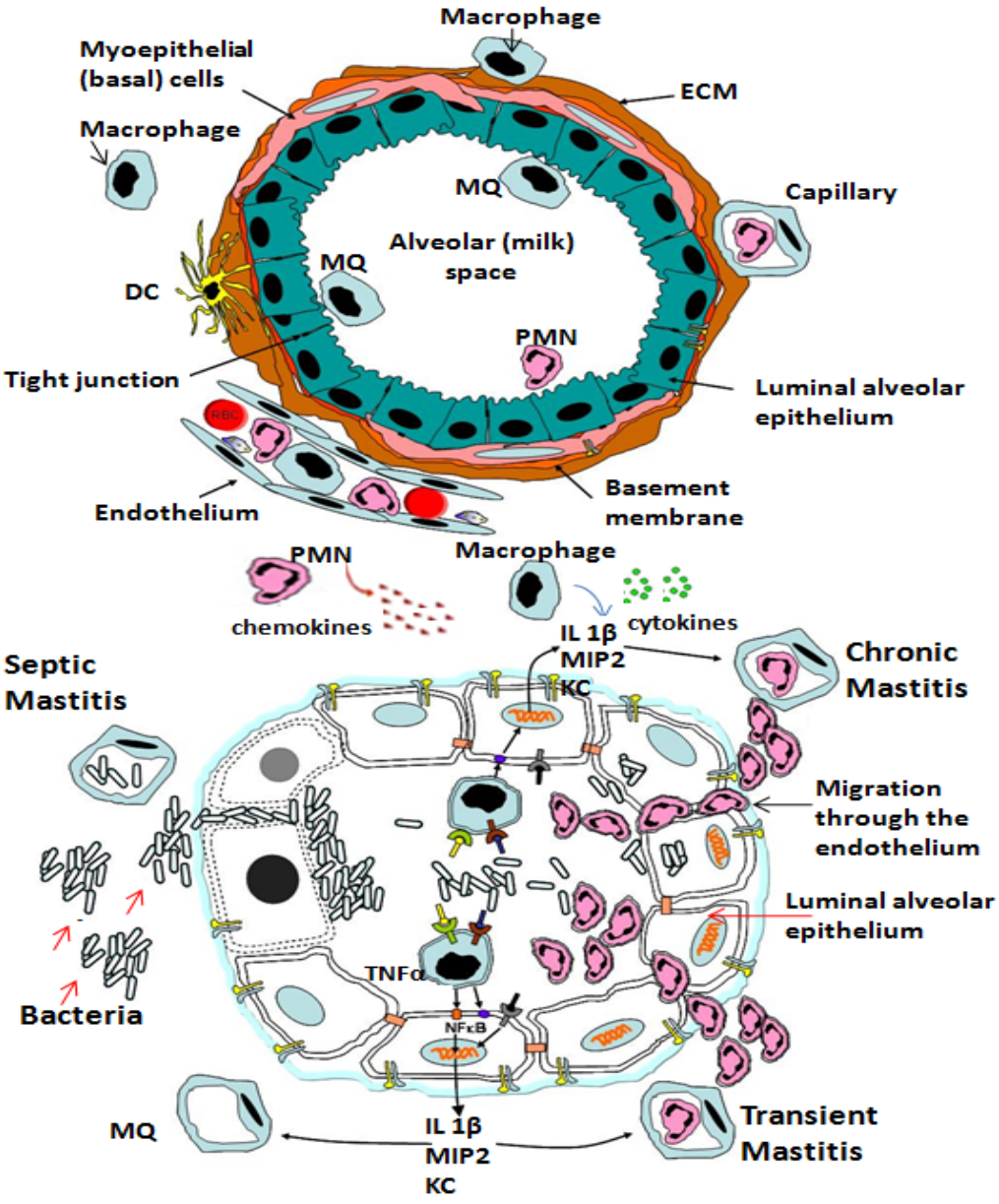

Figure 4. Schematic diagram of the bovine mammary gland alveolus showing: A) cellular and soluble factors that participate in the innate immune response of the mammary gland. Macrophages located in the alveoli phagocytize bacteria that enter the mammary gland cistern. Activated macrophages release cytokines such as TNF-α, IL-1β and IL8 (MIP2 & KC). B) Endothelial cells from blood vessels adjacent to alveoli express adhesion molecules in response to pro-inflammatory cytokines; this, in turn, facilitates neutrophil recruitment from the bloodstream to the site of infection (into the milk places) in order to eliminate the invading bacteria (modified from Elazar *et al*., 2010a).

1.3.2 CELLULAR DEFENSES

When bacteria penetrate the teat sphincter and the teat canal, the second line of defense consists of neutrophils, macrophages, natural killer (NK) cells and lymphocytes. These cells regulate both innate and acquired immune responses (Sordilo *et al.*, 1997; Sordilo and Streicher, 2002; Soltys and Quinn, 1999). To these leucocytes, another cell type should be added, the mammary epithelial cells (MEC), which is at the interface between the body and its environment. The cells which contribute to the innate defense of the udder are either tissue cells or milk cells. Milk from a perfectly healthy bovine udder should contain very few cells, since the gland is not a holocrine secretory organ. Milk cell concentrations vary widely as a function of the lactation cycle (Rainard and Riollet, 2006).

In mammary gland defense against bacterial infection, tissue or milk macrophages recognize the invading pathogen and initiate the inflammatory response. In this response, pro-inflammatory cytokines induce neutrophil recruitment to the mammary gland (Zhang and Issekutz, 2002; Rainard, 2003) (Figure 4). Healthy mammary gland tissue and milk contain mainly macrophages, whereas infected tissue and secretions contain mainly neutrophils. When macrophages recognize bacteria, they release pro-inflammatory cytokines such as TNF-α and IL-1β, stimulating the bactericidal activity of neutrophils and also producing prostaglandins and leukotrienes, which increase the local inflammatory reaction (Bannerman *et al.,* 2004; Bannerman, 2009; Boulanger *et al.,* 2003; Stein *et al.,* 2003). MG epithelial cells however, play an important role in neutrophil recruitment to the infection site. Adhesion of bacteria to epithelial cells as well as the interaction of bacterial toxins with them induces the synthesis of TNF-α, IL-6, and the chemokine IL-8 (Rainard and Riollet, 2003).

Pro-inflammatory cytokines and chemokines secreted by macrophages or epithelial cells activate in turn the expression of cellular adhesion molecules (E-selectin), intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 by endothelial cells. This causes binding of blood neutrophils to the endothelium, their migration through the epithelial and subepithelial matrix, and their further localization at the infection site or in milk. For neutrophil recruitment, participation of chemoattractive molecules such as complement components (C5a and C3a), cytokines (IL-8, IL-12) and even LPS is required (Zhang and Issekutz, 2002; Nishimura *et al.,* 2003; Abbas and Lichtman, 2003; Strindhall *et al.,* 2005). The increase in SCC ($>2 \times 10^5$ cell/ml milk)[2] observed during mastitis has its origin in this transendothelial migration and accounts for the importance of endothelial cells in the pathophysiology of mastitis.

Neutrophils recruited to the site of infection phagocytize bacteria and produce reactive oxygen species, low molecular weight antibacterial peptides, and defensins, which eliminate a wide variety of pathogens that cause mastitis (Sordillo and Streicher, 2002; Mehrzad *et al.,* 2002; Paape *et al.,* 2003). During the period following parturition, the decrease in neutrophil activity is associated with a higher incidence of mastitis (Yamaguchi *et al.,* 1999). If the invading bacteria survive, neutrophil infiltration is replaced after a short period with T and B lymphocytes as well as monocytes; however, neutrophils remain as the most important type of cell found in chronic Mastitis (Rainard *et al.*, 2003). Lymphocytes can recognize a variety of antigenic structures through membrane receptors that define the specificity, diversity, and the immune system memory characteristics. T lymphocytes are classified in two main groups: Tαβ includes CD4+ (helpers), CD8+ (suppressors) and Tγδ. In healthy mammary gland, CD8+ lymphocytes are the prevailing type, whereas in mastitis CD4+ lymphocytes are predominantly activated by molecular complex recognition. This molecular complex is formed between the antigen and major histocompatibility complex class II (MHCII) molecules or by antigen-presenting cells, B lymphocytes, and macrophages (Park *et al.,* 2004; Ohtsuka *et al.,* 2004).

CD8+ lymphocytes act by eliminating the host cell or by controlling the immune response during bacterial infection. Furthermore, these lymphocytes have been considered as 'scavengers' because they eliminate old or damaged cells and their secretions, leading to an increase in MG susceptibility to bacterial infection (Sordillo and Streicher, 2002; Dosogne *et al.,* 2002; Burchill *et al.,* 2003). Although Tγδ lymphocytes have not been well characterized, they are tightly associated with the epithelial surface, where they destroy damaged epithelial cells (Yamaguchi *et al.,* 1999). The main function of B lymphocytes is to produce antibodies against invading pathogens. In contrast to macrophages and neutrophils, B lymphocytes use their membrane receptors to recognize specific pathogens and in the same way as dendritic cells and macrophages, they function as antigen-presenting cells, internalizing, processing and presenting the antigen to CD4+lymphocytes. Finally, NK cells can destroy both Gram-positive and Gram-negative bacteria; for this reason, they are fundamental to the prevention of MG infections (Sordillo and Streicher, 2002; Rainard and Riollet, 2006).

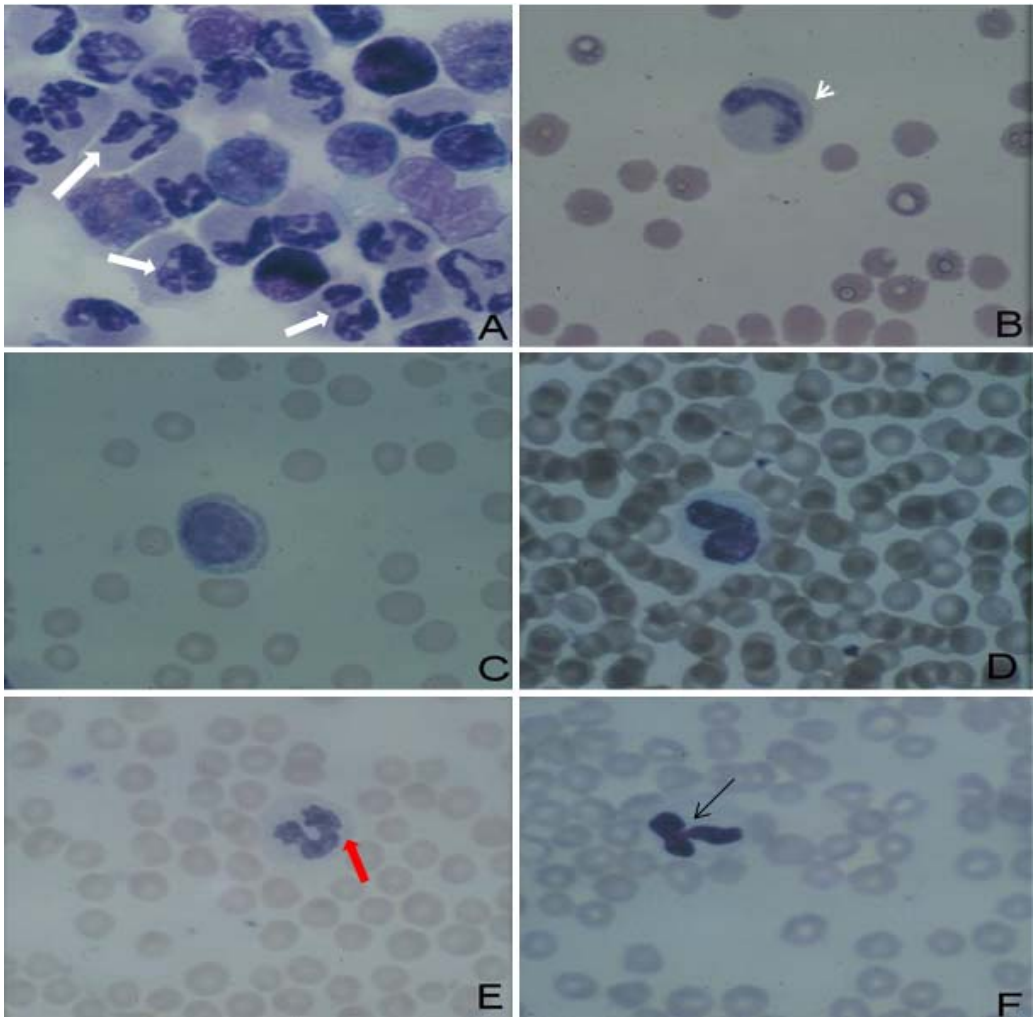

Figure 5. Light micrographs of normal mature bovine polymorphonuclear neutrophil (PMN) and immature and abnormal bovine PMN commonly found in mastitis (Wright's stain, x 1250). (**A**) Cytospin preparation of isolated blood leukocytes (85% PMN). Mature PMN contain the characteristic polymorphic segmented nucleus (white arrows). (**B**) Immature blood PMN (band) with typical horseshoe shaped nucleus (arrow-head). (**C**) Immature blood PMN (myelocyte) with typical large spherical nucleus. (**D**) Immature blood PMN (metamyelocyte) with typical kidney-bean shaped nucleus. (**E**) Endotoxin induced abnormal PMN showing evidence of delayed maturation (red arrow). (**F**) Endotoxin induced apoptotic PMN with bizarre nuclear pattern (thin arrow) (Paape *et al.*, 2003).

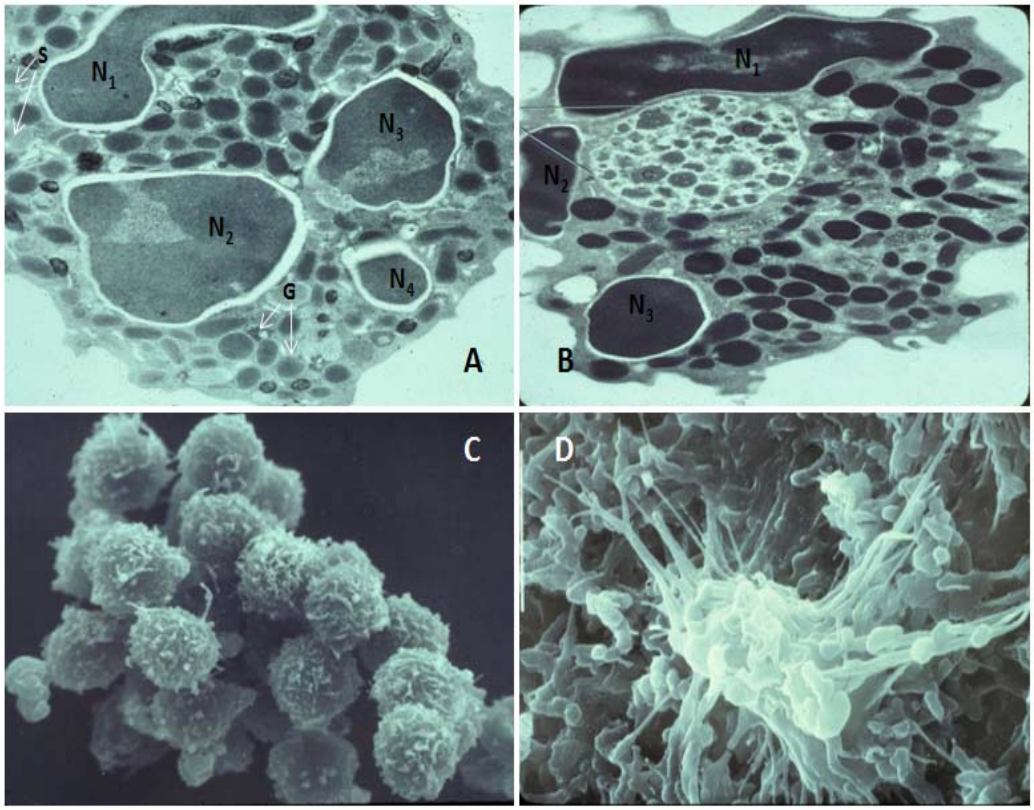

Figure 6. Bovine PMN under transmission electron microscopy (A, B) and scanning electron microscopy (C, D). A (blood PMN): multilobed nucleus (N1 to N4), glycogen granules (G), specific granules (S) x15000; B (milk PMN): large phagocytic vacuole (arrow), surface of milk PMN is smooth and spherical due to loss of pseudopods caused by internalization of membrane material to forms the phagosomes. C (blood PMN): About 98% of these cells are spherical and have a highly convoluted cell membrane that forms protruding pseudopods, x 2500; D: polymorphonuclear leukocyte (PMN) with extended pseudopodia phagocytosing *Staphylococcus aureus*. Numerous individual bacteria are seen in the process of being phagocytosed, x 10 000 (Paape *et al*., 2003).

1.3.3 SOLUBLE FACTORS IN MG DEFENSES

The activity of immune system cells in the pathophysiology of bovine mastitis is regulated by pro-inflammatory cytokines that increase macrophage and neutrophil bactericidal capacity, promote the recruitment of neutrophils towards the site of infection, induce the maturation of dendritic cells, and control the acquired immune response (Alluwaimi and Cullor, 2002; Sordillo *et al.,* 1991; Hornef *et al.,* 2002).

1.3.3.1 CYTOKINES

In fact, cytokines are soluble proteins that affect an array of biological processes, including inflammation and immunity. Almost every mammalian cell type has the capability to produce and respond to cytokines (Dinarello, 2007). Although the majority of cytokines are secreted, some are restricted to the cellular membrane. Most cytokines have more than one function and often have redundant effects with other cytokines (Taniguchi, 1995). One of the primary mechanisms by which they exert their effects is through the activation of intracellular signal transduction pathways and the corresponding upregulation of gene expression (Gouwy *et al.,* 2005).

Although cytokines play an essential role in the host response to infection, they can have deleterious effects on the host. Thus, there is a fine balance between the positive and negative effects of cytokines on the host that is dictated by the duration, amount, and location of their expression. At present, a variety of cytokines, such as interleukins (IL-1, IL-2, IL-6, IL-8, IL-10, IL-12), transforming growth factor beta (TGF-β), colony-stimulating factors (CSF), interferon gamma (IFN-γ), and TNF-α have been found in healthy and infected MG (Sordillo and Streicher*,* 2002; Alluwaimi, 2004; Bannerman, 2009).

TNF-α

TNF-α is produced by multiple cell types such as macrophages, lymphocytes, neutrophils, and epithelial cells (Angelini *et al.,* 2005). Tumor necrosis factor-α is a highly proinflammatory cytokine with both beneficial and injurious properties (van der Poll and Lowry, 1995). This cytokine participates in neutrophil chemotactic activity, because it induces the expression of adhesion molecules by endothelial cells. In fact, neutrophil recruitment to the milk spaces is: (i) mediated through TNFα, which is produced by alveolar macrophages in response to LPS/TLR4 signalling and (ii) is dependent on IL8 and IL1β signalling (Elazar *et al.,* 2010). The systemic effects of TNF-α include the induction of fever and acute phase protein synthesis. Although these local and systemic effects are beneficial to the host innate immune defense against infection, TNF-α is associated with heightened inflammatory responses that can threaten the life of the host. Specifically, TNF-α has been shown to induce endotroxic shock, tissue injury, vascular leakage, multiorgan failure, and dysregulated coagulopathy in acute mastitis caused by *E. coli* (Havel,1989; Tracey and Cerami, 1994; van der Poll and Lowry, 1995; Slebodzinski *et al.,* 2002; Persson *et al.,* 2003). Tumor necrosis factor-α is able to exert its effects directly or indirectly by stimulating the production of secondary mediators. Inducers of TNF-α production include viral, fungal, and parasitic pathogens, bacterial wall products and toxins, other cytokines such as IL-1 and IFN-γ, and complement components.

Tumor necrosis factor-α is undetectable in healthy quarters; however, elevated concentrations of this cytokine have been detected in both milk and blood after intramammary infection (Shuster *et al.,*1995, 1996, 1997; Riollet *et al.,* 2000; Lee *et al.,* 2003; Bannerman *et al.,* 2004c; Dernfalk *et al.,* 2007). In cows with naturally acquired *E. coli* intramammary infections, milk TNF-α concentrations have been shown to range from 100 pg/mL to 100 ng/mL (Hisaeda *et al.,* 2001; Slebodzinski *et al.,* 2002). Similar to *E. coli*, intramammary infections with other gram-negative bacteria, including *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens*, evoke similar TNF-α responses (Bannerman *et al.,* 2004a, 2005). The finding of greater TNF-α in cows with more severe mastitis that developed after experimental infection is consistent with reports of a correlation between the concentration of this cytokine and the severity of naturally occurring coliform mastitis (Ohtsuka *et al.,* 2001), and other bacterially mediated diseases (Waage *et al.,* 1987; Girardin *et al.,*1988). Besides, gram-negative bacteria, gram-positive bacteria or wall-less bacteria have also been reported to evoke TNF-α response and upregulation of TNF-α can be detected in milk cells isolated from *S. aureus* infected glands (Riollet *et al.,* 2001; Alluwaimi *et al.,* 2003).

INTERFERON (INF-γ)

Interferon (IFN)-γ plays a role in linking the innate and adaptive arms of the immune system and is critical for host immunity against intracellular pathogens (Strichman and Samuel, 2001; Nonneche *et al.,* 2003). Cellular sources of IFN-γ include CD4+/CD8+ lymphocytes, natural killer cells, and cells of monocytic lineage as a response to mitogenic and antigenic stimuli (Schroder *et al.,* 2004; Schoenborn and Wilson, 2007). The influence of IFN-γ on the innate immune system is most evident from its effects on macrophages and neutrophils. Interferon-γ enhances the microbicidal activity of these cells by increasing receptor-mediated phagocytosis, inducing respiratory burst activity, and priming nitric oxide production (Ellis and Beaman, 2004; Schroder *et al.,* 2004). Interferon-γ also up regulates cell-surface major histocompatibility complex (MHC) class I molecule expression, thus promoting the induction of cell mediated immunity by increasing the likelihood of cytotoxic T-cell recognition of presented foreign peptides (Schroder *et al.,* 2004). Further, IFN-γ upregulates the MHC class II antigen presentation pathway and corresponding CD4+ T-cell activation. At the transcript level, increases in IFN-γ mRNA have been detected in cells isolated from the milk of mammary glands infected with *E. coli* (Lee *et al.,* 2006), and *S. aureus* (Riollet *et al.,* 2001). During the course of naturally occurring mastitis (Hisaeda *et al.,* 2001), as well as in the setting of experimental mastitis induced by *E. coli*, *M. bovis*, *S. aureus*, *P. aeruginosa*, *S. marcescens*, and *S. uberis* (Bannerman *et al*., 2004a,b,c, 2005; Kauf *et al.,* 2007),

increases in milk protein concentrations of IFN-γ have also been detected. Interestingly, the greatest concentrations of IFN-γ have been detected in intramammary infections characterized by persistent infection with increased numbers of recoverable bacteria (Bannerman *et al.,* 2004a; Kauf *et al.,* 2007). This may reflect an attempt by the host to heighten cell-mediated immune responses to eradicate pathogens that are not readily eliminated by earlier activated host innate immune defense mechanisms.

INTERLEUKIN-1

Interleukin-1 is a proinflammatory cytokine and one of the most potent endogenous inducers of fever (Dinarello, 1998). A variety of cells have been identified as sources of IL-1, including monocytes, macrophages, dendritic cells, lymphocytes, endothelial and epithelial cells, and fibroblasts (Barksby *et al.,* 2007). This cytokine plays a critical role in the host defense against infection, but dysregulation of its expression can have deleterious consequences to the host. Many of the biological effects of IL-1 on host innate immune responses to infection are similar to those of tumor necrosis factor-α (TNF-α), including activation of endothelial cells and leukocytes, and systemic induction of fever and acute phase protein synthesis (Pruitt *et al.,* 1995; Dinarello, 1996). Correspondingly, the deleterious effects of IL-1 overlap those of TNF-α and include the ability to induce shock, vascular leakage, and multiorgan failure. The expression of IL-1 is induced in response to bacterial, viral, fungal, and parasitic infections, as well as nonmicrobial sources, including TNF-α, IL-12, and complement component 5a (Goodman *et al.,* 1982; Dinarello, 1996). It induces the production of TNF-α, IL-6, IL-8, and IL-12 thus act synergistically to enhance their effects.

Interleukin-1 is expressed as either IL-1α or IL-1β, 2 structurally and functionally similar polypeptides (Pruitt *et al.,* 1995; Dinarello, 1996). Because of differential expression of leader peptide sequences and proteolytic processing, IL-1α predominantly serves to regulate intracellular events and mediate local inflammation, whereas IL-1β mediates both local and systemic inflammatory responses. During the inflammatory response, IL-1β regulates the expression of adhesins by endothelial cells and neutrophil chemotaxis in infections caused by *E. coli*. The induction of IL-1β is quicker in *E. coli* mastitis but delayed after intramammary infection with gram-positive or wall-less bacteria (Shuster *et al.,* 1997; Riollet *et al.,* 2000; Yamanaka *et al.,* 2000; Bannerman *et al.,* 2004a; Bannerman *et al.,* 2004c; Kauf *et al.,* 2007). At the mRNA level, IL-1β transcription has been determined to be upregulated in milk cells isolated from quarters chronically infected with *S. aureus* (Riollet *et al.,* 2001; Zhang and Issekutz, 2002).

INTERLEUKIN-2

IL-2 is produced by CD4+ lymphocytes and was initially described as T cell growth factor. This cytokine regulates the acquired immune response, because it stimulates growth and differentiation of B lymphocytes, increases thymocyte proliferation, activates NK cells, and induces T lymphocyte activation. Alterations in IL-2 production cause a decrease in the mammary gland immune response capacity, which contributes to bacterial diseases such as mastitis (Sordillo *et al.*, 1991; Sordillo and Streicher, 2002).

INTERLEUKIN-6

Interleukin-6 is a pleiotropic cytokine, expressed by a variety of cells, including lymphocytes, monocytes, macrophages, neutrophils, endothelial cells, epithelial cells, and fibroblasts, and its expression is induced by bacteria and viruses, as well as by cytokines, such as TNF-α and IL-1β (Biffl *et al.*, 1996; van der Poll and van Deventer, 1998). Interleukin-6 is involved in acute septic shock during mastitis caused by coliforms or *S. aureus*. It modulates both innate and adaptive immunity via inducing fever, B-cell differentiation and corresponding immunoglobulin production, T-cell activation, and enhanced proinflammatory responses of neutrophils (Biffl *et al.,* 1996; Keller *et al.,* 1996).This cytokine facilitates the exchange of neutrophils for monocytes in the mammary gland, which is necessary for reduction of the deleterious effects of neutrophils. Interleukin-6 is a prominent inducer of hepatic synthesis of acute phase proteins, many of which facilitate host clearance of infectious pathogens, the down regulation of the inflammatory response, and the restoration of physiological homeostasis (Moshage, 1997; Papanicolaou *et al.,* 1998; Slebodzinski *et al.,* 2002; Ohtsuka *et al.,* 2001). Similar to transcript abundance (mRNA level), comparable increases in IL-6 protein have been detected among cows infected with *E. coli*, as well as with gram-positive bacteria, including *S. aureus* and undefined species of *Streptococcus* (Hagiwara *et al.,* 2001; Lee *et al.,* 2006). Severe mastitis linked higher concentration of IL-6 is highly correlated with increased mortality (van der Poll and van Deventer, 1998; Song and Kellum, 2005).

INTERLEUKIN-8

Interleukin-8 is a chemotactic cytokine (i.e., chemokine) that is upregulated in response to infection. This cytokine is produced by an array of cell types, including cells of monocytic lineage, endothelial and epithelial cells, fibroblasts, neutrophils, and T-lymphocytes, and can correspondingly be generated in any tissue (Matsukawa *et al.,* 2000). In contrast to the more transient effects of other chemoattractants, IL-8 is able to exert a longer lasting effect, presumably because of its resistance to proteolytic

degradation and slower clearance from tissues (Baggiolini and Clark-Lewis, 1992; Hebert and Baker, 1993). Its expression is induced by both exogenous (e.g., bacteria, viruses, fungi, parasites, and products derived from these pathogens) and endogenous (e.g., TNF-α and IL-1β) proinflammatory stimuli (Matsukawa *et al.,* 2000; Mukaida, 2003).

In addition to its role in attracting neutrophils to sites of infection, IL-8 can activate or augment neutrophil respiratory burst activity, degranulation, and generation of arachidonate metabolites (Mukaida *et al.,* 1998). Thus, IL-8 preferentially recruits and enhances the functioning of neutrophils and to lesser extent T lymphocytes (Harada *et al.,* 1994; Kobayashi, 2008). Increases in IL-8 mRNA transcription have been confirmed in milk cells isolated from *E. coli* infected glands (Lee *et al.,* 2006). Similar to *E. coli*, other gram-negative bacteria, including *K. pneumonia*, *P. aeruginosa*, and *S. marcescens*, have been shown to induce increases in milk IL-8 concentrations within 20 h of intramammary infection (Bannerman *et al.,* 2004a, b, 2005). In contrast to gram-negative bacteria, intramammary infections with gram-positive or wall-less bacteria have been shown to induce a delayed or diminished IL-8 response (Persson *et al.,* 2003; Alluwaimi, 2004; Yang *et al.,* 2008).

INTERLEUKIN-10

Interleukin-10 plays a central role in limiting inflammation and influencing the nature of the adaptive immune response to infection. It is produced by various cell types, including type 2 helper T lymphocytes (TH2), B cells, eosinophils, mast cells, and cells of monocytic lineage, the latter of which are considered to be the major in vivo source of the cytokine (Asadullah *et al.,* 2003). Interleukin-10 exerts a broad anti-inflammatory effect on monocytes, macrophages, and neutrophils by inhibiting their production of proinflammatory cytokines, chemokines, and eicosanoids (Moore *et al.,* 2001). Interleukin- 10 also induces the upregulation of IL-1 receptor antagonist and soluble TNF receptors, which impair the ability of the proinflammatory cytokines IL-1 and TNF-α, respectively, to exert their effects. In terms of its influence on adaptive immunity, IL-10 impairs the ability of monocytes and macrophages to present antigen to T cells by downregulating MHC class II expression. Further, this cytokine is involved in altering the type 1 helper T lymphocyte (TH1)/TH2 balance by suppressing the production of IFN-γ and IL-12, which are involved in promoting a TH1-type response (Moore *et al.,* 2001; Conti *et al.,* 2003; Mocellin *et al.,* 2004). By virtue of its ability to stimulate B cell function while impairing TH1-type responses, IL-10 shifts the nature of the adaptive immune response to one that is humorally mediated.

Intramammary infections by diverse bacterial pathogens, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. marcescens*, *S. uberis*, and *M. bovis*, have been shown to evoke an increase in milk concentrations of IL-10 (Bannerman *et al.,* 2004a,b,c, 2005; Kauf *et al.,* 2007). In contrast, detectable increases in IL-10 expression are not evident in response to *S. aureus* intramammary infection (Bannerman *et al.,* 2004c). During those infections in which an IL-10 response was observed, initial production of IL-10 was preceded by increases in milk TNF-α concentration. In *S. aureus*-infected quarters, the lack of an IL-10 response corresponded with the lack of induction of TNF-α. However, initial and maximal increases in IL-10 production are consistently detected earlier in response to gram-negative bacteria than to gram-positive or wall-less bacteria, and these are similar to findings with TNF-α. Interestingly, in cows with the greatest persistent concentrations of bacteria (i.e., *S. aureus*, *S. uberis*, *M. bovis*) in milk, induction of IL-10 is absent or delayed (Bannerman *et al.,* 2004a, c; Kauf *et al.,* 2007). This may indicate that earlier induction of IL-10 is beneficial to the ability of the cow to limit bacterial growth and eradicate the pathogen (Bannerman, 2009).

INTERLEUKIN-12

IL-12, similar to IFN-γ serves to bridge the innate and adaptive arms of the immune system, and plays an essential role in modulating the host immune response to bacterial and parasitic intracellular pathogens. . Monocytes and dendritic cells are believed to be the major sources of IL-12 (Langrish *et al.,* 2004). Neutrophils produce IL-12 to a lesser extent than monocytes; however, their presence in such large numbers at sites of infection presumably renders them a pathophysiologically relevant source of this cytokine (Trinchieri, 1998). By virtue of its ability to stimulate the production of IFN-γ by T cells and natural killer cells, IL-12 contributes to the activation of macrophages (Gately *et al.,* 1998; Trinchieri, 2003). Interferon-γ can, in turn, induce the production of IL-12 by phagocytes, resulting in a positive feedback loop. In addition to IFN-γ, IL-12 has been demonstrated to upregulate other cytokines, including TNF-α, IL-8, and IL-10 (Gately *et al.,* 1998). Through its ability to heighten formation of cytoplasmic granules, as well as to increase granule contents involved in pathogen killing, IL-12 enhances the cytotoxic activity of cytotoxic T cells and natural killer cells (Trinchieri, 1998). In terms of adaptive immunity, IL-12 plays a critical role in altering the balance between TH1 and TH2 responses by promoting the differentiation of T cells into IFN-γ producing TH1 cells (Hornef *et al.,* 2002; Langrish *et al.,* 2004).

Interleukin-12 also alters antibody responses by enhancing the production of immunoglobulins involved in both opsonization and the facilitation of cell-mediated

responses, while impairing the production of immunoglobulins involved in mediating TH2 humoral immune responses (Gately *et al.,* 1998). Increases in IL-12 mRNA abundance have been detected in cells isolated from cows experimentally infected with *E. coli* or *S. aureus* (Alluwaimi *et al.,* 2003; Lee *et al.,* 2006), as well as in those with naturally derived cases of *S. aureus* mastitis (Riollet *et al.,* 2001). At the protein level, increases in IL-12 have been detected in the milk of cows with experimentally induced mastitis. These increases have all been detected within 32 h of experimental intramammary infection with *E. coli*, *S. aureus*, *P. aeruginosa*, *S. marcescens*, and *S. uberis* (Bannerman *et al.,* 2004a,b,c, 2005), and within 96 h of intramammary infection with *M. bovis* (Kauf *et al.,* 2007). Because IL-12 and IFN-γ are known to induce reciprocal expression, the finding that IL-12 is increased in response to such diverse bacteria is consistent with reports that these bacteria all evoke IFN-γ expression.

TGF-β

The cytokine, transforming growth factor-β, has a well-described effect on cell growth and differentiation. At different stages of development of the mammary gland, TGF-β has been reported to regulate ductal growth and patterning, as well as alveolar functional differentiation (Daniel *et al.,* 2001). The ability of TGF-β to regulate these events is largely attributed to its growth inhibitory effects on epithelial cells and stimulatory effects on fibroblasts and other stromal cells (Kolek *et al.,* 2003; Musters *et al.,* 2004). In addition to effects on mammary gland development, TGF-β is reported to exert proinflammatory properties and modulate mastitis (Letterio and Roberts, 1998; Ashcroft, 1999). The immunoregulatory effects of TGF-β include 1) inhibiting macrophage cytokine and nitric oxide production and respiratory burst activity; 2) limiting IFN-γ production; 3) increasing IL-1 receptor antagonist expression; and 4) enhancing macrophage clearance of injured parenchymal cells, inflammatory cells, and bacterial debris. Thus, TGF-β is involved in mediating mammary gland physiological and pathological processes that are associated with development and inflammation, respectively.

This cytokine is also considered as one of the key modulators of ovarian forlliculogenesis in cow, human and other species (Trombly *et al.,* 2009).Transforming growth factor-β is expressed by a variety of leukocytes (Letterio and Roberts, 1998) as well as other cell types, including epithelial cells (Kwong *et al.,* 2004; Zarzynska *et al.,* 2005). In situ hybridization has been used to confirm that the 3 known mammalian isoforms, TGF-β1, TGF-β2, and TGF-β3 (McCartney- Francis *et al.,* 1998), are all expressed in the bovine mammary gland (Maier *et al.,* 1991). Increases in both TGF-β1 and TGF-β2 have been detected during the course of intramammary infection by diverse bacterial pathogens,

including *E. coli*, *M. bovis*, *P. aeruginosa*, and *S. aureus* (Bannerman *et al.,* 2005, 2006; Chockalingam *et al.,* 2005; Kauf *et al.,* 2007). In comparison with the increases in other proinflammatory cytokines, the induction of TGF-β is markedly delayed. Based on the inherent pleiotropic properties of this cytokine in regulating mammary gland development and inflammation, it is likely that TGF-β plays a role in modulating the inflammatory response to intramammary infection and the ensuing fibrotic tissue repair (Bannermand, 2009).

1.3.3.2 OTHER SOLUBLE MEDIATORS

The bovine complement system is present in serum and milk, and has an important part in the defense mechanisms of the mammary gland against mastitis. Complement activation, which is triggered by antibody immune complexes, bacterial cell surface sugars, and LPS, is characterized by a cascading series of proteolytic events leading to the deposition of a pore-forming complex on the surface of bacteria and concomitant bacterial killing (Gasque, 2004). Complement proteins are predominantly produced by hepatocytes, though they are also produced by some monocytes and macrophages in different tissues. Complement molecules are not only needed to recruit neutrophils to the mammary gland, but also to opsonize and kill bacteria. Gram-negative bacteria (e.g. *E. coli)* are sensitive to complement lytic action, while Gram-positive bacteria (e.g. *S. aureus*) are resistant; however, all bacteria are susceptible to the opsonizing action of C3b and C3bi. The bactericidal and hemolytic activities of complement are increased in inflamed mammary gland quarters, and the intensity of these activities is related to the inflammatory response. It is known that hemolytic activity and C3 concentration in milk are higher in mammary gland with mastitis than in healthy MG (Rainard, 2003; Korhonen *et al.,* 2000; Barrio *et al.,* 2003).

In addition to that other mediators of inflammation, acute phase proteins produced in response to bacterial wall products and cytokines (e.g., TNF-α, IL-1β, and IL-6) have varying, and in some cases poorly defined, roles in moderating mastitis. Increases in milk and blood concentrations of serum amyloid A (SAA), haptoglobin, and LPS-binding protein (LBP) have been reported in response to naturally acquired and experimentally induced intramammary infections (Hirvonen *et al.,* 1999; Eckersall *et al.,* 2001; Gronlund *et al.,* 2003, 2005; Bannerman *et al.,* 2004c). Besides these, fatty acid-derived mediators, the eicosanoids (thromboxane A2, Prostacyclin, PGE2, PGF1α and PGF2α), are involved in regulating an array of inflammatory processes in udder infections, in which the causative agent was identified as either *E. coli, S. uberis*, *S. aureus*, *S. dysgalactiae*, or *Micrococcus* species (Giri *et al.,* 1984; Atroshi *et al.,* 1986, 1987).

Mammary gland also contains non-specific bacteriostatic factors that may act independently or in association with immunoglobulin (Ig). One of these factors is the protein lactoferrin, which is produced by epithelial cells, macrophages, and neutrophils; it binds free iron ions present in milk, making them unavailable to bacteria that need this metal as a growth factor or an iron source (e.g. *E. coli, K. pneumoniae*). However, there are other bacteria that use lactoferrin as an iron source (e.g. *S. agalactiae*) (Sordillo *et al.,* 2002). The main function of lactoferrin is to protect the mammary gland against infection by coliforms, especially in the involution stage, due to the activation of phagocytosis and the complement system (Sordillo *et al.,* 2002; Lee *et al.,* 2004; Kai *et al.,* 2002).

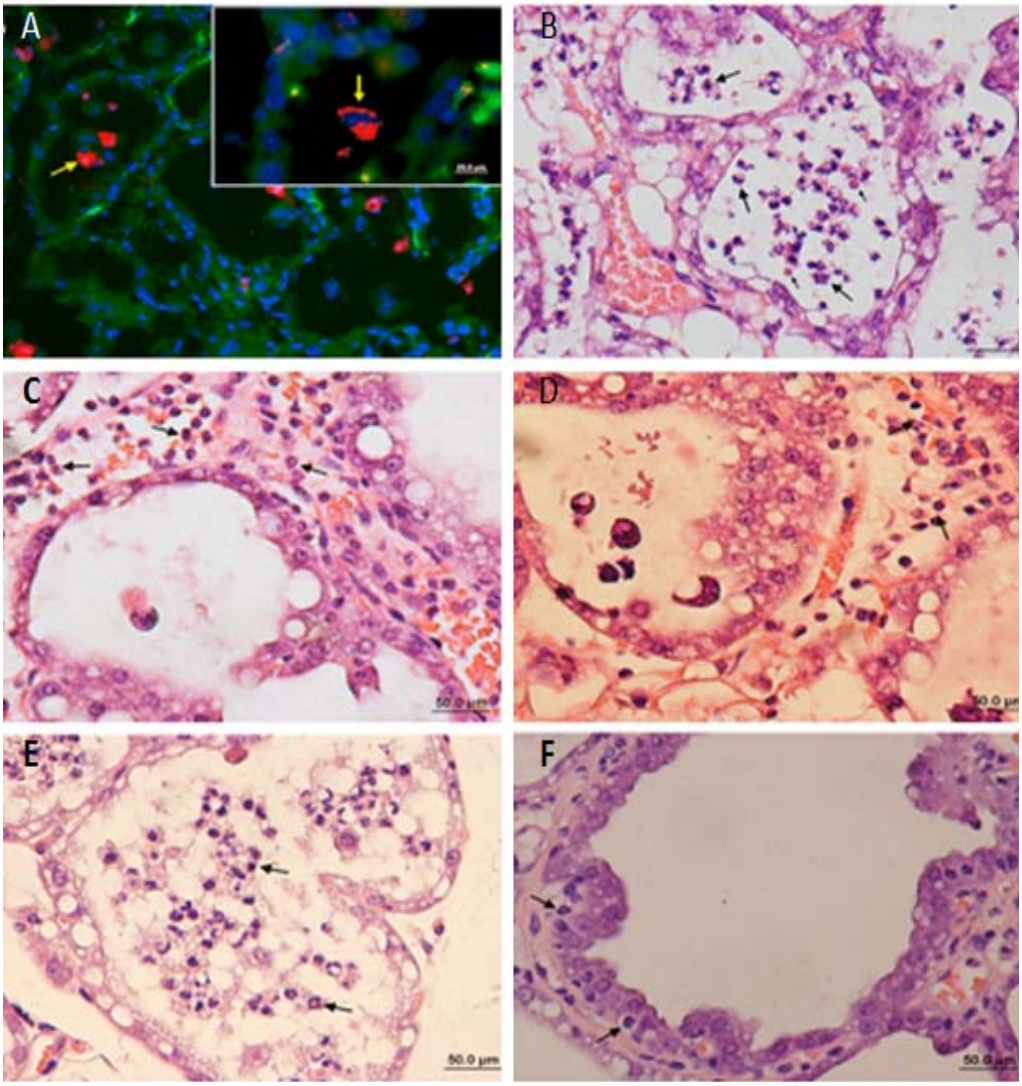

Figure 7. Signalling of the soluble factors in the mammary gland elicited by bacterial virulence factor LPS: A) alveolar macrophages are demonstrated in the normal mammary gland by fluorescence staining with DAPI (arrows in A); B) cytokine TNF-α mediating neutrophil recruitment into the alveolar space; (C &D) IL1β and IL8 are essential for neutrophil recruitment across the alveolar epithelium, recruited neutrophils (arrows) are trapped in congested capillaries and interstitium unable to traverse the mammary epithelium in IL1 receptor type 1 (IL1R −/−) and IL8 receptor (IL8R −/−) murine mastitis. E) massive recruitment of neutrophil into the alveolar space of mammary glands of lactating IL18R (CXCR2) mice, while (F) marked reduction of PMN recruitment is seen in IL18R −/− mastitis (Elazar *et al.,* 2010b).

Table 2. Cytokines associated to the immune response of bovine mammary gland infected with *Escherichia coli* or *Staphylococcus aureus* (Oviedo-Boyso *et al.*, 2007)

Cytokine source		Function	Type of mastitis pathogen
IL-1β	Macrophages and epithelial cells	Neutrophil recruitment to the mammary gland	Clinical mastitis *Escherichia coli*
IL-2	CD^+ lymphocytes	-Induce growing and differentiation of B lymphocytes -Activate NK cells -Activates $CD8^+$ lymphocytes	Subclinical mastitis *Staphyl. aureus*
IL-6	Macrophages	-Regulates acute phase protein synthesis -Favors the influx of monocytes to the mammary gland	Clinical mastitis *Escherichia coli*
IL-8	Monocytes, T lymphocytes, Macrophages, epithelial and endothelial cells	-Chemokine important in neutrophil recruitment to the mammary gland	Clinical mastitis *Escherichia coli*
IL-12	Dendritic cells and T lymphocytes	-Regulates differentiation of T lymphocytes	Subclinical mastitis *Staphylococcus aureus*
INF-γ	$CD4^+$ and $CD8^+$ lymphocytes, and NK cells	-Activates T lymphocytes -Induces production of IL-12 -Mediates activation of neutrophils	ND
TNF-α	Macrophages, neutrophils, and epithelial cells	-Induces expression of adhesion molecules in endothelial cells	Clinical mastitis *Escherichia coli*

(Interleukin (IL; IL-1β, IL-2, IL-6, IL-8, IL-12); Interferon gamma (IFN-γ); Tumor necrosis factor alfa (TNF-α); ND, not determined).

1.3.4 MG DEFENSES AROUND PARTURITION

The transition from the dry cow period to lactation is a high risk period for the modern dairy cows. The biggest challenge at that time is mastitis. Environmental bacteria are the most problematic pathogens around parturition (McDougall *et al.,* 2007). From 2 weeks prior to calving, until about 2–3 weeks after calving, is the most critical period for the health of the mammary gland (Oliver and Sordillo, 1988; Valde *et al.,* 2004). During the periparturient period, hormonal and other factors make the dairy cows more or less immunocompromised. During colostrogenesis, the susceptibility of the mammary gland to infections increases as the teat canal starts to open and leaks mammary secretion (Oliver and Sordillo, 1988). At the same time, the protective effect of dry cow therapy (DCT), if used, has disappeared (Oliver *et al.,* 1990). Hormonal changes include steep rise of the concentration of 17*β*-oestradiol in the plasma during the last week of gestation, peaking during the last days before parturition and a simultaneous drop of progesterone in circulation. Blood cortisol increases about five fold at the day of parturition (Burton *et al.,* 2005). Around parturition, many functions of the PMN are impaired and the numbers of immature neutrophils in circulation increases where as the numbers of mature neutrophils in the blood and milk are at the lowest (Paape *et al.,* 2003; Burvenich *et al.,* 2007). The production of reactive oxygen species (ROS) to kill bacteria is reduced from 1 week before parturition over the first 2 weeks after calving (Hoeben *et al.,* 2000; Mehrzad *et al.,* 2002).

The rapid rise of the concentration of blood cortisol induces changes in the function of PMN, supporting their extended life span in the blood and increasing release from the bone marrow. The change in the respiratory burst activity has been found to be parallel with peripartum increase of concentrations of 3β-hydroxybutyric acid (3-BHB), bovine pregnancy-associated glycoprotein and bilirubin (Hoeben *et al.,* 2000). At that time, neutrophils favor tissue remodeling over defense against infections as their primary task (Burton *et al.,* 2005). At parturition, large numbers of leucocytes are recruited to the reproductive tract and placenta. The ability of the cells to marginate on and migrate through endothelium to infected peripheral tissue in other sites than uterus decreases. After parturition, cortisol down-regulates its own receptors in neutrophils and the system returns to normal function (Burton *et al.,* 2005). The effect of steroid hormones on bovine PMN function was studied by Lamote *et al.,* (2004), who showed that 17β-oestradiol treatment decreased the number of viable cells but progesterone had no effect. In periparturient cows, a loss of expression of critical neutrophil adhesion molecules has been seen (Monfardini *et al.,* 2002), and this loss has been associated with the elevated

cortisol levels (Weber *et al.,* 2004). The same was demonstrated by external glucocorticoid administration (Burton *et al.,* 2005). The proportion of PMN expressing, for example, the adhesion receptor L-selectin, which is necessary for penetration to the sites of infection, is diminished (Diez-Fraile *et al.,* 2004). Neutrophil extracellular traps have recently shown to have a role in killing of bacteria and also to be fully capable to function in the milk environment. The impaired efficiency of this system during the periparturient period may be one more explanation for the immunosuppression of the dairy cows at that time (Lippolis *et al.,* 2006).

CD4+ T lymphocytes that normally activate lymphocytes or macrophages to secrete cytokines for facilitating either cell-mediated or humoral immune response, alters in post-partum, and their numbers and cytokine production declines substantially from that in mid-lactating cows (Sordillo, 2005; Rainard and Riollet, 2006; Shafer-Weaver *et al.,* 1996). Macrophages are the dominant cell type in milk of healthy, lactating gland. During infection, macrophages initiate the immune response by releasing cytokines and other substances augmenting local inflammatory process (Rainard and Riollet, 2006). Bovine macrophage numbers are highest in the mammary gland during the last week of gestation, but their phagocytic capacity is decreased (Sordillo, 2005). Innate and specific soluble factors represent an important part of the defense in the mammary gland; complement, lactoferrin, lysozyme and antimicrobial peptides are the most common (Rainard and Riollet, 2006). Lactoferrin is most active during the steady state of involution (Smith and Schanbacher, 1977). During that time, lactoferrin efficiently prevents growth of bacteria with a high demand of iron such as coliforms (Todhunter *et al.,* 1991). Complement is present in high concentrations in colostrum, and seems not to be a limiting factor in the defence of the mammary gland at that time (Rainard, 2003). The most important factors of the specific immune response are opsonizing immunoglobulins (Ig) produced by antigen-activated B lymphocytes. IgG_1 is the primary isotype present in the healthy mammary gland, but IgG_2 increases during inflammation. The concentration of IgGs in the bovine serum is lower around parturition and in particular, the lack of the IgG_2 isotype is associated with the increased incidence of mastitis (Mallard *et al.,* 1998).

1.4 METABOLIC PREDISPOSITION FOR MASTITIS

Milk production itself is an imposed ‘force’ and is often sustained at high levels for a long period. Metabolic stress arises when the load results in a situation (mismatch of the nutrients actually required for milk production compared with intake) that is physiologically unsustainable (cows of high genetic merit mobilize more body tissue in early lactation than cows of average genetic merit) and results in a compromise of some

processes. Its magnitude is affected by the duration and intensity of an imbalance. For example, there may be a reduction in fertility (since timing of ovulation is related to energy balance), a drop in milk yield or the manifestation of some diseases (Thomas *et al.,* 1999; Pryce *et al.,* 2001). Thus, in dairy cattle, it would appear that there is a greater metabolic predisposition for disease for cows of high genetic merit.

The most important metabolic disturbances occurring shortly after calving are milk fever, ketosis and abomasal displacement. Hypocalcaemia affects the digestive system and pre-disposes the cow to concomitant diseases. It may affect the teat end sphincter and thus increase the risk for mastitis. Cows with periparturient hypocalcaemia are reported to have greater chance of developing coliform mastitis (Curtis *et al.,* 1983).

Negative energy balance and perhaps protein imbalances in early lactation contribute to the impaired immune defence (Spain and Scheer, 2006). Disturbance in fat metabolism and severe negative energy balance may lead to fatty liver and ketosis. Accumulation of fat in the liver disturbs production of humoral immune factors and is also associated with decreased functional capacity of PMN (Zerbe *et al.,* 2000). There is evidence for a decreased capacity for phagocytosis and killing of bacteria in cows suffering from ketosis and fatty liver (Leslie *et al.,* 2001). High concentrations of ketone bodies such as BHB and acetoacetate found at parturition have been shown to inhibit the proliferation of hematopoietic cells, impair their migration and phagocytic activity, enhancing the susceptibility of mammary gland to mastitis pathogens (Hoeben *et al.,* 2000; Smith *et al.,* 1985; Huszenicza *et al.,* 2004). The course of mastitis was severe in all ketotic cows regardless of the chemotactic response before infection (Kremer *et al.,* 1993b). The same factors predispose the cow also for retained placenta and/or the bacterial complications of uterine involution (Kimura *et al.,* 2002; Jánosi *et al.,* 2003).

1. 5 ENDOCRINE ASPECTS IN PATHOGENESIS OF MASTITIS

The successful establishment and persistence of an intramammary infection are mediated by both intrinsic virulence factors of the bacterial pathogen (Hornef *et al.,* 2002) and the rapidity and nature of the immune response of the cow to the pathogen (Burvenich *et al.,* 2003). In contrast to adaptive immune system, which requires several days to become capable of exerting a protective response to infection, the innate immune system is poised to respond immediately to the earliest stages of infection and recognize pathogens that have not been encountered previously (Uthaisangsook *et al.,* 2002). Thus, the innate immune system represents the first line of active defense against invading pathogens once they have penetrated the physical barrier of the teat canal.

Cytokines and other mediators of inflammation are known to play critical roles in the innate immune response to intramammary infection.

Intramammary administration of endotoxin or experimental infection with GN mastitis pathogens induces intense release of cytokines including interleukins (IL-s), tumor necrosis factor-α (TNFα) among others, eicosanoids (like the luteolytic prostaglandin F2α) and oxygen radicals (nitric oxide and others) in the udder (Giri *et al.,* 1984; Sandholm *et al.,* 1995; Sordillo and Daley, 1995), which mediate all the systemic and local symptoms of mastitis. Differences in their production and kinetics may cause wide variation in these clinical signs (Hoeben *et al.,* 2000). Although the outer membrane of GP mastitis pathogens does not contain endotoxin, many layers of a mucopeptide (peptidoglycan) are located in their cell wall. Like endotoxin, this component of GP pathogens has been reported to possess the capability for inducing pyretic and cytokine (TNFα) responses (Salyers and Whitt, 1994; Sordillo and Daley, 1995).

When the proinflammatory cytokines (TNFα, IL-1) and other products of LPS-activated immune cells (mainly of macrophages) reach the central nervous system, these mediators initiate pyretic reaction and lead to marked changes in secretory pattern and/or serum level of numerous hormones. These cytokine-mediated neural and endocrine changes play key roles in the induction of systemic symptoms of mastitis, e.g. fever, lethargy, loss of appetite (anorexia) and many catabolic changes in energy (lipid, carbohydrate), protein and mineral metabolism. In more severe (usually GN) cases simultaneous alterations are seen also in cardiovascular, pulmonary and gastrointestinal functions (hypotension, tachycardia, decreased cardiatic output, respiratory distress, diarrhea), as well as in blood cell counts and blood coagulation system (Cullor, 1992; Sandholm *et al.,* 1995; Sordillo and Daley, 1995). These endotoxin or cytokine-induced endocrine alterations may be dose-dependent and sometimes, also species-dependent.

The endotoxin-mediated cytokine (TNFα and IL-1) response increases the hypothalamic release of corticotrophin-releasing hormone that activates the pituitary-adrenal axis (Kakizaki *et al.,* 1999). Both the intravenous (IV) and intramammary forms of endotoxin challenge are followed by dose-dependent temporary (≤6–8 h) elevation of plasma cortisol level also in lactating cows (Hirvonen *et al.,* 1999; Waldron *et al.,* 2003). Peak levels are seen 2–4 h after challenge (Soliman *et al.,* 2002; Waldron *et al.,* 2003; Lehtolainen *et al.,* 2003), and no significant differences are detected in endotoxin-induced cortisol response of early- versus late-lactating cows (Lehtolainen *et al.,* 2003). The experimental administration of purified recombinant bovine TNFα (rbTNFα) induces similar but shorter elevation in plasma cortisol level (Soliman *et al.,* 2002).

After intravenous endotoxin administration, increasing prolactin levels were seen in rats (Rettori *et al.,* 1994). In cows obvious increase in prolactin production was observed only after iv but not after intramammary LPS challenge and it was supposed to play a role in the immunmodulation, but not in the milk production of the udder (Jackson *et al.,* 1990). In sows, experimental endotoxin administration was capable of decreasing both the prolactin level and milk production in the first week after farrowing but not later (Smith and Wagner, 1984, 1985). In rat models, a variety of other LPS-related changes occur also in the central nervous system, many of which are probably mediated by endotoxin-induced cytokine release, such as alterations of central and peripheral catecholamine levels (Song *et al.,* 1999; Wang and White, 1999), and alterations in neurotransmitter release in different areas of the brain (Mohan-Kumar *et al.,* 1999).

Experimental LPS treatment decreased plasma growth hormone (GH) levels in rats (Rettori *et al.,* 1994), whereas in cattle (Elsasser *et al.,* 1995, 1996; Nikolic *et al.,* 2003), LPS induced mild to moderate elevations in circulating GH concentrations, and a slower but marked reduction in plasma levels of insulin-like growth factor-I (IGF-I). This uncoupling of the GH-IGF-I axis was accompanied by a decrease in the production of one of the IGF-I binding proteins (IGFBP-2). A simultaneous decline in IGF-I and a moderate elevation in IGFBP-1 plasma levels contrasted with the increase in GH secretion, suggest that endotoxin causes a state of resistance to GH, which is exacerbated by a simultaneous reduction in IGF-I bioavailability (Briard *et al.,* 2000; Waldron *et al.,* 2003). In a recent trial, the iv LPS challenge failed to induce any change in GH and IGF-I levels of multiparous lactating dairy cows; however, the possibility of IGFBP-s influencing the bioavailability of this hormone was not examined in the study (Waldron *et al.,* 2003).

The endotoxin-induced endocrine cascade also involves the glucoregulatory pancreatic hormones. Simultaneous with the TNFα and cortisol elevations, a significant but transient increase in insulin levels (Steiger *et al.,* 1999; Waldron *et al.,* 2003), and a clearly dose-dependent and more obvious growth in glucagon levels (Waldron *et al.,* 2003) were reported to occur. Following a temporary hyperglycemia, insulin resistance and decreased glucose concentrations were observed 6 and 24 h after the LPS challenge (McMahon *et al.,* 1998). Similar changes in glucose and insulin levels, as well as in insulin resistance were induced also by iv administration of rbTNFα (Kushibiki *et al.,* 2000; Soliman *et al.,* 2002). The exact mechanism inducing these LPS-related alterations in the pancreatic endocrine function has not been fully understood. However, the effect of nitric oxide and cytokines (TNFα), the early elevation of plasma cortisol and

catecholamine levels, and the sympathetic neuroendocrine changes, have all been implicated in these LPS-related alterations in endocrine functions (Waldron *et al.,* 2003).

Endotoxin mediated infectious and inflammatory diseases like mastitis have been implicated to be associated with changes in the thyroid status, interrupt basal metabolism that eventually leads to decrease productive performance. This systemic non thyroidal illness consists of decreasing plasma concentration of the active thyroid hormone (3,3',5-triiodothyronine, T3) with a simultaneous elevation in its inactive metabolite (3,3',5'-triiodothyronine, synonym: reverse-triiodothyronine; rT3) and, in severe cases, a reduction in thyroxin (T4) and thyroid stimulating hormone (TSH) concentrations. Most of these changes are caused by a lower T3 production rate mainly due to the diminished extra thyroidal enzymatic activation (e.g. outer-ring deiodination) of T4 by 5'-deiodinase (5'D), in combination with a decreased rT3 clearance rate (Wartofsky and Burman, 1982). TNFα and IL-1 may also inhibit the TSH release from pituitary cells and decrease the activity of type-I 5'D in thyroid and liver tissues (Haastaren *et al.,* 1994; Rettori *et al.,* 1994; Hashimoto *et al.,* 1995), reducing the production and circulating level of T4 and inhibiting the T4 transformation to T3 (Bartalena *et al.,* 1998; Bertók, 1998). Correspondingly, decreased 5'D activity has been reported in the liver of LPS-treated cows, which resulted in lower T3/T4 ratio and declining plasma concentrations of thyroid hormones (Kahl *et al.,* 2000).

A recently identified cytokine-like protein hormone, the Leptin, is produced mainly by white adipocytes (Houseknecht *et al.,* 1998). In mice and rats the experimental administration of endotoxin evoked a rapid and long-lasting increase in plasma leptin concentrations with the first detected elevation within 10 min (due to a LPS-stimulated release of stored hormone from the pinocytotic vesicles of adipocytes), and a plateau of its doubled circulating level from 2 to 6 h as evidenced by the highly significant increase in leptin mRNA (Finck *et al.,* 1998; Mastronaldi *et al.,* 2000). The plasma leptin levels were elevated also in human survivors of acute sepsis (Bornstein *et al.,* 1998). Contrary to these findings, infusion of LPS in cows resulted in unchanged leptin level or showed a mild declining tendency in mastitic cows, regardless of the identified pathogens. These reports suggest that alterations in the plasma leptin might be responsible for the anorexia associated with infection-induced inflammatory response in ruminants (Waldron *et al.,* 2003; Kulcsár *et al.,* 2004).

1.6 DAMAGE OF MAMMARY TISSUE DURING MASTITIS

Both bacterial factors and host immune reactions contribute to mammary epithelial tissue damage. Specifically for bovine mammary studies, lactate dehydrogenase activity and *N*-

acetyl-*β*-D-glucosaminidase (NAGase) activity have commonly been used as markers for tissue damage (Zhao *et al.,* 2008). Necrosis of the mammary epithelium occurs during severe, naturally occurring clinical *E. coli* mastitis, as well as during severe experimental *E. coli* mastitis. In moderate cases of *E. coli* mastitis, there is minimal alveolar tissue damage, as shown by Frost *et al.,* (1980). In the most severe form of *E. coli* infection with uncontrolled bacterial multiplication, all lactiferous sinus epithelia were lost, interstitial tissue became hemorrhagic, and often the animal died of toxemia within a few hours of infection (Burvenich *et al.,* 2003). Conclusive and direct evidence for involvement of apoptosis during mastitis has been provided by Long *et al.,* (2001). *Escherichia coli* infected mammary glands were biopsied, with the resulting tissues processed for RNA, protein, and histological examinations. Both mRNA and protein analyses indicated up-regulation of the proapoptotic factors Bax and IL-1*β*-converting enzyme, and a down-regulation of the antiapoptotic factor Bcl-2 (Long *et al.,* (2001). It was reported a 5-fold induction of a putative marker of apoptosis, testosterone-repressed prostate mucin-2 mRNA, after experimental infection of the bovine mammary gland with *Strep. agalactiae* (Sheffield, 1997). In vitro studies indicated that *Staph. aureus* caused apoptosis in a bovine mammary cell line (Bayles *et al.,* 1998).

It has been reported that PMN are involved in the mammary tissue damage (Zhao *et al.,* 2008). Following detection of pathogen invasion into the mammary gland, macrophages and epithelial cells release chemoattractants. These agents trigger the migration of leukocytes, mainly PMN, from the blood toward the mammary gland and increase their proportions from a basal level of 5 to 25% to approximately 90% of total cells in the milk (Leitner *et al.,* 2000; Riollet *et al.,* 2000a). PMN may promote tissue injury and disturb mammary function, via reactive oxygen metabolite such as hydroxyl radical generation (i.e., the respiratory burst) and granular enzyme release i.e., degranulation (Paape *et al.,* 2002; Boulanger *et al.,* 2002). This oxidative stress can damage all types of bimolecular (e.g., DNA, proteins, lipids, and carbohydrates) and therefore induce tissue injury. Bovine PMN have primary (azurophilic), secondary, and tertiary granules (Paape *et al.,* 2002, 2003). These intracellular granules contain bactericidal peptides, proteins, and enzymes such as elastase, other proteinases, and myeloperoxidase that are released into phagocytic vacuoles or the extracellular environment (Faurschou and Borregaard, 2003). The direct involvement of proteases in epithelial cell damage was demonstrated by the fact that coincubation of normal mammary tissue with mastitic milk, but not normal milk, caused tissue degradation (Mehrzad *et al.,* 2005). Therefore, proteases released by PMN are likely involved in mammary tissue damage during mastitis.

There is increasing evidence that pathogens use various mechanisms to impinge upon cell death pathways. A number of pathogens are armed with an array of virulence determinants, which interact with key components of a host cell's death pathways or interfere with regulation of transcription factors monitoring cell survival. These virulence factors induce cell death by a variety of mechanisms, which include 1) pore-forming toxins, which interact with the host cell membrane and permit the leakage of cellular components; 2) toxins that express their enzymatic activity in the host cytosol; 3) effector proteins delivered directly into host cells by a highly specialized type-III secretory system; 4) superantigens that target immune cells, and 5) other modulators of host cell death (Weinrauch and Zychlinsky, 1999). *Escherichia coli* produces a number of proteinases, including collagenolytic enzymes, which contribute to the degradation of ECM components as well as causes apoptosis and necrosis of the mammary epithelium (Haddadi *et al.,* 2005, 2006). LPS increases expression of urokinase-type plasminogen activator induces apoptosis or necrosis in the mammary epithelial cells through induction of proteases or proinflammatory cytokines (Ohta *et al.,* 2000).

Staphylococcus aureus produces toxins that destroy cell membranes, directly damage milk-producing tissue, and induce necrosis in bovine mammary glands (Sordillo and Nickerson, 1988; Trinidad *et al.,* 1990). Initially, the bacteria damage tissues lining the teat and gland cisterns within the quarter. If unchecked, they invade the duct system and establish deep-seated pockets of infection in the milk-secreting cells (i.e., alveoli). This is followed by the walling-off bacteria by scar tissue and the formation of abscesses. Among all, the *Staph. aureus* cytotoxins, *α*-hemolysin, nonspecifically absorbs to the lipid bilayer and forms larger pores in the membrane that are Ca^{2+} permissive, which results in massive necrosis without DNA fragmentation (Weinrauch and Zychlinsky, 1999). Staphylococcal enterotoxin C causes cellular invagination and cytoplasmic vacuolation of mammary epithelial cells (Kuroishi *et al.,* 2003). Cytokines, such as tumor necrosis factor- *α* and IL-1, induce apoptosis in a variety of cell types, including bovine endothelial cells (Mebmer *et al.,* 1999). Several bacteria, such as *Staph. aureus*, *E. coli*, and *Salmonella typhimurium*, express a plasminogen receptor on their surfaces, which, through immobilization of plasminogen, enhances plasminogen activation into plasmin (Lahteenmaki *et al.,* 2001).Plasmin directly degrades matrix proteins such as fibrin and laminin and also activates MMP precursors, such as pro-MMP-3, MMP-9, and MMP- 13 (Green and Lund, 2005). The increase in plasmin activity during mastitis is linked to the permeability of the milk epithelial barrier during inflammation. Lactoserum from mastitic cows exfoliated the cells and surrounding proteins, leaving a nude dense collagen network (Mehrzad *et al.,* 2005).

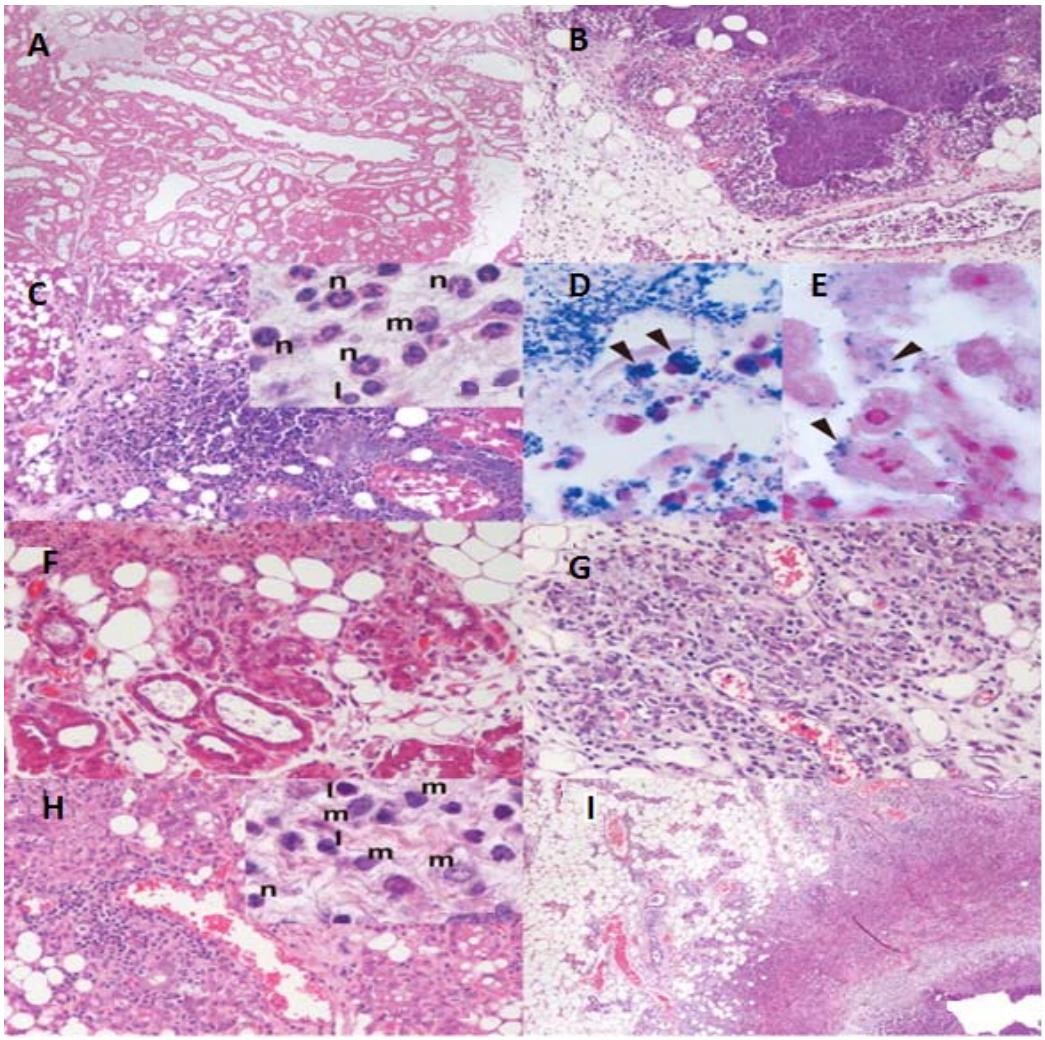

Figure 8. Microscopic alterations and damage of the mammary gland following experimentally induced mastitis by *Streptococcus agalactiae*: (A) normal mammary tissue (H& E); (B) large bacterial colony with PMN infiltration; (C) mammary necrosis with multiple bacterial colonies; (D) blue stained cocci free or inside PMNs; (E) blue stained cocci free or inside epithelial cells; (F) mixed inflammatory infiltrate surrounding glandular structures; (G) intense interstitial inflammatory cell infiltration; (H) mild to moderate interstitial fibrosis and intense inflammatory cell infiltration, macrophages (m) and lymphocytes (l) largely predominate over neutrophils (n) and (I) large pyogranuloma with central necrosis and neutrophil accumulation surrounded by a band of mononuclear cells, replacement of the mammary tissue by adipocytes and fibrous tissue at the periphery (Trigo *et al.,* 2009).

1.7 SOMATIC CELL DYNAMICS AND MASTITIS

Somatic cells in milk are a pivotal part of mammary gland immunity and are vital for protection against intramammary infection as well as a convenient and useful indicator of both clinical and subclinical mastitis and milk quality. The leading cell type present in the milk of uninfected quarters is the macrophage but small percentages of epithelial cells are sometimes found (NMC, 1999). Through the secretion of chemokines, macrophages are responsible for recruiting the large numbers of neutrophils which predominate in infected glands and which are required to fight infection at the time of intramammary challenge (Sordillo *et al.,* 1997). The exact role of somatic cells in the control of clinical mastitis, absolute numbers of cells present in milk and speed of recruitment of additional cells following challenge has been an area of hot debate over a number of years; however milk from uninfected quarters generally have SCC of less than 200,000 cells/ml. About 50% of uninfected quarters will have SCC below 100,000/ml. Somatic cell counts that are greater than 200,000 generally indicate an abnormality and the likelihood of irritation in the udder; elevated SCC are mostly due to microorganisms infecting the gland. As diapedesis of leukocytes is localized, then only the udder quarter that is infected will have a significant increase in concentration of leukocytes (SCC).

It would appear that both initial numbers and speed of migration of leukocytes are important in a cow's defense against clinical mastitis, and that these two phenomena appear to be correlated. However, other factors, in particular poor vitamin E and selenium status as well as negative energy balance have been demonstrated to influence the speed of neutrophil recruitment and migration (Smith and Hogan, 1993; Suriyasathaporn *et al.,* 1999). Studies also reveal that factors such as stage of lactation or age generally do not result in significant increases in SCC above 200,000 if the gland is uninfected (NMC, 1999).

Apart from quarter SCC, the bulk tank milk SCC has also several desirable attributes, especially monitoring subclinical mastitis prevalence in dairy herds with contagious mastitis pathogens or in herds experiencing clinical outbreaks due to environmental pathogens. However, herds that have controlled contagious pathogens and have bulk tank SCC less than 300,000 may experience significant clinical mastitis and losses due to environmental pathogens without elevating the bulk tank SCC. This situation may occur because the overall prevalence of environmental pathogen may be low at any time, the infections are short duration, and infections may be associated predominantly with the dry period and calving. Occasional clinical mastitis outbreaks in such herds may cause an elevation in the bulk tank SCC (NMC, 1999).

Somatic cell count (SCC) in bulk milk is usually determined frequently for every dairy herd that delivers milk to a processing plant. In herds that do not participate in dairy herd improvement (DHI) milk testing, bulk milk SCC is the only parameter available to monitor the udder health situation but the association between bulk milk SCC and the prevalence of subclinical mastitis is far from perfect (Lievaart *et al.,* 2007b). Although this is an important parameter but may not often reflect the udder health situation accurately because the milk from some cows may be withheld from the bulk milk owing to milk quality or food safety regulations regarding high somatic cell count (SCC) milk, antibiotic residues or contagious pathogens (Van Schaik *et al.,* 2002; Olde Riekerink *et al.,* 2006). Therefore, the average yield-corrected SCC of all animals in the herd (CHSCC) has been suggested to be a better parameter for monitoring the udder health situation on farms participating in a DHI programme (Lievaart *et al.,* 2007b). The proportion of dairy herds that participate in a DHI programme ranges from 2% in Poland to 90% in Denmark (International Committee for Animal Recording, 2002). Research on the sampling variation of bulk milk SCC is limited to assessing the required agitation time to ensure that the milk composition will stay as homogeneous as possible (Goodridge *et al.,* 2004; Servello *et al.,* 2004) or to studying annual variations due to management practices of the farm (Berry *et al.,* 2006; Green *et al.,* 2006; Lievaart *et al.,* 2007a; Olde Riekerink *et al.,* 2007).

1.8 FERTILITY

Fertility in dairy cows has been defined as: 'the ability of the animal to conceive and maintain pregnancy if served at the appropriate time in relation to ovulation' (Darwash *et al.,* 1997). Failure to establish a successful pregnancy could arise from failure to show or detect oestrus, failure to ovulate, inappropriate patterns of ovarian cyclicity, embryo or foetal loss (Royal *et al.,* 2000a). Although, there is no clear consensus regarding the mechanism of the effect of milk yield on fertility, it is becoming increasingly evident that fertility is declining with rising yields. There exists a high complexity among genetic (high genetic merit for production), nutritional (an imbalance of nutrients or diets not matched to performance), physiological and management factors (faulty management that leads to disease like mastitis) for the milk yield versus poor fertility in cow (Prycea, 2004). Physiological reasons for the antagonism have not been fully elucidated. To maintain or recover high fertility in modern dairy cows calls for a two-pronged approach involving both inclusion of fertility in broader breeding goals and adjustment of management practices.

1.8.1 A BRIEF OVERVIEW OF THE PHYSIOLOGICAL BACKGROUND

The physiological background of dominant follicles turnover during the bovine estrous cycle has been comprehensively overviewed recently (Ireland *et al.,* 2000; Aerts and Bols, 2010). Here, we will outline the major underlying physiology. Conception and maintenance of pregnancy in dairy cattle involves a synchrony between management effects and physiological processes (Darwash *et al.,* 1999). Management issues include heat detection, timing of insemination and disease prevention. Physiologically, the fundamental prerequisite is the production of an ovum capable of being fertilised and a uterus capable of supporting pregnancy (Darwash *et al.,* 1999). Development of follicle is controlled primarily by a feedback system involving gonadotrophin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinising hormone (LH), oestrogens, androgens and progestins and proteins (inhibin-related proteins secreted from the ovaries; Webb *et al.,* 1992, 1994). Through the control of these, follicles grow in distinct waves that last 7 to 10 days within an oestrous cycle of 21 days. Each wave involves the recruitment of a cohort of 5–7 primordial follicles of which one will become larger while the others regress.

Determining how the selection process results in the selection of a single follicle from a cohort, as the others undergo atresia, is an area of intense research. However, many studies have been put forward two hypotheses and models on this subject. One maintains that, dominance enables a single follicle to prevent the growth of other follicles; while the other interprets the results as dominant follicle becomes capable to grow in a hormone milieu that is unsuitable for other follicles (Ireland *et al.,* 2000). Loss of dominance results in atresia of the dominant follicle, thus initiating growth of a new follicular wave. However, if the later growth of the dominant follicle coincides with luteolysis, it undergoes rapid maturation and ovulates. If fertilization occurs successfully, the cow embryo remains free-living in the reproductive tract until implantation on about day 19 of pregnancy (Wathes and Wooding, 1980).

1.8.2 WAYS OF MEASURING FERTILITY IN PRACTICE

Service sires and service and calving dates are commonly recorded by most milk recording agencies as this information is needed by farmers for management purposes and to provide parentage details of offspring. Fertility measures calculated from calving and service dates can be divided into two categories as fertility scores and interval traits. Fertility scores include, for example, non-return to first service, which is determined by whether another service follows within a pre-determined number of days, such as 56 or 90 days, and conception at first service, which is determined either through pregnancy

diagnosis or a subsequent calving. Interval measures include, for example, days from calving to first service or heat (DFS and DFH, respectively), days open (DOPN) and calving interval. More recently, it has been possible to use endocrine measures to determine the fertility of the cow; these include, amongst others, commencement of luteal activity determined using progesterone (Darwash *et al.,* 1997; Royal, 1999; Royal *et al.,* 2000a, c, 2002a, b; Veerkamp *et al.,* 1998).

In all circumstances, good fertility in dairy cows can be defined as the accomplishment of pregnancy at the desired time. The limitation in using insemination data is that there is considerable variation in the quality of recording, which is a result of these data being used to calculate drying off and calving dates. For example, if a cow is inseminated twice in a month, then to calculate a calving date only the second date is required, thus the first insemination date may be discarded. Assessing the quality of data is an important issue, as this affects the way in which data are used and for what purpose (e.g. whether or not a trait is suitable for genetic evaluation). So, it is mandatory to record all calving dates. However, fertility is the primary reason for culling; failure to conceive accounts for 44% of culls in first lactation animals (Esslemont and Kossaibati, 1997). Thus, only the most fertile animals calve for a second time, which introduces bias. Some farms may also deliberately extend lactations for a proportion or entire part of the herd.

One of the advantages in using physiological measures is that they are more indicative of the cow's inherent ability to be fertile (Darwash *et al.,* 1997). Milk progesterone levels indicate when an animal ovulates, reflect the formation and life-span of the corpus luteum, and indicate whether the estrous cycle is atypical (Royal et al., 2000b). It has been suggested that although measures may be affected by environmental factors or by metabolic load/stress, they are unlikely to be affected by management decisions. Presently, the potential advantages of incorporating endocrine data into a fertility index are being investigated (Royal, 2003).

1.8.3 RELATIONSHIP BETWEEN FERTILITY AND MILK PRODUCTION

Dairy selection objectives have centered on milk production, and today's dairy cows on modern commercial farms produce an amount of milk that has roughly doubled over the past four decades. First parity cows on large commercial dairy farms typically peak at 40 to 45 kg/d, while second and later parity cows typically peak at 50 to 55 kg/d. Furthermore, each group typically sustains daily milk production of 40 kg/d or more during the first seven months postpartum. Since little attention has been given to health and fertility, a negative genetic trend in both traits is expected; given that, the genetic correlation between milk yield and fertility is unfavorable i.e. average fertility will decline

with increasing genetic merit for milk yield. The phenotypic trends indicate a decline of ~1% per year in pregnancy rates to first service (Royal *et al.,* 2000a). Estimates of correlations with calving interval range from 0.22 to 0.59 (Kadarmideen *et al.,* 2000; Pryce *et al.,* 1997), days open from 0.16 to 0.64 (Dematawewa and Berger, 1998), days to first service 0.22 to 0.44 (Kadarmideen *et al.,* 2000; Pryce *et al.,* 1997) and conception rate to first service -0.62 to 0.05 (Kadarmideen *et al.,* 2000; Pryce *et al.,* 1997). In addition, early estimates of the genetic relationship between milk yield, fat, protein and commencement of luteal activity (an endocrine measure of luteal activity) are unfavourable (Royal *et al.,* 2000c) and the estimated genetic correlation between peak milk yield and commencement of luteal activity was 0.36 (Royal *et al.,* 2002a).

Breeds selected for high production are more likely to be in severe negative energy balance in early lactation and thus genetic correlations will be more unfavorable in these breeds, which might suggest that energy balance is inextricably linked with fertility (Lindhe and Philipsson, 1998). Generally, fertility is better in maiden heifers than in lactating cows and the physiological status of cows and heifers are very different in that heifers reach puberty typically between 9 and 12 months of age, but are not usually inseminated until 15 months of age. On the other hand, the postpartum cow has a shorter period in which to re-establish ovarian cyclicity before insemination. Likewise, the genetic control of maiden heifer and cow fertility may be different; genetic correlations between fertility in maiden heifers and lactating cows range between 0.3 and 0.8 (Roxstrom *et al.,* 2001). For example, it has been observed that conception rates to first service of 64% and 71% in lines of maiden heifers of high and average genetic merit, while conception rates were 39% and 45% for lactating cows of high and average genetic merit in the same herd (Pryce *et al.,* 2002). In both lactating and maiden heifers differences between the genetic lines for conception rate were significantly different from zero ($P < 0.05$). However, following parturition, beside activation of hypothalamo–pituitary-ovarian-axis, the post-partum cow must undergo a series of additional recovery events, comprising recovery from pregnancy, a reduction in high exposure to placental hormones, and escape from inhibition of gonadotrophins (caused by suckling) (Malven, 1984).

1.9 MASTITIS AND FERTILITY

1.9.1 EFFECT OF MASTITIS ON REPRODUCTIVE PARAMETERS

Reproductive inefficiency is quickly becoming the biggest and frustrating challenge in dairy operations. Mastitis is conceivably the single most important disease affecting lactating dairy cattle and the consequence is reduced reproductive performance. Cows with clinical mastitis have poorer fertility, but timing and severity of infection together with the type of pathogen influence its extent.

Most of the studies in which the effects of mastitis on reproductive performance were evaluated, are retrospective and diagnosis of clinical mastitis as well as recording of mastitis events were performed by farm personnel. Therefore, it is important to note that inherent variations in diagnosis and recording of mastitis events among studies exist and may account for differences in reported findings. Regardless of possible variations in diagnosis and recording among studies, however, the majority of researches demonstrate a negative correlation between mastitis and fertility.

One of the first studies to demonstrate a correlation between occurrence of mastitis and altered reproductive pattern of dairy cows was performed by Moore *et al.,* (1991). In this study, Holstein cows from two dairy herds were classified as experiencing or not experiencing mastitis between artificial inseminations (AI) or estrus. Cows in herd 1 were affected by *Staphylococcus aureus* (gram-positive bacteria) while cows in herd 2 were affected by coliform mastitis (gram-negative bacteria). The outcome as evaluated was inter-estrus interval and cows were classified as having an abnormal inter-estrus interval when it was smaller than 18 d or greater than 24 d. This parameter has commonly been used as an indicator of early embryonic wastage or altered luteal function. Although there was no effect of mastitis on inter-estrus interval in herd 1, cows in herd 2 that experienced mastitis were more likely to have an altered inter-estrus interval compared with cows that did not experience mastitis (Moore *et al.,* 1991). From these findings authors suggested that the gram-negative IMI observed in herd 2 could have affected the inter-estrus interval through endotoxemia (absorption of bacteria produced toxins by the mammary gland), fever, and systemic secretion of hormones (i.e. prostaglandin F2α and cortisol) that affect luteal and ovarian function (Moore *et al.,* 1991).

A study conducted at the University of Tennessee Dairy Experiment Station research herd further explored the correlation between occurrence of mastitis and reproductive efficiency of dairy cows. Barker *et al.,* (1998) classified lactating Jersey cows as experiencing no mastitis or as experiencing mastitis before first postpartum artificial insemination (FPAI), between FPAI and pregnancy diagnosis, and after pregnancy

confirmation. Cows were followed until 150 days in milk (DIM) and pregnancy was confirmed by palpation per rectum 50 to 65 d after AI. The interval between calving and FPAI was greater for cows that had mastitis prior to FPAI compared with the other cows combined (93.6 ± 5.6 and 71.0 ± 2.2 d, respectively). Cows that experienced mastitis between FPAI and pregnancy diagnosis had increased services per conception (SPC, 2.9 ± 0.3 d) compared with cows that had mastitis prior to FPAI (1.6 ± 0.3) and cows that had mastitis after pregnancy confirmation or those that did not have mastitis (1.7 ± 0.1 d). Furthermore, cows that had mastitis prior to FPAI (113.7 ± 10.8 d) and between FPAI and pregnancy diagnosis (136.6 ± 13.3 d) had greater interval from calving to conception (DOPN) compared with cows that experienced mastitis after pregnancy confirmation or those that did not have mastitis (92.1 ± 4.6 d). Interestingly, the correlation of mastitis and fertility was independent of type of bacteria causing the mastitis (gram-positive or gram-negative).

The same group from the University of Tennessee conducted a subsequent study in which they evaluated the correlation between subclinical and clinical mastitis and fertility. In this study, Jersey cows were grouped as not experiencing mastitis, as experiencing subclinical mastitis (bacteria isolated from milk samples but no alterations in milk or mammary gland), or as experiencing clinical mastitis (Schrick *et al.,* 2001). Cows were further divided according to mastitis type (gram-positive or gram-negative) and to the timing of the mastitis event (before FPAI, between FPAI and pregnancy diagnosis, or after pregnancy confirmation). Cows that did not experience mastitis were grouped together with the cows that experienced it after pregnancy confirmation. Similarly to the findings of Barker *et al.* (1998), cows that experienced clinical or subclinical mastitis before FPAI had extended interval between calving and FPAI (75.7 ± 1.8 d) compared with cows that were uninfected or cows that had mastitis after pregnancy confirmation (67.8 ± 2.2 d). Cows that had mastitis between FPAI and pregnancy diagnosis had intermediate calving to FPAI interval (75.2 ± 4.4 d). Furthermore, the SPC and DOPN were greater for cows that experienced mastitis between FPAI and pregnancy diagnosis (3.1 ± 0.3 and 143.5 ± 11.4 d, respectively) followed by those cows that experienced mastitis prior to FPAI (2.0 ± 0.1 and 106.2 ± 4.8 d, respectively) and those that had no mastitis or had mastitis after pregnancy confirmation (1.6 ± 0.2 and 85.4 ± 5.8 d, respectively), respectively. Similarly to the study by Barker *et al.,* (1998), the correlation between mastitis and reproductive efficiency was not dependent on type of bacteria isolated from milk samples.

In a large study performed in the Central Valley of California (Santos *et al.,* 2004), 1001 lactating Holstein cows from two dairy herds were also classified according to the timing of occurrence of the first mastitis case during the lactation as: no clinical mastitis (CON), mastitis occurring before FPAI (MG1), mastitis occurring between FPAI and pregnancy diagnosis (MG2), or mastitis occurring after pregnancy confirmation (MG3). Cows were followed during the first 320 DIM and reproductive performance was evaluated. The conception rates (CR) after FPAI and the proportion of cows pregnant after 320 DIM were significantly smaller for MG1 and MG2 cows compared with CON and MG3 cows .Cows in the MG2 group had the greatest SPC compared with the other three groups. Furthermore, cows that experienced mastitis, regardless of timing of the event, had increased incidence of abortion between 42 ± 7 and 180 ± 14 d after AI. Cows in the MG1 and MG2 groups had extended interval from calving to conception compared with CON and MG3 cows. When cows were grouped as either not experiencing mastitis (CON) or experiencing mastitis (MG1, MG2, and MG3), those that experienced mastitis had longer interval from calving to conception. Similarly to the studies by Barker *et al.,* (1998) and Schrick *et al.,* (2001), the type of bacteria responsible for the mastitis event did not affect the correlation between mastitis and reproductive performance.

Chebel *et al.,* (2004) evaluated factors that affect conception rates (CR) and pregnancy loss in lactating Holstein cows from three different dairy herds in the Central Valley of California. In this study a total of 7,633 AI were used for evaluation of factors affecting CR and 1,465 cows diagnosed pregnant by ultrasonography at 31 d after AI and re-examined 14 d later were used for evaluation of factors affecting pregnancy loss. Among the factors evaluated was occurrence of mastitis between AI and pregnancy confirmation at approximately 45 d after AI. Cows that experienced mastitis during this interval had similar conception rates compared with those cows that did not experience mastitis. However, when cows had mastitis between AI and pregnancy confirmation they were 2.80 times more likely to experience pregnancy loss between 31 and 45 d after AI compared with those cows that did not have mastitis. In a subsequent study, the same group evaluated the correlation between subclinical mastitis and pregnancy maintenance (Moore *et al.,* 2005). Cows were classified as experiencing subclinical mastitis when they had LSCC > 4.5 in the test day immediately prior to the AI but had no clinical signs of mastitis (Moore *et al.,* 2005). Pregnancy was diagnosed at 28 d after AI by ultrasonography and at 35 d after AI by palpation per rectum. Cows classified as experiencing subclinical mastitis immediately prior to AI were 2.40 times more likely to lose the pregnancy between 28 and 35 d after AI compared with those cows that had LSCC < 4.5. In another large study, McDougall *et al.,* (2005) evaluated the reproductive

performance of 2,004 lactating cows from 10 pasture-fed dairy herds in New Zealand. Cows that had received FPAI within 16 d after the initiation of the breeding season were examined for pregnancy status 29-45 d after AI and at 6, 8, 10, 14, and 22 weeks of gestation. Cows were followed during the entire lactation. In this study, the risk for pregnancy loss was 1.57 times greater for cows that experienced mastitis at any time during the lactation compared with those that never experienced mastitis. In summary, we can conclude that the reproductive efficiency is greatly compromised due to the pathogenetic effect of chronic mastitis in dairy cow.

1.9.2 MASTITIS – FERTILITY (GENETIC CORRELATION)

Knowledge of the genetic parameters and the genetic relationship with important production and functional traits is crucial for successful implementation of survival traits in the breeding objective, as well as for prediction of expected genetic improvement (Holtsmark *et al.,* 2008). During the past decades, several studies have considered the relationship between fertility and other health traits, such as mastitis. In fact, recent reviews have shown a drastic decrease in dairy cattle fertility rates. Conception rate per AI has decreased from 66 percent in 1951 to about 50 percent in 1975, down to 40 percent in 1997 and still decreasing today (Fricke, 2009).

It has been reported that estimated genetic correlations between clinical mastitis and calving interval, services per conception, conception rate, and days to first service is 0.28, 0.41, -0.21, and 0.32 respectively; all of these relationships suggest that cows with more mastitis tend to have poorer fertility (Kadarmideen *et al.,* 2000). Another study reported estimated genetic correlations of -0.58 between clinical mastitis and conception rate and 0.29 between clinical mastitis and calving interval (Heringstad, 2006). Likewise, genetic correlations between milk yield and reproductive measures in dairy cows are unfavorable. This suggests that successful genomic selection for higher yields may have led to a decline in fertility (Pryce *et al.,* 2004). Correspondingly, the estimated genetic correlation between somatic cell score (SCS) and conception rate was -0.40, and the estimated correlation between SCS and calving interval was 0.14 (Konig *et al.,* 2006) . Thus, it appears that cows with mastitis tend to have lower conception rate and longer calving interval.

From the aforementioned study, it is clear that there is a correlation between mastitis and fertility. These findings have led to the hypothesis that the inflammatory and immune responses to the IMI affect reproductive performance by preventing ovulation and resumption of cyclicity after calving (i.e. extended calving to FPAI interval), by reducing fertilization rates and embryo development (i.e. reduced CR and increased SPC), and by

compromising embryonic development and pregnancy establishment and maintenance (i.e. reduced embryonic/fetal survival and increased incidence of abortions)(Chebel *et al.*, 2007). It is important to note, however, that it is not possible to rule out the possibility that cows that are prone to developing mastitis are more debilitated and consequently more prone to develop reproductive disorders that may ultimately affect their fertility.

1.9.3 MECHANISMS BY WHICH MASTITIS MAY AFFECT FERTILITY

The correlation between mastitis and infertility has been studied and demonstrated empirically. Although recently, studies in cattle have led to the emergence of the idea that infectious disease outside the reproductive tract interrupts normal reproduction, we are, however, still far from a specific and detailed understanding of the mechanisms mediating the phenomenon. There are an abundance of likely mechanisms by which mastitis could negatively affect fertility but mostly alterations appear to follow activation of multiple pathways that disrupt the reproductive axis at several points including the hypothalamic–pituitary axis, ovary, oocyte and the embryo (Hansen *et al.*, 2004).

1.9.3.1 HYPERTHERMIA AN INTERRUPTER OF FERTILITY

One of the possible mechanisms for the reduction in fertility of lactating dairy cows that develop mastitis is the elevated body temperature (pyrexia or fever), which can result from both gram -positive and gram-negative infections of the mammary gland (Wenz *et al.,* 2001). In vitro studies have demonstrated that a smaller proportion of oocytes and embryos cultured under heat stresses develop to the blastocyst stage (Edwards and Hansen, 1997; Krininger *et al.,* 2002). Furthermore, when lactating dairy cows and dairy heifers were exposed to heat stress, the fertilization rate and the proportion of excellent/good quality embryos were dramatically smaller for lactating dairy cows compared to heifers (Sartori *et al.,* 2003). This indicates that exposure of oocytes and embryos to heat stress compromises fertilization and development. Aside the direct effect of elevated body temperature on oocyte and embryo quality and development, fever can indirectly affect reproductive performance because cows that experience it have decreased feed intake and body condition (Maltz *et al.,* 1997). Therefore, if a cow develops mastitis and fever during early postpartum, a period of the lactation in which cows are already predisposed to reduced feed intake, greater loss of body condition and more pronounced negative energy balance may occur, which could interrupt reproductive function and delay resumption of ovarian cyclicity (Buttler, 2000).

1.9.3.2 MEDIATORS OF INFLAMMATION AS DISRUPTORS OF FERTILITY

Another possible mechanism by which mastitis may affect fertility in lactating dairy cows is through the production of substances that affect folliculogenesis, oocyte and embryo development, uterine environment, and various aspects of ovarian function. These substances are mainly cytokines and among them interleukin (IL)-1α, IL-1β, IL-6, IL-10, IL-12, and tumor necrosis factor-α (TNF-α) have been isolated from milk-derived cells from infected mammary glands (Riollet *et al.,* 2001). Furthermore, challenge of lactating cows with components of the cell wall (lypopolysaccharide, LPS) of *Escherichia coli* (gram-negative bacteria) resulted in increased milk concentrations of IL-1β, IL-8, and TNF-α (Blum *et al.,* 2000; Waller *et al.,* 2003; Elazar *et al.,* 2010). Other studies have also demonstrated that cows that experience mastitis have increased blood concentrations of TNF-α, IL-1, and IL-6 (Hoeben *et al.,* 2000; Nakajima *et al.,* 1997). Moreover, mastitis is also correlated with increased concentrations of molecules important for influencing the reproductive system, such as nitric oxide (NO), IFN-γ and prostaglandin F2α (PGF2α) in milk as well as mastitic cows challenged with oxytocin had increased blood concentrations of PGF2α metabolite (Blum *et al.,* 2000; Bouchard *et al.,*1999; Giri *et al.,*1984; Hockett *et al.,* 2000). Although the implication of cytokines and other bioactive molecules is still largely unknown, from these findings, it is obvious that mastitis associated systemic immune response greatly intervenes in various aspects of the reproductive processes.

1.9.3.2.1 OOCYTE MATURATION, FERTILIZATION & EMBRYONIC DEVELOPMENT

Most of the studies conducted to evaluate the effects of compounds produced because of IMI on oocyte and embryo development were based on in vitro models. Even if results from *in vitro* studies are not always confirmed *in vivo*, they provide insightful information about possible mechanisms by which mastitis may affect fertility.

It's recognized that metabolic changes are mostly due to the formation of mediators of immune response that circulate in the blood (Hansen *et al.,* 2003), but the mechanism by which they act is unclear. The implication of cytokines in this mechanism is still unknown; but studies in cyclic heifers receiving an intravenous endotoxin challenge in the follicular phase the basal LH pulsatility was impaired, the plasma level of estradiol-17β was significantly decreased; and the formation of preovulatory LH peak was delayed or completely blocked (Suzuki *et al.,* 2001). Studies indicate that mastitis delayed the first postpartum ovulation of the animal still in acyclic state, or lengthened the follicular phase and induced pre-mature luteolysis in already cyclic cows. In fact, after the mastitis outbreak, almost none of the cyclic cows showed visible estrous signs, regardless of the

affected phases and/or the form of the ovarian alterations (Huszenicza *et al.,* 2005). Moreover, certain cytokines released during mastitis can also have significant effects on the ovary such as IL-6 that blocks follicle stimulating hormone-induced estradiol secretion from bovine granulosa cells (Alpizar *et al.,* 1994).

Maturation of bovine oocytes in the presence of TNF-α or addition of lipopolysaccharide of *E. coli*, to cumulus-oocyte complexes resulted in reduced proportion of fertilized oocytes developing to the blastocyst stage (Soto *et al.,* 2003). Furthermore, embryos cultured in the presence of TNF-α, PGF2α, or NO had either increased number of apoptotic cells or compromised development to the blastocyst stage (Pampfer *et al.,* 1994; Wuu *et al.,* 1999; Soto *et al.,* 2003; Chen *et al.,* 2001; Hobbs *et al.,* 1999). Furthermore, administration of PGF2α to cows supplemented with progesterone resulted in poorer quality embryos and decreased pregnancy rates, reinforcing the idea that PGF2α may have a direct effect on embryo development (Buford *et al., 1996*).

1.9.3.2.2 CHANGES IN REPRODUCTIVE FUNCTION MEDIATED BY CYTOKINES

As mentioned previously, endometrial synthesis of prostaglandins is under the control of several cytokines including TNF-α, TNF-β and IL-1c (Skarzynski *et al.,* 2000; Davidson *et al., 1995*). Prostaglandin F2α produced by the endometrium is responsible for luteolysis, which occurs at the end of the estrous cycle if an embryo is not present. TNF-α, can increase PGF2α secretion from cultured bovine stromal endometrial cells (Skarzynski *et al.,* 2000) and IL-1β, can increase secretion of PGF2α and PGE2 from endometrial stromal and epithelial cell (Davidson *et al.,* 1995). Thus, it is possible that the release of cytokines into the bloodstream during mastitis could lead to induction of endometrial PGF2α release and consequently pre-mature luteolysis, which hampers cyclicity of animal or result in embryonic/fetal death. Endotoxin caused an increase in serum concentrations of PGFM when administered intravenously (although not significant change when administered in the mammary glands) (Jackson *et al.,* 1990). Moreover, cows in which mastitis was induced by *Streptococcus uberis* infusion experienced a greater rise in circulating PGFM in response to oxytocin treatment than control cows (Hockett *et al.,* 2000). Intrauterine infusion of live *E. coli* into heifers on days 7–9 of the estrous cycle caused luteal regression and shortened estrous cycles (Gilbert *et al.,* 1990). The cytokine IFN-α, when injected between the 13th and 19th days after insemination in cow raises the body temperature, inhibits secretion of luteal hormone and reduces the concentration of progesterone in the circulation and eventually interrupts the pregnancy (Hansen *et al.,* 2004). Cytokines could also exert other effects on endometrial or oviductal tissue that impede embryonic development. IL-1β, for example, reduced

proliferation of endometrial stromal cells and IFN-α reduced proliferation of oviductal epithelial cells (Kamwanja *et al.,* 1993).

1.9.3.2.3 HYPOTHALAMIC-PITUITARY-OVARIAN AXIS

Reproductive cycles in cows are regulated by hormones produced in the hypothalamus (GnRH = gonadotropin releasing hormone) and pituitary (FSH = follicle stimulating hormone and LH = luteinizing hormone), which are glands located in the brain, and ovaries (E2 = estradiol and P4 = progesterone). Briefly, GnRH is responsible for stimulating the secretion of FSH and LH. Follicle stimulating hormone is responsible for stimulating initial growth of follicles in the ovaries, while LH is responsible for maturation of follicles and ovulation and also for stimulation of P4 secretion by the corpus luteum. Estradiol, produced by the follicles, is responsible for stimulating the peak of LH that causes ovulation, while P4 is responsible for stimulating embryo growth and maintenance of pregnancy. Therefore, it is clear that the disruption of production or secretion of one of these hormones may dramatically affect the fertility of dairy cows.

One possible reason for increased number of services per conception in cows with mastitis is inhibition of gonadotropin secretion leading to reduced gonadotropin support for ovulation, oocyte maturation, folliculogenesis and luteal function. It has been demonstrated that certain cytokines such as IFN-β decrease the secretion of LH (McCann *et al.,* 2000). Furthermore, mastitis and exposure of cows to endotoxins secreted by gram-negative bacteria result in increased blood concentrations of cortisol, a hormone that blocks the release and the peak of LH (Stoebel *et al.,* 1982; Li *et al.,* 1983; Padmanabhan *et al.,* 1983). The decrease or lack of LH secretion may result in compromised follicle development, lack of ovulation, and suboptimal luteal function. Some of the cytokines produced during mastitis also have a direct effect on the ovaries, Interleukin-6, for example, blocks follicle stimulating hormone-induced estradiol secretion from bovine granulosa cells, especially from cells isolated from small follicles which can lead to reduced LH secretion(Alpizar *et al.,* 1994). TNF-β and IFN-δ are cytotoxic to the bovine luteal cells and could cause reduction in concentrations of P4 (Fairchild *et al.,* 1991; Petroff *et al.,* 2001).

It is clear that a correlation between mastitis and reproductive failure exist. Reproductive performance of lactating dairy cows that experience mastitis could be affected in many ways. The longer interval from calving to first postpartum AI could be the result of extended period of anovulation following calving caused by reduced energy balance as a consequence of fever or blockage of GnRH-LH secretion. Mastitis linked immune activation could severely destabilize ovarian folliculogenesis leading to anovulation or

delayed ovulation with weak estrous signs. Production of poorer quality oocytes or embryos because of exposure to fever or to compounds that are deleterious to their development could be manifested by reduced conception rates and increased services per conception. Longer interval from calving to conception could be the result of the conditions mentioned above and increased embryonic/fetal loss or abortions. Therefore, the appropriate management of lactating dairy cows to minimize the incidence of mastitis should increase the profitability of dairy herds not only by improving milk quality, reducing the use of antibiotics, reducing the amount of milk discarded, and reducing involuntary culling but also by improving reproductive performance.

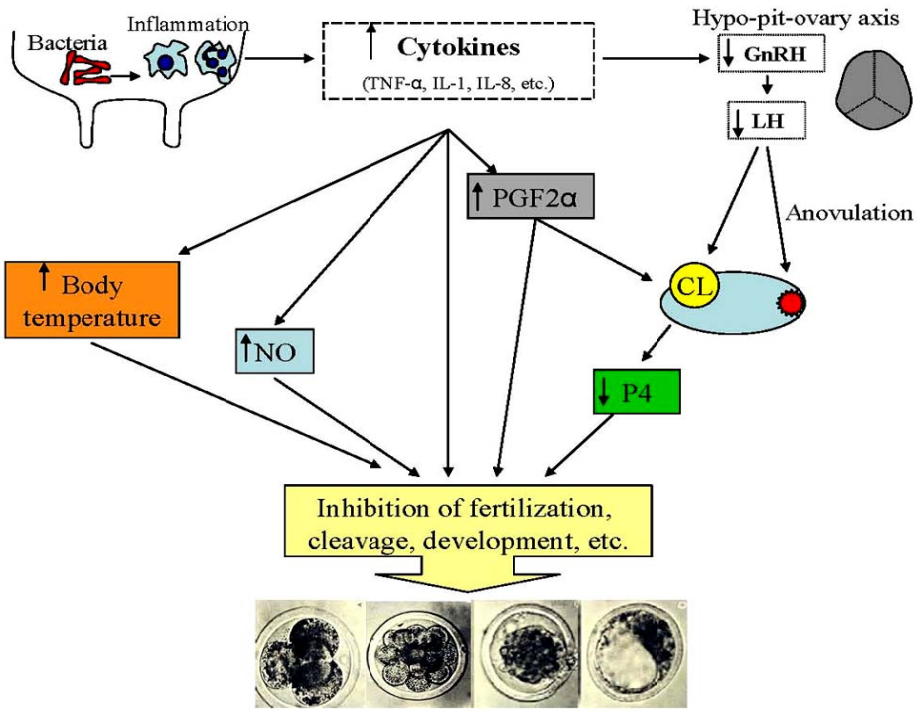

Figure 9. Potential pathways by which mastitis can compromise fertility. According to this model, the central phenomenon leading to reduced fertility associated with mastitis is increased secretion of cytokines (either produced in the mammary and draining lymph nodes and secreted or produced in other tissues in response to mammary-derived signals) that in turn modulate reproductive function at several levels (Hansen *et al.,* 2004).

B. OVARY AND FOLLICULAR DYNAMICS

1.1 BOVINE OVARY AND ITS STRUCTURE

The oocyte-producing ovary in female mammals is functionally homologous to the testes in the male. The two ovaries are located in the pelvis of every cow, one on the left and one on the right side, each approximately 3.8 to 4 cm long and 1.8 to 2 cm wide, with a weight of 2-4 grams. The ovaries are suspended from the broad ligament near the end of the oviduct and lie near the tips of the curved uterine horns.

Ovarian cortex, the dense layer of the ovarian stroma where the matrix is primarily composed of intermingled fibroblasts, collagen and elastin fibers, among which scattered follicles in various stages of development are embedded (Figure 10). Ovarian medulla is centrally located loose texture and consists of fibrovascular, nerve fibers and lymphatic tissues, extending without any clearly defined border to ovarian cortex. The ovary is enclosed by a single layer of cuboidal germinal epithelium and directly beneath the outermost layer of ovarian stromal epithelium lays a layer of dense collagenous connective tissue, called tunica albuginea (Figure 11).

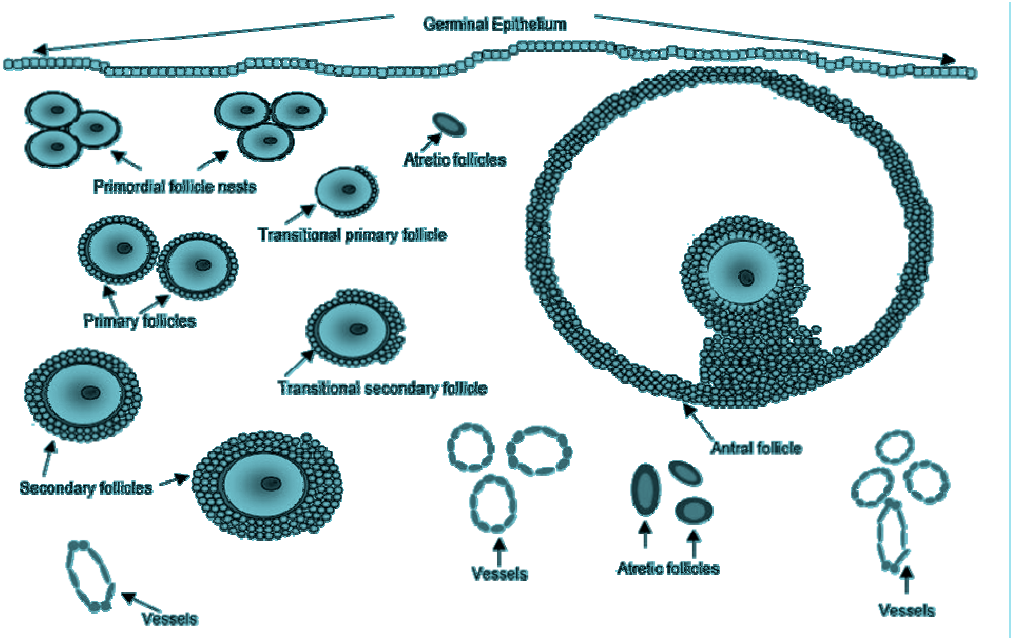

Figure 10. The basic morphological components of the ovarian cortex (Carlssson, 2008).

The oocyte and surrounding cells of the follicle constitute the fundamental reproductive unit of the ovary. The cortex of young animals consists primarily of such follicles, whereas in older cow most of these follicles have been replaced by fibers as a consequence of ovulation and atresia. As the follicles grow in size they initially migrate deeper into the cortex and subsequently migrate to the surface as the time for ovulation approaches. Thus, the surface of a young ovary appears smooth, while upon aging this surface becomes scarred from repeated ovulation.

The several tasks performed by the ovary include housing and nurturing the oocytes, and the secretion of hormones that promote follicle maturation and the development of secondary sex characteristics. It is the follicle that provides a protective cover and a suitable environment for the oocyte. During the fertile period of cow's life, in comparison to its reserve only small numbers of follicles mature and ovulate, whereas the remainders become atretic.

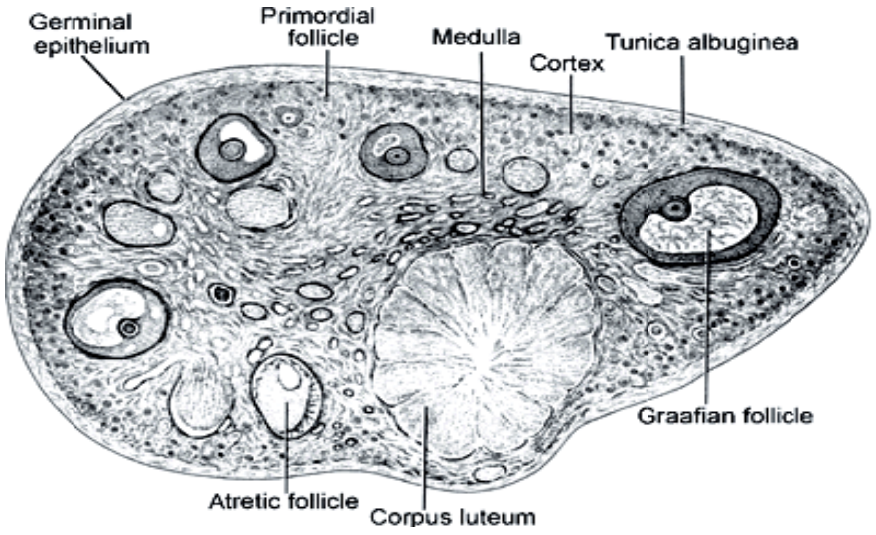

Figure 11. Schematic representation of the morphology of a fully developed bovine ovary (Junqueira *et al.,* 1993)

1.2 DEVELOPMENT OF OVARY AND FOLLICULAR RESERVE

The gonads originate at the ventro-medial side of the rudimentary nephritic organ or mesonephros. Proliferation of the coelomic epithelium and a concomitant condensation of the underlying mesenchyme leads to the formation of a swelling, called the genital ridge or gonadal crest. In bovine embryos, the gonadal ridges develop by day 28 to 32 of gestation (Noden and de Lahunta, 1985; Russe and Sinowatz, 1991). Initially, these genital crests do not contain any primordial germ cells, which at that time are still located in the epithelium of the yolk sac, close to the base of the allantois. A migratory phenotype of the primordial germ cells reaches the gonadal ridges along the wall of the hindgut and dorsal mesentery through amoeboid movements and with the aid of pseudopodia. In the bovine species this migration usually occurs between day 30 and 64 of gestation (Russe and Sinowatz, 1991). Several lines of investigation have led to the assumption that the migratory pattern of primordial germ cells is controlled by chemotactic signals produced by the genital ridge (Oktem and Oktay, 2008).

The invasion of epithelial proliferations in the mesenchyme of the genital ridges gives rise to gonadal or medullar cords. These terms are usually employed as synonyms, but according to Smitz and Cortvrindt (2002), the epithelial cords can be further distinguished by their origin, whereby cells derived from the mesonephros form primitive medullar cords and cells from coelomic epithelium form sex cords. The sex cords disaggregate into cell clusters and develop into the rete ovarii (Lin *et al.,* 2002) and the vascular stroma of the ovary (Lin *et al.,* 2002; Smitz and Cortvrindt, 2002). In male embryos, the counterpart rete testes develop into the tubuli seminiferi.

The foetal bovine ovary from Day 40 to Day 70 is apparently without internal organization, but somatic and germinal elements are distinguishable microscopically (Erickson, 1966a). As the primordial germ cells arrive in the gonads, they are incorporated into primary sex cords (Smitz and Cortvrindt, 2002). These, however, degenerate and are replaced by new epithelial components. During the development of the secondary or cortical cords, the primordial germ cells are embedded in the proliferating tissue (Lin *et al.,* 2002). Around day 170-180 of gestation, prominent medulla is formed from these medullar cords and cortex with germinative tissues from the cortical cord (Erickson, 1966a). Fetal ovarian mass increases markedly after day 180 of gestation (Tanaka *et al*., 2001). When the ontogenesis of the ovary is completed the follicles are embedded in the stromal matrix.

1.2.1 OOGENESIS

The process of meiotic oocyte development from mitotic oogonia is termed oogenesis, involves several processes, including oocytogenesis and ootidogenesis, with folliculogenesis being one aspect of the latter. Upon their arrival in the gonads and embedment in primary sex cords, mitotically active diploid germ cells are termed 'oogonia' (Kaipia and Hsueh, 1997; Oktem and Oktay, 2008). Clusters of germ cells are formed, consisting of a number of oogonia surrounded by somatic cells, which are considered to be precursors to granulosa cells. Mitoses of oogonia within a single cluster of germ cells run synchronous, but with incomplete division of the cytoplasm (Pepling and Spradling, 1998). Hence, the oogonia remain interconnected by cytoplasmic bridges and constitute a syncytium. Mitotic activity of the oogonia is a major determinant of the size of the oocyte pool. Following successive rounds of division, initiation of pre-meiotic DNA synthesis marks the end of the oogonial stage, after which the germ cells are called oocytes. **Oocytogenesis**, i.e., the transformation of oogonia into primordial oocytes, is completed prior to birth in bovine.

Ootidogenesis is the process by which primordial oocytes develops into a primary oocyte and involves entry of oogonial DNA into meiosis and arrest at prophase I, called dictyate. Following instigation of meiosis, epithelial cells from the sex cords send out cytoplasmic processes between the interconnected oogonia, dividing the cluster in individual oocytes surrounded by a single layer of flattened pre-granulosa cells. Concurrently, a basal lamina is deposited on the surface of the granulosa cells, effectively isolating the newly formed primordial follicles from the ovarian stroma. Finally, the primordial follicles are detached from each other by cytoplasmic processes from the stroma cells between neighbouring basal lamina (Merchant-Larios and Chimal-Monroy, 1989). In the cow, fully fledged primordial follicles are present after day 90 of gestation (Yang and Fortune, 2008) (Figure 12).

The primary oocyte then remains in this state until puberty, when a few such oocytes are recruited into the growing pool and become secondary oocytes. This process involves disappearance of the nucleus, (also known as the gemninal vesicle); completion of the first meiosis by extrusion of the first polar body; immediate entry into a second round of meiosis; and arrest in the meta phase of this meiosis II until fertilization occurs (if ever does) and the second polar body is extruded.

Clearly, a detailed understanding the mechanisms that regulate the formation of the pool of primordial follicles together with their oocytes as well as their transition to the growing phase is essential for the manipulation of this pool for practical purposes; especially

would be enormously helpful in connection with our attempts to investigate the effects of mastitis on the ovarian reserve and follicular dynamics in vivo.

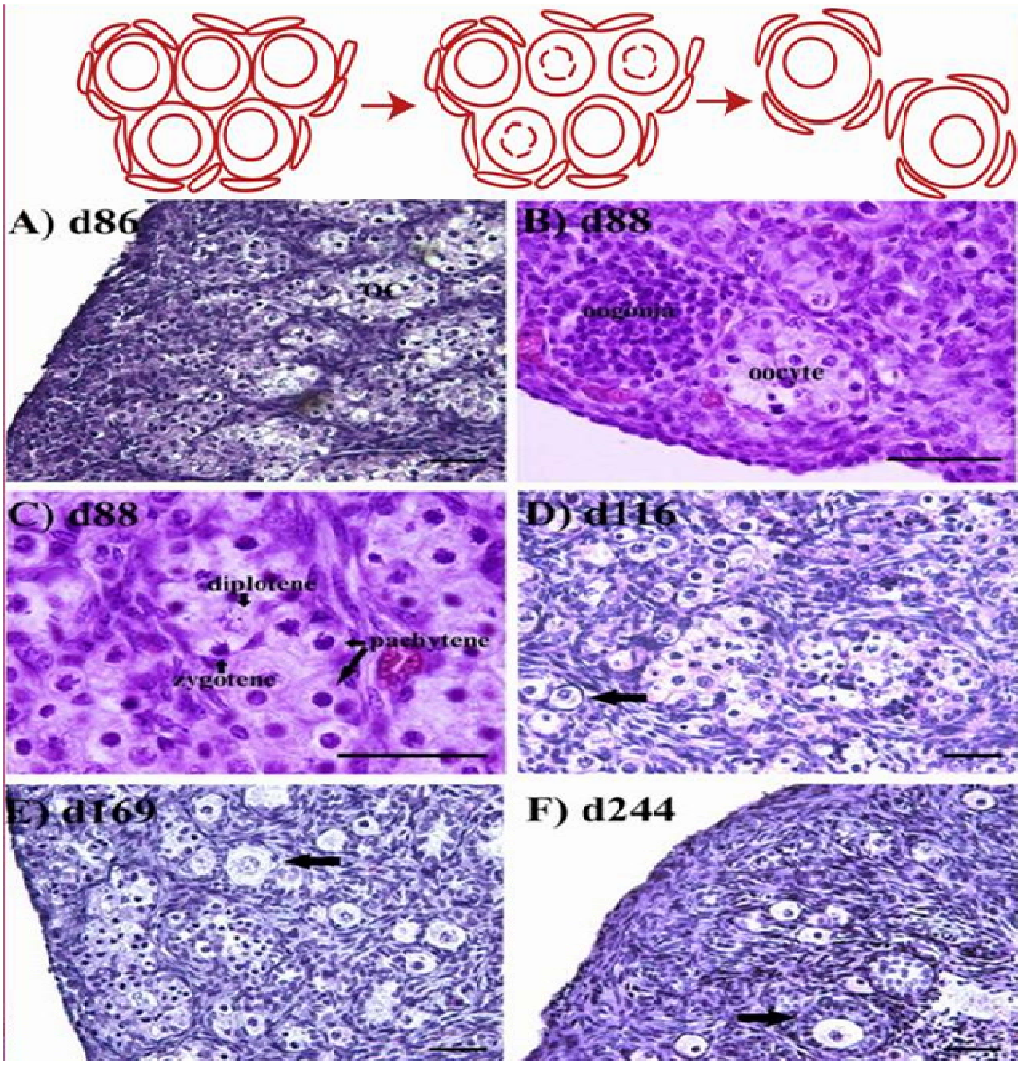

Figure 12. Representative photomicrographs of paraffin sections of whole fetal bovine ovaries stained with hematoxylin and eosin and showing morphology of ovarian cortex at various gestational ages (estimated from the Crown to Rump length). **A**) Day 86 of gestation (d86); cortex filled with ovigerous cords (OC) containing germ cells; no primordial follicles are present. **B**) Day 88 of gestation (d88); oogonia and oocytes within ovigerous cords in the ovarian cortex. **C**) Day 88 of gestation; oocytes in the ovigerous cords at various stages of first meiotic prophase. **D**) Day 116 of gestation (d116); many ovigerous cords still present, but some primordial follicles (arrow) have formed. **E**) Day 169 of gestation (d169); many ovigerous cords have regressed; more primordial follicles are present, and primary follicles have appeared (arrow). **F**) Day 244 of gestation (d244); ovigerous cords have almost completely disappeared; secondary follicles are present (arrow). Bars = 50 μm. At the very top: schematic of primordial follicular assembly (Yang and Forune, 2008).

1.2.2. OVARIAN RESERVES

The female reproductive system contains a non-renewable reserve of germ cells. However, this dogma has been contradicted recently on the ground of unearthing germ stem cells in the surface epithelium of the ovary (Johnson *et al.,* 2004). Initially, these investigators proposed that the germ-line stem cells responsible for this neo-oogenesis are located in the ovarian surface epithelium (Johnson *et al.,* 2005); but later they proposed that these stem cells are present in the bone marrow or circulation, rather than the surface epithelium (Johnson *et al.,* 2005). Later, it was reconfirmed that neo-oogenesis does not occur in ovary (Liu *et al.,* 2007).

In line with the recognized concept, the number of ovarian follicles is largest prior to birth, declines gradually throughout early young life and drops rapidly in adults. In the cow, following the migration from yolk sac to the gonads, the maximum number of primordial germ cells is reached at the time of transition from mitosis to meiosis and was estimated at (2.7×10^6), which is reduced to on average (133×10^3) at birth (Gondos, 1978; Erickson, 1966 a, b). This event usually takes place in the course of approximately 60 days (day 50 to day 110 of gestation) and oogonial mitosis is discontinued at or near days 150 of gestation (Erickson 1966 a). However, according to another study, at day 91, the number of germ cells in fetal bovine ovaries reaches a peak (2.8×10^6). Then after 91 days, the number of germ cells decreased to (38080) at day 160 and (162×10^3) at day 285 (Tanaka *et al.,* 2001).

Numbers of primordial follicles for a given individual apparently remain stable (133×10^3 average) until about the fourth year of life and decline thereafter until the near zero point is reached in ovaries from Hereford cows 15 to 20 years of age (Erickson, 1966 b). In humans, this maximum is established during month 5 of fetal development with (7.1×10^6) primordial germ cells; of which only (2×10^6) remain at birth (Pepling and Spradling, 2001). Apoptosis seems to be a universal mechanism for dramatic reduction of the oocytes, as all vertebrate species that have been examined to date are born with much fewer oocytes than their maximum number during foetal development (Vaskivuo, 2002). It is important to realize that not only do follicle numbers decrease with age, but the quality of the follicle and the oocyte it contains also declines, with an increasing frequency of structural damage and aneuplody (de Bruin *et al.,* 2004).

1.2.3 NUCLEAR MATURATION DURING MEIOSIS

During meiotic cell divisions by which germ cells (oocytes and sperm) are produced, the genetic material contained in each daughter cell is halved completing two nuclear divisions, but only a single replication of the nuclear DNA. The first cell division

associated with meiosis is divided into prophase I, metaphase I, anaphase I and telophase I; while the second cell division, which results in gamete formation, is divided analogously into prophase II, metaphase II, anaphase II and telophase II. Prior to the first meiotic cell division, the oocyte itself is called a germinal vesicle (GV) oocyte as the nucleus it contains is also known as a germinal vesicle. The GV/nucleus disappears as the cell enters metaphase I (MI) and at metaphase II (MII) the first polar body is extruded. Final maturation of the oocyte, including extrusion of the second polar body, occurs immediately after fertilization. This process is referred to as nuclear maturation (Figure 13).

1.2.4 CYTOPLASMIC MATURATION

While nuclear maturation is easy to follow under the light microscope, the parallel process of cytoplasmic maturation cannot be seen in this way. Cytoplasmic maturation includes reorganization, accumulation of various species of mRNA and protein, and epigenetic modifications. Thus, during oocyte maturation, large amounts of RNA are stored in the cytoplasm (Neilson *et al.,* 2000) and organelles are formed and reorganized, with the number of mitochondrial profiles increasing dramatically (Wassarman and Josefowicz, 1978). RNA molecules and proteins that are accumulated in the oocyte cytoplasm during its growth phase are used to sustain the early phases of embryonic development before embryo DNA transcription begins. This makes the oocyte a very special cell, quite different from somatic cells where RNA and proteins usually undergo a rapid turnover (Brevini and Gandolfi, 2001).

Recent investigations reveal the change of mitochondrial profiles especially mitochondrial translocation is highly related with the oocyte developmental competence (Brevini *et al*. 2004, 2005), and mitochondria show three unique distribution pattern such as: peripheral, semi-peripheral and diffused (Brevini *et al.,* 2007). During cytoplasmic maturation, the cytoskeleton plays a key role by shuttling organelles and mRNAs to specific sites within the oocyte cytoplasm. Localization is driven by specific molecular motors belonging to the kinesin superfamily and requires the involvement of the RNA targeting molecule Staufen (Brevini *et al.,* 2007). Infact, during cytoplasmic maturation process, significatnt changes in adenosine triphosphate (ATP) concentration and distribution taking place and these events are closely associated with oocyte developmental competence(Brevini *et al*., 2007). In addition to RNA molecules and proteins, the oocyte also stores lipids and other granules required for the formation of new membranes in its cytoplasm post-fertilization (Picton *et al.,* 1998; Obata and Kono, 2002).

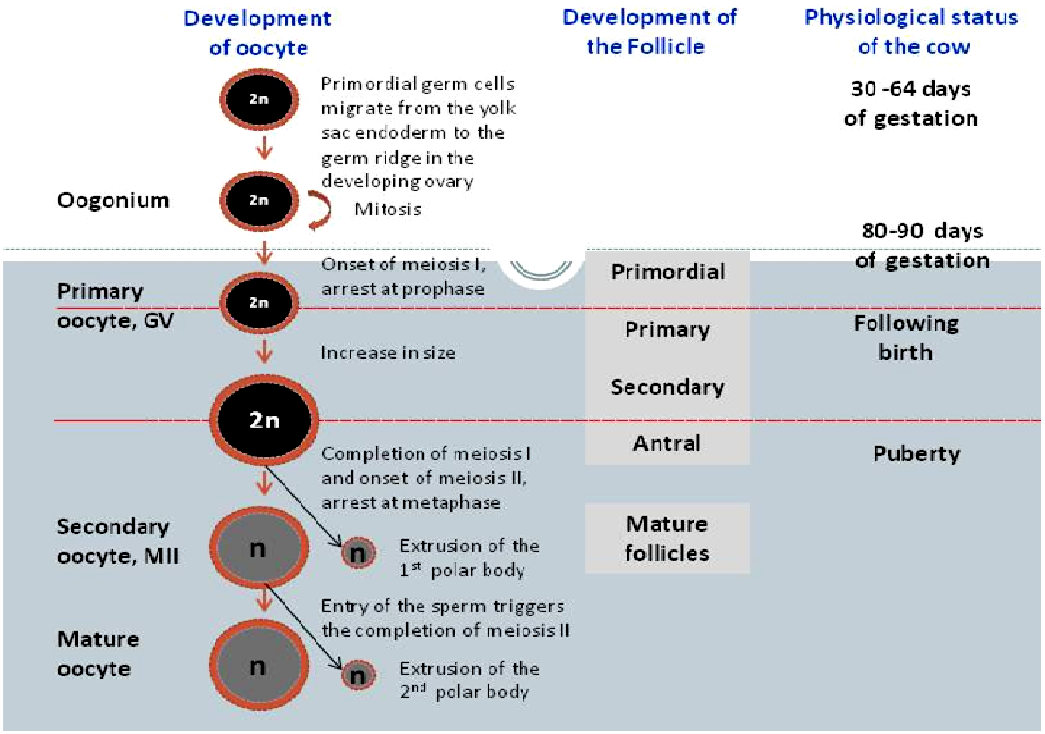

Figure 13. Temporal relationship between the development of the oocyte and follicles with the physiological status of the cow.

1.3 FOLLICULOGENESIS

The process by which a follicle matures from the primordial to the preovulatory stage, with many steps in-between is referred to as folliculogenesis. This development involves two major processes, i.e., recruitment of the follicle into the growing pool and the proliferation and differentiation of the granulosa and theca cells. The first of these processes is regulated by paracrine and autocrine signals produced in the ovary itself; while the second is controlled both by this internal signaling and by endocrine signals from outside the ovary.

Throughout reproductive life, the majority of follicles in mammalian ovaries are nongrowing, primordial follicles with an oocyte surrounded by a single layer of flattened granulosa cells. Individual primordial follicles remain quiescent for variable lengths of time. During development, the proliferating GCs provide nutrients and various molecular

signals to the oocyte, which increases in size. Re-organization of the follicle and the differentiation and proliferation of the GCs results in the formation of an antrum prior to ovulation (Eppig, 1991). In these ways, the follicle supports the oocyte, both chemically and physically. During this developmental process the follicle migrates from the cortex to the medulla and then back again as ovulation approaches.

Gonadotropins, primarily follicle stimulating hormone (FSH), play important roles in the growth of and sustained steroidogenesis by follicles. The “two cell, two gonadotropin theory” proposes that, the interstitial theca cells are stimulated by luteinizing hormone (LH) to produce aromatizable androgens and that these androgens are subsequently transported to the GCs, where they are converted into estrogens by aromatizing enzymes which are regulated by FSH (Hillier *et al.,* 1994). When the follicle has reached a size of 200 µm, it becomes more dependent on FSH for growth and its rate of steroid production increases.

After the wave emergence, estradiol content in the follicular fluid of the selected growing dominant follicle increases dramatically followed by a surge in LH which triggers the dominant follicle to ovulate (Singh J *et al.,* 2003). After ovulation, this follicle transforms into the corpus luteum, which is responsible for the production of progesterone and maintenance of the early phase of pregnancy. This cycle is repeated continuously (with interruptions during periods of gestation) until the pool of follicles is exhausted. At any one time-point, the ovary of a fertile cow contains follicles in all stages of development.

1.3.1 INITIATION AND GROWTH OF FOLLICLES

The majority of the assembled primordial follicles degenerate in pre- or post-natal life, while still in a quiescent state and never embark on the complex developmental pathway that may, or may not, culminate in ovulation. Of those ‘resting’ primordial follicles that survive and are recruited into the growing follicle population, very few (<0.1%) are destined to ovulate; the vast majority will degenerate at some point along this lengthy developmental continuum (duration >3 months in bovine). Unfortunately, the factors and hormones which stimulate or inhibit initiation of this process remain to be fully elucidated.

In addition to gonadotropins, a complex network of cell-cell interactions regulates the transition of primordial to primary follicles. Progression through successive stages of follicle development requires bi-directional communication between oocyte and granulosa cells, and granulosa and theca cells; many of the extracellular signaling molecules implicated in this state of folliculogenesis belong to the transforming growth factor-β (TGF-β) superfamily (Durlinger *et al.,* 1999, Eppig, 2001). Only a few follicles respond to

the cyclic gonadotropin stimulation that occurs after puberty to reach the preovulatory stage. The preovulatory gonadotropin surges associated with each reproductive cycle cause the dominant preovulatory follicle to release its mature oocyte for potential fertilization.

1.3.2 MORPHOLOGICAL CORRELATES AND STAGES OF FOLLICULAR DEVELOPMENT

The primordial follicles in most species are identified histologically on the basis of a quiescent or non-growing oocyte, without a zona pellucida and surrounded by a single layer of flattened granulosa cells, all of which are separated from the surrounding somatic cells by a basement membrane (Figure 14, A-D; Fair *et al.,* 1997, Gosden *et al.,* 2002). The follicles appear this way because the oocyte is not enlarging and the granulosa cells are not replicating. A typical primordial follicle in bovine first appears after days 90 of gestation and has a diameter of approximately 30 µm (Yang and Fortune, 2008). However, according to another study, the appearance of first bovine primordial follicle is much earlier as of day 74 of gestation (Tanaka *et al*., 2001).

Once these follicles are activated or recruited into the growing pool i.e. the transition from quiescence to the growth phase is characterized by a change in shape of the granulosa cells from flattened to cuboidal, while continuing to surround the oocyte as a single layer. Oocyte starts to enlarge and initiate paracrine signaling between GCs (Fair *et al.,* 1997). At this stage the GCs are presumably cuboidal because they have commenced replicating and cells 'round-up' at the prophase and metaphase allowing spindle formation for division to subsequently occur (Boucrot and Kirchhausen, 2008; Rosenblatt, 2008). In bovine ovaries, 83% of follicles that are less mature than the primary stage are ellipsoid in shape with cuboidal cells located at the poles of the follicle (Figure 14-E, H). These activated follicles are in an intermediate or transition stage from primordial to primary (Westergaard *et al.,* 2007). However, for the sake of major classification they were interpreted to be primordial follicles with an ellipsoid shape imposed upon them by the surrounding bundles of collagen fibrils (van Wezel and Rodgers, 1996). In the humans, this intermediate form has also been observed in which some granulosa cells (usually on one side of the follicle) are cuboidal in shape (Figure 14F; Gougeon and Chainy, 1987).

At this stage, GCs begin to express marker for proliferation, as well as expression of FSH receptor initiates in the follicle (Wandji *et al.,* 1996). A typical bovine transitional primary follicle has a diameter of approximately 50-60 µm. The GCs secrete mucopolysaccharides around the oocyte to form the Zona pellucida, a thick layer of

glycoproteins and acid proteoglycans situated between the oocyte and GCs themselves. It is interesting to note that while the origin of the ZP is controversial, at this stage, ZP proteins have been detected in both the oocyte and GCs (Gook *et al.,* 2008). GCs are devoid of any vascular supply, which necessitates the intercellular contact with neighboring cells via gap junctions. Extensive gap junctions not only allow an integral functional syncytium, but are also important in metabolic exchange and transportation of molecules among neighbouring cells. Moreover, the microvilli of the cumulus cells penetrate through the zona pellucida to form gap junctions with the oocyte plasma membrane (Li *et al.,* 1995) for bidirectional transfer of nutrients, metabolic precursors and signal molecules, including growth factors with inhibitory and stimulatory meiotic signals (Eppig, 1991; 1992). A typical primary follicle in bovine first appears around days 140 of gestation and has been observed that there is a 50 days gap between the first appearance of primordial versus primary follicles (Yang and Fortune, 2008). However, the appearance of primary follicle has been reported to be formed as early as of days 91 of gestation (Figure 15, A-D; Tanaka *et al*., 2001).

In case of secondary follicles, the granulosa cells proliferate and form multiple layers around the oocyte, which also becomes larger (Figure 16). In bovine, first secondary follicles develop around 210 days of gestation (Russe and Sinowatz, 1991; Yang and Fortune, 2008). However, it has also been reported that first secondary follicles in bovine fetus develop much earlier and around 120 days of gestation (Tanaka *et al.,* 2001). Only after development has proceeded to the early secondary stage when the second layer of cuboidal granulosa cells begin to appear do the first theca cells begin to differentiate in the theca interna. The theca cells differentiate from a population of unspecialized mesenchymal cells in the ovarian stroma. The theca interna is a layer of highly vasuclarized steroidogenic cells adjacent to the basal lamina in contrast to the theca externa which is a loosely orgnanized band of non-steroidogenic cells between the theca interna and the interfollicular stroma. Thus, at this point the follicle starts to receive a blood supply and is exposed to circulating factors (Reynolds *et al.,* 1992). The diameter of the follicle is now 100-200 µm approximately.

Once the oocyte has completed its growth, granulosa cells proliferate further and arrange themselves into several layers, producing a primary multilaminar follicle (Figure 16-D). This developmental step from secondary follicle is dependent on FSH, which both stimulates proliferation of the granulosa cells and increases the number of FSH receptors expressed at their surface, thereby magnifying its own effects (Erickson and Danforth, 1995). The differentiation of the theca cells surrounding the follicle results 3-5 cells layers thick envelope of elongated endocrine cells immediately adjacent to the basal lamina

(Magoffin, 2005). The GCs present in a secondary follicle demonstrate very high mitotic activity as their number increases.

These multilaminar secondary follicles in bovine and also human can be classified into two groups depending on the morphological phenotype of the follicular basal lamina (Irving-Rodgers and Rodgers, 2000; Irving-Rodgers *et al.,* 2008). In both species, some follicles have a conventional basal lamina of a single layer aligned to the surface of the basal granulosa cells, which at the preantral stage in both species are substantially thicker or even partially laminated than either the primordial or the antral follicles. Secondary follicles with additional layers of basal lamina have also been observed. Loops of basal lamina have been reported in cross sections and are connected to additional layers closer to the granulosa cell surface (Figure 18). Cellular projections emanating from the basal surface of the basal granulosa cells and membrane bound vesicles often at the end on these processes and adjacent to the basal lamina have also been reported (Irving-Rodgers and Rodgers, 2000).

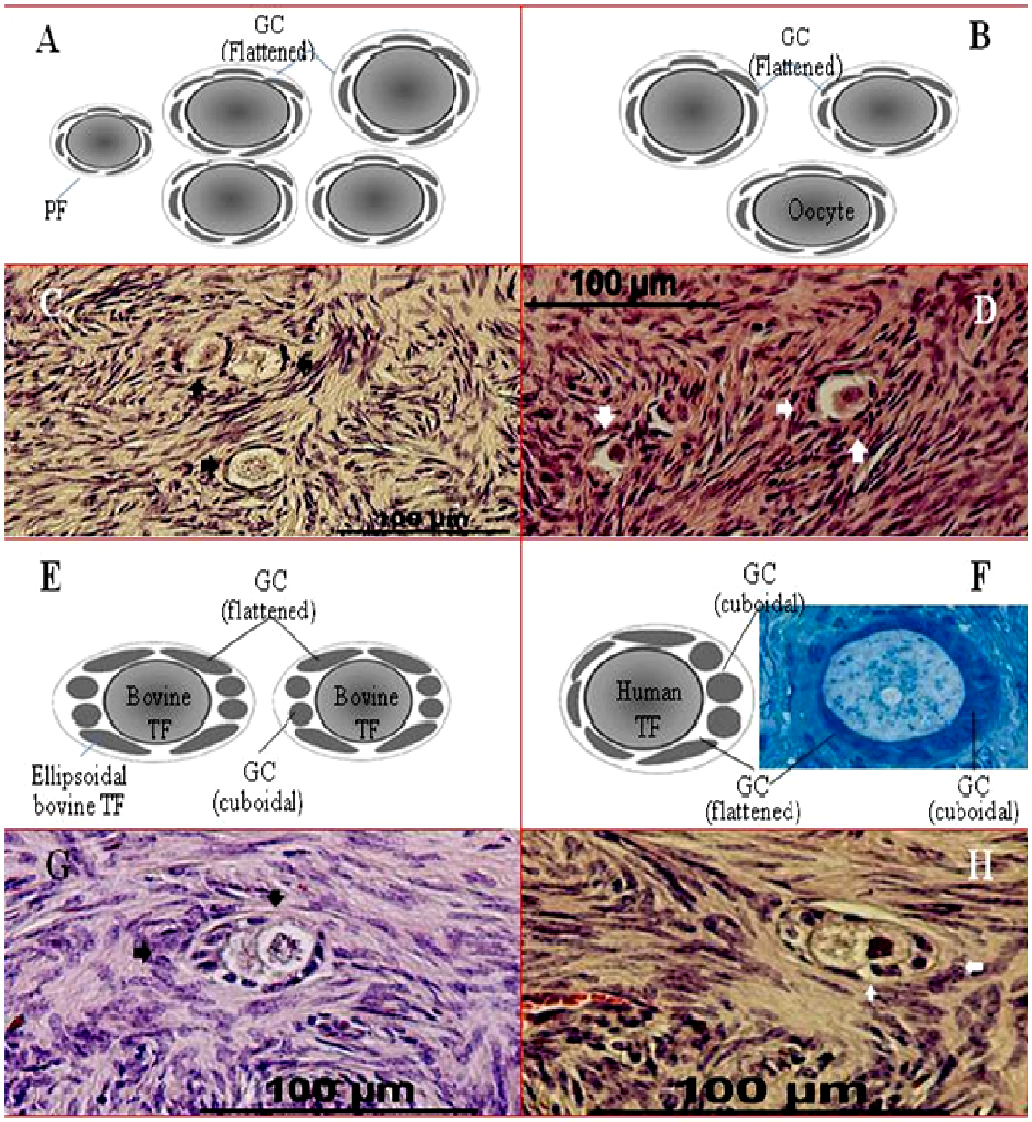

Figure 14. Schematic (A, B) and light micrographs of sections of paraffin embedded, Hemtoxylin and Eosin stained bovine ovarian cortical tissues with follicles illustrating the classical primordial shape (C, D); whereas schematic (E) and light micrographs (G, H) are the common ellipsoid shaped bovine transitional follicle involved in the formation of primary from primordial follicle (original magnification, 20X). F- Schematic and methylene blue stained human transitional follicle (Rodgers and Ivring-Rodgers, 2010).

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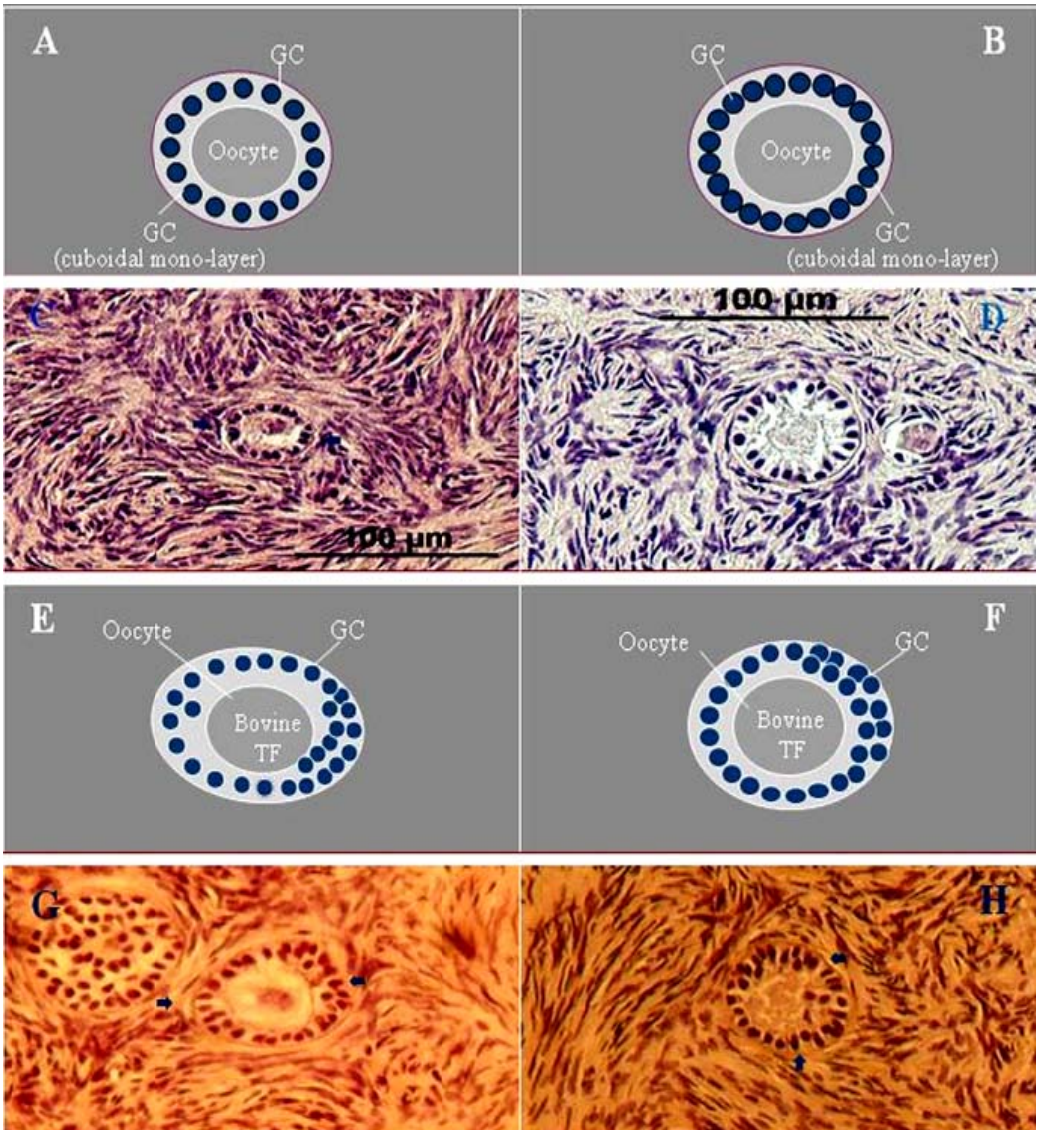

Figure 15. Schematic (A,B) and light micrographs of sections of paraffin embedded, Hematoxylin and Eosin stained bovine ovarian cortical tissues with follicles illustrating the classical primary shape; whereas schematic (E, F) and light micrographs of transitional stage (G, H) involved in the formation of a secondary from primary follicle (original magnification, 20X).

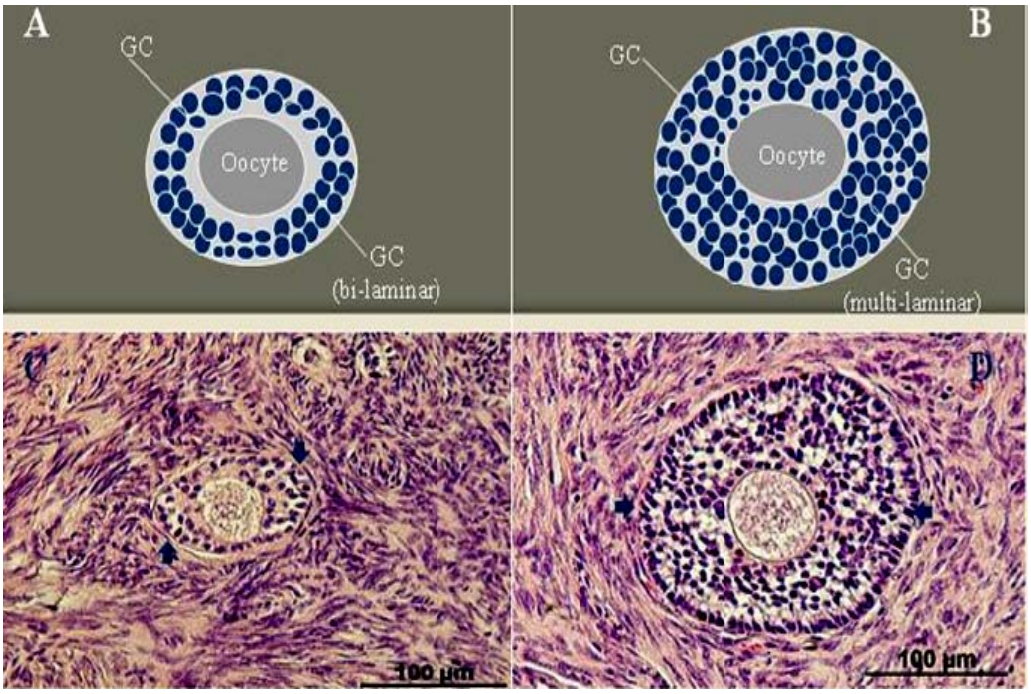

Figure 16. Schematic (A, B) and light micrographs of sections of paraffin embedded, Hematoxylin and Eosin stained bovine ovarian cortical tissues with follicles illustrating the classical secondary shape (C, D; original magnification, 20X); whereas schematic (E, F) and light micrographs (G, H) illustrating the classical early and late antral follicles respectively.

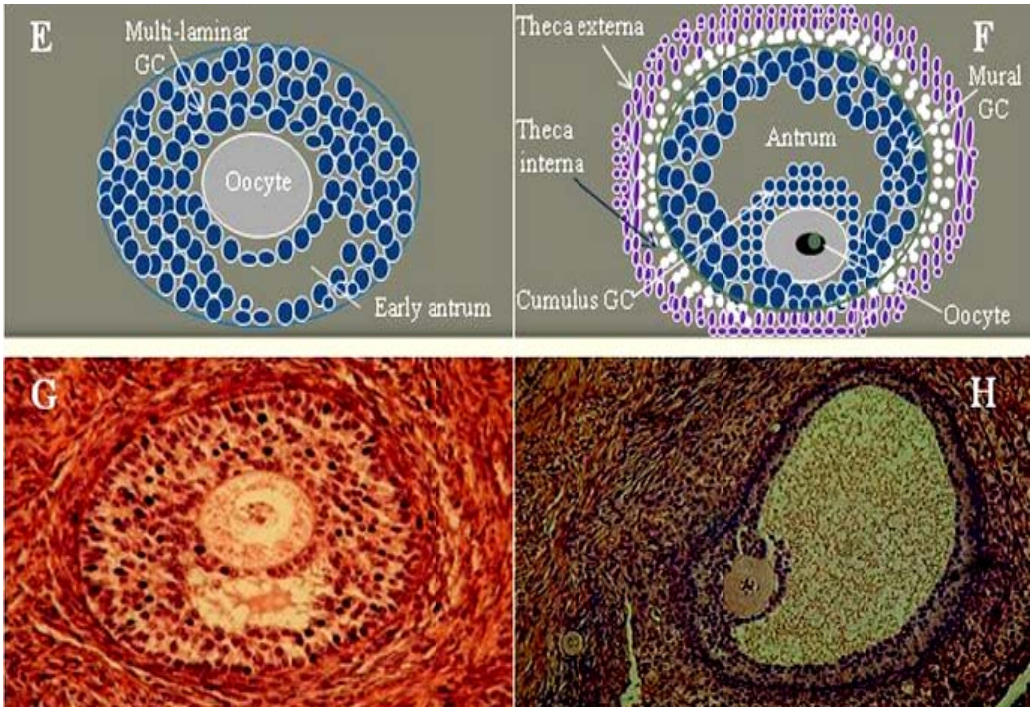

Figure 17. Schematic (A, B) and light micrographs of sections of paraffin embedded, Heamtoxylin and Eosin stained bovine ovarian cortical tissues with follicles illustrating the classical secondary shape (C, D; original magnification, 20X); whereas schematic (E, F) and light micrographs (G, H) illustrating the classical early and late antral follicles respectively.

Recently, based on the morphological and functional criteria two follicle phenotypes have been identified in bovine. One phenotype has the 'loopy' basal lamina and columnar basal granulosa cells (Irving-Rodgers and Rodgers, 2000) and expression of the RNA subunit of telomerase (TERC) in the antrally situated cells (Lavranos *et al.,* 1999; Rodgers *et al.,* 2001). The other phenotype has an aligned single layer of follicular basal lamina and rounded basal cells that express TERC. In bovine antral follicles up to 5 mm in diameter, both phenotypes occur in equal proportions, while over 5 mm in diameter only the latter phenotype is observed (Irving-Rodgers and Rodgers, 2000). It has been observed that follicles with a loopy follicular basal lamina are slower growing, producing excess basal lamina that is subsequently shed from the basal surface of the granulosa cells (Rodgers *et al*., 2001) and contained substantially poorer quality oocyte compared to the follicles with aligned basal lamina (Irving-Rodgers *et al.,* 2009).

In their early phase, antral follicles contain small fluid-filled spaces that gradually coalesce to form antrum, the development of which is limited only by the level of FSH present (Figure 17). In both human and cow an antrum emerges in follicles that have attained 2% of their preovulatory diameter (Aerts and Bols, 2008). During antral follicle growth, replication of granulosa cells and formation and expansion of the follicular antrum containing follicular fluid occurs simultaneously. However, this concept has been challenged that granulosa cell replication and follicular antrum expansion can be regulated differentially (Rodgers *et al.,* 2001). In a growing dominant follicle reaching preovulatory stage, the fluid filled antral space creates a compartment that separates the granulosa cells surrounding the oocyte from those lining the follicle wall.

It appears that FSH plays a predominat role in cumulus expansion and COC mucification (Russell *et al.,* 2007). The basement membrane separates these granulosa cells from the theca interna containing cuboidal, steroid-secreting cells and the external theca externa consisting of vascularized fibrous connective tissue. The interaction of LH with its receptor on the surface of the theca cells stimulates these cells to produce androgen, which is subsequently aromatized in the GC's to yield estrogen. The follicular fluid consists of blood exudates, local secretions and osmotically active proteoglycans hyaluronan and versican (Clarke *et al.,* 2006). The follicular diameter is approximately 500 µm at early antral stage that reaches approximately to 12-20 mm at preovulatory stage.

During a period of approximately 200 days the diameter of the oocyte expands from about 35 µm to its full size of 120 µm (Gougeon, 1986; 1996; Picton *et al.,* 1998) and this cell matures in other ways as well, including the accumulation of both RNA and protein in

the cytoplasm and nucleus. Synthesis of mRNA and proteins is rapid during the early phase of oocyte growth and slowly attenuates thereafter.

It has been argued (Rodgers *et al.,* 1999) that during follicular development there is on average a net 19 doublings in the surface area of the follicle (from a primordial to an 18 mm bovine follicle, calculated from van Wezel and Rodgers (1996), and a net 21 doublings of granulosa cell numbers (if 40 million granulosa cells were present as reported by McNatty *et al.,* (1984). Thus, it is predicted in bovine that cell layers in the membrana granulosa would increase from one layer, as in primordial follicles, to four (calculated as $(21–19)^2$=4) layers (Rodgers *et al.,*1999), and this is close to the number of layers observed in bovine preovulatory follicles (Irving-Rodgers *et al.,* 2001). However, there is considerable variation in the numbers of layers per follicle during follicle growth (van Wezel *et al.,* 1999), consistent with a reported variation in the number of granulosa cells obtained from follicles of the same size (McNatty *et al.,* 1979). On the basis of these observations, it was suggested that the rate of granulosa cell proliferation and maturation is not tightly or co-ordinately regulated with the rate of antrum expansion (Rodgers *et al.,* 2001).

It has been suggested that the shape of the basal granulosa cells reflects the dynamics of the follicle as a whole, rather than individual differences between the cells within the membrana granulosa (Rodgers *et al.,* 1999; van Wezel *et al.,* 1999). It was predicted that if the follicular antrum expansion is slow relative to granulosa cell replication, the layers become compacted leading to one or more of the basal layers of cells being columnar (Figure 19; Rodgers *et al.,* 2001). Oocyte-derived growth factors, such as GDF9/GDF9B, are known to affect these follicular cells (Hussein *et al.,* 2005; McNatty *et al.,* 2006). Oocyte from follicles with a loopy follicular basal lamina might not adequately stimulate replication of granulosa cells or antrum expansion leading to the loopy follicular basal lamina phenotype (Rodgers *et al.*, 2010).

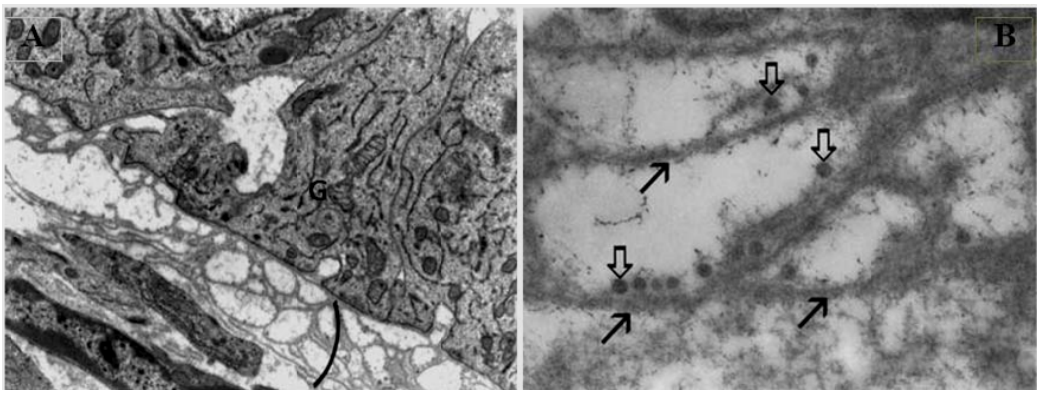

Figure 18. Electron microscopic images of (A) a follicle with a loopy basal lamina (bracket) located at the base of granulosa cells (G) and of (B) a loopy follicular basal lamina with matrix vesicles (arrowheads) located on the granulosa cell side of basal lamina layers (arrows) (Rodgers and Ivring-Rodgers, 2010).

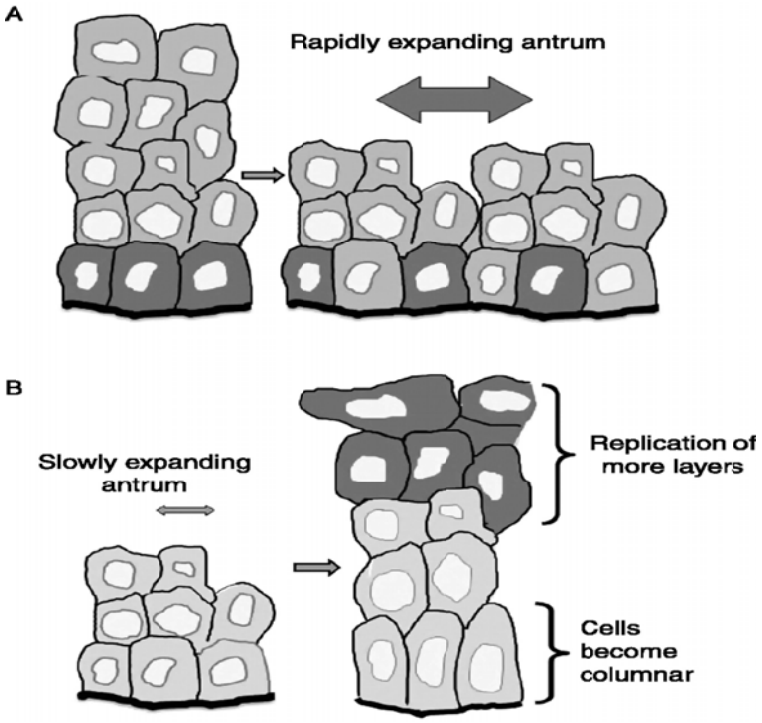

Figure 19. A scheme illustrating the effects of differential rates of antrum expansion. (A) A fast rate reduces the numbers of layers and, with ample room to accommodate the cells; they take up their natural relaxed rounded shape. (B) A slow rate of antrum expansion leads to a build-up of layers and, with little room to accommodate cells, the basal cells pack in and become columnar in shape (Rodgers and Ivring-Rodgers, 2010).

Prior to reaching preovulatory stages, subordinate follicles undergo atresia sometime post deviation when expansion ceases and those that do remain compete for available FSH. Since the estrogen and inhibin secreted by these follicles suppress the action of FSH, follicles expressing lower levels of the FSH receptor do not survive. In fact, healthy follicles have higher levels of E_2 than atretic follicles, and atretic follicles have higher levels of progesterone or thecal products such as testosterone or androstenedione for the same size of follicle (McNatty *et al.,* 1984; Jolly *et al.,* 1994).

Atresia of follicles leads to loss of the whole follicle, even if the death of one cell, such as the oocyte, is observed early in the process. Both the oocyte and GCs undergo ultra structural and morphological changes in connection with this process (de Bruin *et al.,* 2002). In fact, it is an active cellular process with resorption of the follicle involving macrophage infiltration, phagocytosis, and fibroblastic invasion of the follicular cavity from the theca along with production of collagen, which are some of the processes observed in wound healing (Martin, 1997; Schultz and Wysocki, 2009).

Recently, atresia has been classified as antral (apical) and basal atresia, based upon which granulosa cells die first (Irving-Rodgers *et al*., 2001). Antral atresia is characterized by early destruction of the layers of the membrana granulosa closest to the antrum, while the most basal cells remained intact until later (Figure 20- A, E). Numerous pyknotic nuclei are first observed in the most antral or apical layers and in the antrum close to the membrana granulosa. This is the classic description of atretic follicles and is observed in many species and occurs at all sizes of follicle development in the bovine and almost universally in large follicles (>5 mm in diameter), including non-ovulating dominant follicles (Rodgers *et al.,* 2010).

Basal atretic follicles are as prevalent as the antral atretic follicles in sizes up to 5 mm in diameter (Irving-Rodgers *et al.,* 2001) and are characterized by initial destruction of the most basal layer of granulosa cells, whereas the cells in the most antral layers remain associated with each other and are predominantly healthy until later in atresia. The phenotype of the basal atretic follicles observed in bovine is substantially different to the antral atretic follicles (illustrated in Figure 20-B, F).

Atresia can occur at any time from gestation to older cow and is independent of pregnancy. Of the many follicles that start to grow, only one will be ovulated in the end. While more than 99% undergo atresia via apoptosis (Morita and Tilly, 1999), most often at the antral stage of development (Gougeon *et al.,* 1994; Amsterdam *et al.,* 2003; Malhi

et al., 2005), which explains why the number of follicles in the ovary drops significantly with aging.

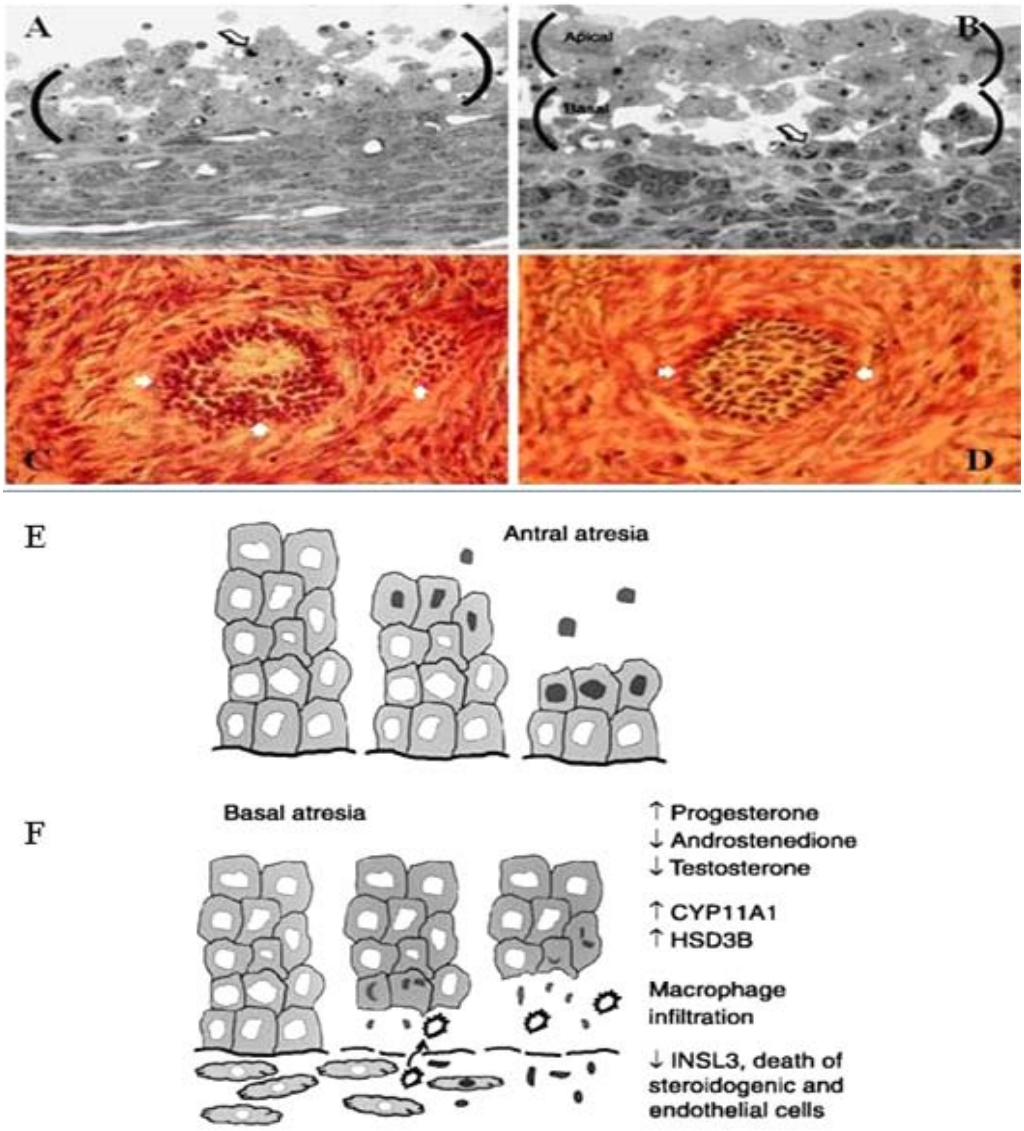

Figure 20. Light micrographs of bovine antral (A) and basal (B) atretic follicles showing the location of granulosa cells (brackets) and examples of dying cells (arrowheads), which in the basal atretic follicles are in the basal areas of the membrana granulosa, while the apical area shows granulosa cells of increased size; whereas C, D's are light micrographs of the alike follicles (Hematoxylin and Eosin stained, original magnification, 20X). Whereas, schematic of antral (E) and basal (F) atresia, illustrating cellular death progresses either from the apical region or from the basal region (modified from Rodgers and Ivring-Rodgers, 2010).

1.4 FOLLICULOGENESIS DURING ESTROUS CYCLE

1.4.1 FOLLICULAR WAVES

In most domesticated animal species (e.g. cow, buffalo, sheep, goat and horse), follicular development during the estrous cycle occurs in a wave-like fashion and that the majority (i.e., >95%) of estrous cycles in cattle are comprised of two or three such waves (Evans *et al.,* 1994; Adams, 1999). However, cycles with one or four waves have also been observed (De Rensis and Peters, 1999). Short cycles with a single growth wave appear more commonly postpartum and during puberty (Evans *et al.,* 1994).

In both two-and three-wave estrous cycles, emergence of the first follicular wave occurs consistently on the day of ovulation (Day 0). Emergence of the second wave occurs on Day 9 or 10 in two-wave cycles, and on Day 8 or 9 in three-wave cycles. In three-wave cycles, a third wave emerges on Day 15 or 16 (De Rensis and Peters, 1999; Mapletoft *et al.,* 2002). Table 3 presents an overview of the average growth wave duration according to the wave pattern. Under the influence of progesterone (e.g., diestrus), dominant follicles of successive waves undergo atresia. The dominant follicle present at the onset of luteolysis becomes the ovulatory follicle, and emergence of the next wave is delayed until the day of the ensuing ovulation. In sheep and goats, double ovulations occur – albeit exceptionally – whereby the DF from the last but one growth wave also ovulates (Ginther and Kot, 1994; Bartlewski *et al.,* 1999; Gibbons *et al.,* 1999). The CL begins to regress earlier in two-wave cycles (Day 16) than in three-wave cycles (Day 19) resulting in a correspondingly shorter estrous cycle (19–20 days versus 22–23 days). Hence, the so-called 21-day-estrous cycle of cattle exists only as an average between two and three wave cycles (Pierson and Ginther, 1987; Sirois and Fortune, 1988) (Figure 21).

Growth wave	two-wave pattern	three-wave pattern
1st	8.7 ± 0.3	7.2 ± 0.7
2nd	10.8 ± 0.3	7.7 ± 0.4
3rd	NA	9.5 ± 0.4
NA = Not applicable.		

Table 3: Duration of bovine follicular growth waves (days, average ± SEM) (Noeiser, 2003)

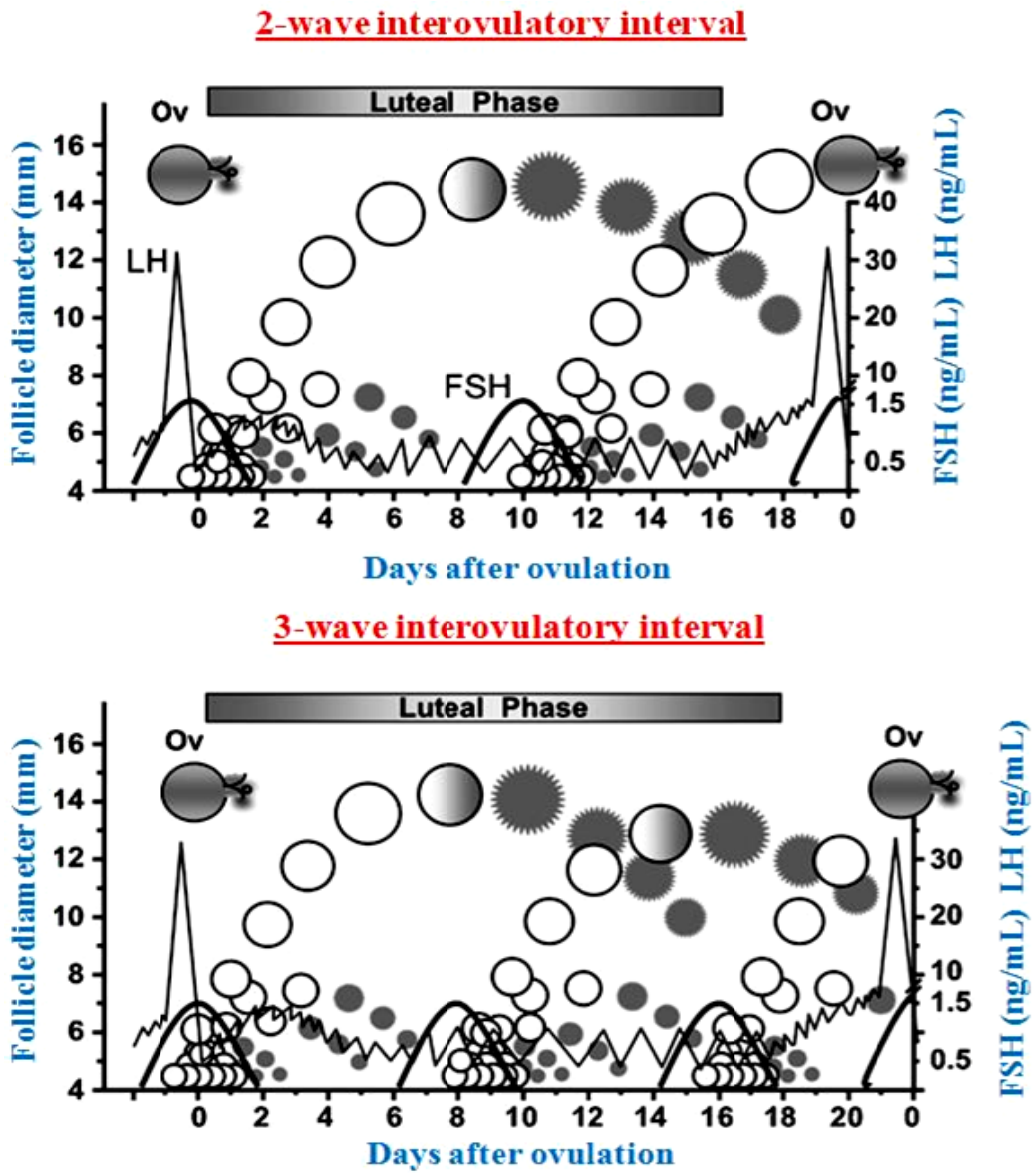

Figure 21. Dynamics of ovarian follicular development and gonadotropin secretion during two-and three-wave estrous cycles in cattle. Dominant and subordinate follicles are indicated as open (viable) or shaded (atretic) circles. A surge in circulating FSH concentrations (thick line) precedes emergence of each wave. A surge in circulating LH concentrations (thin line) precedes ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency as a result of low-circulating progesterone concentrations (i.e., period of luteolysis and luteogenesis, respectively) (Adams *et al.,* 2008).

1.4.2 WAVE DYNAMICS AND HORMONAL INTERPLAY

1.4.2.1 FOLLICLE RECRUITMENT

Every growth wave can be subdivided in a recruitment, selection and dominance phase (Figure 22). Recruitment implies that a cohort of antral follicles escapes apoptosis because of the increased levels of circulating FSH. In the cow, a developing cohort typically consists of 5–10 follicles (Driancourt, 2001), but can contain up to 24 follicles (Mihm and Austin, 2002). Regression of the DF during a growth wave, or ovulation at the end of an oestrous cycle, causes a transient elevation of circulating FSH. Indeed, loss of the DF is accompanied by decreased levels of hormones synthesized by the follicle, such as oestrogen and inhibin, resulting through a negative feedback mechanism in a temporary increase of FSH secretion by the pituitary gland. Follicle stimulating hormone acts as a survival factor for early antral follicles, the stage at which most follicles under physiological conditions perish by atretic degeneration (Chun *et al.,* 1996). In consequence, FSH is chiefly responsible for the recruitment of a new cohort of antral follicles for the next growth wave.

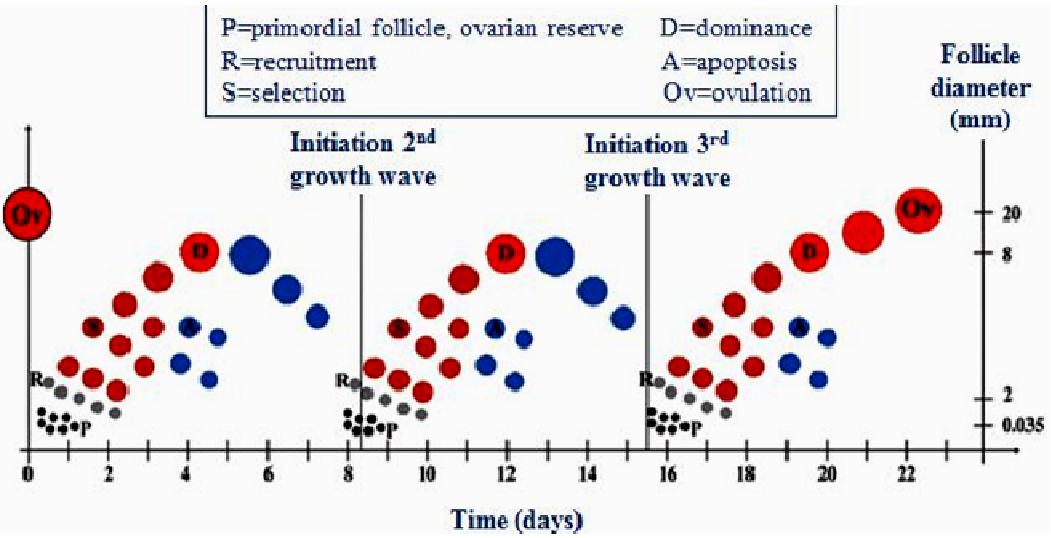

Figure 22. Graphical depiction of recruitment, selection, and dominance phase in a three-wave follicular development during a bovine estrous cycle (Aerts and Bols, 2010b).

1.4.2.2 FOLLICLE SELECTIONS

During each estrous growth wave, the recruited follicles are subjected to a selection process, whereby in monovular species– such as cattle and horses –normally a single follicle develops into a DF, while the remaining follicles regress (Ginther *et al.,* 2000). The transient FSH rise responsible for the initiation of a follicular growth wave peaks when the largest bovine follicles attain a 4–5 mm diameter, and diminishes afterwards (Kulick *et al.,* 1999). Maximum FSH levels in the first growth wave of the cycle are typically expressed 28 h after the onset of oestrus (Mihm and Austin, 2002). Production and secretion of estradiol and inhibin by the growing follicles results in suppression of FSH release, notwithstanding that these follicles are still dependent on FSH for their continued growth. In the bovine species, experiments have revealed that follicles with a 3 mm diameter do not suppress FSH secretion, but acquire the capacity to suppress FSH when the follicle reaches a 5 mm diameter (Gibbons *et al.,* 1999).

1.4.2.3 FOLLICLE DOMINANCE

When the largest follicle of the wave, after approximately 3-5 days of growth (emergence at 1 mm), attains a diameter of on average 8.5 mm, a differentiation transpires between the future DF and the remaining subordinate follicles. This defining moment is termed ‘deviation’ by Ginther and co-workers (Ginther *et al.,* 1997, 1998, 1999; Beg and Ginther, 2006). Before deviation all growing follicles retain the capacity to become DF; after deviation the largest follicle develops into a DF, while the others regress. Experiment with heifers reveals that all antral follicles have the potentiality to develop into a DF and ablation of the largest follicle just before deviation results in the second largest follicle becoming a DF (Gibbons *et al.,* 1997, Ginther *et al.,* 1997).

Acquisition of LH receptors in granulosa cells of the DF results in rapid divergence in growth rates; the DF continues to grow as the subordinates cease and begin to regress in a milieu of minimal FSH. The low FSH concentrations have the advantage of precluding the emergence of a new cohort of growing follicles. Investigations have revealed that demarcation between DF and subordinate follicles usually a very abrupt event (Ginther *et al.,* 1997), whereby the DF in a critical period of 8 h around the deviation mark suppresses FSH, and thus provokes atresia of the remaining antral follicles (Ginther *et al.,* 2000).

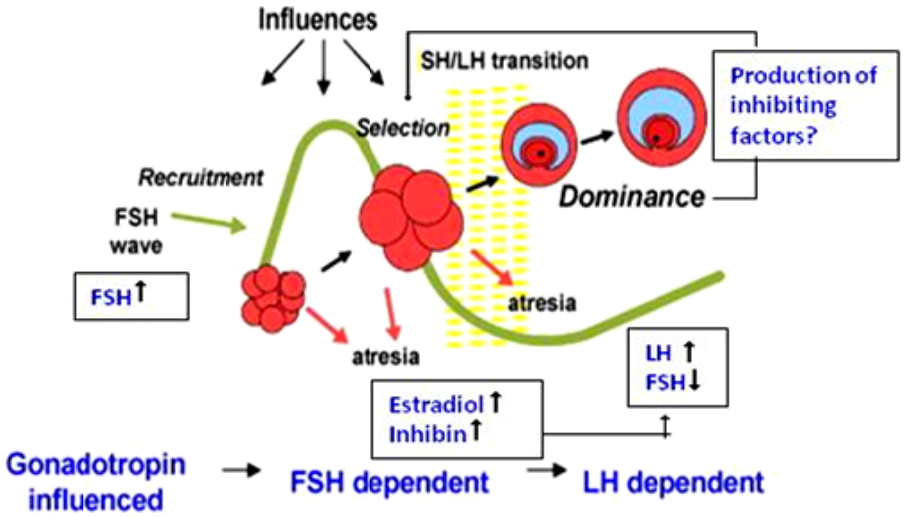

Figure 23. The mechanism describes a follicular wave whereby the growing follicles inhibit FSH secretion through production of estradiol and inhibin, while being dependent on FSH for their own growth and when the dominant follicle(s) transfers its dependence from FSH to LH (modified from Webb, 2007).

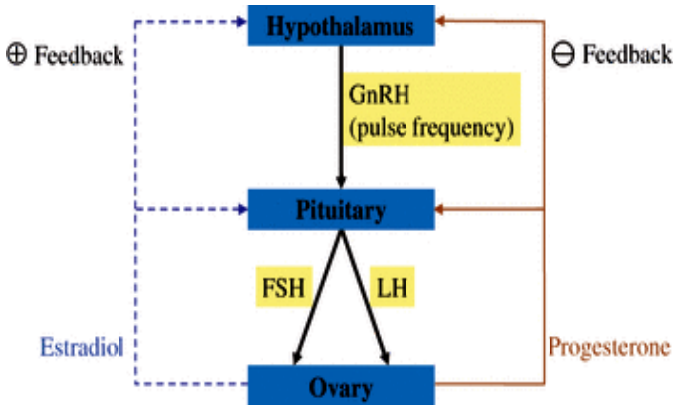

Figure 24. Feedback mechanism of estradiol and progesterone. At plasma concentrations above a threshold level, estradiol increases GnRH pulse frequency through a positive feedback mechanism, and causes elevated LH secretion. Progesterone executes a negative feedback effect on pulse frequency, therefore the LH surge is absent in the presence of a functional corpus luteum (Aerts and Bols, 2010b).

During selection, a multiple follicle–FSH–coupling exists, whereby the growing follicles inhibit FSH secretion through production of estradiol and inhibin, while being still dependent on FSH for their own growth. At the start of deviation, the relation changes to a single follicle-FSH-coupling, providing restricted levels of FSH that enable only the future DF to thrive. LH performs key role in this phase and DF undergoes a transition in gonadotrophin dependency from FSH to LH (Mihm *et al.,* 2006). In heifers, LH receptors emerge in granulosa cells of the future DF 8 h before the onset of deviation, when the follicle has acquired approximately 8 mm diameter (Ginther *et al.,* 2001).

During dominance, functional changes of the DF, such as a decline in the intrafollicular ratio of Estradiol: progesterone (Mihm *et al.,* 2006) – are aimed at preparing for ovulation. The effect of GnRH is determined by its pulse frequency: fast frequencies promote LH release and slow frequencies promote FSH release (Nicol *et al.,* 2008). The specific functions of the various modulators on the pulsatile pattern of GnRH release remain to be exactly defined. However, progesterone acutely inhibits GnRH pulse frequency, and it is regarded as the primary effector of LH (Bergfeld *et al.,* 1996; McCartney *et al.,* 2007). FSH release is affected – independently of LH – by members of the transforming growth factor-β superfamily (Nicol *et al.,* 2008).

The feedback effect of estradiol on gonadotrophin secretion is function of a threshold level: at plasma concentrations below the threshold, estradiol exerts a negative feedback effect; above the threshold a positive feedback effect (Bouchard *et al.,* 1988; Svensson, 2000; Wiltbank *et al.,* 2002). Increased peripheral estradiol concentrations thus positively affect GnRH pulse frequency, resulting in elevated LH secretion. In the absence of a functional corpus luteum (CL), this will give rise to the LH surge, this surge, together with follicle derived prostaglandins of the E series induces the oocyte to be released in the periovarian space, ovulation and leutinization (Filion *et al.,* 2001).

1.5 OOCYTE COMPETENCE AND FOLLICULAR STATUS

During the primordial follicle formation, the oocyte is arrested in the first meiotic prophase. The process of oocyte competence implies the gradual acquisition of a number of capabilities. It is set in a stepwise fashion that commences with primordial follicle activation and is only completed days before ovulation. Throughout follicular development, the oocyte diameter enlarges from on average 30 to 130 µm, which is almost equivalent to a 100-fold volume expansion. Such a substantial increase in volume is achievable only because of the fact that by blocking meiosis, the oocyte can grow without division.

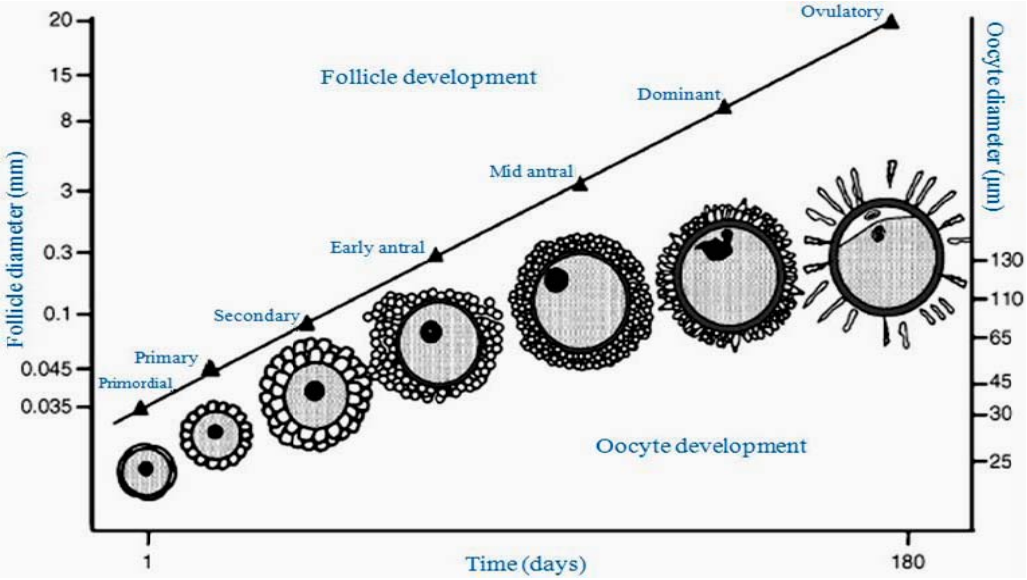

Figure 25. Graphical depiction of the relationship between follicle and oocyte development

The transition of pre-antral to antral follicle is marked by the development of meiotic competence. Pre-antral follicles, being meiotically incompetent, are unable to develop beyond the diplotene stage of meiosis I. In humans and cows, development of meiotic competence is not strictly linked to antrum formation, but the ability to surmount meiotic blockage increases as follicular size increases (Trounson *et al.,* 2001) and oocytes in antral follicles, however, spontaneously resume meiosis when removed from the follicle (Eppig, 2001). Bovine oocytes become meiotically competent at 110 to 115 µm diameter; whereas developmental competence (ability to undergo the first stages of embryonic development) is acquired when oocyte diameter reaches at 120 µm (Fair *et al.,* 1995; Otoi *et al.,* 1997). The ability of in vitro matured oocytes to develop to blastocyst stage keeps a steady pace with oocyte expansion, until an optimum is reached at 135 mm diameter (Armstrong, 2001).

After the preovulatory LH-surge meiosis is resumed, resulting in breakdown of the large oocyte nucleus and release of its nucleoplasm into the cytoplasm, a process known as germinal vesicle breakdown. Subsequently, the chromosomes condensate and the last stages of meiosis I are completed. In most mammalian species, meiosis is thereafter

again blocked in metaphase II. In addition to nuclear processes, egg cell competence is also determined by complex cytoplasmic processes, basic knowledge of which is still largely unknown (Suikkari and Soderstrom-Anttila, 2007). Besides important changes in the oocyte, the LH-surge also induces alterations in the follicle. Cumulus cells start producing hyaluronic acid, a non-sulphated glycosaminoglycan which is bound to the cumulus cells by linking proteins. Hydration of hyaluronic acid causes enlargement of spaces between cumulus cells, and these cells become embedded in a mucified matrix (Eppig, 2001). The process of cumulus expansion is a key feature of the ovulation mechanism and is instrumental in facilitating fertilization (Zhuo and Kimata, 2001).

1.6 INTRAOVARIAN REGULATORS OF FOLLICULOGENESIS

1.6.1 TRANSFORMING GROWTH FACTOR-β SUPERFAMILY

A plethora of growth factors, many belonging to the transforming growth factor-β (TGF-β) superfamily, are expressed by ovarian somatic cells and oocytes in a developmental, stage-related manner and that, in concert with systemic signals, function as intraovarian regulators of folliculogenesis (Knight and Glister, 2006). Almost all of the more than 40 protein hormones presently known to belong to this superfamily, exhibit similar structures including 7 cysteine residues, and are conserved among species. This family can be divided into three major groups, i.e., TGF-β itself; activins and inhibins; and bone morphogenetic proteins (BMPs), the largest of the subgroups. TGF-β signaling involves two sub-types of transmembrane serine/threonine kinase receptors, both of which are required for signal transduction. The protein ligands interact with these cell surface receptors to generate intracellular signals through Smads.

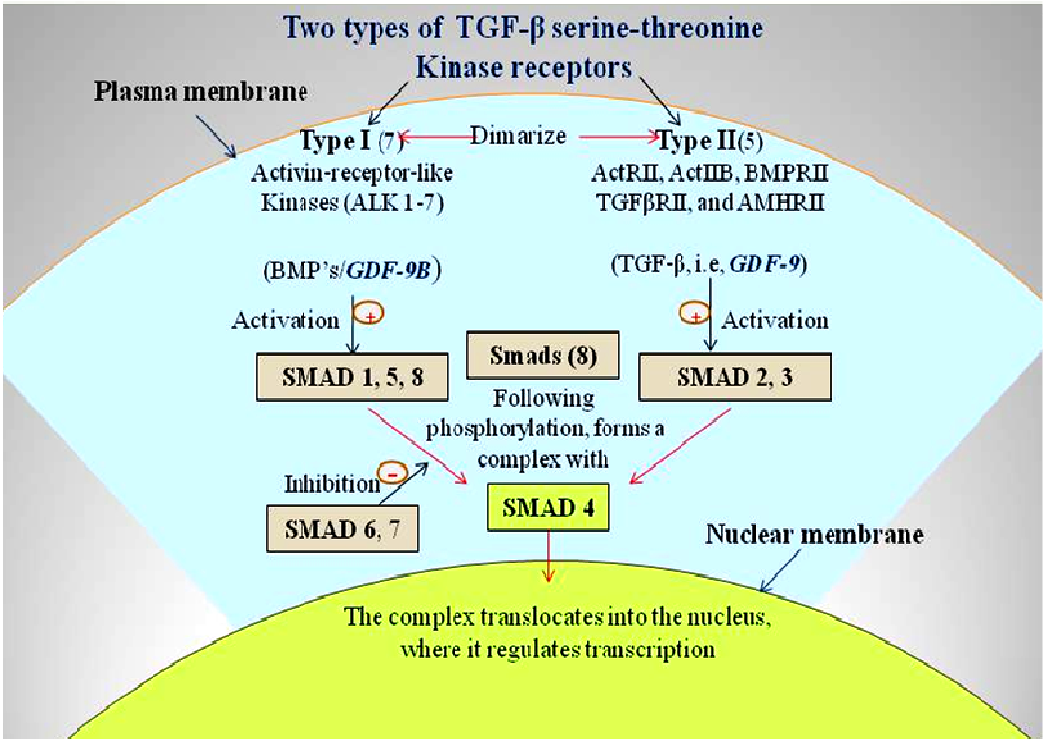

Figure 26. TGF-β activated signaling pathways (proteins in the TGFB family initiate signaling by assembling type I and type II serine/ threonine kinase receptor complexes that activate similar to mothers against decapentaplegics (SMAD) transcription factors).

1.6.1.1 GROWTH DIFFERENTIATION FACTOR-9

One of the major hallmarks in ovarian biology was the discovery of oocyte derived growth differentiation factor-9 (GDF-9) in 1993 in connection with genomic screening for mouse TGF-β family members that highlight the importance of paracrine signaling within the oocyte-granulosa cell complex (McPherron and Lee, 1993; Myers *et al.,* 2010).

In mammalian ovary, oocyte is the unique site for GDF-9 and GDF-9B expression and studies on mutant mice and sheep with GDF9 gene defects, together with in vitro and in vivo research indicate an important role of GDF9 in the stimulation of early follicular growth, cumulus expansion, and fertility (Mazerbourg and Hsueh, 2003; Hanrahan *et al.*, 2004; Huang, 2009). In most species, expression of GDF-9 is detected in non-growing primordial follicles (Bodensteiner *et al.,* 1999; Eckery *et al.,* 2002; Wang and Roy, 2004). However, in the case of sheep GDF-9 transcripts have been detected in fetal ovaries even prior to follicle formation (Mandon-pepin *et al.,* 2003), indicating possible functions for this factor at this earlier stage as well (Juengel *et al.,* 2004). Furthermore, in ovine and bovine, GDF-9 mRNA is expressed at all stages of follicular development

(Bodensteiner *et al.,* 1999). Vitt and co-workers observed enhanced activation of primordial follicles in rats treated with GDF-9 (Vitt *et al.,* 2000).

However, the expression level of GDF-9 increases dramatically when the oocyte undergoes its growth during preantral folliculogenesis, and remains high in the oocyte through ovulation in mice, indicating species dependent variability in GDF-9 expression at various stages of follicular development (McGrath *et al.,* 1995). Consistent with the expression of GDF-9 mRNA, GDF-9 protein is expressed in oocytes of human and other mammals during folliculogenesis from the early primary stage onwards and shows the most intensive immunostaining in primary and preantral follicles (Aaltonen *et al.,* 1999; Elvin *et al.,* 1999).

In humans, GDF-9 enhances the survival, growth rate and recruitment of follicles (Hreinsson *et al.,* 2002) and, moreover, mutations in the GDF-9 gene may be associated with premature ovarian failure (Kovanci *et al.,* 2007). Mice null for Gdf9 had a block in folliculogenesis at the primary stage of development, while the oocyte continues to grow in size until it degenerates, defects in oocyte meiotic competence and that paracrine signaling by GDF9 is required for granulosa cell function during process of cumulus expansion, which is an integral process that must occur in preovulatory follicles to enable successful ovulation (Dong *et al.,* 1996; Bodensteiner *et al.,* 1999; Elvin *et al.,* 1999). In addition, GDF-9 and GDF-9B/BMP-15 act synergistically to promote the growth and differentiation of granulosa and theca cells in mice which in turn supports oocyte maturation. Therefore, they were implying that GDF-9 may be a key element in the oocyte-cumulus regulatory loop (Gilchrist *et al.,* 2004; Gilchrist *et al.,* 2006; Yeo *et al.,* 2008).

Like other members of the TGF-β superfamily, GDF-9 activates type I and II serine/threonine kinase receptors which results in phosphorylation of Smad proteins (Massague, 1998). Thus GDF-9 has been shown to bind both to the activin receptor like kinase 5(ALK5), a type I receptor, and the bone morphogenetic protein receptor II (BMPRII), a type II receptor on the GCs (Vitt *et al.,* 2002; Kavio-Oja *et al.,* 2005; Mazerbourgh and Hsueh, 2006) (Figure 26). GDF-9B/BMP-15 also use BMPRII as its type II receptor, but as a type I receptor it uses ALK6 or BMPRIB (Moore *et al.,* 2003). The biological effect of both of these factors can be blocked by using a fusion protein composed of BMPRII ectodomain coupled to the Fc domain of human IgG (Vitt *et al.,* 2002; Moore *et al.,* 2003). For example, inhibition of the actions of GDF-9 and GDF-9B/BMP-15 partially attenuates the proliferation of GCs induced by oocytes (Vitt *et al.,* 2002; Mazerbourg and Hsueh, 2006).

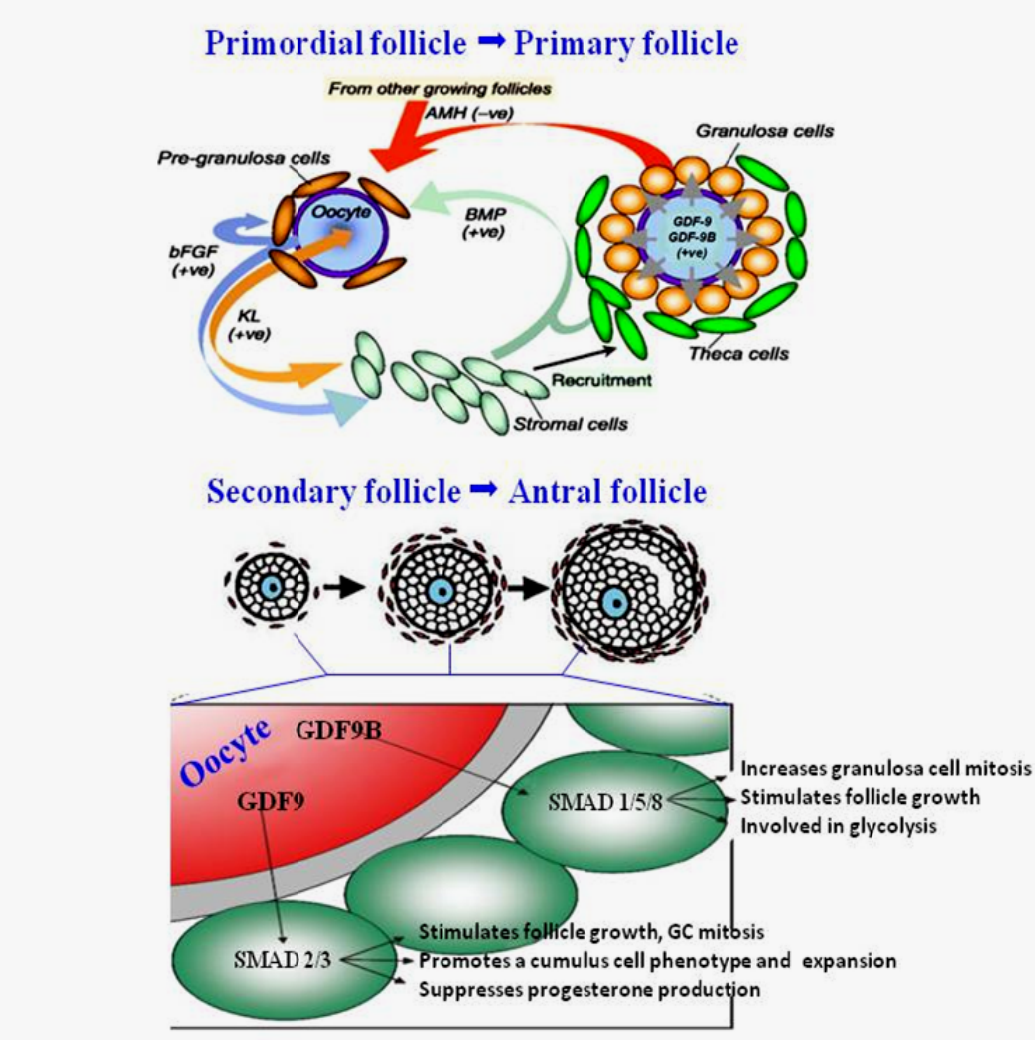

Figure 27 Potential GDF-9/GDF-9B signaling interactions involved in the primordial to primary follicle transition (top) and successive follicular development (bottom). Oocyte secreted GDF of the activated follicle promote granulosa cell proliferation, KL expression and theca formation. Oocyte and pre-granulosa cells secreted kit ligand and basic fibroblast growth factors promote recruitment of theca cells from the surrounding stromal/interstitial cell population. Stromal /interstitial cells and theca cells secrete BMPs (BMP-4, BMP-7), which promote follicle activation and survival. Granulosa cells of the growing follicle secrete AMH that appears to act as a 'brake' on primordial follicle recruitment. In bottom: crosstalk between the oocyte and granulosa cells (both cumulus and mural) is integral for various stages of folliculogenesis. Oocyte-derived GDF-9 and GDF-9B act via SMAD 2/3 and SMAD 1/5/8 respectively, to elicit cellular responses that are essential for successful folliculogenesis and ovulation (Knight and Glister, 2006; Myers and Pangas, 2010).

1.7 GDF-9 AND SPECIFIC INTRAOVARIAN INTERACTIONS

1.7.1 OOCYTE-THECA CELL INTERACTION

GDF-9 stimulates theca cell recruitment, proliferation and differentiation during the early stage of the follicular development (Orisaka *et al.,* 2009). The theca cells differentiate from a population of unspecialized mesenchymal cells in the ovarian stroma only after development has proceeded to the early secondary stage when the second layer of cuboidal granulosa cells begin to appear do the first theca cells begin to differentiate in the theca interna (Magoffin, 2005). Ovaries from GDF-9 null mice exhibit failed theca cell layer formation in early follicles and blocked folliculogenesis from primary stage onwards (Elvin *et al.*, 1999). Nevertheless, GDF-9 is believed to be more important for the differentiation of the theca cells, since the double-mutant (GDF-9 and inhibin α) mouse exhibits secondary to preantral follicles with undetectable selective thecal markers, CYP17A1 and LH receptors (Wu *et al.,* 2004). GDF-9 treatment increases androgen production in cultured rat theca-interstitial cells and promotes ovarian expression of specific theca cell markers (Elvin *et al.,* 1999; Solovyeva *et al.,* 2000). *In vitro* studies have shown that androgens stimulate pre-antral follicle growth, especially transition of primary follicles to secondary stage in cattle (Yang *et al.,* 2006). Also, GDF increases theca cell number and DNA synthesis in theca cells of small bovine follicles (Spicer *et al.*, 2008). This result suggests that GDF-9 promotes preantral follicle growth by up-regulating theca cell androgen production. Furthermore, intraoocyte injection of GDF-9 morpholino antisense oligos suppresses androgen production and CYP17A1 mRNA expression, indicating this intraovarian TGFβ factor is key in theca differentiation during fulliculogenesis especially transition from secondary stage onwards (Orisaka and Jiang *et al.*, 2009).

1.7.2 OOCYTE-GRANULOSA CELL INTERACTION

GDF-9 is the key paracrine regulator of ovarian GC and its deletion in the oocyte results in decreased granulosa cell proliferation, abnormal oocyte growth and failure of follicles to develop beyond the primary stage (Dong *et al.,* 1996). Recent studies demonstrated that GDF-9 down regulation attenuates both basal and FSH-induced follicular growth in vitro, while the addition of recombinant GDF-9 enhances basal and FSH induced follicular growth in rat. In addition, down-regulation of GDF-9 content increases caspase-3 activation and granulosa cell apoptosis (Orisaka *et al.,* 2006). GDF-9 was sufficient to suppress ceramide-induced apoptosis in primary granulosa cells from secondary, but not large/preovulatory follicles, suggesting that GDF-9 is an important granulosa cell survival factor during preantral to early antral transition but might play a lesser role in follicle

survival past antrum formation (Orisaka *et al*., 2006). GDF-9 also promotes development and survival of early follicle in organ culture (Hreinsson *et al*., 2002). GDF-9 is required to maintain FSH receptor expression in the secondary follicles and GDF-9 receptors (BMPRII and ALK-5) are up-regulated by co-treatment of estrogen and FSH (Jayawardana *et al.,* 2006).

1.7.3 THECA-GRANULOSA CELL INTERACTION

Evidence indicates that there is a robust communication between the theca and granulosa cells in developing follicles that is mediated by steroidal and nonsteroidal growth and differentiation factors produced by each cell types on opposite sides of the basement membrane during folliculogenesis (McGee and Hsueh, 2000; Richards, 2001; Nilsson *et al*., 2001; Vitt and Hsueh, 2001; Monget *et al*., 2002). LH receptors are found exclusively on theca cells and FSH receptors exclusively on granulosa cells during preantral follicular development. It has been reported that androgens stimulate early folliculogenesis and granulosa cell mitosis (Vendola *et al.,* 1998). Androgens also enhance FSH action in the follicles by increasing FSH receptors expression, FSH-induced granulosa cell aromatase activity and proliferation, and follicular growth (Hickey *et al.,* 2004; Drummond, 2006). Theca cell steroidogenesis is under the primary control of LH through stimulation of the cAMP signaling pathway, while FSH induces aromatase expression and increases the conversion of theca cell androgen to estrogen (two-cell two-gonadotropin theory) (Magoffin, 2005). Where, GDF-9 plays an important role by positively modulating the LH action (Orisaka and Jiang *et al.,* 2009). The principal steroid product of the theca cells in the most species is androstenedione. Androstenedione diffuses across the basal lamina where it metabolized to estradiol by the granulosa cells (Figure 28).

In the secondary follicle when the theca cell first differentiate, they secrete factors such as TGF-β that mediate signals to the granulosa cells indicating that the theca cells are capable of androgen biosynthesis. In response, factors such as TGF-β and activin are produced by the granulosa cells that inhibit thecal androgen production. This is physiologically an important paracrine signaling because the granulosa cells have yet to express CYP19 and the accumulation of excess androgen is associated with arrested follicle development and infertility (Drummond, 2006). But at the small antral stage when GC begin to express CYP19 and the follicles become dominant, the granulosa cells begin to secrete significantly increased amounts of stimulatory factors that lead to robust metabolism of androstenedione to estradiol. LH surge that triggers ovulation causes a downregulation of the CYP17 enzyme in the theca cells, transforming them from

androgen producing to progesterone-producing cells (Magoffin, 2005). Though the nature of the theca-granulosa cell interaction remains to be determined, recent studies also suggest that theca cell derived growth factors such as EGF, TGF-α, KGF and BMP-7 are involved in the regulation of apoptosis of granulosa cells especially by lowering the apoptotic incidence during the preantral/early antral transition (Tilly *et al.*, 1992; McGee *et al.,* 1999; Lee *et al.,* 2001; Wang *et al.,* 2002).

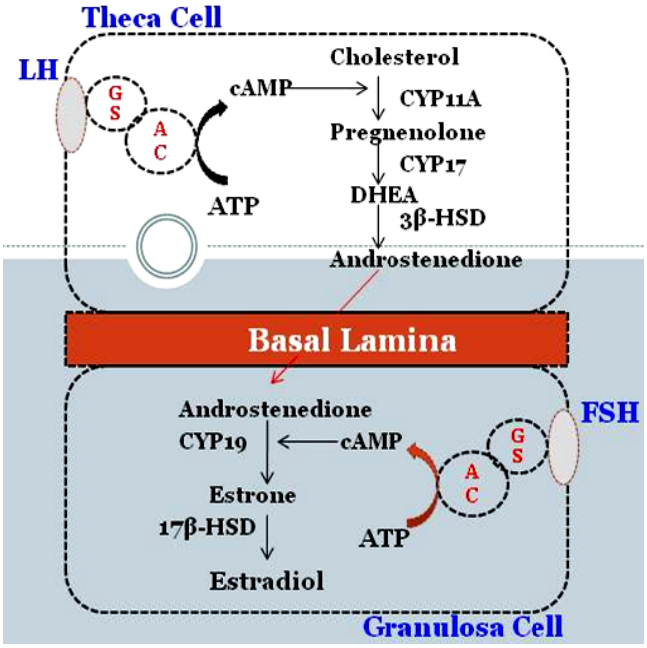

Figure 28. The two-cell, two-gonadotropin concept of follicle estrogen biosynthesis. Luteinizing hormone (LH) stimulates theca cells by a cAMP-mediated mechanism to express cholesterol side-chain cleavage cytochrome P450 (CYP11A), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17α-hydroxylase/$C_{17–20}$ lyase cytochrome P450 (CYP17). The theca cells can then synthesize androstenedione from cholesterol. The androstenedione diffuses across the basal lamina into the granulosa cells. Follicle-stimulating hormone (FSH) stimulates the expression of aromatase cytochrome P450 (CYP19) and 17β-hydroxysteroid dehydrogenase (17β-HSD) in the granulosa cells by a cAMP-mediated mechanism. The granulosa cells can then metabolize the androstenedione to estradiol. Gs, stimulatory G-protein; AC, adenylate cyclase; cAMP, adenosine 3′, 5′-cyclic monophosphate (Magoffin, 2005).

1.8 STROMAL VASCULARIZATION AND FOLLICULOGENESIS

In adult tissues, capillary growth (angiogenesis) occurs normally during repairing of damaged tissues. Conversely, inadequate capillary growth or loss of existing capillaries is associated with various pathological conditions (Folkman and Klagsbrun, 1987; Redmer and Reynolds, 1996). The female reproductive organs (the ovary, uterus and placenta) exhibit marked periodic growth and regression, accompanied by equally striking changes in their rates of blood flow (Reynolds *et al.,* 1992). Ovarian stroma is one of the few adult tissues where neovascularization takes place as a normal process. In fact, angiogenesis refers to the formation of new blood vessels, or neovascularization, and is essential for normal tissue growth and development (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). The angiogenic process begins with capillary proliferation and culminates in formation of a new microcirculatory bed composed of arterioles, capillaries and venules. The initial component of angiogenesis, capillary proliferation, consists of at least three processes: (1) fragmentation of the basement membrane of the existing vessel, (2) migration of endothelial cells (the primary cell type constituting capillaries) from the existing vessel toward the angiogenic stimulus and (3) proliferation of endothelial cells (Folkman and Klagsbrun, 1987). Neovascularization is completed by formation of capillary lumina and differentiation of the newly formed capillaries into arterioles and venules (Klagsbrun and D'Amore, 1991; Redmer and Reynolds, 1996). However, recently it has been reported that the process of neovascularization as well as existing stromal microvasculature alters during injury to the ovarian stroma or endovascular complications associated with antineoplastic agents (Meirow *et al.,* 2007).

The majority of follicles in the mammalian ovary reside in the outer ovarian cortex at resting primordial stage. The signals that activate the follicles and conditions that allow continued growth and development of primary follicles to the secondary stage are still poorly understood, particularly for bovine species. Stromal vascularization is a prominent characteristic that are responsible for an adequate supply of oxygen, nutrients and regulatory signals for folliculogenesis. It has been shown that in the growing follicles, an extensive vascular plexus develops surrounding the avascular basement membrane and granulosa cells (Acosta *et al.,* 2005). Since, resident primordial and early growing follicles in the outer cortex do not possess an independent vascular network (Delgado-Rosas *et al.,* 2009), and are, therefore, mostly dependent on their proximity to stromal vessels that proliferate and give rise to the perifollicular capillary network from the secondary follicle stage onwards. Whilst many studies have been focused on ovarian angiogenesis, providing evidences on the essential role of vascularization for antral follicle and corpus

luteum development and function (Fraser, 2006; Jiang, 2003; Feranil, 2004), so far, no attention has been devoted to the alterations in overall cortical vasculature of the ovaries of bovine affected with chronic mastitis.

It has been shown that intraovarian factors such as growth factors and/or cytokines contribute to early follicular growth (Adashi, 1990; Plendel, 2000). Since some of the intraovarian regulatory cytokines are reported to be produced by activated endothelial cells (Schott *et al*., 1993; Shimizu *et al*., 2003), this attributes a major role of early follicular growth to the microvascular bed of the cortex. The cytokines, with almost exclusive activity on endothelial cells are the members of the vascular endothelial growth factor (VEGF) family as well as the angioproteins belong to the fibroblast growth factor (FGF) family (Ferrara, 1999; Gale *et al.,* 1999; Grazul-Bilska *et al.,* 2007). It has been shown that inhibition of the action of these cytokines results in delayed rate of follicular development, indicating the existence of potential interactions amongst angiogenetic cytokines and folliculogenesis (Zimmermann *et al.,* 2002). As systemic inflammatory stimulus on endothelium extensively affects microvascular bed (Landis, 2009), and modulate interactions amongst cytokines (including angiogenic cytokines like IL-8, TNF-α) (Arici *et al.,* 1996; Sakumoto *et al*., 2000; Bannerman, 2009), in this context, mastitis related changes in the cortex vascularization may have profound effects on both the survival of primordial follicles and on the early stages of follicle growth. Recent availability of endothelial cell markers, like *Bandeiraea simplicifolia*-I (BCL-I, binding specificity: α-*N*-acetylgalactosamine and α-galactose), one of the lectins (specific carbohydrate binding proteins of non-immune origin), now allows histological evaluation of microvascular bed in tissues (Feranil, 2004).

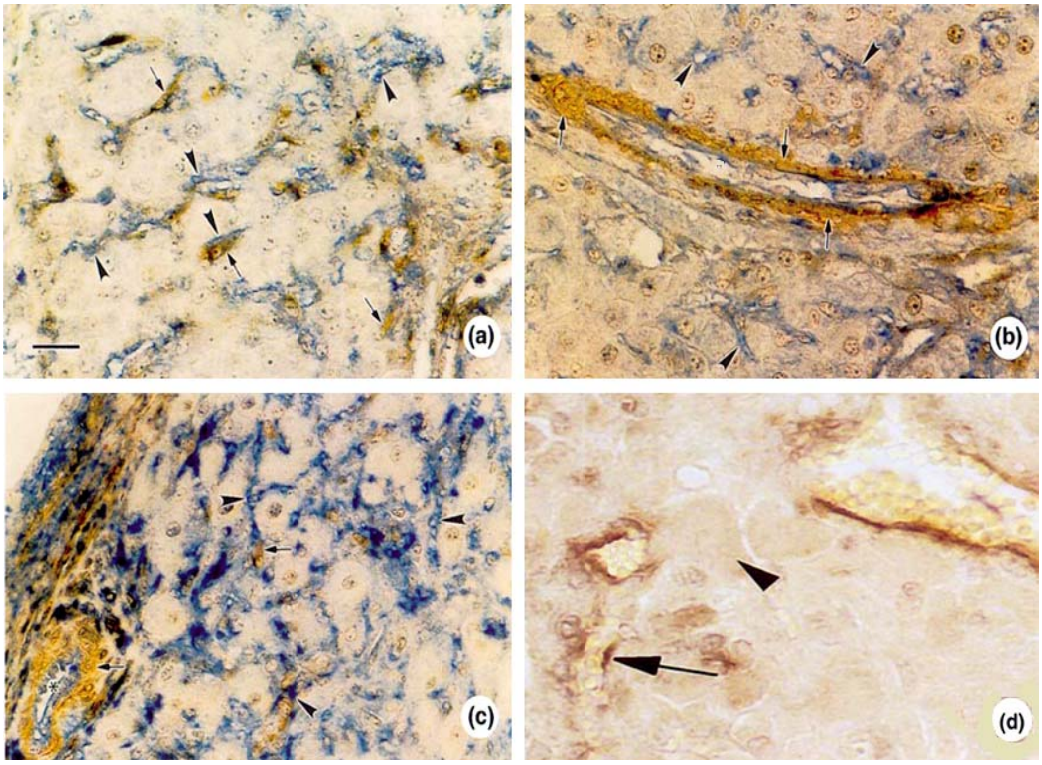

Figure 29. Immunohistochemical localization of the key angiogenic cytokine, VEGF (vascular endothelial growth factor, brownish staining) and Lectin BS-1 (marker for endothelial cells, bluish staining) in the cortical section of the ovary. In (a-c) for the most part, the brownish cytoplasmic staining for VEGF (arrows) does not correspond to the bluish Lectin staining for endothelial cells (arrowheads), but rather primarily seems to be perivascular. The perivascular staining for VEGF is most apparent in the larger microvessels. Whereas in (d), localization of another angiogenic cytokine FGF-1(arrowheads), scale bar represents 25μm (Redmer and Reynolds, 1996).

1.9 CONNECTIVE TISSUE MATRIX AND FOLLICULOGENESIS

Ovarian follicles during the normal process of growth and maturation are accommodated spatially by structural rearrangements within the ovary. This intense proliferation and growth of the parenchymatous elements of the ovary may be directly related to events occurring in surrounding structures, and in particular remodelling in the ground substance of the connective tissue in response to endocrine and paracrine signaling (Smith *et al.,* 1999; Rodgers *et al.,* 1999). Ovarian connective tissue, a type of biological tissue with an extensive extracellular framework, form a matrix, provides structural support for the ovary, and are composed of two major structural protein molecules: collagen and elastin. This property is especially ascribed to the 19 different types of collagen molecules which

undergo self-assembly into various supramolecular structures, fibrils and different kinds of networks onto which other constituents, such as proteogycans and glycoproteins, adhere (Prockop *et al.,* 1995). In fact, the degree of collagen and elastin cross-linking varies between tissues and is constantly shifting in the lamina basalis of developing ovarian follicles (Oksjoki *et al.,* 1999).

Several steps of oocyte maturation take place in intimate contact with ovarian connective tissues. These include differentiation of germinal cells into primordial follicles, their further development into functional ovulatory follicles and rupture, as well as formation of corpus luteum and atresia. The spatiotemporal expression of connective tissue growth factor gene during folliculogenesis and corpus luetum formation brings out the possibility that connective tissue also performs non-structural functions in the ovary (Harlow *et al.,* 2002). Several recent studies have demonstrated changes in the production and activity of matrix degrading enzymes, especially matrix metalloproteinases (MMPs), and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs), during follicular development (Hulboy *et al.,* 1997; Duncan *et al.,* 1998), but surprisingly little is known about mastitis linked possible alterations in the synthesis of matrix connective tissues in the ovary.

In fact, a key feature of persistent inflammation is collagen production. Especially connective tissue cells, fibroblasts enter in the area of tissue injury and then start producing extra cellular matrix components, such as collagen which is necessary to replace the tissue lost during long term inflammation. The overabundance of collagen production over time can lead to permanent distortion of the tissue, interfering with its own function (Atamas, 2002). Availability of special stains, Masson Trichome with Aniline blue, permits assessing the cascades of ovarian stromal collagen components connected with different stages of follicular development of both healthy and mastitic ovaries.

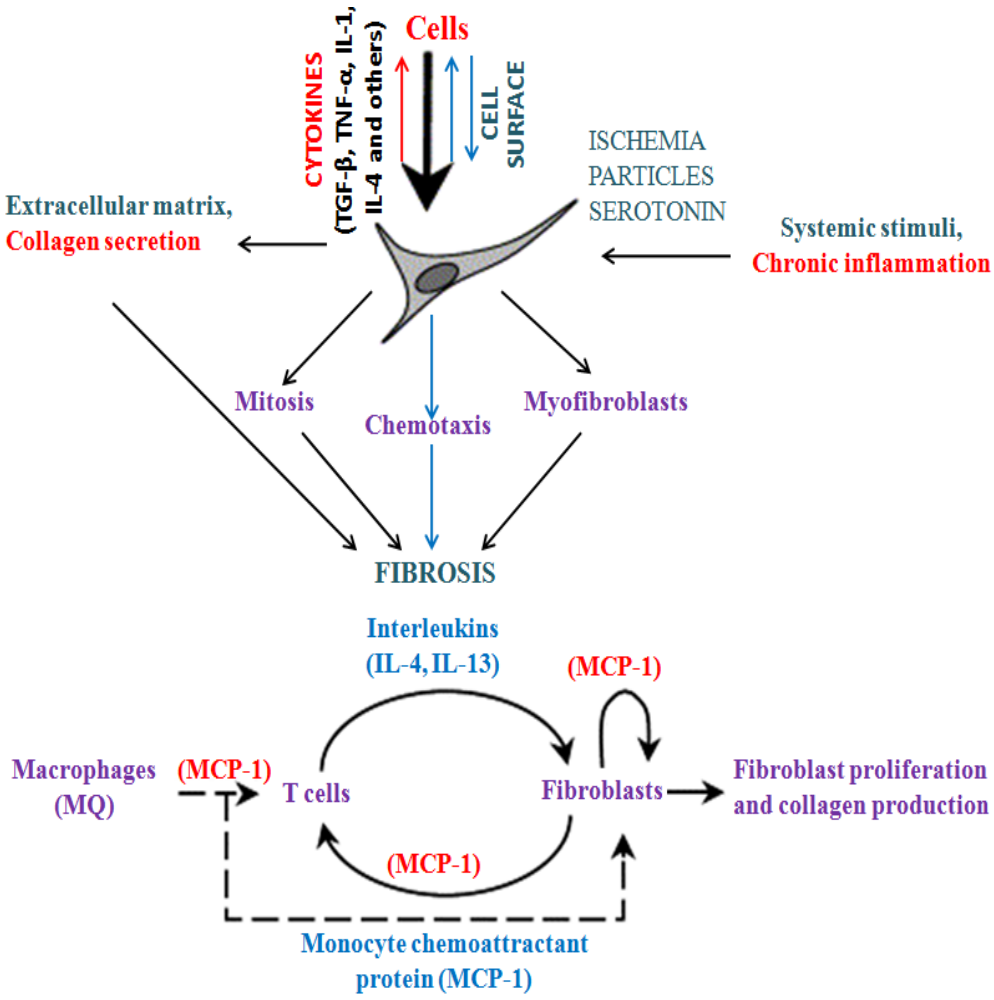

Figure 30. Cytokine regulation of fibroblasts: these cells are subjected to systemic stimuli/chronic systemic inflammation; interact with extracellular matrix and with other cells. Any of these interactions can lead to fibrosis (in the top). MCP-1 directly activates collagen production by fibroblasts as well as acts indirectly by attracting T cells, which stimulate fibroblast proliferation and collagen production by secreting IL-4 and IL-13. Fibroblasts secrete autocrine MCP-1 in response to IL-4 stimulation during chronic inflammation. Fibroblasts and T cells continuously sustain the profibrotic IL-4-MCP-1 loop, even in the absence of activated macrophages (in the bottom) (modified from Atamas *et al.,* 2002).

1.10 CYTOKERATIN 17, VIMENTIN AND FOLLICULOGENESIS

Proliferation of granulosa cells is the key event in the successful folliculogenesis in female. Cytokeratin17 is a member of intermediate filamentous protein subfamily that is expressed in the epithelial cells (Magin *et al.,* 2007). Infact, it is a protein with 432 amino acids involved in the regulation of cell growth and structural support interacting with the adaptor protein 14-3-3-σ in the cellular protein synthetic pathway. It also integrates with cytokeratin 6b forming dense filamental bundle for cellular strengthness and ristance. Mutation or alteration in the gene responsible for cytokeratin results in diminished translation of the protein, eventual down regulation of the Akt/mTOR signalling pathway with compromised epitheliogenesis. Cytokeratin 17 is also a marker that permits indentification of tumoral cells of the larynx and urinary tract. Vimentin is another member of the same intermediate filament family of proteins that is especially found in connective tissues and is responsible for anchoring the position of the organelles in the cytosol (Ortega *et al.,* 2007). Although most intermediate filaments are stable structures, in fibroblasts, vimentin exists as a dynamic structure (Perez-Martınez *et al.,* 2001). During the last decades a number of studies demonstrated that pharmacological agents that induce either disruption or stabilization of cytoskeletal fibres strongly affect steroid horomone secretion (Chen *et al.,* 1994). This factor has been extensively studied in ovarian cells of many species including farm animals (van denHurk *et al.,* 1995; Khan-Dawood *et al.,* 1996; Marettova *et al.,* 2002), and is particularly important in the changes in steroidogenesis that takes place during progressive folliculogenesis (Isobe *et al.,* 2003).

*"Hope is like the sun, which, as we journey toward it, casts a shadow of our burden behind us." —**Samuel Smiles***

02.

SCOPE OF THE WORK

A series of epidemiological studies performed in the past decade indicated that mastitis had a negative effect on the reproductive performance of dairy cows. A few studies reported that mastitic cow has greater risk for reduced conception rate, delayed ovulation with steroidal and gonadotrophin hormonal alterations and increased fetal loss, regardless of clinical case being caused by Gram-positive or Gram-negative bacteria. Apart from that, strongly negative genetic correlation between milk yield and fertility is also evident. However, our knowledge on the possible mechanisms by which mastitis negatively affects fertility is imprecise. Indeed, a sound ovarian environment is the key for optimal ovarian cyclicity in cow. In this respect, we carried out a morphometric and molecular study on the ovaries of healthy and mastitic animals.

A network of locally produced cytokines orchestrates the regulation and recruitment of the leukocytes to any inflammatory site. Several of these cytokines have been proposed to act as regulators in different processes in reproductive physiology. As part of normal ovarian physiology, they regulate the processes of follicular growth and development, ovulation and subsequent repair of the ruptured follicle. Recent investigations however, revealed a massive upregulation and often dysregulation of these soluble inflammatory mediators during severe mastitis. Although cytokines play a critical role in the host defense against infection, but dysregulation of its expression during chronic infection can have deleterious consequences to the host. Thus, one of the aims of our study was to describe the effect of microbial toxin and/or cytokine associated pathogenetic interaction on ovarian physiology.

Any investigation on mastitis-infertility interaction needs molecular and morphometric analysis of the ovary, which is a highly organized organ composed of germ cells (oocytes) and somatic cells (granulosa, theca, stromal cells as well as matrix connective tissue fibers), whose interactions dictate successful folliculogensis in normally fertile cow. It is well established that ovary plays a key role in the reproductive cycle through interregulating various intraovarian factors and systemic hormones by directing feedback mechanisms to the hypothalamus and pituitary. Any interruption in this complex regulation of ovarian cycle could effectively destabilize folliculogenesis leading to compromised fertility in female. Keeping it in mind, we carried out an unbiased estimation of early and growing follicular numbers of the healthy and affected animals.

In fact, ovarian stroma is one of the few adult tissues where neovascularization takes place as a normal process. Similarly, it is well recognized that in healthy ovarian stroma, the process of folliculogenesis is always accompanied by equally striking changes in the stromal angiogenesis. Stromal angiogenesis is a cytokine linked, as well as other locally and systemically regulated complex process, essential for supplying oxygen, nutrients and other stimulatory signals for efficient folliculogenesis in the stroma. Cytokine linked vascular damage is already documented in various tissues. In this respect, cytokine or other inflammatory mediator associated alterations in the stromal vasculature could greatly affect cow fertility. Using recently available endothelial cell biomarkers, histological evaluation of ovarian stromal microvascular bed in both healthy and sick animals provided us a new avenue for inferring a correlation between mastitis and infertility.

Morphometric assessment of stromal connective tissue especially estimating matrix collagen is another important aspect of knowing ovarian follicular environment. In fact, stromal connective tissue apart from structural support also performs non-structural function, and in effect ovarian folliculogenesis takes place in intimate contact with ovarian connective tissues. In view of the fact that, any persistent irritation results in increased collagen production in that tissue, we investigated the effect of chronic mastitis on cortical stromal collagen in the affected ovaries.

Bidirectional signaling between the oocyte and surrounding somatic cells is absolutely essential for successful germ cell development in mammals. Oocytes secrete proteins that are necessary for granulosa cells growth and differentiation, whilst granulosa cells regulate oocyte development and integrate ovarian function with the rest of the body by orchestrating gonadal steroidogenesis**.** GDF-9, a member of TGF**-**β superfamily, has been recently recognized as a key regulator transmitting the signaling between the oocyte and the surrounding granulosa cells. Thus, we investigated the expression of GDF-9 in the healthy and mastitic ovaries.

Keeping the aforesaid fact in mind and side by side for the fulfillment of the requirement of my PhD thesis, in the present work, we tried to investigate the effect of naturally occurring chronic mastitis on ovarian reserve and its follicular dynamics. In fact, we tried to elucidate if any difference exists at the level of folliculogenesis, stromal micro-vascular bed, stromal collagen as well as ovarian growth factors between the two categories of ovarian samples: one healthy and other from animals having mastitis so that impact of inflammatory process on sensitive biological structures could be insightful in a substantial manner. To accomplish this, supported by available logistics, established protocols and

techniques, we correlated the level of health of slaughtered cows with ovary by macroscopic and microscopic morphology as well as molecular study.

"Knowledge must continually be renewed by ceaseless effort, if it is not to be lost. It resembles a statue of marble which stands in the desert and is continually threatened with burial by the shifting sand. The hands of service must ever be at work, in order that the marble continue to lastingly shine in the sun. To these serving hands mine shall also belong" — ***Albert Einstein***

03.
MATERIALS AND METHODS

3.1 SOURCE AND MANIPULATIONS OF THE SAMPLES

Milk samples and the pairs of ovaries of the corresponding 74 animals having different grades of udder infections were obtained at local abattoir, AL CARNI SNC Padino, Cremona, Italy.

3.1.1 MILK

Milk samples were collected aseptically from udder quarters of cows immediately before slaughter. Briefly, collection of milk were made by beginning from the closest teat and moved to teats on the far side of the udder; and the reverse order was followed for cleaning. Grossly dirty teats were scrubbed and cleaned thoroughly with individual disposable paper towel containing isopropyl alcohol before proceeding with sample collection. The rim of the sample tube was not allowed to come in touch with the teat end. Prior to milk collection, a few streams of foremilk were discarded from the teat and then one to three streams of milk were taken into the tube and tightly secured with cap. The pre-slaughter sample tubes were labeled with cow's identity by permanent ink pen and usually filled less than ¾ full. The milk samples were immediately cooled and stored at 4°C on ice packs and delivered to the infectious disease diagnostic laboratory of DIPAV within 2 hours of collection.

3.1.2 OVARY

Post-slaughter ovaries of the corresponding animals were collected and immediately kept in a thermal container with normal saline set at 32-34°C and delivered to the Laboratory of Anatomy and Histology within 2 hours of collection.

3.2. ANALYSIS OF MILK

3.2.1 CHARACTERIZATION OF BACTERIA

Milk evaluation was carried out at the infectious diseases section of DIPAV. Milk samples were examined following procedures recommended by the National Mastitis Council (NMC, 1999), and as described by Oliver *et al.,* (1994). Briefly, milk samples (10 μL) from each quarter were plated separately onto one quadrant of a trypticase soy agar plate

supplemented with 5% defibrinated sheep blood. In case of clinical samples the volume of milk was enhanced using a sterile cotton swab. Plates were incubated at 37°C and bacterial growth was observed and recorded at 24-h intervals for 3 days. The following day, the colonies on growth, when no more than two different types were present, were isolated on blood agar plate. Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics, and catalase test. Isolates identified presumptively as staphylococci were tested for coagulase by the tube coagulase method.

Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin, and CAMP-reaction. Streptococcal organisms were identified to the species level using the API system (bioMérieux, Lyon, France) upon isolation of the organism from naturally infected mammary glands. Streptococcal organisms isolated were identified by sodium hippurate hydrolysis for *Streptocoocus uberis*. All other *Streptococcus* and *Enterococcus species* were identified using the API Strep System (bioMerieux Lyon, Inc.) for subsequent isolates. Gram-negative isolates were plated on MacConkey's agar (Becton Dickinson Microbiology Systems, Sparks, MD) and evaluated by various biochemical tests. Microbial scoring was performed based on the intensity of bacterial load and the presence of minor or major pathogens in the udder quarter (Table 4).

Microbial scoring	
Type of Microbs	Score
Negative	1
Minor pathogens (CNS)	2
Contaminated	3
Major pathogen	4

Table 4. The parameters used for the microbial scoring of the milk samples

3.2.2 QUARTER SOMATIC CELL COUNT

Somatic cell count was performed by a fluorimetric method on a Bentley Somacount 150® (Bentley instruments, Chaska MN, USA); subsequently quarter health was scored following parameters described in Table 5.

Somatic cell scoring	
SCC × 1000	Score
<200 cells/ml	1
200-500 cells/ml	2
501-1000 cells/ml	3
>1000 cells/ml	4

Table 5. The parameters used for the somatic cell scoring of the milk samples

Finally, the animals were classified according to the two aforementioned parameters on udder health: somatic cell count (SCC) and type of bacteria detected in milk. In fact, for assessing the udder health of each animal, both of the parameters (microbial and somatic cell scores) were merged into single sum score. Based on this sum or total scoring, the samples were graded into 7 groups. Depending on the severity of mastitis, healthy group and severely mastitic groups were considered for intensive study. The groups that we studied in more details were the group (2+3) in one pole and group (7+8) in another pole, in sufficient numbers to comply with the statistical analysis. The group (7+8) consists of animals with severe mastitis, where somatic cells were >1000 x 10^3 and the presence of potential pathogens (*E. coli, Staphyloccus aureus, Streptococcus agalactiae* etc.). In contrast, group (2+3) consists of healthy animals with the absence of mastitis or presence of minor pathogens (CNS) and the somatic cells were < 200 x 10^3 in number.

3. 3. OVARIAN SAMPLE

3.3.1 MACROSCOPIC MORPHOLOGICAL ANALYSIS

In the laboratory, the ovaries were cleaned; adhering tissues were removed, rinsed with sterile saline solution and subjected to macroscopic evaluation by measuring the ovarian volume and counting of the macroscopic follicles, corpus luteum, corpus albicans and corpus hemrrohagicum.

Based on ovarian morphological classification made by Gandolfi *et al.*, (1997), that was preindicative of oocyte developmental competence, ovaries were divided into 3 categories on the basis of: Type A) highly competent ovary, having at least 1 or more follicles >8 mm in diameter, +/- corpus luteum; Type B) intermediary, no follicle >8 mm in diameter, >10 follicles 2-5 mm, +/- corpus luteum; and Type C) low fertile ovary, no follicle >8mm, <10 follicles 2-5 mm, +/- corpus luteum. Therefore, based on the distribution of different combinations of ovaries we categorized each animal as: Highly fertile (AA, AB, BB), Intermediary (AC, BC) and low fertile (CC).

Breslow-Day test was used for assessing the homogencity of prevalence amongst udder health SCC scores, bacteriological scores and macroscopic follicular scores of ovaries. Morphological differences were analyzed by Fisher's exact and Mann–Whitney U rank sum test. The confidence interval for statistical significance was 95% ($p<0.05$).

3.3.2 HISTOLOGY PROCEDURES

3.3.2.1 TISSUE FIXATION

Each ovary was cut through its major axis with scalpel and forceps, starting from one pole to other. After having a set of mutually perpendicular axes the ovarian cortex were fragmented into cubic size of about 20×5 mm^2 and a thickness of ≥5 mm for the inclusion in paraffin. The cortical fragments of each ovary were immediately fixed in 4% (w/v) Paraformaldehyde and 10% (v/v) Formalin.

3.3.2.2 PARAFFIN EMBEDDING

The ovarian cortical fragments were fixed in neutral buffered paraformaldehyde (4% [w/v] paraformaldehyde in PBS) or Formalin (10% [vol/vol] formalin in PBS) for 48 hours. They were then dehydrated by bathing them successively in a gradually increasing grade of mixtures of ethanol and water (50%, 70%, 90% and 100%). The samples were immersed in xylene and then passed through paraffin: xylene (50:50) solutions overnight. On the following day, cortical pieces were washed for 3 times, each for 1 hour with melted paraffin (60°C). They were then isolated and embedded in the plastic blocks. Finally, the paraffin embedded blocks were preserved at -20°C.

3.3.2.3 PREPARATION OF HISTOLOGICAL SECTIONS

Serial sections (thickness, 5 µm) were cut from each ovary at a regular interval of 100 µm using the microtome (Microm, HM-335E, Bio-Optica, Stumentazioni Scientifica, Milan, Italy) at cooling. They were then transferred on slides and put on the hot rack for 24 hours.

3.3.2.4 STAINING OF THE SLIDES

The ovarian sections, processed and included in paraffin, obtained by cutting with microtome didn't have a definable image contrast. Therefore, it became necessary to color the sections in order to allow the subsequent morphological analysis. For the staining of the slides, first we eliminated the paraffin with the solvent xylene and then we removed the solvent by bathing them successively in a gradually decreasing grade of alcohols (100%, 90%, 70% and 50%) as direct change from absolute ethanol to water would lead too rapid swelling of the cells.

At this stage staining were carried out with common organic dyes: Hematoxylin (Emallueme Carazzi, 05-M06012, Bio-Optica, Milan, Italy) combined with an acid dye Eosin (Eosin 1% aqueous solution, 05-M 10002, Bio-Optica, Milan, Italy) according to well-established procedures (Presnell and Schreibman, 1997). Excess dyes were removed to allow the preservation of the tissue and this was achieved by re-passing the slides through an increasing grade of alcohols (50%, 70%, 90% and 100%) until return to absolute Xylene. The samples still wet with xylene were mounted with synthetic based mounting media, Biomount® (Bio-Optica, 05- BM250, Milan, Italy) and covered with microscopic cover-slip.

3.3.3 MICROSCOPIC MORPHOLOGICAL ANALYSIS

Morphological characteristics were recorded for every twentieth section (100 µm apart) using the following criteria: 1) only the follicles having visible oocyte nuclei (to avoid duplicate counting in each section), 2) number of primordial follicles, 3) number of primary follicles, and 4) number of secondary follicles. Follicles were characterized (using Leica Galen-III 317506 EU microscope at bright field under 20× or 40× magnification) considering the following stages of follicular development into account: primordial follicles (a small oocyte is surrounded by a single layer of flattened pre-granulosa/granulosa cells); classical primary follicles (enlarged oocyte with a visible nucleus and a single layer of granulosa cells, the number of granulosa cells ranging from <20-60); and secondary follicles (oocyte surrounded by two or more layers of cuboidal granulosa cells, a fine or stratified theca cell layer also distinct). Secondary follicles were further sorted as small if they contained fewer than four layers of granulosa cells and large if they contained four or more layers of granulosa cells but during quantitative analysis these two subtypes were considered as single category (Figure 32).

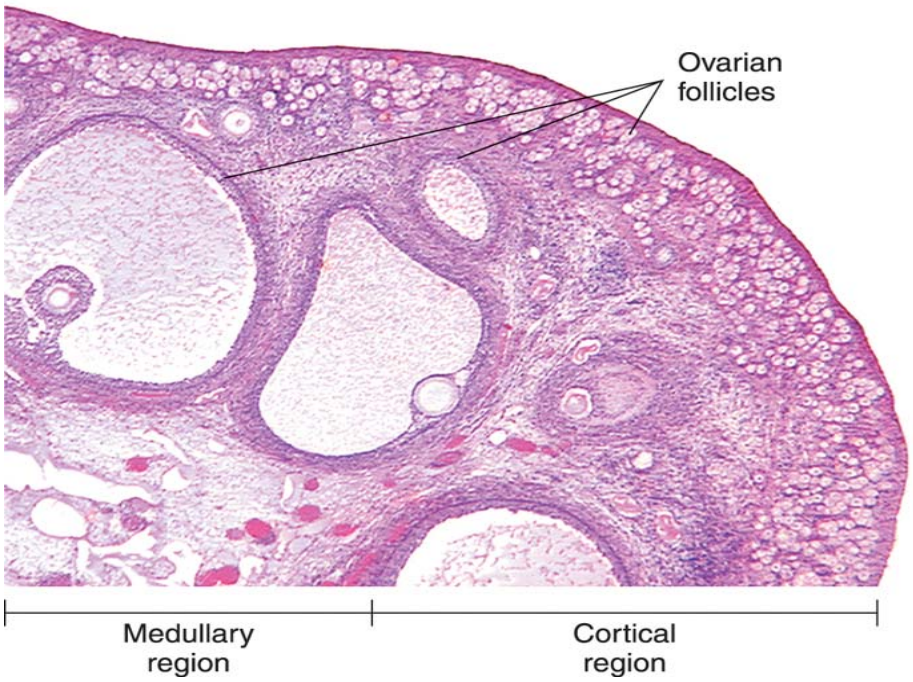

Figure 31. Photomicrograph of part of an ovary showing the cortical and medullary regions (Hematoxylin and Eosin staining; Low magnification).

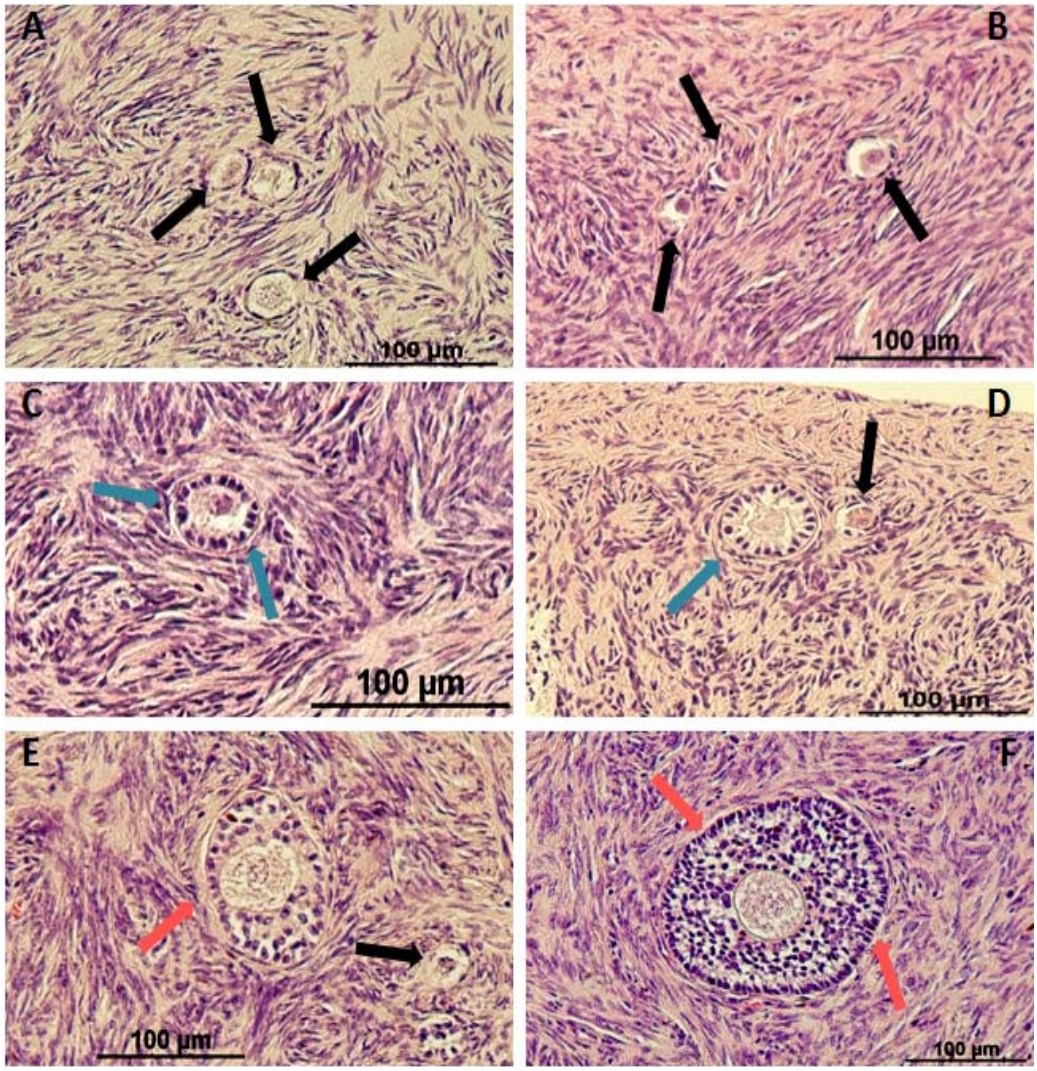

Figure 32: Representative photomicrographs of ovarian follicles in the cortical stroma. Color variations in the arrows indicate the key morphological criterias used for morphometric strudy in this experiment. A, B = primordial follicle (with black arrows); C, D = primary follicle (with dark cyan arrows); E, F = secondary follicle (with deep coral arrows). (Hematoxylin and Eosin staining; original magnification, 20x).

3.4 QUANTITATIVE ANALYSIS OF FOLLICLES

The population dynamics of ovarian follicles was estimated as the density of small follicles (primordial, transitional primary and classical primary) as well as larger ones (secondary onwards) per unit area of cortical stroma, in sections stained with hematoxylin and eosin. Total follicle counts for each ovary were obtained by adding the values found from every section of the two hemi ovaries. For each ovary 4 or 5 sections were analyzed. To eliminate the repeated counting of the same follicles (if any) in the subsequent sections of the same ovarian cortical fragment, in every section all the follicles having visible oocyte nucleus were counted and classified. The ovarian sections counted for follicles were not uniform, to achieve that we photographed the slides with camera (Nikon Coolpix 990, Tokyo, Japan), by using an automated image analyzer (MacBiophotonics image J, NIH, USA) measured the sections on the slides and then determined the follicle density per cm^2 of tissue surface (for each animal surface area mean ± SD= 5.65 ± 0.25 cm^2).

Fisher's exact test was used for analyzing the respective follicular thresholds; associations between the values of healthy and mastitic groups were assessed by Odds Ratio (OR). After descriptive analysis, Kruskal-Wallis test was also performed to determine the statistical significance. The confidence interval for statistical significance was 95% ($p<0.05$).

3.5 SPECIFIC BLOOD VESSEL IMMUNOSTAINING

After deparaffinization with xylene, the sections were rehydrated through a series of graded volumes of ethanol in distilled water (100, 90, 70 and 50%), washed in PBS (5 min) and incubated with H_2O_2 (0.3% [v/v] in methanol for 30 min) to suppress the endogenous peroxidase activity, rinsed in PBS (5 min), non-specific bindings were blocked with 5% BSA (30 min), then incubated with 10 µg/ml biotinylated *Griffonia (Bandeiraea) simplicifolia* Lectin- I (GSL-I/BSL-I, B-1105; Sigma) at room temperature (30 min) in a humidified chamber. After washing with TPBS (PBS+0.05%Tween™ 20) for 8 min (4 min × 2), the lectin binding was detected by using VECTASTAIN® elite ABC kit (peroxidise, Cat. No. PK-6100) (Vector Laboratories Inc., Burlingame, CA, USA) according to manufacturer's protocol. In brief, the lectin binding was visualized by incubating the sections with a peroxidase substrate- chromogen solution consisting of 3', 3'-diaminobenzidine (ImmPACT™ DAB, Cat. N. SK-4105) for 2-10 min. After development of immunoreaction, the slides were counterstained with hematoxylin (Emallueme Carazzi, 05-M06012, Bio-Optica, Milan, Italy)) for 10 min. To determine the specificity of lectin binding, staining of negative control was carried out by substituting the

substrate medium with buffer (PBS) without lectin. The sections were mounted with synthetic based mounting media, Biomount® (Bio-Optica, No. 05 BM250, Milan, Italy). They were then studied and photographed on Nikon microscope and camera respectively (Nikon Eclipse E600, Digital Sight DS-2MBWc, Tokyo, Japan) (Figure 37).

3.6 ASSESSMENT OF LECTIN-POSITIVE CAPILLARY VESSELS (LPCV)

Three sections per ovary (approximately 1.5 mm apart from each other), and equidistant microscopic fields per section with the ×20 objective were analyzed. Lectin binding of capillary vessels was counted using an automated image analyzer (MacBiophotonics image J, NIH, USA). The application consisted of the following modules: photographed image, manual thresholding, morphologic filtering algorithm, LPCV area identification and image analysis. Photographed images of ovarian stroma stained with Lectin contained excess light and that's why they were subjected to pre-processing, thresholding yielded a binary image representing different types of blood vessels (BV) and stromal cells. Threshold image elements of interest were quantified in μm^2, and the percentage LPCV area in the stroma was calculated as follows: LPCV = 100 × VA/IA, where VA is the vascular area and IA, is the image area (area of the whole image). Microscopic fields were selected at random in the cortical zone following a systematic procedure throughout the ovarian section. The observation was conducted in 10 equidistant cortical areas in the superficial and deep cortex of different sections (with area mean ± SD= Value as μm^2, as well as VA percentage with respect to whole stroma) and, therefore, approximately a total area of 2897.413 to 3268.372 mm^2 of stromal tissue per ovary was used for LPCV estimation procedures. The area of capillary vessel contained both endothelial cells and lumen of vessel. Both ovaries of the two intensively studied animal groups were considered when available.

Outer cortical stroma was recognizable by the presence of densely packed stromal cells, the presence of primordial and early growing follicles and low density of blood vessels. Whereas deep cortical stroma (DCS) was composed of less densely packed stromal cells together with more abundant and larger blood vessels. Volume density of deep cortical blood vessels was estimated by applying the same image analyzing process except discarding the areas corresponding to the theca layer of antral follicles, corpora lutea or avascular corpora albicantia. The medullary zone containing densely packed larger blood vessels, as a continuum with the ovarian hilus, was not considered. Possibility of cyclic changes, i.e. density of cortical blood vessels in the follicular and luteal phases of the estrous cycle was not taken into account.

After assessing the normal distribution and the descriptive analysis, student's *t*- test, Pearson Product Moment correlations as well as Mann-Whitney U test were used in order to compare and delineate the correlation between the two intensively studied groups. The confidence interval for statistical significance was 95% ($p<0.05$).

3.7 GDF-9 IMMUNOHISTOCHEMISTRY

To detect comparative expression of GDF9 in the ovarian follicles of both healthy and mastitic animals' immunohistochemistry was performed as described below. Briefly, the paraffin-embedded follicle sections (thickness, 5µm) were mounted on positively charged slides (Superfrost® plus, Menzel GmbH & Co KG, Braunschweig, Germany; no. 21998), deparaffinized (xylene- 15 min, Xylene: Ethanol-8, Absolute Ethanol-10 min, 95% Ethanol-5 min, 70% Ethanol-5 min, 50% Ethanol-5 min), hydrated (5 min) and immersed in PBS (5 min). The sections were then subjected to antigen retrieval by microwave in 0.001 M Ehylenediamine-Tetraacidic acid (E-9884, EDTA, PH 8.0) for 10 minutes. The sections were then allowed to cool down at room temperature for 10 minutes. They were sequentially incubated in rinsing solutions PBS with 0.1% (vol/vol) Polyoxyethylene Sorbitan Mono Laurate (Tween 20) (P-9416, Sigma Aldrich, St. Louis, MO, USA) for 5 min in room temperature, PBS with 0.025% (vol/vol)Triton X-100 (T-8787, Sigma Chemical Co, St. Louis, MO, USA) for 5 minutes in room temperature and washed in buffer (PBS) (5 min, RT). They were treated with serum buffer solution (10% Donkey serum) (D-9663, Sigma Aldrich, St. Louis, MO, USA) for 40 minutes as a blocking agent to nonspecific binding.

The sections were immunostained using affinity purified goat polyclonal GDF9 (C-18) antibodies (raised against a peptide mapping near the C-terminus of GDF-9 of human origin) (sc-12244, Santa-Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibody were used at 1:100 dilution in buffer (Ab: PBS) and incubated overnight at +4°C in humified chamber. On the next day they were washed in buffer (2 wash with PBS +0.1% Tween-20 and 1 wash with only PBS, 5 minutes each at RT) and subjected to incubation with secondary antibodies. The secondary antibodies, labeled Donkey Anti-Goat IgG-Alexa Fluor®594 (H+L) (A-11058, Molecular probles™, Eugene, OR, USA) were applied at 1:100 dilution with buffer (PBS) and incubated for 1 hour at RT in dark room. Simultaneous to Alexa Fluor® 594, the sections were also incubated with DNA binding fluorescent stain, 4', 6-diamidino-2-phenylindole (DAPI) (D-8417, Sigma-Aldrich, St. Louis, MO, USA) for the same period of time. The sections were then washed with PBS (3 wash, 5 minutes each, with slight shaking) followed by mounted with Anti-fading mounting kit (P-7481, Molecular probe, Eugene, Oregon, USA) for photography. The

specificity of primary antibody was verified by incubating with equivalent dilution of blocking peptide (sc-12244 p, 100µg peptide in 0.5 ml PBS containing <0.1% sodium azide and 0.2% BSA, Santa Cruz Biotechnology, CA, USA). For negative control, the primary antibodies were omitted and the condition was substituted with PBS. The sections were photographed on a Nikon microscope (Nikon Eclipse E600) equipped with epifluorescent optics (Nikon, DS-2MBWc, Tokyo, Japan) (Figure 51).

3.8 QUANTIFICATION OF GDF9-POSITIVE FOLLICLES

The percentage of GDF-9 expressed area was assessed in compare to the whole cortical area studied. In fact, two sections per ovary (approximately 1.5 mm apart from each other) with the ×20 objective were analyzed. Assessment was carried out by an automated image density analyzer (MacBiophotonics image J, NIH, USA). Threshold image elements of interest were quantified in μm^2, and the percentage area of GDF expressed follicles (GEF) was calculated as follows: GEF = 100 × GEA/IA, where GEA is the GDF expressed area and IA is the image area (area of the whole image). GDF-9 immunostain in 20 oocytes for each healthy and mastitic animal group was semiquantified using a relative intensity scale: 0, 1 and 2 for no (Figure 51- panel G, H, I), weak (Figure 51- panel D, E, F) and strong (Figure 51- panel A, B, C) staining respectively. Observation was conducted in stromal areas of different sections with total cortical area of 13227 mm^2.

After assessing the normal distribution and the descriptive analysis, student's *t*- test, Pearson Product Moment correlations as well as Mann-Whitney U tests were performed in order to compare and delineate the correlation between the two intensively studied groups. The confidence interval for significance was 95% ($p<0.05$).

3.9 CONNECTIVE TISSUE HISTOCHEMISTRY

The connective tissue histochemistry was performed to assess follicular environment, i.e. overall texture of stromal fibrous tissue of affected and healthy animals. Staining was carried out with Masson trichrome e aniline blue (Bio-Optica, 010802, Milan, Italy) in line with the principles like: Weigert's iron hematoxylin for nuclei, picric acid for erythrocytes, acid fuchsin-ponceau de xylidine for cytoplasm and aniline blue for connective tissue following manufacturer's protocol. In brief, the sections were deparaffinized with xylene, rehydrated through a series of graded volumes of ethanol (100%, 90%, 70% and 50%), and brought to distilled H_2O (5 min). Then equal amount (6 drops) of two different Weigert's iron hematoxylin (A and B) were put on the sections and left to act for 10 min. Without washing, the slides were drained and 10 drops of picric acid alcoholic solution (C) was put and left to act 4 minutes. Following quick wash (3-4 seconds) in distilled

H_2O, 10 drops of ponceau acid fuchsin (D) was put on the sections and left to act for 4 min. After washing with distilled H_2O, 10 drops of phosphomolybdic acid solution (E) was put on the slides and left to act for 10 minutes. Finally, without washing the slides, the connective tissue stain (10 drops), Masson aniline blue (F) was put on the sections and left 5 minutes to act followed by washed in distilled water, dehydrated rapidly through ascending alcohols and xylene, mounted with synthetic based mounting media, Biomount® (Bio-Optica, No. 05 BM250, Milan, Italy), studied and photographed on Leica Galen-III microscope and Nikon camera respectively (Nikon Coolpix 990, Tokyo, Japan) (Figure 55)

3.10 ASSESSMENT OF STROMAL CONNECTIVE TISSUE (OSCT)

Two sections per ovary (approximately 200μm apart from each other), with the ×20 objectives were analyzed. Both ovaries of two intensively studied animal groups were considered when available. Assessment of connective tissue in the ovarian stroma was carried out with a computer adapted program, automated image analyzer (MacBiophotonics image J, NIH, USA). The image analysis application consisted of the same principles as LPCV area assessment including the following modules: photographed image, pre-processing of the images, and manual thresholding for converting it to binary image, morphologic filtering algorithm, OSCT area identification and image analysis. Threshold image elements of interest (collagen in the extracellular matrix) were quantified in μm^2, and the percentage area of ovarian stromal connective tissue (OSCT) was calculated as follows: OSCT = 100 × CA/IA, where CA is the connective area and IA, is the image area (area of the whole image). The observation was conducted in six (6) similar cortical areas of different sections for each animal. Therefore, approximately a total stromal tissue area of 1695.71 mm^2 (mean ± SD) was used for OSCT area assessing procedures.

After assessing the normal distribution and the descriptive analysis, student's *t*- test, Pearson Product Moment correlations as well as Mann-Whitney U tests were performed in order to compare and delineate the correlation between the two intensively studied groups. The confidence interval for statistical significance was 95% ($p<0.05$).

3.11 CYTOKERATIN-17 HISTOCHEMISTRY

To detect mastitis linked alterations in the expression of cytokeratin-17 in the ovarian granulosa cells, immunohistochemistry was performed. Briefly, the paraffin-embedded follicle sections (thickness, 5μm) were mounted on positively charged slides (Superfrost® plus, Menzel GmbH & Co KG, Braunschweig, Germany; no. 21998), deparaffinized (xylene- 15 min, Xylene: Ethanol-8, Absolute Ethanol-10 min, 95% Ethanol-5 min, 70%

Ethanol-5 min, 50% Ethanol-5 min), hydrated (5 min) and immersed in PBS (5 min). The sections were then subjected to antigen retrieval by microwave in 0.001 M Ehylenediamine-Tetraacidic acid (E-9884, EDTA, P^H 8.0) for 10 minutes. They were then allowed to cool down at room temperature for 10 minutes. Then subjected to rinsing in PBS with 0.1% (vol/vol) Polyoxyethylene Sorbitan Mono Laurate (Tween 20) (P-9416, Sigma Aldrich, St. Louis, MO, USA) for 5 min in room temperature, PBS with 0.025% (vol/vol)Triton X-100 (T-8787, Sigma Chemical Co, St. Louis, MO, USA) for 5 minutes (RT) and only PBS (5 min, RT). They were treated with serum buffer solution (10% Donkey serum) (D-9663, Sigma Aldrich, St. Louis, MO, USA) for 40 minutes as a blocking agent to nonspecific binding.

The sections were immunostained with monoclonal mouse anti-keratin-17 antibody (MAB1677, Chemicon® international, MA, USA). Primary antibody was used at 1:400 dilution in buffer (Ab: PBS) with overnight incubation at +4°C in humified chamber. On the next day, followed by 3 washes (2 washes with PBS +0.1% Tween-20 and 1 with PBS, 5 minutes each at RT) sections were subjected to incubation with secondary antibodies. The secondary antibodies, labeled Donkey Anti-Mouse IgG-Alexa Fluor®594 (H+L) (A-21203, Invitrogen®, USA) were applied at 1:200 (Ab: PBS) and incubated for 1 hour at RT in dark room. Simultaneous to Alexa Fluor® 594, the sections were also incubated with DNA binding fluorescent stain, 4', 6-diamidino-2-phenylindole (DAPI) (D-8417, Sigma-Aldrich, St. Louis, MO, USA) for the same period of time. Followed by 3 wash with PBS (5 minutes each, with slight shaking) the sections were mounted with Anti-fading mounting kit (P-7481, Molecular probe, Eugene, Oregon, USA) for photography. For negative control, the primary antibodies were omitted and the condition was substituted with PBS. The sections were photographed on a Nikon microscope (Nikon Eclipse E600) equipped with epifluorescent optics (Nikon, DS-2MBWc, Tokyo, Japan).

3.12 VIMENTIN HISTOCHEMISTRY

For assessing the intermediate filament proteins of ovarian stromal connective tissues, vimentin immunohistochemistry was performed as below. In brief, the paraffin-embedded follicle sections (thickness, 5µm) were mounted on positively charged slides (Superfrost® plus, Menzel GmbH & Co KG, Braunschweig, Germany; no. 21998), deparaffinized (xylene- 15 min, Xylene: Ethanol-8, Absolute Ethanol-10 min, 95% Ethanol-5 min, 70% Ethanol-5 min, 50% Ethanol-5 min), hydrated (5 min) and immersed in PBS (5 min). The sections were then subjected to antigen retrieval by microwave in 0.001 M Ehylenediamine-Tetraacidic acid (E-9884, EDTA, P^H 8.0) for 10 minutes. They were then allowed to cool down at room temperature for 10 minutes. Then subjected to rinsing in

PBS with 0.1% (vol/vol) Polyoxyethylene Sorbitan Mono Laurate (Tween 20) (P-9416, Sigma Aldrich, St. Louis, MO, USA) for 5 min in room temperature, PBS with 0.025% (vol/vol)Triton X-100 (T-8787, Sigma Chemical Co, St. Louis, MO, USA) for 5 minutes (RT) and only PBS (5 min, RT). They were treated with serum buffer solution (10% Donkey serum) (D-9663, Sigma Aldrich, St. Louis, MO, USA) for 40 minutes as a blocking agent to nonspecific binding.

The sections were immunostained with monoclonal mouse anti-keratin-17 antibody (MAB1687, Chemicon® international, MA, USA). Primary antibody was used at 1:500 dilution in buffer (Ab: PBS) with overnight incubation at +4°C in humified chamber. On the next day, followed by 3 washes (2 washes with PBS +0.1% Tween-20 and 1 with PBS, 5 minutes each at RT) sections were subjected to incubation with secondary antibodies. The secondary antibodies, labeled Donkey Anti-Mouse IgG-Alexa Fluor®594 (H+L) (A-21203, Invitrogen®, USA) were applied at 1:200 (Ab: PBS) concentration and incubated for 1 hour at RT in dark room. Simultaneous to Alexa Fluor® 594, the sections were also incubated with DNA binding fluorescent stain, 4', 6-diamidino-2-phenylindole (DAPI) (D-8417, Sigma-Aldrich, St. Louis, MO, USA) for the same period of time. Followed by 3 wash with PBS (5 minutes each, with slight shaking) the sections were mounted with Anti-fading mounting kit (P-7481, Molecular probe, Eugene, Oregon, USA) for photography. For negative control, the primary antibodies were omitted and the condition was substituted with PBS. The sections were photographed on a Nikon microscope (Nikon Eclipse E600) equipped with epifluorescent optics (Nikon, DS-2MBWc, Tokyo, Japan).

3.13 STATISTICAL ANALYSES

The statistical analyses were performed with Sigma stat 3.1 (Systat Inc., San Jose, California) as well as SPSS 17.0 (SPSS Inc., Chicago, USA) software.

"The crew of the caravel 'Nina' also saw signs of land and a small branch covered with berries. Everyone breathed afresh and rejoiced at these signs" —***Christopher Columbus***

04.
RESULTS

It was not possible to collect the milk of 5 of the 74 cows involved in this study due to severe injury to the teat opening or teat canal or complete fibrosis in the teat and/or udder quarters and they were excluded. Further 3 animals showed either one or two quarters and/or teats fibroses or allowed partial collection of milk. Moreover, ovaries of 3 animals were discarded after the discovery of pregnancy. So, it was not always possible to collect milk and ovary of the same animal therefore only 63 cows were included in the study.

4.1 BACTERIOLOGICAL ANALYSIS OF MILK AND SOMATIC CELL COUNT

Based on the well established protocols (NMC, 1999), the major pathogens were characterized and somatic cells were counted. Mastitis pathogens isolated from mammary glands of the infected cows as well as the somatic cell numbers (SCN) are presented in Table 6. The majority of the clinical infections were caused by *E. coli* and *Staphylococcus aureus*, whereas subclinical chronic infections were caused by *Steptococcus dysgalactiae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and coagulase-negative staphylococci (CNS). Other organisms such as *Proteus* sp., yeasts as well as milk contaminant *Corynebacterium pyogenes* were also observed in the collected samples (summarized in Table 7).

Mammary scores were determined as an indicator of the mammary status (severity or absence of disease) of the udder quarters. A total score (TS) representing the sum of the microbiological score (BCC) plus the somatic cell count score (SSC) was calculated for each animal. Animals were divided into 7 groups based on the TS value (Table 6)

The milk quality of the animals with a TS ≥ 6 appeared extremely compromised with the presence of flakes or clots and watery or unusual appearance. It is worth mentioning that in the present study animals were graded into different groups based on the intensity of mastitis regardless of pathogen types (gram-positive or gram-negative). However, it was observed that gram-negative *E. coli* with BCC (≥3) causes greater elevation of SCC, with total count of 36675 for all the four quarters of an animal. Followed by *Staphylococcus aureus*, *Streptococcus dysgalactie* and *Klebsiella* sp., and in each animal total SCC were

16115, 13247 and 12890 respectively. Indeed, in the healthy group, SCC value of an animal was as low as 39 to 1647 with absence or mild contamination (BCC ≤1) by non pathogenic *Staphlylocci or Corynebacterium bovis*. The SSC values and respective scores in each animal group are presented in the Table 8.

		Anterior		Left	Anterior		Right	Posterior		Right	Posterior		Left			
Animal N°	**Date of Birth**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**SCC score**	**Bact score**	**Sum score**
56	7/6/2006	nrs		113	Cor. bov	3	50	nrs		9	nrs		44	1	1	2
74	12/15/2006	nrs		11	Stf. spp	3	11	nrs		8	nrs		9	1	1	2
3	11/15/2004	nrs		100	Cor. bov	3	844	nrs		488	nrs		152	2	1	3
5	12/24/2005	Stf. spp	3	55	Stf. spp	3	115	Stf. spp	3	53	Stf. spp	3	17	1	2	3
32	12/15/2004	nrs		78	nrs		61	Stf. spp	2	267	Contam.		113	2	2	3
37	1/1/2004	nrs		454	nrs		468	nrs		227	nrs		798	2	1	3
72	12/3/2006	nrs		82	nrs		49	nrs		34	nrs		31	2	1	3
73	24/3/2006	nrs		73	polimic		58	polimic		219	polimic		126	1	2	3
18	11/7/2004	nrs		478	nrs		25	polimic		284	Stf. spp	3	4088	2	2	4
40	2003	nrs		7497	nrs		3649	nrs		217	Stf. spp	3	271	3	1	4
53	2002	nrs		598	Stf. spp	3	588	nrs		607	Stf. spp	3	575	2	2	4
64	2003	Stf. spp	3	858	nrs		30	polimic		15	polimic		42	2	2	4
69	Mar-07	Stf. spp	3	5785			6000	nrs		5356	polimic		2884	2	2	4
71	Sep-05	nrs		937	Stf. spp	2	2846	nrs		2295	nrs		199	2	2	4
1	11/22/2004	nrs		5741	polimic		4534	nrs		5677	Abs.	Abs.	Abs.	4	2	5
2	3/21/2002	nrs		677	Proteot.	2	7123	Abs.	Abs.	Abs.	nrs		2511	4	1	5
6	1/21/1998	nrs		5	Pseud aer.	2	9890	Stf. spp	3	1265	polimic		64	3	3	5
11	10/15/2000	Stf. spp	3	2944	Stf.spp	3	62	polimic		337	polimic		98	2	3	5
13	7/29/2003	nrs		4221	nrs		2877	nrs		3257	nrs		4127	4	1	5
24	4/27/2003	nrs		890	polimic		905	polimic		1457	nrs		562	3	2	5
30	9/16/2006	nrs		1554	nrs		5222	nrs		6345	nrs		1488	4	1	5

Table 6. Details of the microbiological evaluation of Milk samples of all the animals (n=63): the somatic cell count and characterization of bacteria present in the samples. (Where, nrs-not relevant, contam- bacterial crowding with contamination, abs-absent, cod 1: <5 colonies; cod 2: >10 pure colonies, cod 3 : >10 contaminated colonies; SCC-somatic cell count, Bact. score-Bacteriological scoring).

		Anterior		Left	Anterior		Right	Posterior		Right	Posterior		Left			
Animal N°	**Date of Birth**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**SCC score**	**Bact score**	**Sum score**
31	6/1/2003	Abs.	Abs.	Abs.	nrs		1298	polimic		352	nrs		1583	3	2	5
36	10/20/2001	Str. spp	3	553	Str. spp	3	331	Str. spp	3	703	Str. spp	3	535	3	2	5
49	2004	nrs		636	nrs		556	nrs		625	polimic		1062	3	2	5
58	2005	nrs		4801	nrs		3585	nrs		2796	nrs		2034	4	1	5
62	2005	nrs		263	Str. dysg.	2	75	nrs		1471	polimic		638	3	2	5
63	2004	nrs		72	nrs		77	Str. dysg.	3	3087	A. pyogen.	3	3086	3	3	5
66	Sep-02	nrs		853	nrs		7441	E. coli	3	904	polimic		7554	4	1	5
4	11/9/2002	Stf. spp	3	3108	polimic		5709	nrs		2614	polimic		2161	4	2	6
7	5/21/2005	contam.		6357	nrs		5813	nrs		6659	contam.		5406	4	2	6
10	11/10/2002	nrs		2609	nrs		3110	nrs		2415	polimic		1593	4	2	6
12	3/13/2003	Stf. spp	3	1217	polimic		5700	Stf. spp	3	1196	Stf. spp	2	9234	4	2	6
14	9/1/2004	polimic		4387	Stf. spp	3	990	polimic		585	nrs		633	3	2	6
17	8/1/2001	nrs		2683	nrs		3122	Stf. spp	3	2716	Stf. spp	3	2672	4	2	6
20	6/11/2003	Stf. spp	3	1833	Stf. spp	3	1162	nrs		4019	E. coli	2	4905	4	2	6
22	10/5/2001	polimic		6658	Cor. bov	2	6941	nrs		9999	polimic		9027	4	2	6
25	8/28/2004	Cor. bov	3	2770	Cor. bov	3	3597	nrs		2355	Cor. bov	3	3801	4	2	6
27	6/22/2004	nrs		564	S. aureus	6	60	S. aureus	3	661	S. aureus	1	742	3	3	6
29	2/26/2002	Abs.	Abs.	Abs.	nrs		1711	polimic		686	polimic		897	3	2	6
38	6/5/2000	polimic		4177	nrs		3822	nrs		2828	nrs		3729	4	2	6
42	2004	S. aureus	6	1055	nrs		1992	nrs		3074	nrs		1266	4	2	6
43	2005	nrs		2867	Cor. bov	2	2309	Cor. bov	2	4085	Cor. bov	2	2702	4	2	6

Details of the microbiological evaluation of Milk samples of all the animals (n=63) (continued).

		Anterior		Left	Anterior		Right	Posterior		Right	Posterior		Left			
Animal N°	**Date of Birth**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**Bacteria**	**Cod**	**SCC**	**SCC score**	**Bact score**	**Sum score**
47	2000				Contam.		2648	nrs		2605	polimic		4214	4	2	6
50	2003	polimic		1489	Str. dysg.	3	2834	Stf. spp	3	1075	polimic		42	3	3	6
52	2006	polimic		3576	nrs		2701	nrs		2298	nrs		1497	4	2	6
54	2003	Str. uberis	3	1924	nrs		128	nrs		6004	Str.uberis	3	3322	3	3	6
68	Dec-03	nrs		322	nrs		165	S. aureus	3	2177	nrs		191	3	3	6
8	10/8/2004	polimic		1749	Abs.	Abs.	Abs.	Kleb. pne.	2	5786	nrs		5355	4	3	7
9	4/28/1999	Stf. spp	3	1040	Abs.	Abs.	Abs.	Str. dysg	3	6681	polimic		8045	4	3	7
15	12/20/2003	nrs		7360	polimic		9500	polimic		5594	polimic		4163	4	3	7
16	1/27/2006	contam.		1680	Pseud aer.	2	5834	Abs.	Abs.	Abs.	polimic		4065	4	3	7
19	10/7/2004	nrs		9999	E.coli	2	7764	E. coli	2	8913	E. coli	3	9999	4	3	7
23	6/21/2003	S. aureus	2	9171	S. aureus	3	1466	S. aureus	3	150	Stf. spp	3	399	3	4	7
26	8/17/2001	nrs		5618	nrs		4648	E. coli	3	8505	E. coli	2	9999	4	3	7
28	7/3/2001	polimic		2300	polimic		887	polimic		4216	polimic		3260	4	3	7
35	5/13/2006	S. aureus	2	6178	nrs		1446	Stf. spp	3	4451	polimic		4040	4	3	7
41	7/16/2006	nrs		2789	Contam.		1652	S. aureus	6	2298	S. aureus	2	3777	4	3	7
45	5/13/2001	Stf. spp	2	8081	Stf.spp	2	7743	E. coli	2	5752	E. coli	2	2211	4	3	7
60	1/6/2005	polimic		1130	polimic		4750	polimic		5178	polimic		5733	4	3	7
61	8/22/2005	Prot.mir	3	5431	S. aureus	1	1255	Str. dysg.	3	5168	nrs		1393	4	3	7
34	1/14/2005	Str. agal	1	6208	S. aureus	3	2224	Str. agal	3	7096	Str. agal	3	5861	4	4	8
51	6/28/2003	no milk	-	-	no milk	-	-	Proteus mir.	3	3316	contam.	-	2458	4	4	8
59	7/19/2004	E. coli	2	8177	polimic	-	3946	E. coli	3	5750	E. coli	3	2950	4	4	8

Details of the microbiological evaluation of Milk samples of all the animals (n=63)

Bacteriological Analysis			
Animal Groups	Bacteria	Code	Bacteriological Score (average)
Animal group 2	*Corynebacterium bovis*, *Staphylcoccus* spp.	3	1
Animal group 3	*Corynebacterium bovis*, *Staphylcoccus* spp.	2, 3	1.67
Animal group 4	*Staphylococcus* spp., contaminated	2, 3	1.83
Animal group 5	*Staphylococcus* spp., Proteus sp., *Pseudomonas aer., Streptococcus dysgalactiae, Actinomyces pyogenes, E. coli*	2, 3	1.86
Animal group 6	*S. aureus, Staphylococcus spp., Cor. bovis, Streptococcus uberis, Streptococcus dysgalactiae, E. coli*	1,2,3,6	2.21
Animal group 7	*E. coli*, S. *aureus*, *Staphylococcus* spp., *Pseudomonas aer.*, *Klebsiella pne.*, , *Proteus mir.*, *Streptococcus dysgalactiae*	1,2,3	3.08
Animal group 8	*E. coli, Proteus mir., Streptococcus agalactiae, S. aureus*	1,2,3	4

Table 7. Detailed microbiological analysis of all the animal groups (where cod 1: <5 colonies; cod 2: >10 pure colonies, cod 3 : >10 contaminated colonies)

Somatic cell counts (SCC)		
Animal Groups	SCC (mean±SD)	SCC Score (average)
Animal group 2	127.5 ±125.2	1
Animal group 3	827.1 ±746.8	1.67
Animal group 4	7687.3 ±7091.5	2.17
Animal group 5	9430.6 ± 7336.8	3.36
Animal group 6	11220.8 ± 7606.2	3.68
Animal group 7	18046.3 ±8310.1	3.92
Animal group 8	16028.7±8798.8	4

Table 8. Detailed somatic cell counts (mean ±SD) and total scores in all the groups

4.2 ANALYSIS OF MACROSCOPIC MORPHOLOGY OF THE OVARIES

Each matched category of ovarian follicles in different groups was compared statistically. Analyses on macroscopic follicle counts of category (1-3 mm) of different groups reveal no (P>0.05) difference amongst groups. In the follicle category of 4-7 mm, significant statistical difference was noticed especially between less affected group 4 vs. severely mastitic group (group 7 or group 8) (3.09±0.53 vs. 0.84±0.16 or 0.67±0.33; P<0.05) (Table 9). This difference seemed much wider in follicular category (>8 mm), where follicular mean (±SEM) values were significantly reduced in severely mastitic animals [healthy (group 2 or group 3) vs. severely mastitic (group 8) was (1.5±0.87 or 0.94±0.29 vs. 0.17±0.16; P<0.05)]. Similar difference (P<0.05) was also noticed between the macroscopic median values of healthy (group 2 or group 3) and severely affected animal groups (group 7 or group 8) (Mann–Whitney test).

Table 9. Influence of mastitis on ovarian macroscopic morphology in all the animals (n=63) studied.

Total score value	N° Follicle 1-3 mm (mean±SEM)	N° Follicle (4-7 mm) (mean±SEM)	N° Follicle (>8 mm) (mean±SEM)
2	5.25±2.98^{ab}	1.25±0.75^{ab}	1.5±0.87^{a}
Group-3	7.37±1.12^{ab}	1.44±0.32^{ab}	0.94±0.29^{a}
Group-4	8.4±3.12^{ab}	3.09±0.53^{a}	1.09±0.39^{ab}
Group-5	7.58±0.83^{ab}	1.28±0.39^{ab}	0.35±0.11^{ab}
Group-6	7.57±0.96^{ab}	1.08±0.29^{ab}	0.37±0.12^{ab}
Group-7	5.88±0.82^{ab}	0.84±0.16^{b}	0.4±0.12^{ab}
Group-8	9.5±2.45^{ab}	0.67±0.33^{b}	0.17±0.16^{b}

^{a,b}Different superscripts within the same column indicate significant differences of respective category between groups (P≤0.05)

Based on previously described criteria (Gandolfi *et al.*, 1997), ovaries were arbitrarily divided in three categories: A, B and C, with A and B corresponding to ovaries containing oocytes with high developmental competence whereas ovaries of type C contain oocytes of lower competence. On this basis fertility of each animal was classified as high (H) when the two ovaries were in one of the following combinations: AA, AB, BB; intermediate (I) when ovaries where AC or BC and low (L) when both ovaries were of type C.

In fact, we noticed a tendency of alteration in cows' fertility due to the effect of mastitis; however, at statistical level the differences were not wider (Figure 33). That's why, for investigating existence of subtle differences, we carried out intensive microscopic study on the healthy and affected animal groups.

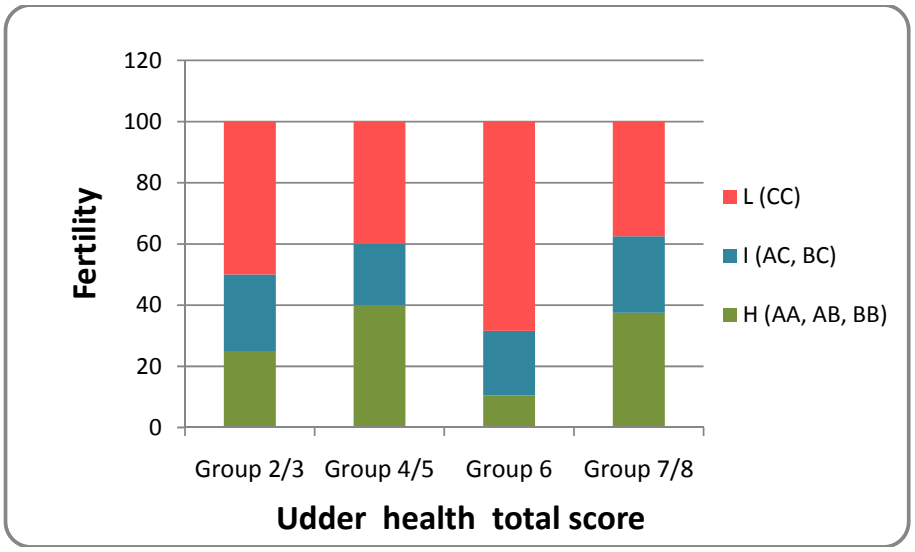

Figure 33: The graph explicates the relationship between fertility and Udder health total score

Since ovarian follicle population is influenced by the age of the animal we investigated the normality of the age distribution amongst the different degrees of mammary infection. We classified cows ≤ 4 years old as young (23 cows) and cows of >4 years as old (40 cows). Our result reveals that age has no relationship with the udder health total score. In otherwords, irrespective to age, both young and adult animals are susceptible to severe mastitis. So, in our study, observed follicular alteration at macroscopical level was due the effect of mastitis.

4.3 ANALYSIS OF THE MICROSCOPIC MORPHOLOGY OF THE OVARIES

Based on the results of milk analysis (SSC and bacterial counts) described above, we selected the animals with the higher and lower score of the sum of these two values to perform more detailed morphological analysis.

All the slides prepared from two intensively studied groups: healthy (Total score= 2 or 3) (n = 8) and affected by chronic mastitis (TS= 7 or 8) (n = 9) were studied under light microscope and the follicles were categorized and recorded. In all slides, the most predominant type of follicle observed in the stroma was the primordial follicle (healthy vs. sick: 88.87% vs. 90.92%; $P>0.05$). At the same time, primordial follicles were observed having a tendency to lie in clusters in the cortical fragments rather than being evenly distributed throughout the tissue (Figure 34). The next available type was transitional and classical primary follicles (healthy vs. sick: 9.14% vs. 8.11%; $P>0.05$), then early and classical secondary (healthy vs. sick: 1.99% vs. 0.98%; $*P<0.05$) followed by progressively developing stages. The variability of the density of follicles within the cortical fragments from ovaries of two intensively studied animals is illustrated in table 10.

An average cortex area of 5.65 cm^2 per animal was analyzed with a standard deviation of 0.25 (i.e. mean ± SD= 5.65± 0.25 cm^2, for a total area of 96.08 square centimeters and total counted follicles of 2437). The follicular density varied markedly within the ovary; primordial type ranging from 4.8 to 56.67 follicles/cm^2 in healthy, and from 10.74 to 54.16 follicles/ cm^2 in affected groups. Same trend observed in the primary follicles, ranging from 0.69 to 3.56 follicles/cm^2 in healthy, and from 0.37 to 4.11 follciles/cm^2 in mastitic animals. This variability was exeedingly higher in case of secondary follilcs in affected ovaries; ranging from 0 to 1.31 follicles/cm^2 and in >55% cases follicles were absent in the respective ovarian stroma.

The numbers of primordial, primary and secondary follicles were divided in 2 classes by the statistical software, based on respective frequency distribution, the Fisher's Exact test with Odds ratio (OR) reveals no differences in the number of primordial and primary follicles between the intensively studied animal groups; (Primordial follicle N°<100, OR =2.083; $P>0.05$) and (Primary follicle N°<12, OR =0.50; $P>0.05$) between healthy and mastitic animal groups (Figure 34, 35). In contrast, the number of secondary follicles was significantly lower in sick animals (Odds ratio 10.50*; $P<0.05$), indicating a 10 times higher risk for a mastitic animal to have less than 2 secondary follicles per square centimeter of stromal area than healthy animals (Figure 36).

Table 10. Values of the follicles counted, average and percentages of the total number, estimates of the areas measured and the relations between the two data of both healthy and sick animals.

Healthy Animals								
N° Animals	Date of Birth	Area (cm^2)	Primordial follicles	Primary follicles	Secondary follicles	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
3	15/11/2004	5.127	56	6	5	10.92	1.17	0.98
5	24/12/2005	5.812	102	15	3	17.55	2.58	0.52
32	15/12/2004	5.837	28	4	1	4.8	0.69	0.17
37	01/01/2004	5.925	63	11	2	10.63	1.86	0.34
56	06/07/2006	5.827	96	12	4	16.48	2.06	0.69
72	03/12/2006	5.634	241	17	3	42.78	3.02	0.53
73	24/03/2006	5.812	62	15	1	10.67	2.58	0.17
74	12/08/2006	5.894	334	21	3	56.67	3.56	0.51
		5.734 [a]	982[+] (122.75[a]) (88.87%)	101[+] (12.63 [a]) (9.14%)	22[+] (2.75 [a]) (1.99%[1])	21.31[a]	2.19 [a]	0.49 [a]*
Sick Animals								
N° Animals	Date of Birth	Area (cm^2)	Primordial follicles	Primary follicles	Secondary follicles	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
8	08/10/2004	5.391	88	12	1	16.32	2.23	0.19
16	27/01/2006	5.491	59	3	0	10.74	0.55	0
19	10/07/2004	5.453	111	2	0	20.36	0.37	0
35	13/05/2006	5.352	160	22	7	29.9	4.11	1.31
41	16/07/2006	5.727	72	7	0	12.57	1.22	0
51	28/06/2003	5.393	135	8	1	25.03	1.48	0.19
59	19/07/2004	5.582	96	11	0	17.2	1.97	0
60	06/01/2005	5.708	159	23	0	27.86	4.03	0
61	22/08/2005	6.111	331	20	4	54.16	3.27	0.65
		5.58 [a]	1211[+](134.56[a]) (90.92%)	108[+] (12 [a]) (8.11%)	13[+] (1.4 [a]) (0.98%[1])	23.79 [a]	2.14 [a]	0.26 [a]*

[+]sum of a specific values of all the animals within a group; an average of a specific values of all the animals within a group; [1,*] indicate statistical differences (P<0.05) between two intensively studied groups.

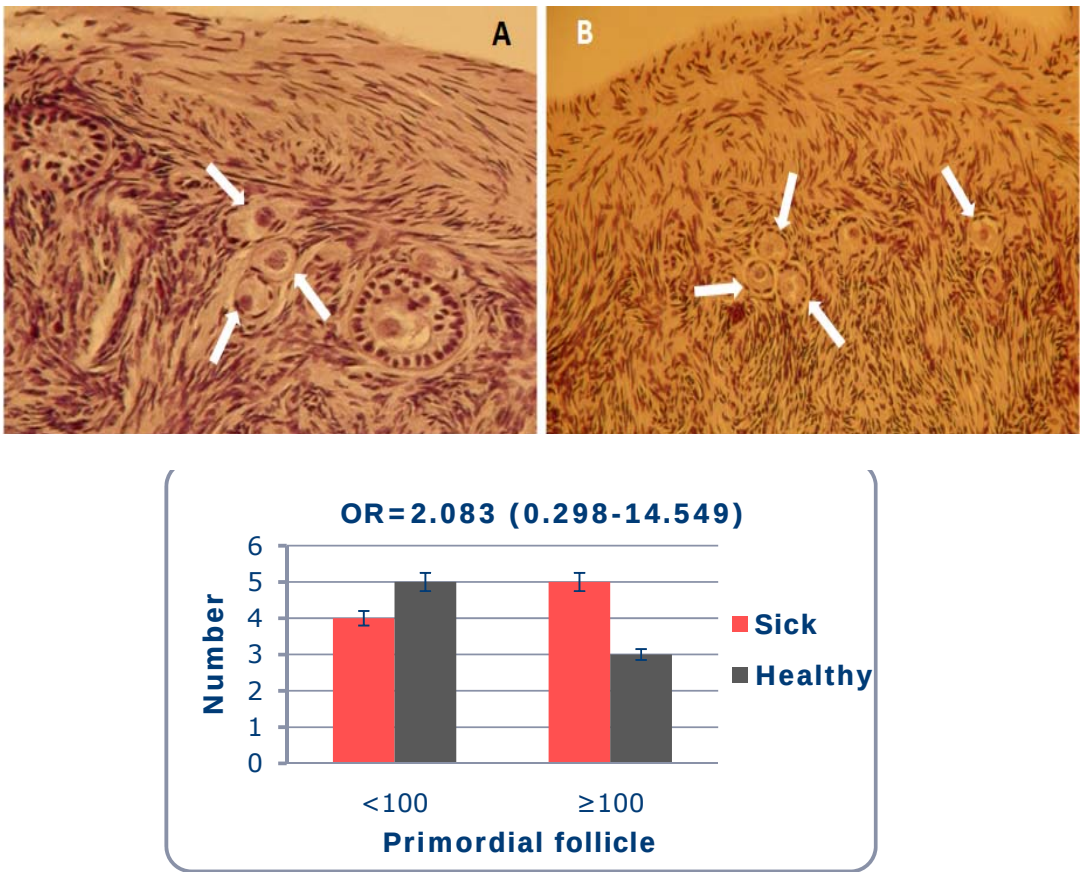

Figure 34. In top: photomicrographs of the bovine primordial follicles (arrows in A, B); primordial follicles having a tendency to lie in clusters in the upper cortical stroma rather than being evenly distributed throughout the tissue (Hematoxylin and Eosin). In bottom: sick (crimson bars) and healthy (dark bars) corresponding to the respective follicle thresholds; odds ratio value (estimate of the risk that the sick animal has <100 follicles) = 2.083 (0.298-14.549).

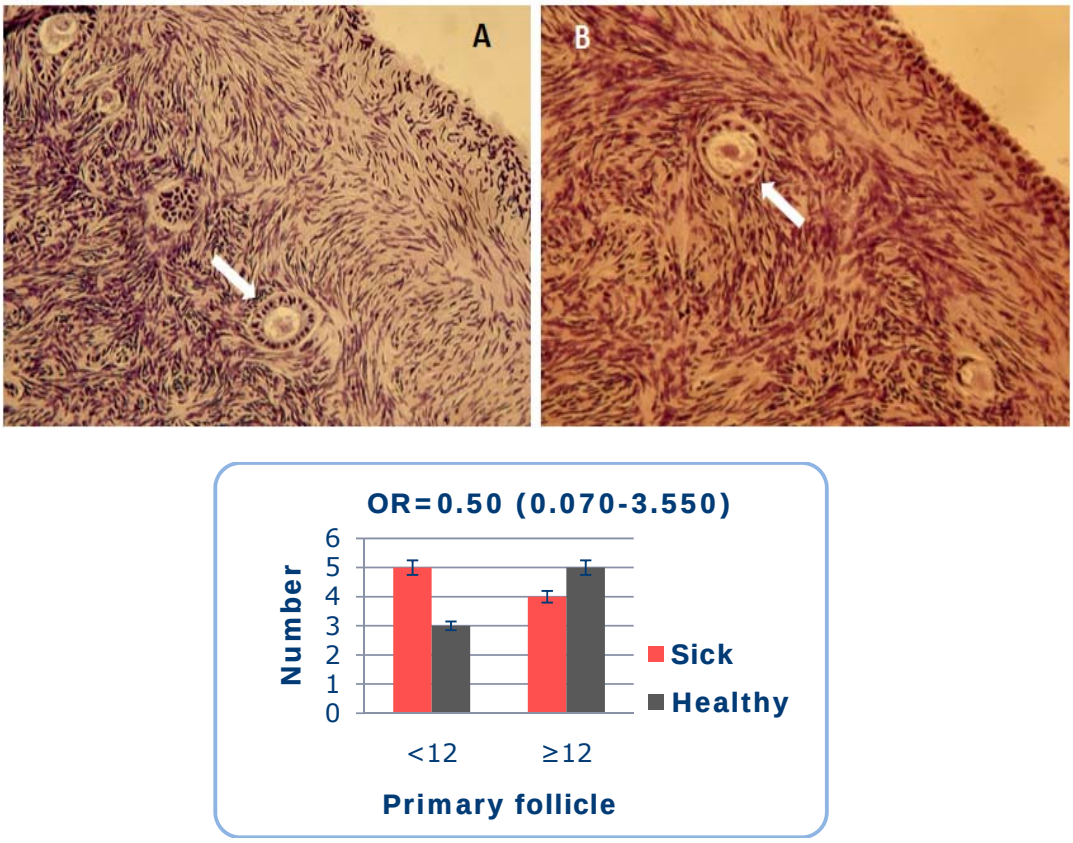

Figure 35. In top: photo-micrographs of primary follicles (arrows in A and B), in the ovarian cortical stroma with Hematoxylin and Eosin. In bottom: sick (crimson bars) and healthy (dark bars) corresponding to the respective follicle thresholds; odds ratio value (estimate of the risk that the sick animal has <12 follicles) = 0.50 (0.070-3.550).

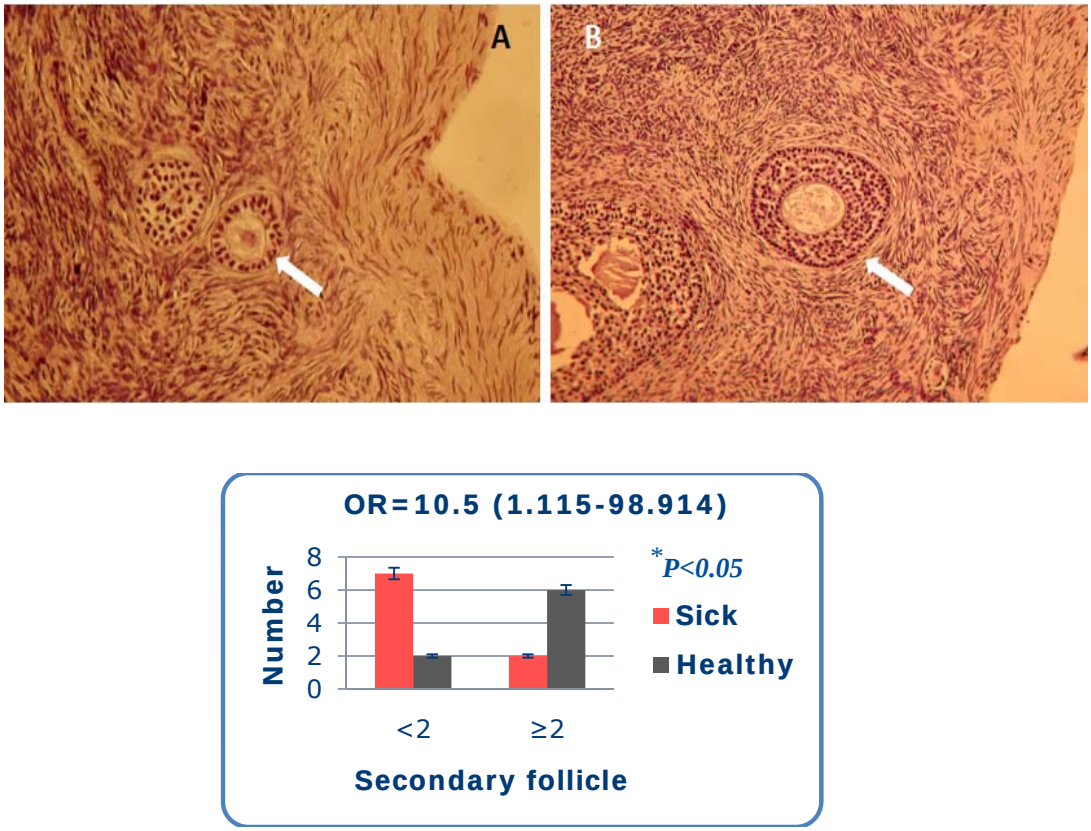

Figure 36. In Top: photomicrographs of transitional secondary (arrow in A) and secondary follicle (arrow in B) in the cow ovary (Hematoxylin and Eosin staining). In bottom: sick (crimson bars) and healthy (dark bars) corresponding to the respective follicle thresholds; odds ratio value (estimate of the risk that the sick animal has <2 follicles) = 10.50 (1.115-98.914) (*P<0.05).

4.4 ANALYSIS OF CORTEX VASCULARISATION IN HEALTHY AND MASTITIC ANIMALS

Cortex vascularisation was assessed by determining the volume density of blood vessels (i.e. the proportion of the ovarian cortical tissue occupied by BV), identified by endothelial cell specific Lectin immunostaining, in the stroma of both uninfected and affected animals. Endothelial cells of stromal BV as well as those in the theca (interna and externa) and in all stages of atresia were bound to the Lectin (Figure 37). Quantification was performed independent of the orientation of blood vessels and changes in both number and size of blood vessels, both in superficial and deep cortex, where primordial and growing follicles were located. In the superficial cortex, blood vessels corresponded mostly to microvessels with a narrow lumen that seemed to be non-homogenously distributed throughout the respective area without any apparent relationship to resting follicles located to the inner superficial cortical zone (Figure 38-A). In healthy ovaries, a well developed capillary network was observed in the deep cortical stroma. As a consequence, direct contact between blood vessels and follicles of secondary stage (SFs) onwards were frequently observed. However, compared with deeper cortex, poorer and many fold decreased vascularisation with a gradient in the blood vessels were evident to outer cortical area both in healthy and mastitic animals (Figure 37-A, 39-A). The capillary vessel areas were lower in the theca externa than interna (Figure 38-C). Simultaneously binding signal of Lectin seemed less intense in affected stromal vessels than that of healthy ones (Figure 38, 39).

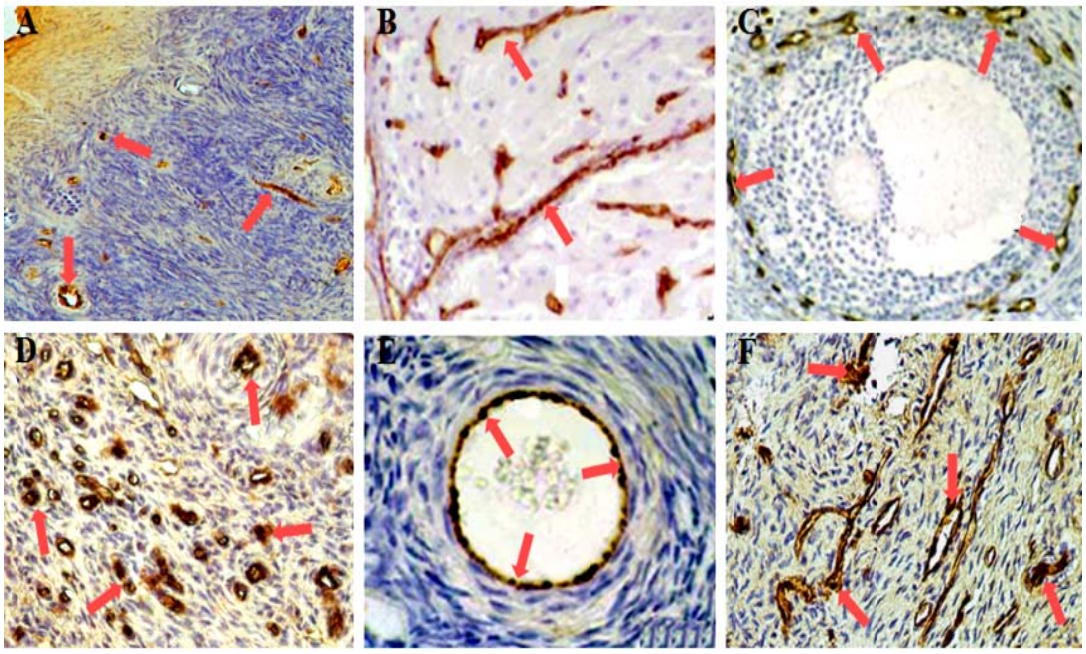

Figure 37. Specific immunostaining of ovarian blood vessels with Lectin. Strong labelling is present in the endothelium (arrows) of (a) superficial cortical stroma (b) corpora lutea, (c) newly formed blood vessels in the theca interna of antral follicles, (d) deep cortical stroma (e) mature arteries in the deep ovarian stroma and (f) small blood vessels in the mid cortex. Haematoxylin counterstaining.

4.5 ASSESSMENT OF MASTITIS RELATED CHANGES IN STROMAL VASCULATURE AND FOLLICLE DENSITY

In order to establish a correlation between microvascular bed and the population dynamics of ovarian follicles (i.e. small: primordial, transitional and classical primary and large: multi-laminar secondary), proportion of the BV occupied stromal area of the respective affected and healthy animals were measured. In fact, quantified mean (± SD) stromal vasculature for each healthy and mastitic animal was (184855.81 ±9903.56 μm^2) and (138579.59±16605.43μm^2), respectively (Table 11). Evidently, this ovarian stromal vasculature represented the 6.38±0.66 % of cortical area in healthy animals vs. 4.24±0.37%; ($P<0.001$) in affected cows (Figure 42). Unlike stromal vasculature, no differences were noticed in the numbers of primordial (21.31±18.35 vs 23.79±13.16; $P>0.05$), transitional and classical primary follicles (2.19±0.95 vs 2.14±1.40; $P>0.05$) between the two intensively studied groups. However, quantitatively there was significant difference in the density of larger follicles between the intensively studied groups

(0.49±0.27 vs 0.26±0.45; (P<0.05). The multivariate correlation analysis demonstrated an existence of a strongly positive correlation (R=0.89, P<0.05) between volume density of blood vessels and the density of stromal follicles in respective healthy ovaries (Figure 41 A). However, this relationship appeared to be altered (r=0.02, P>0.05) during mastitis as demonstrated in the single scatter plot diagram (with 95% confidence interval) in Figure 41B.

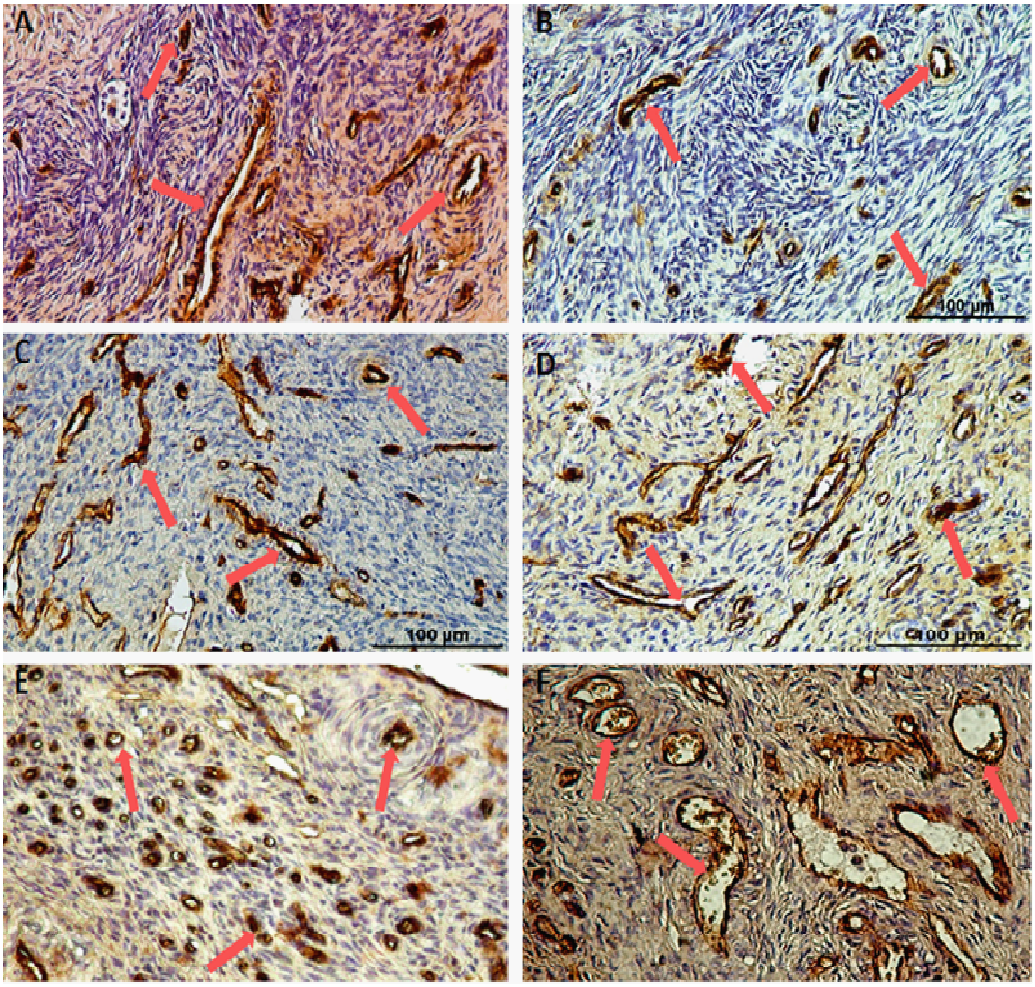

Figure 38. Lectin specific immunostaining of ovarian blood vessels of healthy animals. Strong labelling is present in the endothelium (arrows) of the blood vessels of the ovarian cortex. Vascularisations, comparatively less in upper cortex (A, B), gradually increasing in mid (C, D) and in deep cortex (E, F) were identified; with haematoxylin counterstaining (original magnification, 20x)

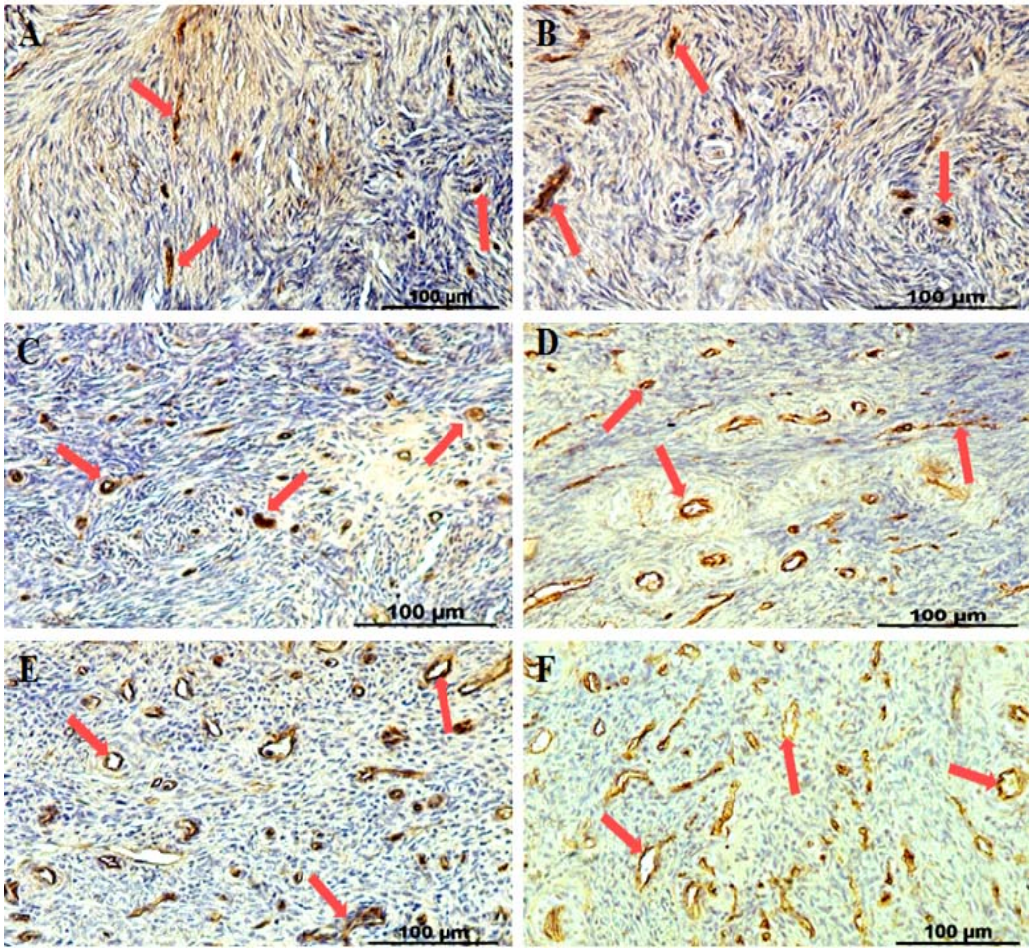

Figure 39. Mastitis associated changes of ovarian blood vessels of affected animals. Lectin specific immuno-labelling (arrows) highlights comparatively less binding signal to the endothelium of the cortical blood vessels of the affected animals. Vascularisations, comparatively less in upper cortex (A, B), gradually increasing in mid (C, D) and in deep cortex (E, F) were identified; with haematoxylin counterstaining (original magnification, 20x)

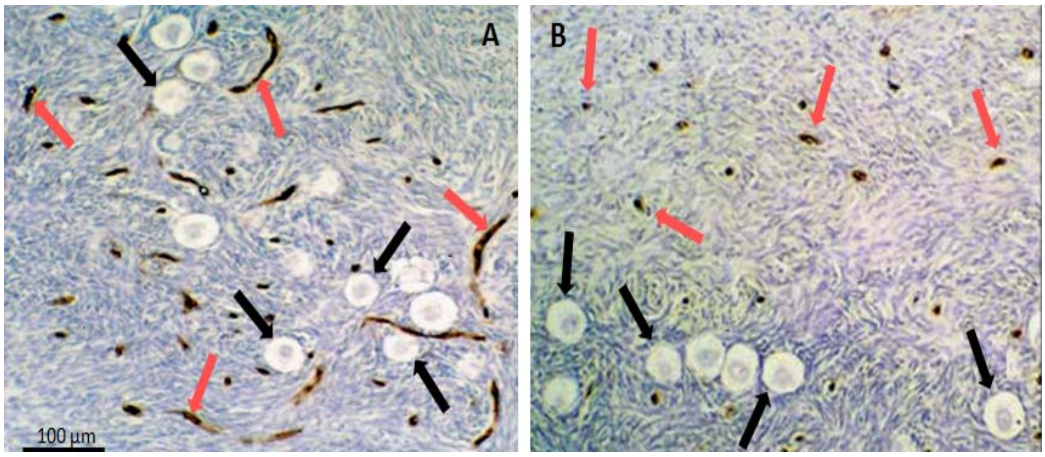

Figure 40. Photomicrographs of the ovarian cortex of healthy (A) and Mastitic (B) animals. Lectin specific immuno-labelling of blood vessels (deep coral arrows) and small (primordial and primary) follicles (black arrows) both are visible in healthy and affected ovaries. Abundant small follicles are seen in the ovarian stroma of both healthy and affected groups. In healthy ovary, blood vessels can be observed surrounding small follicles, even with direct contact between them (arrows); while in mastitic ovary decreasing number of vascular profiles are seen (arrows in B) (with hematoxylin counterstaining).

Table 11. Lectin stained blood vessel (BV) area quantification in uninfected (n=8) and mastitic cows (n=8)(average 10 counts for each animal with area mean ± SD, as well as area percentage with respect to the whole cortical part measured).

Uninfected animals				Mastitic animals		
Serial N°	Anim N°	Mean BV area (μm^2)	Area (%)	Anim N°	Mean BV area (μm^2)	Area (%)
1	3	187827.52±51287.54	5.60	8	138392.23±29445.61	4.40
2	5	177852.93±53143.55	6.59	16	137259.51±33888.91	4.33
3	32	226385.62±45511.42	5.95	19	120639.87±33830.43	3.89
4	37	200427.73±34664.94	5.75	35	141069.98±35149.72	4.37
5	56	225791.98±40410.75	6.69	41	152218.68±13687.69	4.33
6	72	191858.68±38305.97	7.09	59	140381.58±24831.71	4.62
7	73	229736.54±43025.89	5.95	60	148936.37±35096.20	4.35
8	74	256007.12±51631.91	7.39	61	110291.36±29120.46	3.48
		184855.81 ±9903.56	6.38 ±0.66[a]		138579.59 ±16605.43	4.24 ±0.37[b*]

(Anim N°-Animal number; selected animals from all that were classified and intensively studied)

/where as ([a, b*]) indicate significant statistical difference ($p<0.001$)

Table 12. A composite table demonstrates the relations between the quantified vascularised area in the ovarian stroma and follicular estimates of the respective healthy and mastitic animals.

Healthy Animals						
N° Animals	Mean BV area (μm^2)	Blood vessel Area (%)	Cortex area (cm^2)	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
3	187827.52±51287.54	5.60	5.127	10.92	1.17	0.98
5	177852.93±53143.55	6.59	5.812	17.55	2.58	0.52
32	226385.62±45511.42	5.95	5.837	4.8	0.69	0.17
37	200427.73±34664.94	5.75	5.925	10.63	1.86	0.34
56	225791.98±40410.75	6.69	5.827	16.48	2.06	0.69
72	191858.68±38305.97	7.09	5.634	42.78	3.02	0.53
73	229736.54±43025.89	5.95	5.812	10.67	2.58	0.17
74	256007.12±51631.91	7.39	5.894	56.67	3.56	0.51
	184855.81 ±9903.56	6.38 ±0.66[a]	5.73±0.26	21.31±18.35	2.19±0.95	0.49±0.27
Sick Animals						
N° Animals	Mean BV area (μm^2)	Blood vessel Area (%)	Cortex area (cm^2)	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
8	138392.23±29445.61	4.40	5.391	16.32	2.23	0.19
16	137259.51±33888.91	4.33	5.491	10.74	0.55	0
19	120639.87±33830.43	3.89	5.453	20.36	0.37	0
35	141069.98±35149.72	4.37	5.352	29.9	4.11	1.31
41	152218.68±13687.69	4.33	5.727	12.57	1.22	0
51	140381.58±24831.71	4.62	5.393	25.03	1.48	0.19
59	148936.37±35096.20	4.35	5.582	17.2	1.97	0
60	110291.36±29120.46	3.48	5.708	27.86	4.03	0
61	138579.59 ±16605.43	4.44	6.111	54.16	3.27	0.65
	138392.23±29445.61	4.24 ±0.37[b*]	5.58±0.24	23.79±13.16	2.14±1.40	0.26±0.45

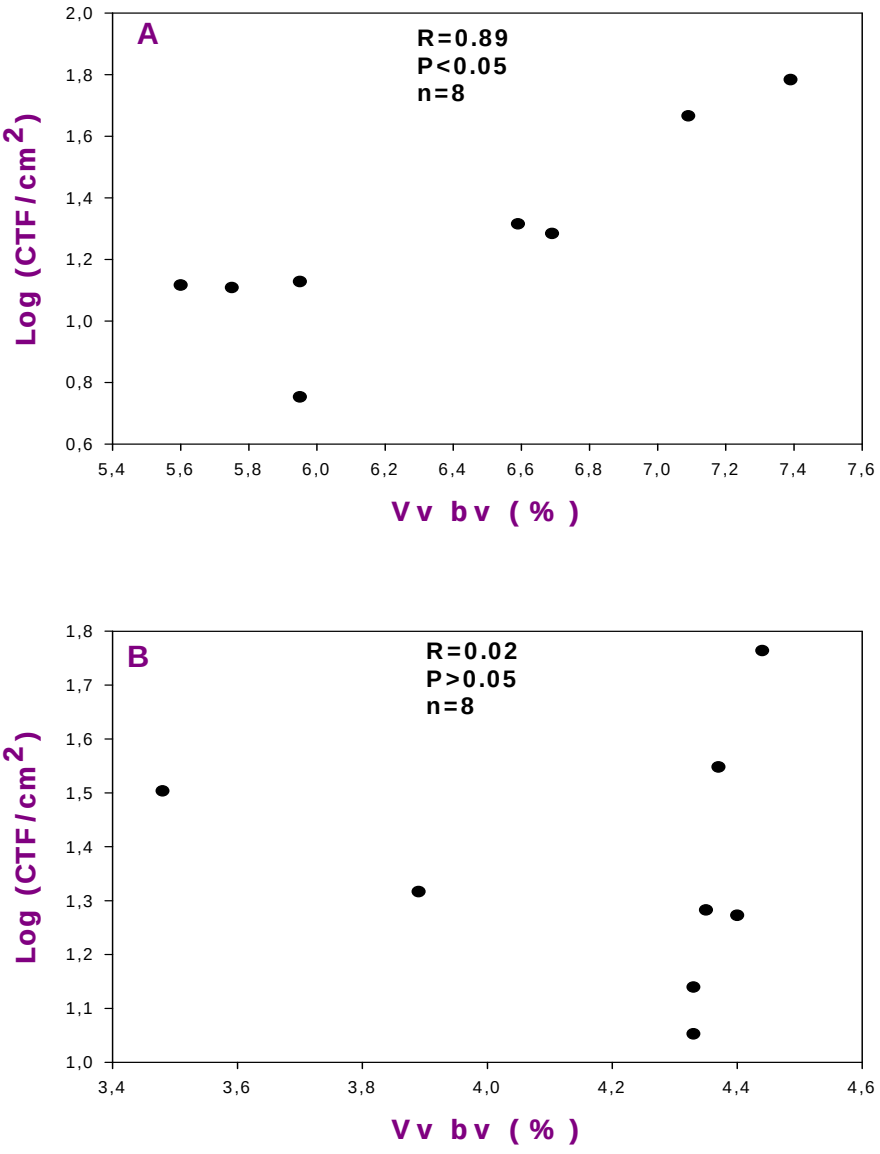

Figure 41. Scatter gram with 95% confidence for correlation analysis between counted total follicles (CTF/cm^2) and quantified BV volume density (Vv Bv) in the ovarian cortex of normal (A) and affected (B) ovaries. Multiple linear regression between the logarithm of the number of counted total follicles (primordial, primary and secondary) per cm^2 plotted against the volume density of the blood vessels; (A) r=0.89(P<0.05), exhibiting a positive interaction between CTF and stromal vascularisation in healthy animals; (B) however, in mastitc animals this relation appeared to be altered(r=0.02, P>0.05).

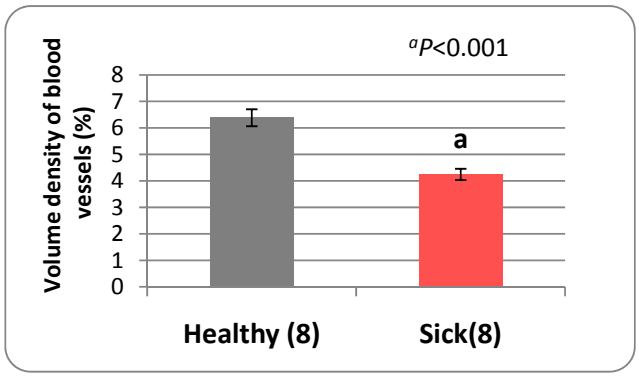

Figure 42. Mastitis related changes in the density of stromal vasculature in the bovine ovaries. The graph reveals the values of quantified vessel area (QVA or Vv Bv) in healthy (dark bars) and mastitic (crimson bars) ovaries. $^{a}P<0.001$(healthy versus sick), indicating a significant decrease in QVA percentage in the affected animal groups (*t*-test, for the numbers are indicated in parentheses).

4.6 EXPRESSION AND LOCALIZATION OF GDF-9 IN THE BOVINE OVARY

Normal ovarian development and ensuing folliculogenesis are regulated by TGFβ superfamily signalling. To determine whether GDF-9, the potent regulator of mitosis, may play a role in these processes, its expression and localization in the healthy and mastitic bovine ovary were determined. For this, ovarian cortical sections of two intensively studied animal groups were immunostained with affinity purified goat polyclonal antibovine GDF-9 antibody. Localization of GDF was confirmed by visualization of fluorescence. It appeared that expression of this factor in bovine ovary was exclusively restricted to the oocyte and no signal was found in the GC, the other part of the follicle or in stroma (Figure 51, A-C). It became also visible that this factor is expressed from primordial to all preantral follicular stages both in healthy and affected groups. However, oocyte of the healthy ovaries expressed high levels of GDF9, as it was evident by strong fluorescence (Figure 47) compared with faint or less fluorescent intensity observed in mastitic ovaries (Figure 48). GDF-9 immunostaining also clearly demonstrated that the density of different types of follicular populations in the healthy ovarian stroma was more than that of diseased animal. Negative control was demonstrated clearly with no fluorescence and complete darkness in the sample (Figure 49, 50; bottom-left).

4.7 QUANTIFICATION OF GDF-9 IN HEALTHY AND MASTITIC OVARY

Quantification was performed independent of the type, size and number of GDF-9 expressed follicles, rather on the basis of total GDF area occupied in relation to whole cortical area measured. An average area of 826697.94 square micrometres per ovary was analyzed with a standard deviation 220216.72 (i.e. mean ± SD= 826697.94 ± 220216.72 μm^2, for a total cortex area of 13227167.10 square micrometres and total GDF expressed area of 72818.42 μm^2). In fact, GDF expressed area (average six counts for each animal) varied markedly within the ovaries of individual animal; ranging from 1315.18 ±205.51 to 27040.53±3836.39 μm^2 in healthy, and from 746.07±322.46 to 7502.99±10272.03 μm^2 in affected groups (Table 13). Same trend observed in the expressed area percentage (with respect to the whole cortical area measured), ranging from 0.77±0.32 to 3.40±0.95%; (P<0.05) in each calculated healthy animals. However, less variability in expressed percentage area, ranging from 0.96±0.29 to 1.20±0.47%; (P>0.05) was observed in diseased animal groups (Table 13). In any case, higher GDF-9 protein expression was noted in disease free samples (mean± SD: 1.97 ±0.83^a) as against the samples from mastitic animals (mean± SD: 1.03 ±0.13), resulting in a significant difference between the two experimental groups (P=0.007, Figure 44). On the other hand, the semi-quantified fluorescent intensity in the oocyte panels were: 2 (strong), 1 (weak) and 0 (no) respectively (Figure 51; panel 2: A-C; panel 1: D-F and panel 0: G-I). The multivariate correlation analysis demonstrated an existence of a significantly high positive interactive effect (R=0.93; P<0.001), between follicular density and the level of GDF9 expression in the follicles of healthy ovaries (Figure 43 A). However, based on the expressed area percentage, weak fluorescent intensity and analysed R value (r=0.84) as demonstrated in the scatter plot diagram (Figure 43 B), this positive correlation appeared to be significantly altered (P<0.05) in the mastitc ovaries (Figure 44).

Table 13. GDF-9 immunostained follicle area quantification in uninfected (n=8) and mastitic cows (n=8)(average 6 counts for each animal with area mean ± SD, as well as area percentage with respect to the whole cortical part measured).

Healthy animals				Mastitis affected animals		
Serial N°	Anim N°	GDF expressed area (μm²)	Area (%)	Anim N°	GDF expressed area (μm²)	Area (%)
1	3	27040.53±3836.39	1.41±0.20	8	891.15±271.78	0.98 ±0.35
2	5	13837.59±20263.55	2.17± 0.83	16	871.56±331.24	0.96± 0.29
3	32	6842.29±10830.45	0.77±0.32	19	908.47±290.09	0.98± 0.33
4	37	1681.26±282.65	1.80 ±0.30	35	1093.56±407.15	1.20± 0.47
5	56	1986.50 ± 1056.21	2.15 ±1.14	41	746.07±322.46	0.83 ±0.34
6	72	2498.57±534.69	2.70 ±0.6	59	961.93±217.36	1.04±0.25
7	73	1315.18 ±205.51	1.40 ± 0.17	60	1090.93 ±461.93	1.08 ± 0.28
8	74	3549.84 ±1479.42	3.40 ±0.95	61	7502.99±10272.03	1.20±0.49
		7343.97 ±8986.99	1.97 ±0.83[a]		1853.68±2493.29	1.04 ±0.11[b*]

(Anim N°-Animal number; selected animals from all that were classified and intensively studied)

/where as ([a, b*]) indicate significant statistical difference ($P<0.05$)

Table 14. A composite table demonstrating the relations between the GDF expressed area and follicular estimates of the respective healthy and mastitic animals.

Healthy Animals						
N° Animals	GDF expressed area (μm^2)	Area (%)	Cortex area (cm^2)	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
3	27040.53±3836.39	1.41±0.20	5.127	10.92	1.17	0.98
5	13837.59±20263.55	2.17± 0.83	5.812	17.55	2.58	0.52
32	6842.29±10830.45	0.77±0.32	5.837	4.8	0.69	0.17
37	1681.26±282.65	1.80 ±0.30	5.925	10.63	1.86	0.34
56	1986.50 ± 1056.21	2.15 ±1.14	5.827	16.48	2.06	0.69
72	2498.57±534.69	2.70 ±0.6	5.634	42.78	3.02	0.53
73	1315.18 ±205.51	1.40 ± 0.17	5.812	10.67	2.58	0.17
74	3549.84 ±1479.42	3.40 ±0.95	5.894	56.67	3.56	0.51
	7343.97 ±8986.99	1.97 ±0.83[a]	5.73±0.26	21.31±18.35	2.19±0.95	0.49±0.27
Sick Animals						
N° Animals	GDF expressed area (μm^2)	Area (%)	Cortex area (cm^2)	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
8	891.15±271.78	0.98 ±0.35	5.391	16.32	2.23	0.19
16	871.56±331.24	0.96± 0.29	5.491	10.74	0.55	0
19	908.47±290.09	0.98± 0.33	5.453	20.36	0.37	0
35	1093.56±407.15	1.20± 0.47	5.352	29.9	4.11	1.31
41	746.07±322.46	0.83 ±0.34	5.727	12.57	1.22	0
59	961.93±217.36	1.04±0.25	5.582	17.2	1.97	0
60	1090.93 ±461.93	1.08 ± 0.28	5.708	27.86	4.03	0
61	7502.99±10272.03	1.20±0.49	6.111	54.16	3.27	0.65
	1853.68±2493.29	1.04 ±0.11[b*]	5.61±0.25	23.64±14.06	2.22±1.47	0.27±0.48

(Selected animals from all that were classified and intensively studied)

/where as ([a, b*]) indicate significant statistical difference ($P<0.05$)

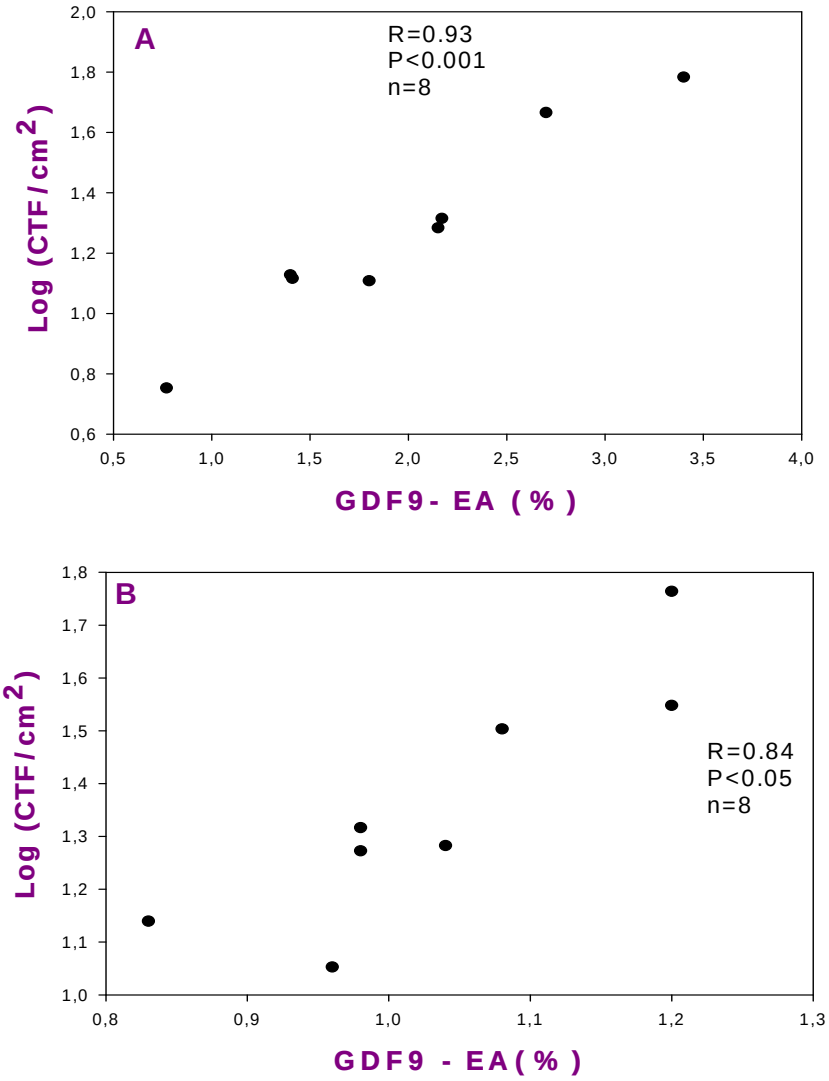

Figure 43. Relationship between GDF expressed area and counted total follicles (CTF/cm^2) in the ovarian cortex of respective healthy (A) and affected (B) animals. Multiple linear regression with 95% confidence band between the logarithm of the number of counted total follicles (primordial, primary and secondary) per cm^2 plotted against the percentage of GDF expressed area; (A) r=0.93 (P<0.001), indicating an existence of significantly high positive correlation between CTF and the level of GDF9 expression in the follicles; (B) however, this positive correlation appeared to be significantly altered in mastitic ovaries(r=0.84, P<0.05).

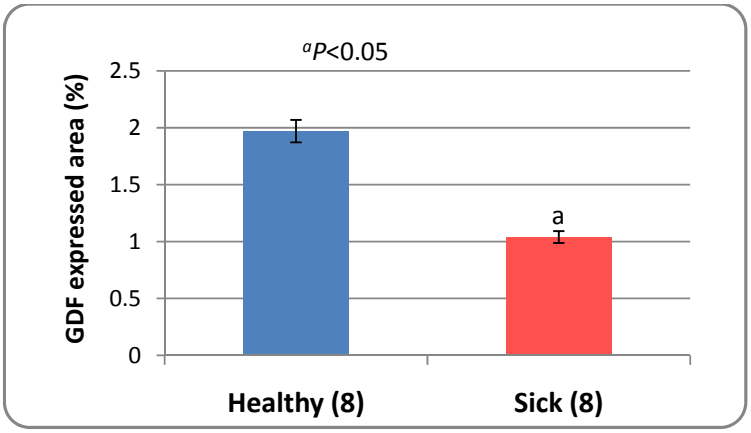

Figure 44. The graph reveals the expression pattern of GDF9 in healthy and mastitic ovaries. The percentage of mean values (± SD) of GDF expressed area in the ovaries of healthy (dark bars) and mastitic (crimson bars) animals. $^{a}P<0.05$ (healthy versus sick), indicating decreased expression of this factor in the affected animals (*t*-test, for the numbers are indicated in parentheses).

4.8 HISTOLOGICAL ASSESSMENT OF STROMAL CONNECTIVE TISSUE IN OVARY

Ovarian stroma, abundantly supplied with blood vessels, consisting for the most part of spindle-shaped cells with a small amount of ordinary connective tissue. Stromal matrix made up of different types of collagen and nonfibrillar proteins plays a prominent role in ovarian function by participating in signalling processes such as cell migration, proliferation, growth and development of follicles. Although some of these signalling processes have been characterized in mammalian ovary, however, the relative quantity and distribution of these proteins as well as the way in which collagen structure affects cell-cell and cell-matrix communication within stroma, and how tissues are changed in development and disease especially during the most predominant bovine disease like mastitis have not been well understood. For this, ovarian sections of healthy and mastitic animals were stained with special connective tissue stains like Masson trichrome (with aniline blue). Successful localization of collagen was confirmed by visualization of blue stained fibrils (Figure 53-55).

It appeared that these proteins are in the form of elongated fibrils, arranged in different combinations and concentrations both in the medullar and cortical stroma of the healthy and affected animals (Figure 53-55). Compared to healthy stroma, the distribution of collagen was consistently high in all stained sections from mastitic animals and heavy staining was observed in deeper cortex, cortico-medullary and medullar parts (Figure 55

A-D). Patches of collagen aggregation encircling at the exterior of the follicular granulosa cells were also evident in the superficial cortex of mastitic animal (Figure 54). It is interesting to note that, in compared to CL of healthy ovaries; collagen expression was abundant in the CL compartment of mastitic animals. In the healthy animal, low level staining was noticed in the ovarian surface epithelial zone, however very less or faint staining was observed in the underlying follicular zone, then again in the deeper cortex low staining was evident (Figure 52 A, B). Taken together, aggregates of collagen molecules in the ovary changes during mastitis and reveals a distinct spatial pattern.

4.9 QUANTITATIVE ANALYSIS OF STROMAL CONNECTIVE TISSUE

Quantification of collagen was performed both on vessel-poor outer cortical area as well as vessel-rich deep cortex. Although cortex showed dense, cell-rich connective tissue, there was no sharp delineating border with medulla, that's why the cortico-medullary zone was not taken into account. An average area of 105981.84 square micrometres per ovary was analyzed with a standard deviation 18437.67 (i.e. mean ± SD= 105981.84 ± 18437.67 μm^2, for a total cortex area of 1695709.38 square micrometres and blue stained total collagen area (CA) of 60204.96 μm^2). In effect, average collagen expressed area (average six counts for each animal) varied markedly within the groups ranging from 1084.372±263.52 to 2382.9263±1550.47 μm^2 in healthy, and from 3648.997±865.39 to 8369.357±2142.43 μm^2 in affected groups (Table 15). Similar tendency was also found in the CA percentage (with respect to the whole cortical area measured), ranging from 0.98 to 3.63%; ($P<0.05$) in each calculated healthy animals. In the affected groups, the CA percentage was consistently higher than healthy animals but less individual variability (3.40 to 8.73%; $P<0.05$) was evident. Altogether, affected ovarian stroma displayed significantly (mean± SD: 1.61 ± 0.90^a vs 6.046 ± 1.85^b; $P<0.001$) higher collagen area than ovaries of healthy animals, indicating mastitis linked ovarian stromal alterations (Figure 46). The multivariate correlation analysis demonstrated a negative correlation (R=-0.59 P>0.05), between follicular density and the level of collagen expression in the healthy ovarian stroma (Figure 45 A). However, this negative correlation appeared to be consistent and extremely high in the mastitic ovaries (r=-0.89, P<0.05) as demonstrated in the scatter plot diagram (with 95% confidence interval) in Figure 45 B.

4.10 ASSESSMENT OF VIMENTIN AND CYTOKERATIN IN THE CORTICAL TISSUE

We could not detect any meaningful differences in the CK and vimentin immunolebelling between the healthy and mastitic ovaries. Infact, we noticed very faint or absence of CK and vimentin immunolebelling in both healthy and mastitic ovaries with available

antibodies. On the whole, failure to obtain quantitative data regarding immunohistochemical reactivity in the follicular wall eventually made us unable to draw any inference on mastitis involvement in the pathology of these cytoskelatal proteins or the mastits linked interference (influence or reduction) on these intermediate filament proteins in bovine ovary.

Table 15. Masson Trichrome stained ovarian stroma: quantified collagen area (QCA), in healthy (n=8) and mastitic cows (n=8)(average 6 counts for each animal with area mean ± SD, as well as area percentage with respect to the whole cortical part measured).

Healthy animals				Mastitic animals		
Serial N°	Anim N°	QCA (μm²)	Area (%)	Anim N°	QCA (μm²)	Area (%)
1	3	1549.739±538.74	1.37	8	6324.07±798.65	7.35
2	5	1162.39±202.61	1.02	16	8369.357±2142.43	8.73
3	32	2382.9263±1550.47	3.63	19	5238.467±1708.42	5.37
4	37	2112.456±209.99	2.03	35	4577.634±2558.49	4.40
5	56	1433.013±1012.78	1.05	41	7553.39266±2073.33	8.02
6	72	1484.4296±142.36	1.06	59	6002.886±3452.51	6.05
7	73	2212.867±933.64	1.74	60	5067.971±791.05	5.05
8	74	1084.372±263.52	0.98	61	3648.997±865.39	3.40
		1677.77±493.38	1.61±0.90[a]		5847.85±1555.90	6.046±1.85[b*]

(Anim N°-Animal number; selected animals from all that were classified and intensively studied); where as ([a, b*]) indicate significant statistical difference (P=<0.001)

Table 16. The composite table demonstrates the relations between the quantified collagen area (QCA) to the follicular estimates in the respective healthy and affected animals.

Healthy Animals						
N° Animals	QCA (µm^2)	Area (%)	Cortex area (cm^2)	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
3	1549.739±538.74	1.37	5.127	10.92	1.17	0.98
5	1162.39±202.61	1.02	5.812	17.55	2.58	0.52
32	2382.9263±1550.47	3.63	5.837	4.8	0.69	0.17
37	2112.456±209.99	2.03	5.925	10.63	1.86	0.34
56	1433.013±1012.78	1.05	5.827	16.48	2.06	0.69
72	1484.4296±142.36	1.06	5.634	42.78	3.02	0.53
73	2212.867±933.64	1.74	5.812	10.67	2.58	0.17
74	1084.372±263.52	0.98	5.894	56.67	3.56	0.51
	1677.77±493.38	1.61±0.90^a	5.73±0.26	21.31±18.35	2.19±0.95	0.49±0.27
Sick Animals						
N° Animals	QCA (µm^2)	Area (%)	Cortex area (cm^2)	Primordial follicles/cm^2	Primary follicles/cm^2	Secondary follicles/cm^2
8	6324.07±798.65	7.35	5.391	16.32	2.23	0.19
16	8369.357±2142.43	8.73	5.491	10.74	0.55	0
19	5238.467±1708.42	5.37	5.453	20.36	0.37	0
35	4577.634±2558.49	4.40	5.352	29.9	4.11	1.31
41	7553.39266±2073.33	8.02	5.727	12.57	1.22	0
59	6002.886±3452.51	6.05	5.582	17.2	1.97	0
60	5067.971±791.05	5.05	5.708	27.86	4.03	0
61	3648.997±865.39	3.40	6.111	54.16	3.27	0.65
	5847.85±1555.90	6.046±1.85^{b*}	5.61±0.25	23.64±14.06	2.22±1.47	0.27±0.48

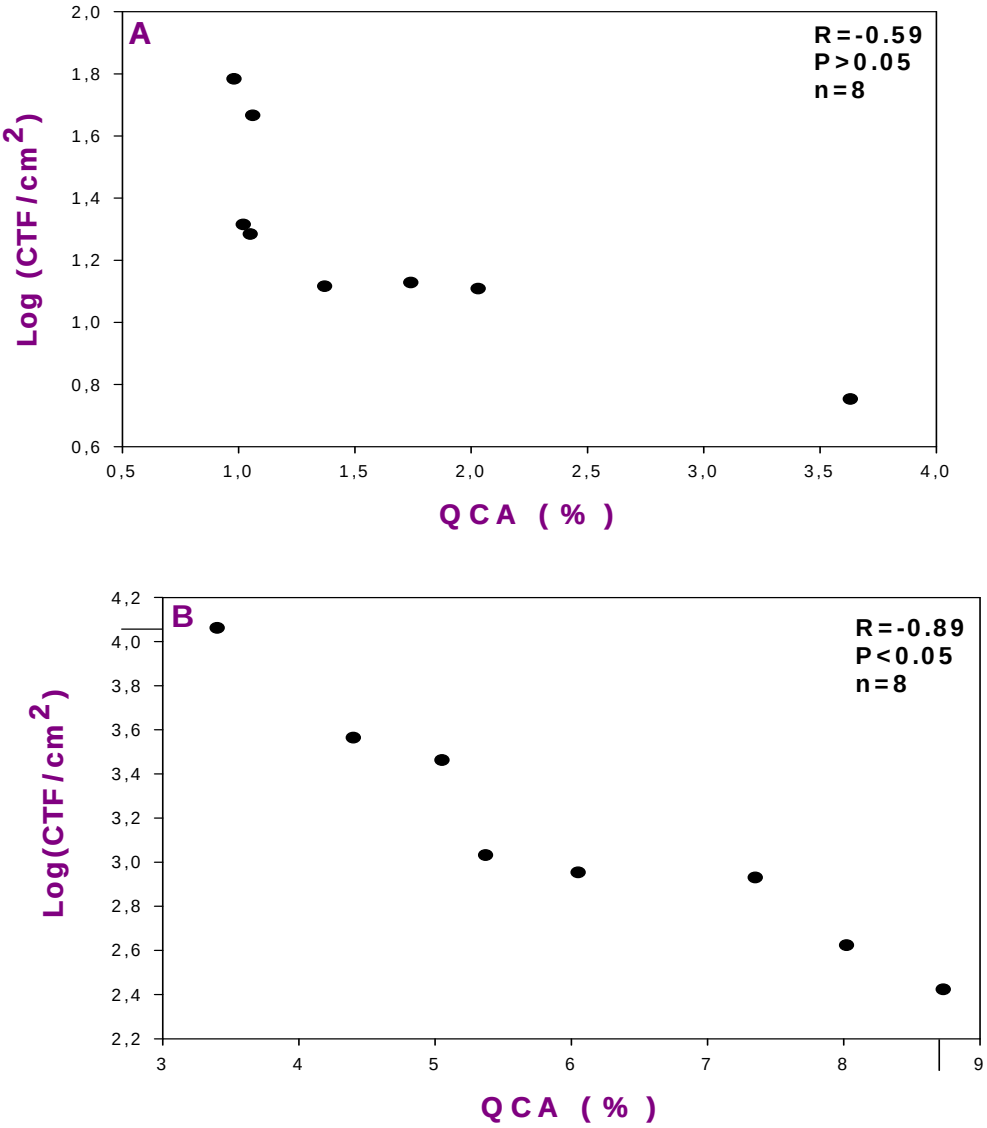

Figure 45. Relationship between expressed collagen area and counted total follicles (CTF/cm^2) in the ovarian cortex of respective healthy (A) and affected (B) animals. Multiple linear regression between the logarithm of the number of counted total follicles (primordial, primary and secondary) per cm^2 plotted against the percentage of quantified collagen area (QCA); (A) R=-0.59 (P>0.05), indicating an existence of negative correlation between CTF and the level of QCA percentage in the ovarian stroma; (B) however, the regression line delineates a significantly high negative correlation in the mastitic ovaries (r=-0.89, P<0.05, 95% CI).

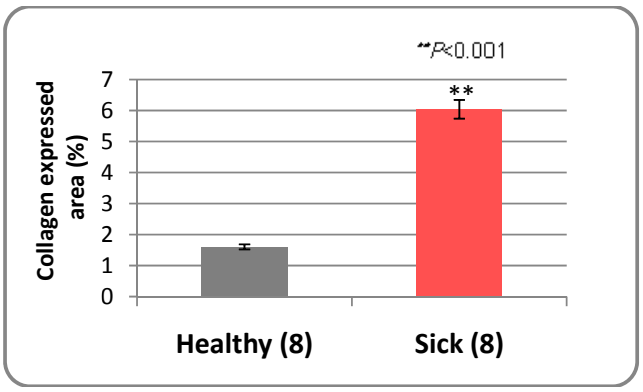

Figure 46. The graph reveals the expression pattern of connective tissue protein, collagen in the ovarian cortical stroma of healthy and mastitic animals. The percentage of mean values (± SD) of collagen expressed area (QCA) in the ovaries of healthy (dark bars) and mastitic (crimson bars) animals. $^{**}P<0.001$ (healthy versus sick), exhibiting a very high expression of this protein in affected animals (the numbers are indicated in parentheses).

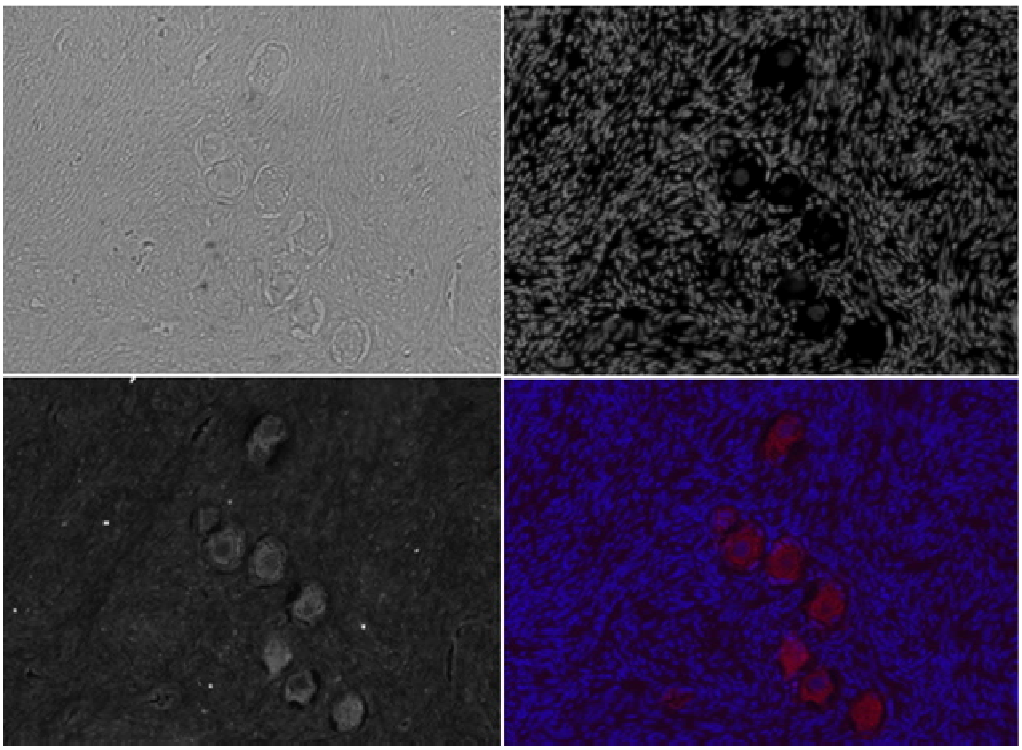

Figure 47. Localization of GDF9 in the ovaries of healthy animals; in top left: Bright field micrograph (campo chiaro); in top right: DAPI; in bottom left: GDF9 conjugated with Alexafluor 594; in bottom right: MERGE

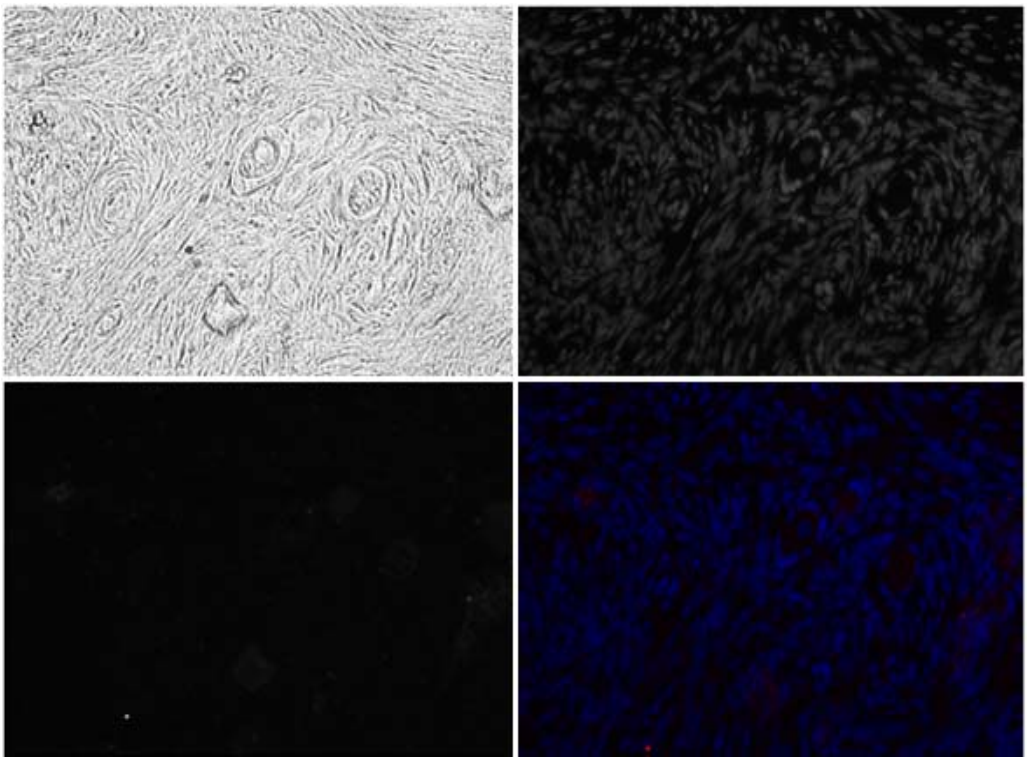

Figure 48. Localization of GDF9 in the ovaries of mastitic animals; in top left: Bright field micrograph (campo chiaro); in top right: DAPI; in bottom left: GDF9 conjugated with Alexafluor 594; in bottom right: MERGE

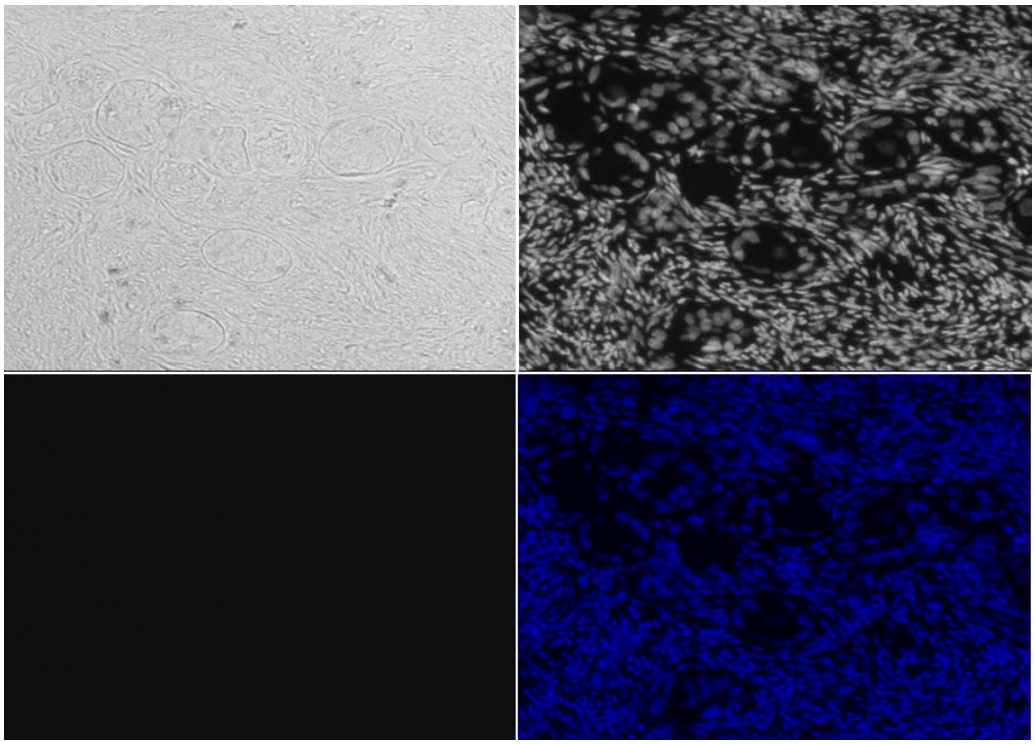

Figure 49. Negative control for GDF-9 expression in the ovary of unaffected animals. In top left: Bright field micrograph (campo chiaro); in top right: DAPI; in bottom left: control with Alexafluor 594; in bottom right: MERGE

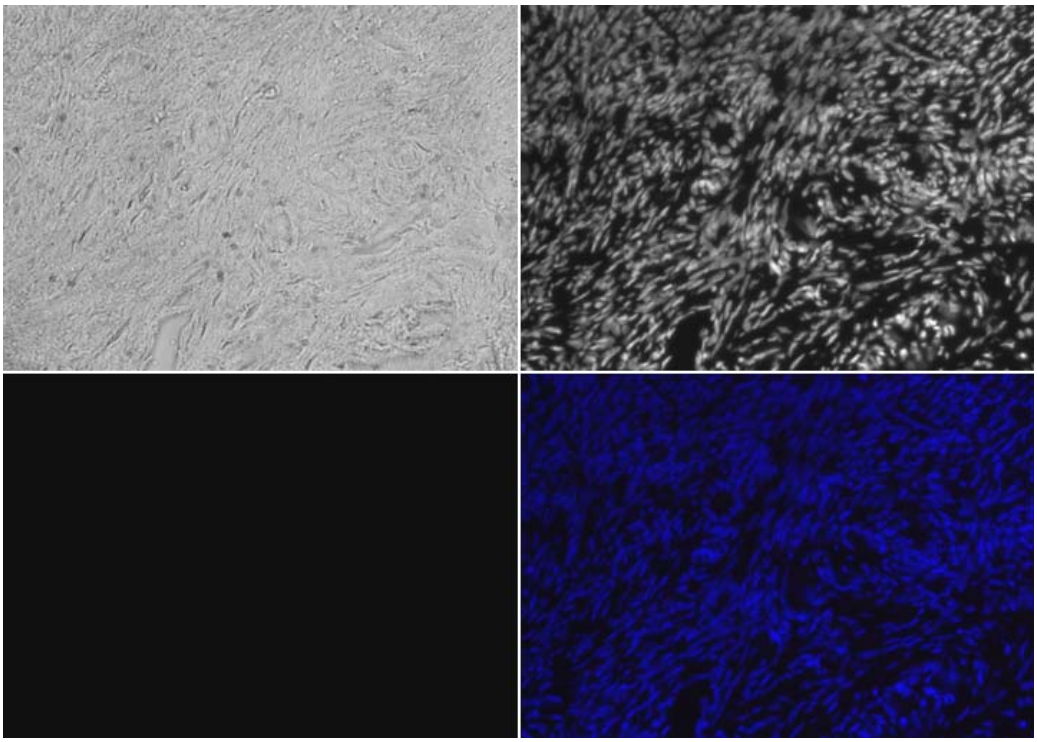

Figure 50. Negative control for GDF-9 expression in the ovary of mastitic animals. In top left: Bright field micrograph (campo chiaro); in top right: DAPI; in bottom left: control with Alexafluor 594; in bottom right: MERGE

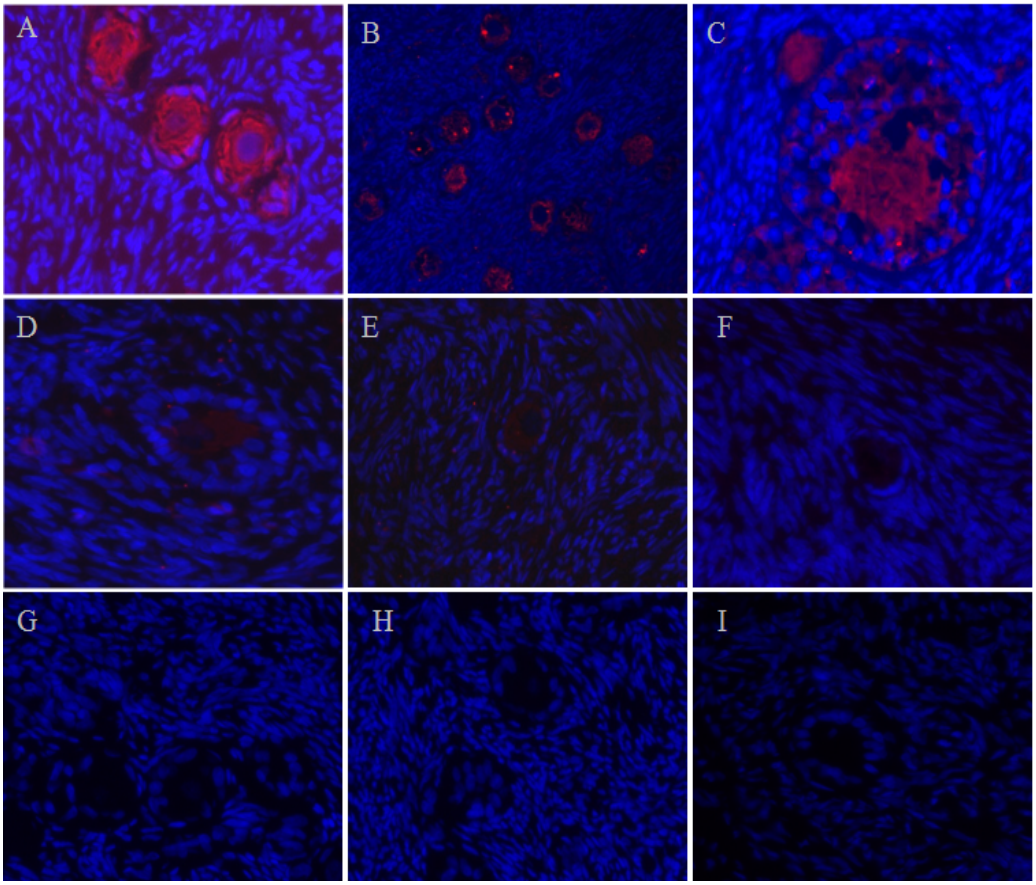

Figure 51. Immunohistochemical localization of GDF-9 in normal and affected animals. Relative intensity has been scaled into 3 panels: 0, 1 and 2 for no (G, H, I); weak (D, E, F); and strong (A, B, C) staining respectively.

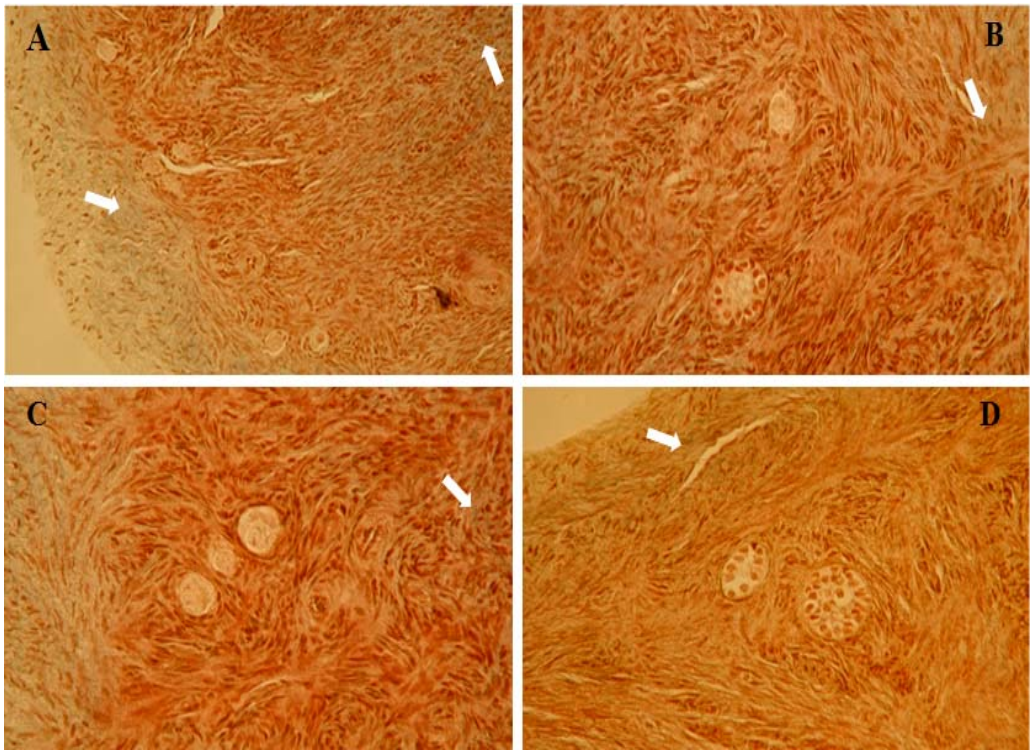

Figure 52. Characteristic healthy ovarian sections stained with special connective tissue stain, Masson trichrome: (A, D) low level expression of blue stained collagen in the tunica albuginea zone (white arrow), no apparent expression of collagen in the underlying superficial cortex, again towards deeper cortex low level expression was observed (arrow); (B, C) healthy primordial and primary follicles are observable, with faint expression of collagen in the mid cortical stroma (original magnification, 20x)

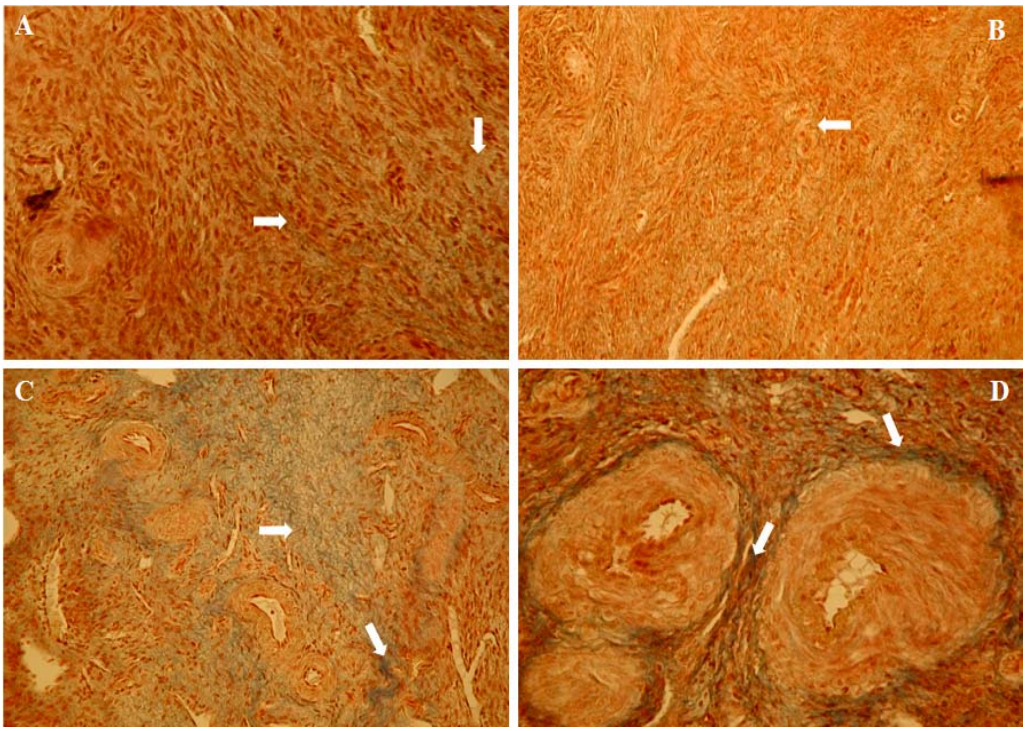

Figure 53. Characteristic healthy ovarian sections stained with special connective tissue stain, Masson trichrome: (A, B) low level of blue stained collagen expression observed in the mid cortex (white arrow); (C) moderate to higher level of collagen expression was seen in the underlying deeper cortical area, (D) deep cortex to cortico-medullary zone, displaying moderate level blue stained collagen (original magnification, 20x)

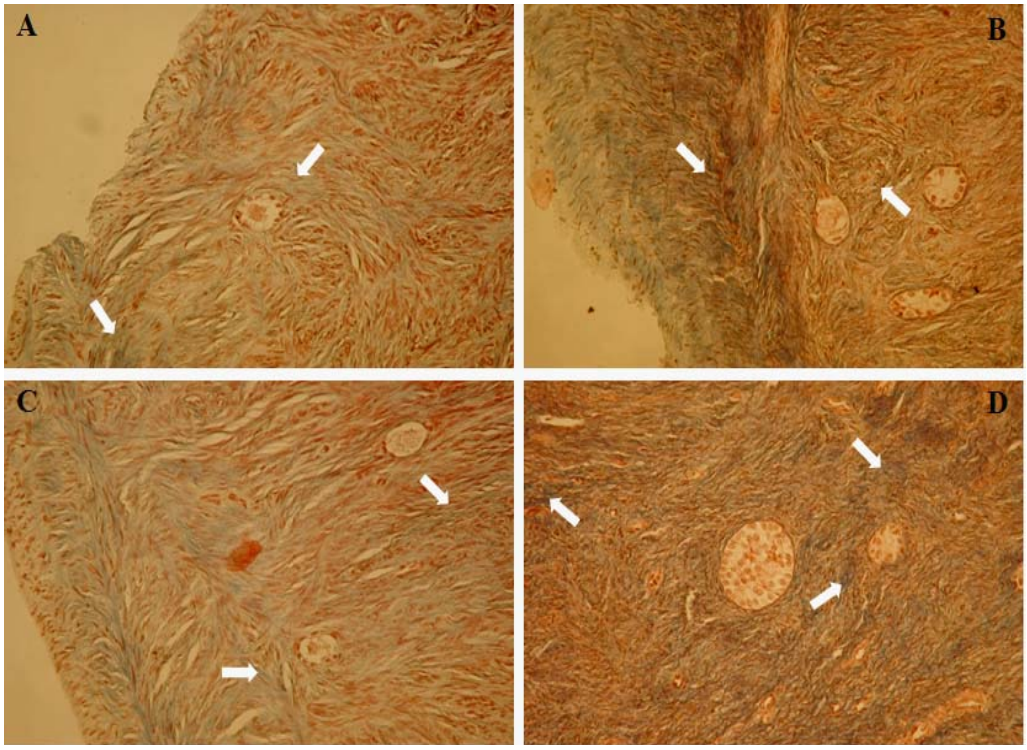

Figure 54. Mastitis related changes in the stromal connective tissues as demonstrated in the affected ovaries. Masson trichrome staining displays higher collagen firbers in the superficial (A, B, C) and mid (D) cortex. It appeared that small follicles (primordial, transitional and primary) are encompassed by blue fibers and residing in a fibrous environment (original magnification, 20x)

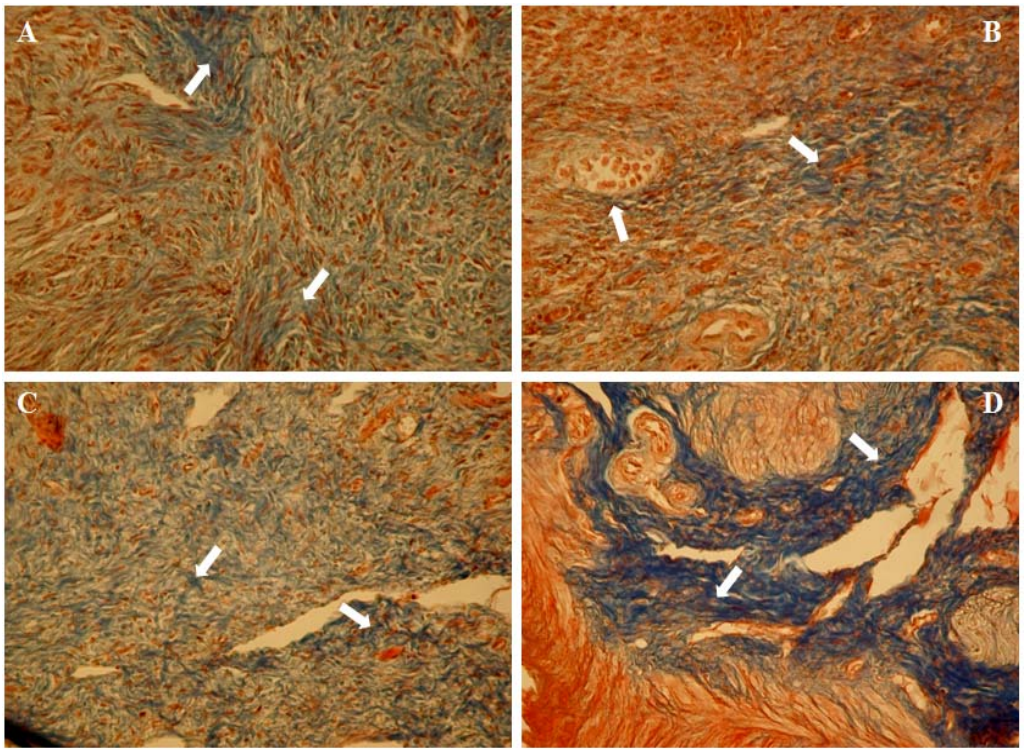

Figure 55. Masson trichrome staining highlights intensely blue staining in the cortical sections of affected animals. Higher distribution of collagen fibres are conspicous: (A) mid cortex; (B, C) deeper cortex and in (D) cortico-medullary zone, indicating mastitis related alteration in the ovarian stroma (Original magnification, 20 x).

"The man who gets the most satisfactory results is not always the man with the most brilliant single mind, but rather the man who can best coordinate the brains and talents of others."

--W. Alton Jones

05. DISCUSSION

We investigated whether mastitis has any interference on reproductive function especially at the level of bovine ovarian stroma with its cells and matrix fibers, follicular growth factors, characteristic micro-architecture of stromal vasculature, as well as growth and development of follicles by macroscopic and microscopic morphological as well as immuno-detection based molecular analysis of the ovaries collected at slaughter. Both at macroscopic and microscopic levels, we noticed existence of differences in the stromal environment and ovarian follicular growth and development between the two intensively studied healthy and mastitic animal groups. These differences were mostly striking, however, in some cases no worth-noticing alterations also appeared.

In fact, in the current study, we observed a wider extent of variations amongst various macroscopic ovarian parameters of the two thoroughly studied groups. Even within the group the degree of unlikeness in terms of normal and pathologic macroscopic appearances was evident. Although no remarkable macroscopic alterations appeared between the comparable parameters of all possible distributions of the two intensively studied groups, however the median value thresholds of the respective total macroscopic follicles (TMF) of each ovary differed significantly ($P<0.05$), indicating a randomly chosen TMF numbers from a healthy population will be higher than that of mastitic animals. Then again, in the mastitic group, presence of higher number of macroscopic medium follicles and the low number of large follicles in the ovary exhibiting mastitis linked retarded folliculogenesis probably resulted from late gain of dominance of the first-wave dominant follicle or could be related to weak dominance of the second-wave dominant follicle. But, these observations should be treated with caution because of the lack of statistical significance in the other macroscopic parameters or that its influence was masked by other factors not specified.

In addition to that, a less significant relationship ($P<0.1$) was also exists especially in the older animals (>4 yrs) with high SCC and bacteriological count (>7), where slightly diminutive ovarian volume coincides with reduced macroscopic follicles were recorded. Seemingly, this is due the fact that older animals had suffered from repeated waves of inflammation that eventually led to slight alteration of macromorphology of the ovaries. The variability among the macroscopic parameters within healthy and the mastitic cows regarding their reproductive responses being altered or depressed is unclear. Our data

however, support Burvenich *et al.,* (2003), who stated that the severity of naturally occurring cases of clinical and subclinical mastitis and the variability in responses among individuals was largely related to cow factors. Accordingly, it is not clear what cow factors caused some cows to be more susceptible to ovarian suboptimal functioning during chronic mastitis. On this limited basis, these findings, however, partially explains mastitis linked alterations in the follicular growth dynamics in the affected animals.

In concurrence with the above, current micro-morphologic analysis also indicated chronic mastitis linked suppression on the follicular growth and development especially at the level of follicular transit towards secondary stage. In most mammals, earlier stage of ovarian follicular development especially transition from primordial to primary stage, follicle somatic cells are neither endocrinologically active nor they express gonadotropin receptors and largely regulated by various intraovarian mediators, among them many are cytokines such as TGF-β, TNF-α, Interleukins and interferons (Nilson *et al.,* 2001; Morrison *et al.,* 2002; Son *et al.,* 2004; Tamura *et al.,* 2001; Hurk *et al.,* 2005; Trombly *et al.,* 2009).

It is well established that in bovine small (primary and secondary) follicles, both granulosa and theca cells contain receptors for TNFα (Sakumoto *et al.,* 2003; Son *et al.,* 2004). In fact, two immunologically distinct TNFα receptors (TNFR-I and TNFR-II) with multiple intracellular signaling pathways have now been identified (Beutler *et al.,* 1994). Through type II receptors, TNFα has been demonstrated to play a role in the regulation of ovarian cell proliferation by modulating basal or gonadotrophin induced steroidogenesis and protein production in the granulosa and theca cells in vitro (Terranova *et al.,* 1997; Korzekwa *et al.,* 2008). In contrast, through type I receptor that contains an intracellular death domain, it regulates signaling pathways associated with apoptosis (Sakumoto *et al.,* 2003). In fact, cytokines, including TNF α induces apoptosis in various cell types, including follicular cells in ovary to establish ovarian atresia in many species (Kaipa *et al.,* 1997; Amsterdam *et al.,* 1998). However, recent studies reveal naturally occurring *E. coli* mastitis (Bannerman, 2009), or exogenous LPS treatment significantly increases TNFα secretion, alters TNFα regulation and directly impair ovarian function by inhibiting ovarian cell steroidogenesis in bovine (Williams *et al.,* 2008).

In spite of the fact that primary follicles can develop to secondary stage independently of FSH availability, but FSH receptors are already present on the granulosa cells during this follicular transition (Okay *et al.,* 1997; Findlay *et al.,* 1999). It is therefore conceivable that optimal development to secondary follicles is attained only in the presence of gonadotropins (Cortvrindt *et al.,* 1997). In fact, it was reported that FSH acts as an

initiator of early folliculogenesis in the bovine fetal ovary (Lee *et al.,* 1999), as well as considerably influence earlier stages of follicular development in adult farm animals (Webb *et al.,* 2003, 2004). FSH activates adenylyl cyclase, leading to production of cAMP and activation of PKA, as well as other signaling cascades independently of cAMP: for example, pathways involving PI3K , RAS or glycogen synthase kinase 3 (Wayne *et al.,* 2007; Gonzalez *et al.,* 2000). Activation of PI3K leads to phosphorylation and activation of AKT, which in turn phosphorylates and thereby inactivates FOXO1; results in better proliferation of granulosa cells with normal folliculogenesis usually for a prolonged period of time (Fan *et al.,* 2008). As it was hypothesized that mastitis severely disrupts hypothalamic-pituitary-ovarian axis (Hansen *et al.,* 2004); in this respect, one of the possible underlying mechanisms responsible for the impediment of primary to secondary follicular transition observed in the affected cows could be microbial toxin linked deranged cytokine or immune interference on hypothalamic-pituitary-ovarian axis leading to inhibition of gonadotropin support.

This point is particularly important in light of earlier studies on mastitis, suggesting that disruptive effect of endotoxin on reproduction involved primarily by a depression of gonadotrophin secretion (Lavon *et al.,* 2008; 2010). Such an example of gonadotropin inhibitory cytokine released during mastitis is IL-6, which blocks FSH-induced estradiol secretion from bovine granulosa cells; especially cells isolated from small follicles (Alpizar *et al.,* 1994). The cytokine TGF-β, on the other hand, normally stimulate secretion of FSH from anterior pituitary cells (Ying *et al.,* 1986); however, during mastitis increased secretion or dysregulation of this cytokine could greatly modulate this interaction (Bannerman DD. 2009). Certain cytokines can directly decrease gonadotropin release, in cattle, for example, INF-α has been shown to have such an action (Barros *et al.,* 1992; McCann *et al.,* 2000). Previous investigation have demonstrated that the progressive growths of the ovarian follicles are accompanied by the development of blood vessels around them (Lunenfeld *et al.,* 1996) that blood flow velocity correlates significantly with changes in gonadotrophin levels (Tan *et al.,* 1996), and that during pituitary desensitization with GnRH agonists, ovarian blood flow is decreased (Faddy and Gosden, 1995).

Furthermore, treatment of heifers with endotoxin near estrus can lead to inhibition of the LH surge, anovulation or delayed ovulation, suppressed expression of estrous and formation of follicular cysts (Peter *et al.,* 1989; Battaglia *et al.,* 1999; Suzuki *et al.,* 2001; Lavon *et al.,* 2008). It was reported that tumor necrosis factor-α inhibited the stimulating action of FSH on LH receptor formation in cultured rat granulosa cells and had an inhibitory effect on FSH-induced cAMP production (Darbon *et al.,* 1989). Another study

(Battaglia *et al.,* 2000) reported that low doses of endotoxin were sufficient to slightly decrease GnRH release, yet LH pulses were completely inhibited in some animals. Therefore, activation of the immune system may alter GnRH release from the hypothalamus and hinder the ability of the pituitary to respond to GnRH signals (Hocket *et al.,* 2005). Accordingly, in mastitic cows, cytokine linked disruption of hypothalamic-pituitary-ovarian axis and a subsequent change in gonadotrophin level weaken stimulatory follicle-GnRH signal transduction that could eventually weaken normal folliculogenesis.

The previous study reveals, oestrogens are pro-angiogenic in vivo and vasculogenesis around follicles are dependent on the concentration of estradiol released around the vessels (Lee *et al.,* 1999). In fact, this steroid modulates angiogenesis, under both physiological and pathological conditions, and was demonstrated to augment angiogenesis induced by FGF-2 (Hyder and Stancel, 1999). Disruption of the oestrogen receptor gene prevents 17-beta-estradiol-induced angiogenesis in transgenic mice (Johns *et al.,* 1996). On the other hand, any disruption of steroidogenesis could generate estrogen metabolite 2-methoxyestradiol, which is known to be a potent anti-angiogenesis factor. It induces apoptosis in endothelial cells and inhibits angiogenesis by modulating stress-activated protein kinase signaling pathway (Yue *et al.,* 1997). Cytokines especially interleukin-6 and TNF-α severely modulates this pro-angiogenic action of estradiol (Purohit *et al.,* 2002) by interfering ovarian steroidogenesis in cow (Chebel, 2007).

On the other hand, cytokine, IL-6 alone has been shown to stimulate COC expansion and induce the expression of specific genes encoding proteins involved in this process (Liu *et al.,* 2009). These observations indicate that in clinical situations where levels of IL-6 are elevated, such as chronic mastitis (Bannerman, 2009), this and other cytokines may disrupt normal granulosa cell and cumulus cell functions (Richards *et al.,* 2010).

Cytokines such as interleukins and TNF-α are key mediators associated with effects of gram-negative and gram-positive bacteria during bovine mastitis (Nathan, 1987; Zerbe *et al.,* 2001). A possible direct effect of mastitis on attenuation of estradiol secretion is supported by the other finding that TNF-α and IL-1β inhibit steroidogenesis (Sakumoto *et al.,* 2003). Addition to TNF-α to bovine granulosa or theca cell cultures lowered steroid production (Spicer, 1998). In fact, TNF-α linked direct deleterious action on oocyte maturation with compromised ability of resultant embryo to develop to the blastocyst stage was reported (Soto *et al.,* 2003). Similarly mastitis associated hyperthermic stress could result depression of steroidogenesis in bovine ovary (Oliver *et al.,* 2000; Roth *et al.,* 2001). Likewise, secretion of LH can also be blocked by cortisol (Stobel *et al.,* 1982; Li *et*

al., 1983; Padmanabhan *et al.,* 1983), a hormone whose secretion can be elevated during mastitis (Hockett *et al.,* 2000) or after endotoxin treatment, resulting prolonged follicular phase (Peter *et al.,* 1989; Kujjo *et al.,* 1995; Suzuki *et al.,* 2001; Lavon *et al.,* 2008). In fact, studies revealed the GN (endotoxin) mastitis had a more severe effect on ovarian activity compared to GP mastitis (Hocket *et al.,* 2005).

Studies revealed few molecules, which are either synthesized by bacteria (LPS) or produced in response to inflammatory cytokines, may mediate some of the deleterious effects of mastitis on ovary (Schrick *et al.,* 2001). Lipopolysaccharide (LPS) can bind granulosa cells recovered from human follicular aspirates (Sancho-Tello *et al.,* 1992), as well as decreases estradiol production from GC of small follicles (Williams *et al.,* 2008). In vivo, systemic immune challenge with LPS results increased apoptosis in rat granulosa cells (Besnard *et al.,* 2001). Perhaps LPS influence oocyte maturation indirectly by altering secretion of one or more cytokines from the COC. NO synthesis is increased by LPS, TNF-α and INF-γ in mouse (Athanassakis *et al.,* 2000), and by $PGF_{2\alpha}$ in rat, which was reported to have a deleterious effect on reproductive processes (Peres-Martinez *et al.,* 1998). Indeed, LPS can increase TNF-α secretion from human granulosa cells and subsequently modulate granulosa cell multiplication (Sancho-Tello *et al.,* 1992), as well as reduces theca cell androstenedione production (Williams *et al.,* 2008). Actions of LPS at extra-ovarian sites could also lead to the production of cytokines or other regulatory molecules that could in turn inhibit follicular development (Soto *et al.,* 2003).

Conformity with the above, other studies also revealed that one of the key molecules released during mastitis that may be involved in disruption of follicular development is $PGF_{2\alpha}$. When $PGF_{2\alpha}$ was added at concentrations of 50 or 100 ng/mL it compromised in vitro oocyte maturation (Soto *et al.,* 2003). Inflammatory cytokines can also increase $PGF_{2\alpha}$ secretion, including IL-1 in granulosa cells (Narko *et al.,* 1997) and by doing so interleukin 1β can greatly modulate bovine granulosa cell proliferation in vitro (Basini *et al.,* 1998). It has been demonstrated that TNF-α also stimulates the $PGF_{2\alpha}$ output of the bovine endometrium or from sensitive CL both in the follicular and luteal phase and disrupts reproductive cycle (Okuda *et al.,* 2002; Skarzynski *et al.,* 2005). Increased release of cytokines including TNF-α, can be expected both in GN and GP mastitis (Salyers *et al.,* 1994; Sandholm *et al.,* 1995; Sordillo *et al.,* 1995); in this respect, TNF-α stimulated release of other inflammatory mediators could destabilize normal ovarian folliculogenesis.

Present study strengthens the above arguments of mastitis associated reproductive disruption since decreased vascular area was observed in the ovarian cortical stroma of

the affected animals. This is the first description of the changes in the density of stromal vasculature in bovine ovary affected with chronic mastitis. Worthy to note that, in our study it revealed that outer ovarian cortex constitutes the poorest vascularized zone of the bovine ovary. Previous studies in human (Suzuki *et al.,* 1998; Gaytàn *et al.,* 1999; Wulff *et al.,* 2001), and primate (Fraser and Wulff, 2003; Fraser, 2006) ovaries have reported that the highest vascular density corresponds to the corpus luteum and, to a lesser extent, to large antral follicles, whereas the ovarian stroma (mostly corresponding to deeper cortex) shows a comparatively lower vascularization (Suzuki *et al.,* 1998). We report herein that in healthy animals a significant gradient of stromal vascularization also exists in the mid zone between the deeper and outer cortex, with the latter showing small and scarce blood vessels. This agrees with a previous study on the vascularization of the bovine (Herrmann and Spanel-Borowski, 1998) and human ovary (Delgado-Rosas *et al.,* 2009). In healthy ovaries this characteristic micro-architecture of the ovarian vasculature should have a physiological significance (Delgado-Rosas *et al.,* 2009). A possibility is that such a poor vascularization of the outer stroma is related to the maintenance of the resting stage of primordial follicles (Herrmann and Spanel-Borowski, 1998), and to the very low growth rate of primary and early secondary follicles (ESFs) (Gougeon,1996).

In the present work, compared to healthy animals, lower vessel density was observed in the whole cortical stroma (outer, mid and deeper) of the ovaries of affected animal groups. The diminished vessel area may indicate that mastitis generated long-standing exposure to potential microbial toxins and immune activation could modify tissue homeostasis leading to structural and functional changes in the cortical stroma and subsequent reduction in the capillaries. Although the existence of bidirectional regulatory loops between the oocyte and granulosa cells is clearly established (Albertini *et al.,* 2001; Eppig, 2001), yet, possible factors mediating communication between the oocyte/primary follicles and the surrounding cortical stroma are largely unknown. Ample microvascular bed however, a key for potential follicle-stroma regulatory loops. It has been shown that in the adult ovary, folliculogenesis; especially around the secondary stage, the angiogenesis and vasodilatation around follicles runs synchronously (Lee *et al.,* 1999). Growing follicles are a source of angiogenic factors involved in the theca layer vascularization; in addition, blood vessels in the theca layer may persist in the stroma after follicle atresia (Jiang *et al.,* 2003), indicating existence of a further complexity in the regulatory loop between folliculogenesis and stromal angiogenesis. In view of the fact that, well developed vascular network is necessary for follicular growth (Jiang *et al.,* 2003), thus compromised vascular area and reduced number of secondary follicles found

in the ovaries of mastitic animals conforms the fact that mastitis weakened follicular development.

Injury related impairment of angiogenesis seems to be a general rule in most tissues and early vascular alterations consisting of hyalinization, mild to moderate intimal fibrosis and thickening of the muscular wall, subsequently severe narrowing and obliteration of the vascular lumen (Meirow *et al.,* 2007). In our study, stromal vascularization was visualized by lectin that immuno-labeled against endothelium. Decreased vessel area in the mastitic stroma observed in this work was possibly due to narrowing or obliteration of the vessel lumen eventually less Lectin immuno-labeling to the endothelium. Increased concentrations of angiogenic factors may affect follicle dynamics by different mechanisms. Indeed, endothelium secreted angiogenic factor VEGF may play a pivotal role in follicle survival and early growth either directly (Yang and Fortune, 2007) or indirectly by increasing angiogenesis and/or vascular permeability (Geva and Jaffe, 2000), or through effects on AMH expression (Thomas *et al.,* 2007). Thus endothelial damage could severely destabilize VEGF secretion and consequently brings a dystrophic effect on folliculogenesis. In fact, it has been shown that, in spite of the lack of independent vascularization, direct contacts between primary/early growing follicles and cortical capillaries were frequently observed, reflecting the inevitability of active vascular link on folliculogenesis (Delgado-Rosas *et al.,* 2009).

Although our knowledge over the mechanisms involved in the depressing effects of mastitis on the ovary is partial and insufficient, but a few studies have examined the cytokine interaction on vascularization. It has been observed that the process of angiogenesis is modulated by various cytokines and quite a number of other factors including transforming growth factor β, tumor necrosis factor α, hyaluronic acid fragments, fibroblast growth factor, interleukin 8, vascular endothelial growth factor, haptoglobin, estrogen metabolites, steroids and collagen synthesis inhibitors (Cockerill *et al.,* 1995). Recent studies, however, imply that most of these angiogenesis modulators are significantly altered during bovine mastitis (Bannerman, 2009), as well as the existence of possible association between inflammatory cytokines and immunological challenge upon the vessel wall has been established (Pay *et al*., 2000). Morphological changes have been shown to occur in cytokine treated endothelial cells. TNF-α, for instance, injure endothelial cells (Bernard *et al.,*1992), and key cytokines currently implicated in the endothelial injury and fibrotic processes are transforming growth factor-β and tumor necrosis factor-α (Coker *et al.,* 1998), and is worth mentioning that TGF-β is a known folliculogenesis mediator too (Trombly *et al.,* 2009). It has been shown that, potentiated by IFN, TNF-α induced vessel apoptosis acting mainly on microvascular

endothelial cells in bovine (Korzekwa *et al.,* 2008). In this respect, microbial toxin or inflammatory cytokine linked endothelium degeneration and subsequent shrinkage or obliteration of stromal microvasculature could results in diminished blood flow, consequently generate a depressive effect on the follicular environment in the ovarian stroma.

Regardless of the mechanisms underlying decreased vascularization in the ovarian stroma of the mastitic animals, a richer microvascular bed is indispensable for the delivery of regulatory signals to resting and early growing follicles, thus affecting their functional status. The activation of primordial follicles and the early stages of follicle growth seem to be dependent on as yet not well characterized local stimulatory and inhibitory factors (Fortune, 2003). Endothelial cell degeneration and subsequent fibrosis with reduction of active microvasculature could severely impede these local stimulatory factors. Since the blood supply to the ovary is an end artery system (Clement, 1997), reduction of the microvascular bed will give rise to poor deliverance or collapse of blood supply to the respective ovarian stroma, consequential low oxygen tension that stimulates COL1A1 transcription through the action of TGF-beta1 as well as collagen synthesis in the ovary leading to compromised stroma along with sustained depressive effect on folliculogensis (Falanga *et al.,* 2002; Meirow *et al.,* 2007).

In the present study, Masson trichrome staining showed lower to moderate degree of fibrosis as evidenced by the proliferation of the collagen fibers in the ovarian stroma of the affected animal groups. Since, the connective tissue growth factor genes have the spatiotemporal expression pattern during folliculogenesis and corpus luetum formation in the ovary (Harlow *et al.,* 2002); in this respect the proliferation of the collagen fibers in the ovarian stroma has great implication on the follicular growth and development in the mastitic animals. The variety of inflammatory cytokines potentially interacts with fibroblast and possibly involved in fibrotic alteration of the ovarian stroma. Previous study reported that during chronic stimulation, fibroblast interacts with a range of cells, including neutrophils, macrophages, T-cells, eosinophils and mast cells with an extremely intricate and redundant manner (Atamas *et al.,* 2002). Members of the growth factors, particularly transforming growth factor-β (TGF-β), are believed to be the most potent profibrotic cytokines. At the same time they regulate a host of function in most cells, including embryonic development of ovary, folliculogenesis, immune response, healing of injured tissue and chemotaxis (Raines *et al.,* 2001; Flanders *et al.,* 2001; Trombly *et al.,* 2009). Produced by a variety of cells, including those typically involved in inflammatory processes, such as macrophages, lymphocytes, endothelial cells, platelets, and

fibroblasts themselves, these cytokines are powerful activators of production of collagen and other extracellular matrix components (Atamas, 2002).

In fact, a key feature of persistent inflammation is collagen production. On the other hand, along with inflammatory stimulation, tissue fibrosis driven by TGF- β may be a result of abnormally activated ‘‘aberrant repair process’’ (Khalil *et al.,* 1991; Gauldie *et al.,* 2002). Members of this group are functionally associated and may mediate each other’s action in an autocrine fashion. For example, connective tissue growth factor (CTGF), an autocrine factor, is associated with TGF- β, expression of which in fibroblasts is always linked with fibrosis (Shi-wen *et al.,* 2000). Production of cytokine, CTGF from fibroblasts is upregulated by TGF-β (Igarashi *et al.,* 1993), an effect that can be attenuated by interleukin (IL)-4 (Rishikof *et al.,* 2002). In fibroblasts, CTGF is an active mitogen and stimulant of collagen and fibronectin production (Frazier *et al.,* 1996). Importantly, CTGF may be a key mediator of critical TGF-β effects, a conclusion drawn from the fact that transfection with antisense CTGF gene blocks TGF-β effects on fibroblasts and so does anti-CTGF antibody (Duncan *et al.,* 1999; Yokoi *et al.,* 2001). Surprisingly, IL-7 inhibits fibroblast secreted cytokine TGF- β production during fibrotic signaling (Huang *et al.,* 2002).

Previous study reported that several members of the cytokine family such as IL-1, IL-4, IL-6, TNF-α and others exert activities on fibroblasts (Atamas, 2002). Fibroblasts themselves, along with other cell types, e.g. monocytes/macrophages, are a major source of these proinflammatory and autocrine cytokines. There is significant similarity between the effects of IL-1 and tumor necrosis factor (TNF)-α on fibroblasts (Mauviel *et al.,* 1991). IL-1, particularly IL-1β, a ubiquitous and pleiotropic cytokine, enhances fibroblast proliferation (Postlethwaite *et al.,* 1984). Similarly, TNF, a primary immune and inflammatory regulator, stimulates fibroblast chemotaxis and proliferation (Postlethwaite *et al.,* 1990). Adenovirus-mediated delivery of TNF-α to lungs, revealed severe pulmonary inflammation followed by accumulation of extracellular matrix, which was mediated by secondary up regulation of TGF-β (Sime *et al.,* 1998). Previous report indicated a low oxygen tension stimulated collagen synthesis along with COL1A1 transcription through the action of TGF-beta1 (Falanga *et al.,* 2002). It was also reported that other cytokines, such as IL-6 and IL-11 stimulate production of growth factors for mast cells by fibroblasts, and mast cells in turn can be stimulatory for fibroblasts (Garbuzenko *et al.,* 2002; Gyotoku *et al.,* 2001).

Several other studies reported that interleukins of both types have polar effects on fibroblasts (Gillery *et al.,* 1992; Yuan *et al.,* 1999; Yamamoto *et al.,* 2000). Generally,

IFN-γ is considered a prototypic type 1 and IL-4 - a prototypic type 2 interleukin. INF-γ is produced almost exclusively by activated NK cells and type 1 T cells, whereas IL-4 is produced not only by type 2 T cells, but also by mast cells, basophils and eosinophils (Atamas, 2002). IL-4 stimulates fibroblast proliferation and production of collagen, chemotaxis, expression of adhesion molecules ICAM-1 and VCAM-1, IL-6, and many other molecules (Feghali *et al.,* 1992; Monroe *et al.,* 1988; Piela-Smith *et al.,* 1992; Postlethwaite *et al.,* 1992; Postlethwaite *et al.,* 1991; Spoelstra *et al.,* 1999). Treatment with anti-IL-4, as well as IL-4 receptor (-/-) mutation prevents collagen deposition in mouse tissue (Ong *et al.,* 1998; McGaha *et al.,* 2001). IL-13 shares the receptor and most biological activities with IL-4, including profibrotic action (Doucet *et al.,* 1998; Murata *et al.,* 1998; Oriente *et al.,* 2000). Because of such powerful profibrotic potential of IL-4, one of its sources, mast cells, have been implicated as a contributor to tissue healing and development of fibrosis (Trautmann *et al.,* 2000; Garbuzenko *et al.,* 2002). Moreover, rapidly expanding universe of proinflammatory chemokines also holds several factors, which are directly active on fibroblast, causing upregulated production of collagen, probably through interaction with autocrine TGF-β and IL-1α (Gharaee-Kermani *et al.,* 1996; Yamamoto *et al.,* 2000).

On the other hand, it has become increasingly evident that members of the TGFβ family play integral roles in the regulation of growth and development of germ and somatic cells during folliculogenesis (Spicer *et al.,* 2008; Orisaka *et al.,* 2009). Our study has highlighted the importance of this factor, in particular GDF-9 in the progression of follicular development in both healthy and mastitic ovaries. Based on the immunohistochemical analysis of ovarian tissue sections using bovine GDF-9 specific antibody we report here that GDF-9 respective proteins are expressed in oocytes during early folliculogensis. It was reported that this factor expressed in the oocyte as a stage dependent manner (McGrath *et al.,* 1995; Elvin *et al.,* 1999; Su *et al.,* 2008). In agreement with previous studies, we also detected this factor in various stages of follicular development, with the most intense expression in primary to preantral follicles. We however, observed a negative correlation between the level of GDF-9 expression and intensity of mastitis as it was evidenced by a decrease in GDF expressed area as well as less fluorescent intensity in the affected oocytes. In the present work, folliculogenesis beyond primary stage seemed affected in the mastitc ovaries. It thus seems reasonable to assume that mastitis linked downregulation of GDF-9 was one the key modulators of this alteration.

In fact, the essential role of this oocyte derived factor was underscored by findings in GDF-null mice in which the theca cells failed to form, GC failed to multiplicate and the

follicles did not develop beyond the primary stage (Elvin *et al.,* 1999). Combined with studies showing that GDF9 treatment causes further development of primary follicles in hamsters (Wang *et al.,* 2004); mice (Vitt *et al.,* 2000), and rats (Orisaka *et al.,* 2006; Nilsson *et al.,* 2002), one can conclude that GDF9 stimulates early follicular growth, GC proliferation, theca cell recruitment, and theca cell proliferation in bovine. It was also reported that in bovine, GDF9 stimulates theca cell formation around early secondary follicles (Spicer *et al.,* 2008); as well as augments androgen production by increasing CYP17A1 mRNA expression in theca cells (Orisaka *et al.,* 2009). This androgen enhances the FSH action in the follicles by increasing FSH receptor expression, FSH-induced granulosa cell aromatase activity and proliferation, and follicular growth (Weil *et al.,* 1999). In addition, previous study on cultured small follicles revealed comparatively less apoptosis in the absence of FSH but more without GDF-9, suggesting that this oocyte-derived factor plays a significant role in early folliculogenesis (Orisaka *et al.,* 2006). In this respect, down regulation of GDF9 could compromise androgen stimulated folliculogensis in mastitic ovaries.

In fact, the TGFβ family ligands initiate signaling by assembling type I and type II serine/threonine kinase receptor complexes that activate two main SMAD pathways (Chang *et al.,* 2002; Mazerbourg *et al.,* 2006; Jayawardana *et al.,* 2006). Studies using granulosa cells indicated that GDF9 interacts with BMPR2 (type II receptors), followed by the specific activation of the type I receptor, ALK5, and subsequent phosphorylation of the downstream SMAD2/3 proteins characterized by the stimulation of the CAGA promoter (Mazerbourg *et al.,* 2004; Sudo *et al.,* 2004), leading to activation of granulosa cell survival and proliferative PI3K/Akt pathway (Franke *et al.,* 2003; Downward, 2004; Thompson *et al.,* 2004; Tilly *et al.,* 2004). The GDF9 activated signaling also increases cell numbers as well as DNA synthesis in the theca cells of small follicles (Spicer *et al.,* 2008). Gonadotrophins and a number of growth factors are known to activate this PI3K/Akt pathway to prevent arrest or apoptosis of granulosa cells (Asselin *et al.,* 2001; Wang *et al.,* 2003; Hu *et al.,* 2004), further strengthening the argument that mastitis linked possible cytokine disruption of hypothalamic-pituitary axis could compromise granulosa and theca cell proliferative pathway and put an inhibitory effect on the progression of bovine folliculogenesis.

In the present study, we could not however, relate the disruptive effects of naturally occurring chronic mastitis on ovarian follicular growth and development in individual cows to the following factors: body condition score, the stage of lactation, state of ovarian cycle and other metabolic and hormonal parameters.

In brief, our results reveal mastitis associated impaired folliculogenesis, which is also in tune with the hypothesis that mastitis compromises reproduction through increased secretion of cytokines (either produced in the mammary and draining lymph nodes and secreted or produced in other tissues in response to mammary-derived signals) that in turn modulate reproductive function at several levels (Hansen *et al.,* 2004). In fact, in our observation, the processes of mastitis induced impaired folliculogenesis don't seem to act directly on follicles rather cytokine linked disruption of hypothalamic-pituitary-ovarian axis with insufficient hormonal milieu, injury to the blood vessels, resulting fibrotic diminution of active microvascular bed, consequential low oxygen tension stimulated slow collagenous shift of the stroma with compromised follicle-stroma-growth factors signaling are the key patterns of mastitis linked intervention on the ovary. Better knowledge of the pathways by which mastitis brings pathological changes in the stroma might help in the development of strategies for ovarian protection from this detrimental disease as well as for providing better fertility in cow.

"I was like a boy playing on the sea-shore, and diverting myself now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me." — ***Isaac Newton***

06. CONCLUSIONS

In this work, we tried to correlate the status of mammary gland infection with macroscopic and microscopic morphology as well as immuno-detection based molecular analysis of the ovaries collected at local abattoir. The choice of working with materials collected during the slaughter, forced by economic considerations and logistics, has proved efficient; and we detected mastitis related specific ovarian alterations. Although this alterations seemed smaller on macroscopic level, however, at microscopic level specific effects of mastitis on ovary was considerably evident.

In particular, we addressed chronic mastitis related diminution of active microvascular bed in the ovarian stroma. Lower to moderate degree of fibrosis as evidenced by the proliferation of the collagen fibers has also been detected in the entire cortico-medullary stroma of the affected ovaries. This was accompanied by considerable alterations in the follicle growth and differentiation with a decreased ability of primary follicles to develop into secondary state as evidenced by reduced number of secondary follicles as well as the down regulation of the follicle differentiation associated factor GDF in affected animals.

The phase of follicular growth affected by chronic mastitis is independent of gonadotropins, but is under the control of various endocrine and intraovarian factors such as INF, TNF-α, TGF-β, VEGF and various IL's (Van den Hurk, Jia Zhao, 2005; Yang & Fortune, 2007; Trombly *et al.,* 2009). Therefore, it is important to note that, these factors are considerably altered by inflammatory processes in general and mastitis in particular (Slebodzinski *et al.,* 2002; Blum *et al.,* 2000; Nakajima *et al.,* 1997, Bannerman, 2009). Therefore, mastitis induced injury to the stromal microvascular bed and subsequent reduction of the active vascular area as well as moderate degree of collagen proliferation could be explained by the action of the same toxin-cytokine interlinked framework.

Our findings can explain, in part, the disruptive effects of mastitis on dairy cows fertility; however, further studies are warranted in order to shed more light on the interaction hypothesized above.

07. BIBLIOGRAPHY

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If you want to go far, go together"
African Proverb

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