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TESI DI DOTTORATO DI RICERCA

Influence of distance from calving on the transcriptional activity of granulosa cells from preovulatory follicles of dairy cows

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Abstract

Negative energy balance (NEB) in dairy cattle early lactation period is associated with a multitude of endocrine, metabolic and immunological changes that not only influence animal health, but also affect fertility, and in particular ovarian function. There is a lack of information on the the transcriptional activity of granulosa cells during the first month after calving, when dairy cow pass from a severe NEB condition to a correct methabolic omeostasis. In this research, GCs of preovulatory follicles have been collected at 30 (30 d), 60 (60 d), 90 (90 d) and 120 days (120 d) after calving from 12 Holstein Freisian cows. To map the differences in genes expression and cellular functions that occur in the follicular microenvironment during the progressive recovery from NEB condition in dairy cow an analysis of the transcriptome was performed using a global bovine oligo array microarray. Considering that after 4 months from parturition the dairy cows have recovered from the NEB condition, the GC samples collected at 120 d were used as a positive control in microarray analysis. The results obtained allowed the identification of a list of differentially expressed transcripts for each GC group contrast: 30 d vs 120 d, 60 d vs 120 d and 90 d vs 120 d. To provide a comprehensive understanding on the interferences of lacatation on the the processes involved in the maturation of ovarian dominant follicle, different gene pathways and molecular and cellular function by Ingenuity Pathways Analysis (IPA) software were used to reveal the different roles of transcripts. The comparison between 30 d and 120 d groups evidenced up and down regulations differences of transcripts in small molecule biochemistry, DNA replication, recombination and repair, and cellular assembly and organization. The contrast analysis between 90 d and 120 d group revealed modifications in up and down regulations genes activities linked to cell cycle progression, cell proliferation and cell interaction, which are indicative of cells preparing for ovulation. The granulosa cells of 60 d group revealed a significant increase in up and down regulations of genes associated with apoptosis, ovarian cancer and a slow cell follicular development compared to the 120 d group; differences which suggest activaton of apoptotic chain typical of cell in suffering conditions more than those of a normal preovulatory follicular stage. Overall the results and findings of the current study lead to the conclusion that microarray analysis is a useful and valid method for the study of the different gene expression profiles in granulosa cells, from collected

preovulatory follicles in dairy cow at different distance from calving. These results offer the opportunity to future studies aimed to the understanding of which molecular mechanisms or external factors negatively influence ovarian development during the time interval between the 30 d and 60 d postpartum period in dairy cow.

Contents

1 Introduction

The ovaries are complex endocrine organs developed from the bipotential gonad (Gillman, 1948). Under the stimulatory actions of the gonadotropins, they are responsible for the production of the sex steroids and are the source of fertilizable ova. The ovary is composed of three distinct regions: an outer cortex containing the germinal epithelium and the follicles, a central medulla consisting of stroma and blood vassels, and a hilum around the area of attachment of the ovary to the mesovarium (Carr, 1998). The steroidogenic cells of the ovary are the granulose cells, which are organized in an avascular cellular compartment surrounding the oocyte, and the theca cells, which reside in the ovarian stroma. These two cellular compartments are separated by the basal lamina (Weakly, 1966).

1.1Bovine ovarian follicle

The adult ovary contains a reserve of inactive primordial follicles. Each contains a small non-growing oocyte and a layer of non-dividing pregranulosa cells encapsulated by the follicular basal lamina. Every day a number of primordial follicles become active, and the oocyte commences growing while the granulosa cells begin to divide. As the granulosa cells divide, the number of layers of cells (called the membrana granulosa or follicular epithelium) around the oocyte increases, and the follicular basal lamina expands. Later in development, a fluid-filled cavity or antrum forms and specialized stromal layers, the theca interna and externa, develop. Only follicles that reach the stage of having a large antrum, and are present in the follicular wave following regression of corpora lutea, can ovulate an oocyte in response to the surge release of LH. Following ovulation, the granulosa cells and thecal cells differentiate into the large and small luteal cells of the corpus luteum, and the vascular supply of the corpus luteum is derived from the capillaries of the theca interna. All non-ovulating follicles undergo atresia and regression. Since the development and regression of follicles are associated with major structural and functional changes, it is important to classify

follicles accurately as healthy or atretic at all stages of development and to subclassify them further into the different isoforms that exist at most stages.

1.2 Follicular development

Folliculogenesis is the process of follicle growth and development, starting with recruitment of resting follicles and ending with the ovulation of dominant follicle(s) (Figure 1). There are approximately 400,000 resting follicles (depending on the species) in the ovaries at the onset of puberty (Baker TG, 1963).

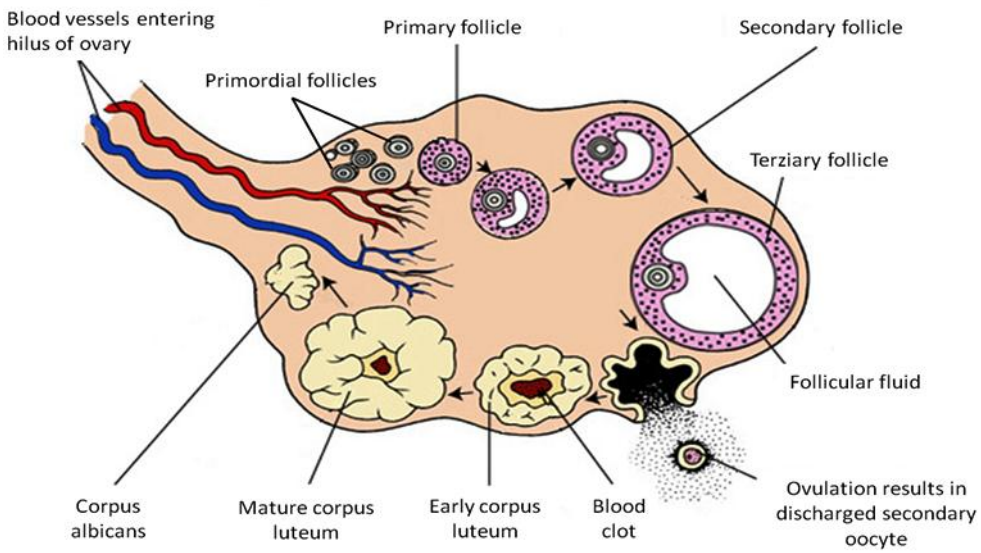


Figure 1. A figure of an ovary showing the different development stages of a follicle

1.2.1 Primordial follicles

Primordial follicles in most species are identified histologically on the basis of a small non-growing oocyte arrested in diplotene stage of meiosis I, without a zona pellucida and surrounded by flattened granulosa cells (Figure 2; Fair et al., 1997).

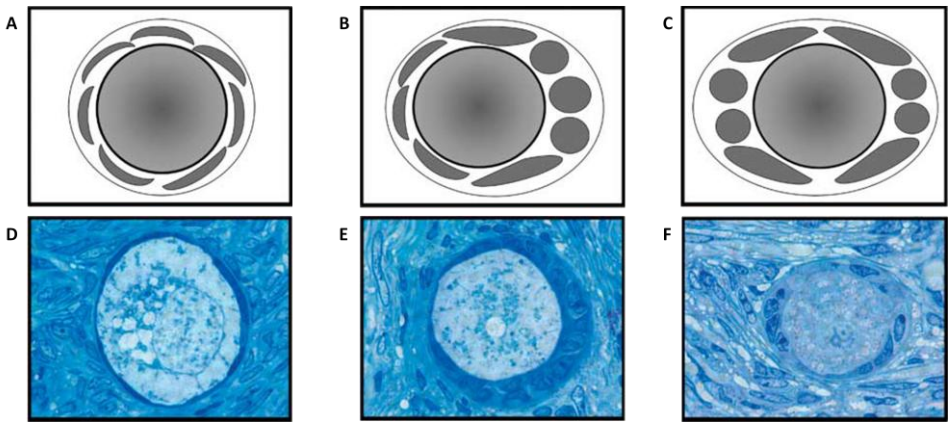


Figure 2. Caartoons and light micrographs (methylene blue-stained section of plastic embedded tissues) of human (D and E) and bovine (F) follicles illustrating the classical primordial follicle shape (A and D), the human transitional shape (B and E) and the common ellipsoid shape of bovine follicles (C and F).

The follicles appear this way because the oocyte is not enlarging and the granulosa cells are not replicating. Once activated, the primary follicles have an enlarging oocyte around which the zona pellucida will form, and a layer of granulosa cells that have become cuboidal in shape (Fair et al., 1997). The cells 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 & Kirchhausen, 2008; Rosenblatt J., 2008). In support of this, it has been observed directly in the mouse ovary that the cuboidal granulosa cells divide more than the flat cells (Da Silva-Buttkus et al., 2008). In both bovine (van Wezel & Rodgers, 1996) and human ovaries (Gougeon & Chainy, 1987), other morphological variations of primordial and primary follicles are also observed. 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 2). These

have been interpreted to be primordial follicles with an ellipsoid shape imposed upon them by the surrounding bundles of collagen fibrils (van Wezel & Rodgers, 1996). The terms 'transitional' or 'intermediate' are commonly used to describe these follicles (Stubbs et al., 2007; Westergaard et al., 2007; Rice et al., 2008).

Factors that have been experimentally demonstrated to arrest or induce the recruitment of primordial follicles are: inhibiting factors include anti-Mullerian hormone (AMH; Durlinger et al., 2002), forkhead transcription factor O3 (FOXO3; Castrillon et al., 2003) a downstream effector of the PTEN/PI3K/AKT signaling pathway of cell proliferation and survival (Cantley and Neel, 1999; Li et al., 2010) and the chemokine (SDF-I) and its receptor (CXCR4) (Holt et al., 2006). The list of activating factors is longer and includes leukaemia inhibitory factor, which is produced by granulosa cells and induces these cells to express the kit ligand (KL) (Parrott and Skinner, 1999) that binds to its cognate receptor c-kit on the oocyte surface and regulates the expression of bone morphogenetic protein (BMP)-15 gene (BMP-15). Other factors are: basic fibroblast growth factor (Skinner, 2005), produced by the oocyte; BMP-4 (Nilsson and Skinner, 2003), a member of the transforming growth factor-β (TGF-β) family of growth factors, which is produced by the theca and stromal cells and also has a central role in follicle survival; keratinocyte growth factor, produced by precursor theca, theca and stromal cells (Kezele et al., 2005); BMP-7 (another member of the TGF-β family) also produced by the precursor theca and stromal cells (Lee et al., 2001); platelet-derived growth factor, expressed by the oocyte (Nilsson et al., 2006); Nobox (newborn ovary homeoboxencoding gene) (Rajkovic et al., 2004), Sohlh I and Lhx8 (Pangas et al., 2006), which are three oocyte-specific genes whose lack of expression in deficient mice correlates with arrest at the transition from primordial to primary follicles; and FOXC1, the product of Tgfb-1 responsive gene, which regulates primordial germ cell migration, follicle formation and development beyond the preantral stage, as well as the responsiveness to BMP – TGFb-related signals (Mattiske et al., 2006).

The involvement of FSH in the recruitment of primordial follicles is a long-debated issue. An early study (Dierich et al., 1998) showed that disruption of FSH receptor does not block the recruitment of primordial follicles into the growing pool, even though folliculogenesis is blocked before antral follicle formation.

These data led to the idea that FSH is not involved at the beginning of oocyte growth. However, a number of studies demonstrated that FSH and its receptor make critical contributions to the transition of primordial follicles from the resting to the growing pool (Roy and Albee, 2000; Balla et al., 2003; Thomas et al., 2005). Although follicles do not have functional FSH receptors at this stage, pregranulosa cells and primordial follicles respond to activators of the cAMP pathway (forskolin and cAMP analogues) with increased expression of aromatase and FSH receptor (McNatty et al., 2007).

1.2.2 Secondary and tertiary (pre-antral) follicles

Follicles with two to eight layers of granulosa cells, termed secondary follicles, begin recruiting fibroblast-like cells, external to the granulose layer. Vascularization of this so called theca layer leads to the first direct exposure of the follicle to the surrounding endocrine milieu. The initial proliferation of the granulosa cells is extremely slow, making it difficult to distinguish resting from early growing follicles and to estimate the extended time passing from recruitment to the later stages of folliculogenesis (Gougeon A. 1984, 2004).

In tertiary or pre-antral follicles, cavitation, i.e., the formation of a fluid-filled cavity, marks the transition to the antral stages of folliculogenesis. Preantral follicles in bovine (Irving-Rodgers & Rodgers 2000) and also human (Irving-Rodgers et al., 2008) can be classified into two groups depending on the morphological phenotype of the follicular basal lamina. 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. Preantral follicles with additional layers of basal lamina have also been observed. Loops of basal lamina are seen in cross sections and are connected to additional layers closer to the granulosa cell surface. 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 are also present (Irving-Rodgers & Rodgers 2000).

At the time of follicle recruitment, growth differentiation factor 9 (GDF-9) and BMP-15 seem to have a co-operative function in regulating follicle cell proliferation (Edwards et al., 2008), an activity performed mainly by GDF-9 (Dong et al., 1996; Vitt et al., 2000) during the early phases of folliculogenesis and by BMP-15 during more advanced phases (Galloway et al., 2000; Yan et al., 2001; Juengel et al., 2002, 2004). The correct growth of the follicle is balanced through the regulation of KL expression, which is inhibited by GDF-9 and activated by BMP-15, this latter being itself inactivated by c-kit termed kit ligand (KL) expression in a negative feedback loop (Otsuka and Shimasaki, 2002; Hutt et al., 2006). KL seems to be the link that co-ordinates the growth of the oocyte and the proliferation of granulosa cells (Wu et al., 2004). Later, in antral follicles, this factor up-regulates the expression of activin in granulosa and theca cells, which in turn positively regulates FSH secretion. FSH secretion is, in contrast, negatively regulated by both inhibin and follistatin (Ying, 1988; Knight and Glister, 2001), this latter acting through affinity binding to activin which neutralizes its function (Knight and Glister, 2001). Insulin-like growth factor (IGF)-I has a role similar to that of activin, controlling FSH receptor expression in granulosa cells (Magoffin and Weitsman, 1994; Zhou et al., 1997). Important structures that appear since the secondary follicle stage of development (Hyttel P. et al., 1997) and that regulate the interactions between oocytes and the surrounding follicle cells, mainly those of the innermost layer bound to the zona pellucida, are transzonal projections that maintain the physical link between the oocyte and the somatic compartment of a follicle. The maintenance of a relationship between oocyte and follicle cells via transzonal projections is under FSH regulation (Combelles et al., 2004) and is required to ensure the growth of a healthy oocyte (Albertini et al., 2001; Eppig et al., 2001).

1.2.3 Antral follicle

The transition from the pre-antral to the antral stage is under the control of both FSH and paracrine factors secreted by the oocyte. A recent study by Diaz et al., 2008 suggested that this transition is still controlled by TGF-β ligands, which might be processed differently depending on the presence of the convertase protein PCSK6 in granulosa cells. Since the levels of the expression of PCK6 protein, as well of GDF-9 and AMH, are

high in the pre-antral stage but decrease during the transition to the antral stage, PCK6 could be consideredan intra-ovarian regulator of GDF-9 and AMH activity.

The antral stage is characterized by the appearance of a fluid-filled cavity, the antrum, which begins to form when follicles reach a critical size (from 180 to 300 µm, depending on the species) and a critical number of granulosa cells (about 2000 in the mouse) (Boland et al., 1994).

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 bovine follicle (from a primordial to an 18 mm bovine follicle, calculated from van Wezel & Rodgers 1996, and a net 21 doublings of granulosa cell numbers (McNatty et al., 1984 and van Wezel & Rodgers, 1996). Thus, it is predicted in bovine that cell layers in the membrane 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., 1999b), consistent with a reported variation in the number of granulosa cells obtained from follicles of the same size (McNatty et al., 1979).

The appearance of the antral cavity establishes the morphological and functional separation of granulosa cells into mural granulose cells, which line the follicle wall, and the cumulus cells, which surround the oocyte. In mice, cumulus cells appear to be more closely related to pre-antral granulosa cells from large secondary follicles than to mural granulosa cells, because the oocyte can regulate a wide range of cumulus cell functions via paracrine control (Su et al., 2009). Although formation of the antrum is not fundamental for the acquisition of full developmental potential, the follicular fluid represents a microenvironment enriched in nutritional and regulatory molecules as well apoptotic factors. Formation of follicular fluid is biochemically very different from the species and it has been suggested in bovine to involve the production of osmotically active proteoglycans hyaluronan and versican (VCAN) (Clarke et al., 2006). It is well known that high concentrations of estradiol and low concentrations of insulin-like growth-factor binding proteins (IGFBP-2, -4, and -5) in the follicular fluid are the hallmark of dominant and pre-ovulatory follicles (Fortune

et al., 2004). As mentioned earlier, the antral phase of follicular development is characterized by dependency on gonadotrophins, FSH and LH, which are cyclically secreted by the pituitary gland. FSH, binding to its receptor, activates the cAMP/protein kinase A pathway (Richards, 2001), thus promoting cell proliferation, the differentiation of follicle cells into cumulus and mural granulosa cells, and the acquisition of meiotic competence.

The final phase of folliculogenesis that leads to meiotic resumption and germinal vesicle break down is triggered by a surge of LH and results from the release from the inhibitory action exerted by the follicle cells surrounding the oocyte and the interruption of the action of cAMP or other inhibitory molecules on the oocyte (Mehlmann, 2005).

1.2.4 Oestrous cycle

In cows, there are two or three waves of follicular development during an oestrous cycle (Fortune et al., 1991; Ginther et al. 1996). Each wave consists of the contemporaneous appearance of three to six follicles > 5 mm in diameter (ovulatory size is 12–20 mm) and, several days after the emergence of a wave, one follicle becomes larger than the rest at a time called deviation which occurs when the follicle is about 8 mm in diameter (Fortune et al., 1991, Ginther et al., 1996). The larger, dominant follicle continues to grow at a faster rate (Ginther et al., 2001), while the smaller follicles in the cohort, referred to as subordinate follicles, will eventually regress (Fortune et al., 1991; Ginther et al., 1996). The corollary of these dynamic changes in follicle growth is that healthy antral follicles are clearly of two types, dominant and subordinate, characterized by whether they are actively growing or growing slowly, respectively.

It has been shown in cows that as follicles grow from sizes smaller to larger than deviation, the expression in granulosa cells of FSH receptor declines, while LH receptor, CYP11A1 (cholesterol side-chain cleavage), 3b-hydroxysteroid dehydrogenase (HSD3B) and CYP19A1 (aromatase) increase as do the concentrations of oestradiol (E2) and progesterone in the follicular fluids (reviewed in Fortune et al., 2001, Knight & Glister 2003, and Beg & Ginther 2006). In comparison with subordinate follicles, dominant follicles have elevated

follicular fluid E2 concentrations (Fortune et al., 2001, Ouellette et al., 2005, Ryan et al. 2007, Sisco & Pfeffer 2007) and synthetic capacity (Fortune et al. 2001, Rhodes et al., 2001), and increased free insulin-like growth factor 1 and decreased IGFBP4 and -5 (Beg & Ginther 2006). The dominant follicle in a first wave of the cycle is also slightly larger than one in a second wave (Manikkam et al., 2001), and these have higher follicular fluid E2 concentrations (Wolfenson et al., 1999). No difference in plasma E2 concentration was found between cows with two or three follicular waves (Parker et al., 2003), while the ovulatory follicle is larger in cows with two versus three follicular waves (Townson et al. 2002; Parker et al., 2003); however this is not due to differences in growth rates (Townson et al., 2002). A significant negative relationship between transforming growth factor b1 (TGFB1) in follicular fluid and follicle diameter occurs at 6.5 mm (before selection), but not at later stages (Ouellette et al., 2005). In addition, TGFB1 inhibits FSH-stimulated E2 secretion from granulosa cells of small antral follicles (Ouellette et al., 2005; Zheng et al., 2008) as well as CYP19A1 expression, but not CYP11A1 (Zheng et al., 2008).

Ovulation is induced by an increase in LH secretion (Kanitz et al., 2001). The LH surge triggers a biochemical cascade. The results of Dieleman S.J and Blankenstein D.M., 1984 and 1985, indicate that in preovulatory bovine follicles inhibition of aromatisation takes place at about 14 h after the preovulatory LH peak and progesterone concentrations increase before ovulation. Also progesterone receptor mRNA expression is upregulated specifically in the granulosa layer of bovine preovulatory follicles following the LH surge (Cassar et al., 2002). Moreover results of Dow et al., 2002 show that mRNA expression and enzyme activity for both plasminogen activators, tissue type and urokinase type (tPA and uPA, respectively) are increased in a temporally and spatially specific manner in bovine preovulatory follicles after exposure to a gonadotropin surge. Increased plasminogen activator and plasmin activity may be a contributing factor in the mechanisms of follicular rupture in cattle. In sum the processes lead to the rupture of the preovulatory follicle, the expulsion of the oocyte and the formation of the Corpora luteum.

Local regulation of ovulation involves the interaction of LH and intrafollicular factors including steroids, prostaglandins, and peptides derived from endothelial cells, leukocytes, fibroblasts, and steroidogenic cells. An increase of prostaglandins (PGE_2 and $PGF_{2\alpha}$) in follicular fluid of preovulatory follicles in the cow has

been demonstrated by Algire et al., 1992. Results from Acosta et al., 1998 and 2000, suggest that interactions among endothelin-1 (ET-1), PGE_2, and cytokines may have key roles in a local intermediatory/amplifying system of the LH- triggered ovulatory cascade in the bovine preovulatory follicle. Fortune al., 1993 found that oxytocin is also part of the LH induced biochemical cascade for ovulation.

1.3 Atresia and cell death during folliculogenesis

Atresia of follicles leads to loss of the whole follicle and not just death of single cells, even if death of one cell, such as the oocyte, is observed early in the process. Additionally, even though the focus is often on cell death, atresia is also an active cellular process with resorption of the follicle involving macrophage infiltration, phagocytosis, migration of fibroblasts from the theca and production of collagen, which are some of the processes observed in wound healing (Martin 1997, Schultz & Wysocki 2009). Which cell(s) die first and perhaps initiate atresia varies during follicular development.

During follicle growth, different cell(s) may vary in their susceptibility to death and if these cells are irreplaceable then this would result in follicular atresia. At the bovine preantral stage, oocytes are reported to be the first to die (Rajakoski 1960), in contrast to the antral stage where granulosa cells die first, and in one form of atresia (discussed below) thecal cells, including steroidogenic and endothelial cells (Clark et al., 2004), also die very early in atresia. When atresia is initiated by a large amount of cell death, this could be due to loss of growth factor support such as TGFα (Wang et al., 2002), or that expression of Fas or Fas ligand (Porter et al., 2000, Quirk et al., 2004) could be involved in initiating the process. But where one cell, such as the ooctye, is involved in the initiation of atresia, this is likely to include a failure in development of the oocyte, whereas failure of a few granulosa cells would not have the same consequences. In fact, cell death is a normal part of tissue homoeostatis. Therefore, a limited amount of cell death of granulosa or thecal cells would not be unexpected in a healthy follicle (Jolly et al., 1994), contributing to the difficulty of

defining precisely when a follicle has commenced atresia, and estimation of the required level of cell death to indicate atresia is imprecise.

Modes of cell death include apoptosis, autophagy, cornification and necrosis; and all forms have been observed in granulosa cells (Jolly et al., 1994, van Wezel et al., 1999a, D'Haeseleer et al., 2006); however, their occurrence does not necessarily indicate atresia. Hence, the term apoptosis should not be used when the term atresia is meant. A consensus statement on the nomenclature of cell death (Kroemer et al., 2009) also recommends that the methods by which cell death has been observed should always be reported when reporting the degree of cell death, e.g. apoptosis as observed by DNA fragmentation or karyorrhexis. Historically, atresia was first classified by gross and morphological criteria. These criteria assessed follicles that were then used to identify biochemical changes, such as steroid hormones in follicular fluid. Concentrations of steroid hormones in follicular fluids have since been used as surrogate markers of atresia; however, this approach should be applied with caution as clearly the levels of hormones change with both growth and development, as well as with atresia (Rodgers, and Irving-Rodgers, 2010)

1.3.1 Antral and basal atresia

Many studies of atresia in the bovine ovary have been confounded because there is more than one type of atresia, and a seminal report on follicular atresia (Rajakoski, 1960) misdescribed one form by misidentifying granulosa cells as theca cells in what is now called 'basal atresia'. Marion et al., 1968 accurately described this major type of atresia misidentified by Rajakoski, but described incorrectly how this form of atresia originated. On re-examination of these forms, they were renamed as 'antral (apical may have been a better term) atresia' 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. 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.

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. The follicular basal lamina of basal atretic follicles is often breached by macrophages, which then phagocytose dying basal granulose cells (Irving-Rodgers et al., 2001). Importantly, in these basal atretic follicles, the surviving antral granulose cells, now detached from the basal lamina, express CYP11A1 and HSD3B (healthy and antral atretic follicles of the same size do not), and these follicles have substantially elevated progesterone levels in follicular fluid (Irving-Rodgers et al., 2003b). In basal atretic follicles, there is also an increased deposition of collagen within the theca, and early death of endothelial and steroidogenic thecal cells (Clark et al. 2004) and reduced expression of insulin-like factor 3 in the theca (Irving-Rodgers et al. 2003a). The early decline of the theca in these follicles is also consistent with reduced levels of thecal-derived androstenedione and testosterone in the follicular fluid compared with healthy and antral atretic follicles of the same size (Irving-Rodgers et al. 2003b). It has been suggested that the basal atretic follicles develop from antral atretic follicles later in the process of atresia (Braw-Tal & Roth 2005).

Bovine antral atretic follicles <5 mm were observed to have no appreciable difference in the levels of progesterone, androstenedione or testosterone to those of healthy follicles of the same size (Irving-Rodgers et al. 2003b). In a latter study significant differences in E2 production by cultured granulosa cells from follicles classified as healthy and atretic were observed (Henderson et al., 1987), indicating that there is a difference in granulosa cells in their capacity to produce E2, at least in vitro, at these follicles sizes. However, differences between follicles in E2 synthetic capacity might not be sufficient to reliably use this method as an indicator of atresia.

In larger bovine follicles (>5 mm in diameter), no basal atresia is evident (Irving-Rodgers et al., 2001), making classification simpler. Numerous studies of bovine follicles have measured steroid hormone concentrations in follicular fluids with different indicators of atresia or cell death used to determine whether the steroid concentrations are indeed good measures of atresia (Ireland & Roche 1983, McNatty et al., 1984, Grimes et al., 1987, Jolly et al., 1994). The consensus is that healthy follicles have higher levels of E2 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. e. The terms 'oestrogen active' or 'inactive' are used to describe healthy or atretic follicles respectively. However, the absolute levels of progesterone, androstenedione and E2 also increase with increasing follicle size up until ovulation (Ireland & Roche 1983), and hence absolute levels can only be used as a basis of comparison between similar sized follicles.

1.4 Regulation of steroidogenesis in bovine preovulatory follicles

Follicle development up until antrum formation is considered to be independent of the cycling levels of the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), whereas antral follicles depend on these trophic hormones for their continued growth. In addition to the gonadotropins, local factors are important in follicular development. The expression of these local factors is often regulated by the gonadotropins.

The two-cells, two-gonadotropin model (Figure 3) for estrogen biosynthesis is operative in cattle and in others species like sheep and swine.

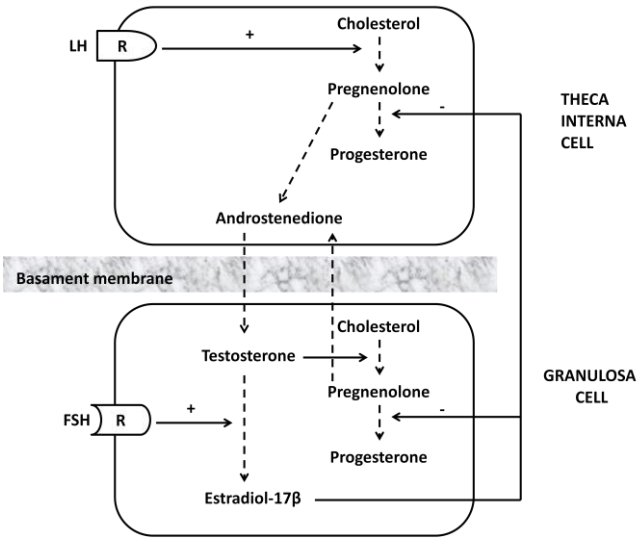


Figure 3. The 2 cells – 2 gonadotropins model of control of steroidogenesis in bovine preovulatory follicles. Theca interna cells use the Δ^5 pathway to make androstenedione, that pregnenolone made by the granulose cell compartment enhances the production of androstenedione by the theca, and that testosterone, made by granulose cells from thecal androstenedione, increases the capacity of granulose cells to make pregnenolone. This model thus postulates that two interactions between theca and granulose cells, in addition to the conversion of thecal androgen to estradiol by granulose cells, serve to increase the capacity of the follicle to make estradiol.

Bovine theca cells secrete androgen primarily in the form of androstenedione, and its production is stimulated by LH, but not by FSH (Fortune, 1986). Conversion of androstenedione to estradiol doesn't happen in theca cells inasmuch they are unable make the conversion, which does happen in granulose cell. These cells cannot synthesize androgens de novo, but they can convert exogenous androgen, which diffuses across the basement membrane from the LH-stimulated theca interna, to estradiol. FSH and LH, acting via the cAMP / protein kinase A (PKA) intracellular signaling pathway, stimulate the steroidogenic capabilities of granulosa and theca cells, respectively, even though the enzymes expressed in the two cell types differ. Among the enzymes induced is steroidogenic acute regulatory protein (STAR), which is considered the ratelimiting step in the production of steroids (Figure 4), as it performs a critical step in the transport of cholesterol from the outer to the inner membrane of the mitochondrion (Dorrington et al., 1975).

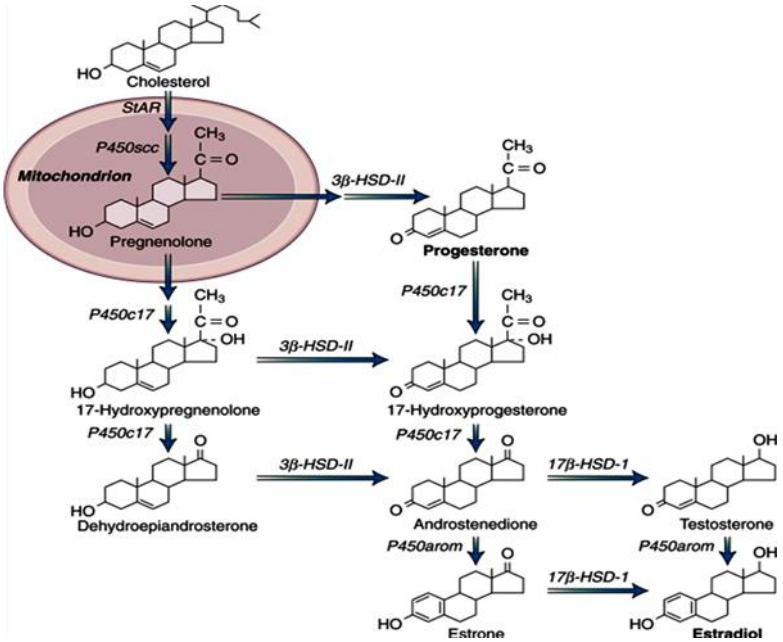


Figure 4. Schematic overview demonstrating the enzymatic steps of steroidogenesis. All steroids are produced from cholesterol, which serves as the basic building block, in the following order: progestagens, androgens and estrogens. Additional steps not included here include, for example, the synthesis of corticosteroids and mineralocorticoids. The carbon atoms in the cholesterol skeleton are numbered as a reference for the enzymatic transitions. All enzymes are indicated by their common names. 3β-HSD-II, hydroxy-delta-5-steroid dehydrogenase 2, 3 beta- and steroid delta-isomerase cluster (HSD3B); 17β-HSD-1, hydroxysteroid (17-beta) dehydrogenase (HSD17B1) 1.

Granulosa cells secrete progesterone, and both LH and FSH increase its production (Fortune, 1984). Estradiol, in addition to being the primary steroid messenger secreted by ovulatory follicles, also acts within the follicle to regulate its development and function. Estradiol acts as a mitogen for granulosa cells and acts with FSH to induce the appearance of LH receptors on granulosa cells (Richards, 1980). In general, the actions of estradiol on follicular cells have the effect of increasing production of estradiol. In bovine follicles estradiol also has effects that positive feedback on its own production. Estradiol inhibits progesterone secretion by granulosa cells (and also by theca cells) in a dose-dependent fashion (Fortune and Hansel, 1979). At the same time, estradiol increases secretion of pregnenolone by granulosa cells as Fortune (1986) has studied. Therefore, in bovine follicles estradiol appears to act as an inhibitor of the 3β-hydroxysteroid dehydrogenase (3β-HSD) that converts pregnenolone to progesterone. Although the doses of estradiol that maximally inhibit progesterone production and increase pregnenolone secretion are quite high in bovine: 1.1 µg/ml (Fortune and Hansel, 1985).

Therefore, differential inhibitory effects of estradiol on progesterone vs androstenedione production could provide a mechanism by which estradiol initially acts as a positive feedback on its own production, by increasing androgen synthesis, but eventually inhibits its own production, by inhibiting 3β-HSD which catalyze the conversion of dehydroepiandrosterone (DHEA) to androstenedione.

In granulose cells, the decline in the concentrations of estradiol may lessen the inhibition of 3β-HSD activity and allow the follicle to shift from androgen-estradiol production to the progesterone production typical of the luteal phase.

1.5 Postpartum anestrus in dairy cow

The postpartum period plays a pivotal role in cattle reproduction. The duration of postpartum anestrus has an important influence on reproductive performance (Lucy MC, 2007). It has been suggested that in high-yielding dairy herds, there is increased incidence of anestrus (Berger et al., 1981; Opsomer et al., 2000). Perhaps increased partitioning of energy to milk production can result in anestrus by delaying resumption of follicular activity. However, factors such as limited energy intake, lower body reserves, and postpartum diseases can also delay the return to cyclicity. A trouble-free calving predisposes to prompt resumption of postpartum ovarian activity (Opsomer et al., 2000). Ideally, this should be followed by a minimal period of negative energy balance (NEB). The duration of postpartum anestrus is not determined by emergence of follicular waves, but rather by follicular deviation and/or the fate of the dominant follicle. Under optimal conditions, there is deviation in follicular growth, selection of a dominant follicle, its growth to maturity, ovulation, and subsequently luteolysis, resulting in reestablishment of cyclical ovarian activity, with an opportunity for becoming pregnant. Failure of any of these processes prolongs postpartum anestrus. In addition, failure of cows to express estrus and apparent anestrus detection efficiency can increase the incidence of anestrus in a herd.

Historically, anestrus was broadly classified into physiologic and pathologic (clinical) types, with the following representing the pathologic type: inactive ovaries (i.e., minimal follicular development, anovulation, and absence of a CL); silent ovulation (i.e., ovulation without behavioral estrus); ovarian hypofunction (i.e., persistent dominant follicle); cystic ovarian degeneration (i.e., follicular or luteinized follicular cyst); and persistent CL (i.e., lack of luteal regression) (Mwaanga et al., 2000). Recently, anestrus has been classified based on ovarian follicular and luteal dynamics (Wiltbank et al., 2002). Follicular wave dynamics involve three main morphologic events: emergence, deviation, and dominance, ending in anovulation or ovulation. Therefore, classification of anestrus or anovulation based on follicle characteristics at emergence, deviation, and dominance provides for a rational diagnosis and treatment of the underlying physiologic condition.

1.5.1 Resuption of ovarian activity during postpartum period.

During the postpartum period, the uterus involutes and the hypothalamo-hypophyseal-ovarian axis resumes cyclic secretions of gonadotropic/gonadal hormones, leading to first postpartum ovulations and regular estrous cycles. Under optimal conditions, these events are completed within 6 weeks after calving. Ninety percent of cows have their first postpartum ovulation within this period (Peter et al., 1986); however, the interval from calving to ovulation can be 3 weeks longer in the present-day dairy cow (Ambrose et al., 2007).

Subclinical and clinical postpartum uterine infection factors affect ovarian activity. Postpartum contamination of the uterine lumen is inevitable; persistence of pathogenic bacteria commonly causes clinical disease or subclinical endometritis. It has been hypothesized that uterine disease suppresses hypothalamic gonadotropin-releasing hormone (GnRH) and possibly pituitary luteinizing hormone (LH) secretion and has localized effects (i.e., delaying folliculogenesis) on ovarian function (Bosu et al., 1987; Mateus et al., 2002). The mechanisms underlying the negative effects of uterine infection on the reproductive tract may involve the inflammatory response (Sheldon et al., 2004; Williams et al., 2007). In

cows with an abnormal puerperium resulting in delayed uterine involution, the resumption of ovarian activity is also delayed. High circulating concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the first 3 weeks postpartum due to subclinical infection serve as a uterine signal, preventing premature onset of ovarian cyclicity until the puerperal infection has been largely eliminated (Peter and Bosu, 1988; Peter AT et al., 1990; Sheldon et al., 2002). In that regard, it is important for the uterus to be free of infection to ensure functional/physiologic follicular and luteal stages of the estrous cycle. Hence, prolonged uterine secretion of $PGF_{2\alpha}$ in cattle with severe metritis has a role in prolonging postpartum anestrus.

The anatomic and physiologic return of the genital tract to the pregravid state and the events leading to the optimal function of the hypothalamo-pituitary-ovarian axis in a postpartum cow have an obligatory delay to optimize fertility (Bilodeau et al., 2003) and to direct energy toward milk production (Bauman et al., 1980). Reestablishing pregnancy within 85 d after calving requires supreme cooperation among the involuting uterus, the hypothalamus, the pituitary, and the ovaries, manifest as the resumption of ovarian cyclicity, expression of estrus, and conception after a timely breeding.

After parturition in dairy cows, sequential follicle-stimulating hormone (FSH) increases (2 to 3 d duration) are initiated in the first week postpartum (Beam et al., 1999), resulting in emergence of the first postpartum follicular wave (3 to 5 follicles, 4 to 6 mm in diameter) within 10 to 14 d after calving (Rajamahendran et al., 1990). As FSH concentrations decline, one follicle is selected for continued growth to become the dominant follicle. This dominant follicle is believed to suppress FSH secretion and the emergence of a new wave, likely due to production of inhibin and estradiol. The ability of this follicle to grow and ovulate depends on the changes in the concentrations and the availability of many growth factors within the follicle like, insulinlike growth factors and their binding proteins (Fortune et al ., 2004), as well as increased pulsatility of LH (Canfield et al., 1990). Another key molecular event in the dominant follicle is differential gene expression in granulosa cells, including increased mRNA expression for LH receptor, 3-b hydroxysteroid dehydrogenase, p450 side-chain cleavage, and p450 aromatase enzymes (Bao et al., 1998). These changes within the dominant follicle are necessary to stimulate steroidogenesis, aromatase activity, and induce LH receptors on granulosa cells. Ovulation, followed by the formation of a CL, is a normal

physiologic progression. In the absence of ovulation, the dominant follicle either becomes atretic or cystic; cows in which the dominant follicle undergoes atresia have new follicular waves, leading to the selection of new dominant follicles.

1.5.2 Types of anestrus.

Anestrus is a broad term that indicates the lack of expression of estrus (or absence of estrous signs), despite efficient estrus detection. High-producing dairy cows have inherently low expression of estrous signs (Lucy MC, 2007), particularly during the early postpartum period (Harrison et al., 1990). Therefore, overt signs of estrus may not always precede an ovulation, in particular the first postpartum ovulation (Roche et al., 2001). Furthermore, in intensively managed modern dairy herds, the efficiency of estrus detection can be extremely poor (Ambrose et al., 2007), resulting in unobserved estrus.

Historically, anestrus was broadly classified into physiologic and pathologic (clinical) types, with the following four types representing the latter: silent ovulation, cystic ovarian disease, ovarian hypofunction, and ‘‘persistent’’ CL (Mwaanga & Janowski, 2000).

In the first type of anestrus, silent ovulation, there is growth of follicles to emergence without further deviation or establishment of a dominant follicle. The pathophysiology of this condition is not well understood, but it is presumed to be due to extreme undernutrition. In that regard, undernutrition and severe energy deficit may cause this condition through a lack of essential LH support to sustain follicular growth and dominance (Jolly et al., 1995). Ovaries associated with this type of anestrus may fall under the classic description of ‘‘inactive ovaries’’ (Fielden et al., 1980). Roche et al., 2001 has studied the effects of NEB on the resumption of follicular activity in the early postpartum period. The reduction in LH pulse frequency may be the result of increases in the negative feedback effect of estradiol on LH pulse frequency. This can occur due to increased availability of estradiol receptors in the hypothalamus or increased sensitivity of hypothalamus to the negative feedback effect of estradiol, as well as other factors (Sheldon et

al., 2002). In addition, there may be suppression of GnRH pulses, and it can be hypothesized that there is decreased GnRH neuronal activity (similar to prepuberal anestrus).

During cystic ovarian disease development, second type of anestrus, there is deviation and growth, followed by either atresia or regression. In certain cases, the regression or atresia occurs only after a follicle has reached a dominant status. Regression of this follicle results in the emergence of a new follicular wave 2 to 3 days later. In these cases, there are sequential follicular waves prior to first ovulation, which may be delayed for a prolonged interval. Some follicles grow further and regress prior to ovulation (McDougall et al., 1995). These cows may have low LH pulse frequency (<1 per 3 to 4 h). These dominant follicles produce very low peripheral estradiol concentrations; hence, there is either insufficient estradiol production or failure of positive feedback from basal estradiol production by the dominant follicle. Subsequent follicular waves emerge within 1 to 2 days after this follicle regresses. It is noteworthy that there may be up to nine waves of follicular growth before first ovulation can occur (McDougall et al., 1995).

For these first two types of anestrus conditions, remedial measures involve correcting the NEB, which is very difficult to achieve through nutritional management. However, recent studies indicate that a reduction in the length of dry period or its elimination (Grummer, 2007) can attenuate the detrimental effects of NEB and potentially reduce anestrus. Furthermore, feeding certain dietary fatty acids during the dry period (without altering its length) can also reduce the interval from calving to the first postpartum ovulation (Colazo et al., 2007). Much has been discussed regarding the first and second type of anestrus in cow at 50 to 60 days after calving (Opsomer et al., 2000, Rhodes et al., 2003). If energy requirements meet the demands of production, hormonal treatment can be used for these two ‘‘static follicles’’ (Rhodes et al., 2003). In general, exogenous progestins are considered appropriate for noncyclic or anestrous postpartum cows (Yániz et al., 2004). The development of controlled intravaginal progesterone-releasing devices, or an intravaginal progesterone insert (IPI), hastened resumption of ovarian follicular activity. The IPI facilitated hormone treatments and circumvented delivery problems associated with feeding or injecting progestins. At the end of IPI treatment, hormones such as equine chorionic gonadotropin (Schmitt et al., 2000), estradiol, or $PGF_{2\alpha}$ have been given to maximize the response (resumption of follicular activity).

Furthermore, estradiol or GnRH analogues have been used prior to IPI treatment (Rhodes et al., 2003). Various combinations of GnRH analogues and $PGF_{2\alpha}$ have been used to initiate ovarian cyclicity. It is noteworthy that cows in the very early postpartum period or those with low reserves of body fat are unlikely to respond to hormonal treatments. Although exogenous progestins are considered the most appropriate therapy for noncyclic or anestrous (first and second type) postpartum dairy cows, any hormonal method to induce ovulation should be done in conjunction with correction of management problems. Pretreatment of anestrous cows with progesterone for 5 to 9 days is a prerequisite for the concomitant expression of estrus at first ovulation. However, the need for additional exogenous hormones to ensure ovulation of a dominant follicle is dependent on energy status, body condition score, and the postpartum interval (which regulate LH pulse frequency).

In the third type of anestrus, ovarian hypofunction, there is deviation, growth, and establishment of dominant follicle, but it fails to ovulate and becomes a persistent follicular structure. A single follicular structure >8 mm in diameter was observed in the absence of a CL or cyst in two ultrasonographic examinations 7 days apart (Marrkusfeld O, 1987; Lopez-Gatius et al., 2001). This may be due to insensitivity of the hypothalamus to the positive feedback effect of estradiol or to altered follicular responsiveness to gonadotrophic support, mediated via metabolic hormones (e.g., insulin-like growth factor and insulin) (Beam et al., 1999). Persistent follicular structures may become follicular cysts or they may luteinize (luteal cysts). The latter occurs in 10% to 13% of the cases (Peter AT et al., 2004). Follicular cysts may either regress or persist as an anovulatory structure. Depending on its structural/functional status, this anovulatory structure may or may not suppress the emergence of a subsequent postpartum follicular wave for a variable interval (Sakaguchi et al., 2006).

Cows with persistent ovarian follicles can be successfully treated with progesterone, GnRH, and $PGF_{2\alpha}$ and subsequently timed-inseminated (Lopez-Gatius et al., 2001). Estradiol benzoate, given in association with progesterone, reduced the persistence of dominant follicles in both cycling and anestrous cows but delayed subsequent follicular development in some anestrous cows (Rhodes et al., 2002). For follicular cysts, agents that induce the release of LH from the anterior pituitary (e.g., GnRH) or have LH-like action (e.g., hCG) can

be used. Regardless of the type of agents used, if cysts become luteinized, luteal regression must be induced with $PGF_{2\alpha}$. A combination of GnRH and $PGF_{2\alpha}$ can also be used (Day 0, GnRH; Day 7, $PGF_{2\alpha}$; and Day 9, GnRH), with or without timed-insemination (Bartolome et al., 2002; Crane et al., 2006). Other researchers have treated cows with progesterone per se (Halter et al., 2006) or with concurrent GnRH followed by $PGF_{2\alpha}$ (Abrose et al., 2004). Progesterone treatment can reestablish hypothalamic function and normal cyclicity in cows with follicular cysts; presumably, it can induce estrogen receptor a in the mediobasal hypothalamus, thereby facilitating a GnRH/ LH surge in response to follicular estrogen (Gümen et al., 2002). The incorporation of progesterone into a timed-insemination protocol for reproductive management of cows with cystic ovarian follicles has considerable potential (Crane et al., 2006) and must be evaluated under field conditions to determine its efficiency and usefulness. Before these treatments can be recommended, further studies are needed with larger herds to determine the economics of their use, which has to be considered in relation to the reduction in open interval in these cows (De Vries et al., 2006). It is noteworthy that regulatory policies may prohibit the use of these hormones in lactating dairy cows. For luteal cysts, $PGF_{2\alpha}$ or its analogues are the treatment of choice; they induce regression of luteinized cysts, with estrus occurring within 8 days in 87% to 96% of treated cows (Peter AT, 2004).

The last type of anestrus is due to a prolonged luteal phase. These cows have normal estrus, ovulation, and formation of a CL, with prolonged luteal function due to a lack of luteal regression. A contributing factor may be the lack of an estrogenic dominant follicle at the expected time of luteal regression (Wiltbank et al., 2002). In that regard, estradiol from a dominant follicle is believed to induce the formation of uterine oxytocin receptors, leading to pulsatile release of $PGF_{2\alpha}$ (Thatcher et al., 1989; Knickerbocker et al., 1986). Many factors have been suggested to increase the risk of a prolonged luteal phase (Opsomer et al., 2000), including parity, dystocia, health problems during the first month of lactation, heat stress, and perhaps ovulation soon after calving. Uterine infection (Mateus et al., 2002) or pyometra can prolong the life of the CL (Sheldon et al., 2006).

For this type of anestrus, $PGF_{2\alpha}$ is used to terminate the life of the CL and initiate the estrous cycle. If the persistence of CL is due to pyometra, treatment with $PGF_{2\alpha}$ will also be beneficial (Farin et al., 1993). In

certain cases, depending on the severity and amount of collection of purulent material in the uterus, uterine lavage and/or intrauterine or systemic antibiotic treatments are recommended (Földi et al., 2006).

1.6 Negative Energy Balance (NEB) in dairy cows

Negative energy balance (NEB) is a frequent condition occurring in high producing dairy cows some days after calving. It consists of an imbalance between diet energy supply and production requirements.

In the early post-calving period, cow face a strong increase in milk production and a decrease in appetite resulting from calving stress, and the possible quick change in diet compared to the last stage of pregnancy. In high yielding dairy cows diet is unable itself to support the loss of nutrients related to milk production. Therefore cows often mobilize their lipid reserves accumulated during dry period and use them as further energy source. Fatty acid relased are metabolized to Acetil COA, that is metabolized into Krebs Cycle (Figure 5) for energy production (Nelson et Cox, 2002).

The large amount of Acetil COA accumulated after lipomobilization, cannot be completely utilized in this metabolic pathways, because of the lack of oxalacetate resulting from carbohydrates catabolism and glucose depletion.

As alternative energetic pathways the body produces an excess of ketone bodies, which leads to typical clinical symptoms of post-calving ketoacidosis, like decreasing in ruminal activity and milk production.

As a result, several negative events can be observed: deterioration of milk characteristics, decreased immune response (mastitis, lameness, respiratory diseases and metritis) and reduced reproductive efficiency in the post-partum period.

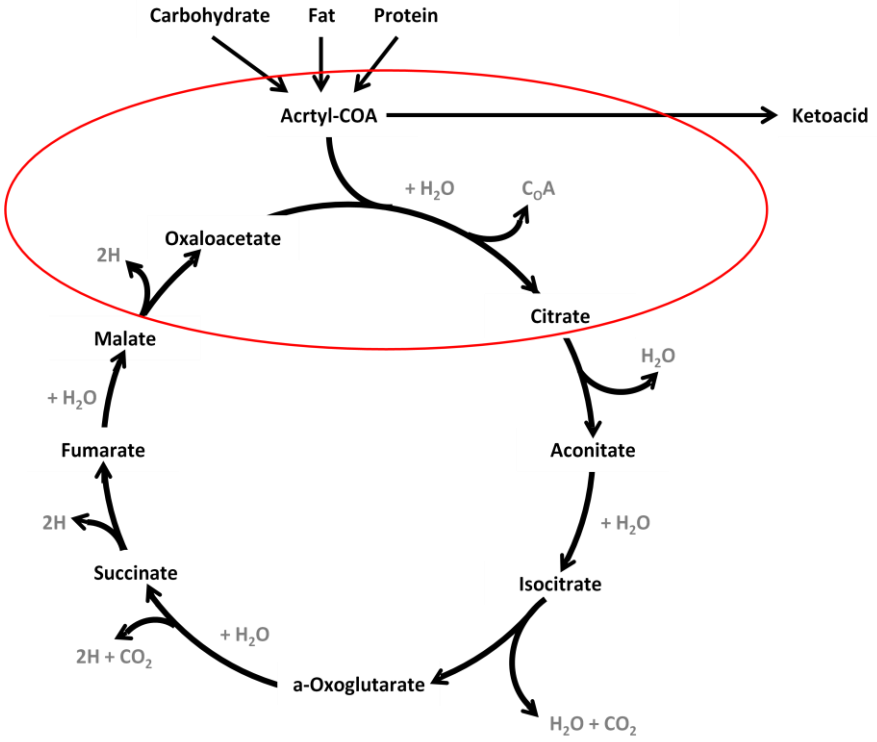


Figure 5. Effect of Acetil COA excess on the Krebs Cycle. NEB condition in dairy cow often mobilize their lipid reserves accumulated during dry period and use them as further energy source. Fatty acid released are metabolized to Acetil COA, that is metabolized into Krebs Cycle for energy production. The large amount of Acetil COA accumulated after lipomobilization, cannot be completely utilized in this metabolic pathway, because of the lack of oxalacetate resulting from carbohydrates catabolism and glucose depletion. As alternative energetic pathway the body produces an excess of ketone bodies, which leads to typical clinical symptoms of post-calving ketoacidosis, like decreasing in ruminal activity and milk production.

Negative energy balance can affect cow reproductive performances through some biological mechanisms: metabolic hormonal modifications regulated by pituitary-hypothalamic axis (as LH, FSH, GH, insulin, leptin, IGF-1, oestrogen and progesterone); interactions between blood metabolites and ovarian activity (glucose, NEFA, β-OHB); relationship between uterine functionality and immune response during pregnancy and transition period.

The oocyte is the structure that suffers more because of the changes in energy requirements of the body. In early development stage, the blood-oocyte barrier is very permeable and oocyte is almost in directly contact with the bloodstream. The permeability of this barrier seems to increase when follicles are in pre-ovulatory stage, but in this period blood metabolites are able to easily penetrate into follicular fluid and directly act on the oocyte (Fernandez et al., 2006).

The lipomobilization that distinguishes this disease, due to the low plasmatic level of insulin and to the high concentration of GH (growth hormone), is the basis of the increase of non-esterified fatty acids (NEFA) in

blood. They metabolites have a direct toxic effect on the follicoles with induction of cumulus cells apoptosis, necrosis and follicular development arrest (Jorritsma et al., 2004; Friggens, 2003). A probable negative effect is also supported by ketone bodies (particular β-hydrossibuthyrate β-OHB) although metabolic alterations are still unknown (Reist et al., 2003).

In the immediate pre-calving stage, low leptin plasmatic levels can serve as reference to highlight a delayed first post-partum ovulation and a longer duration of the calving-conception interval. In this stage, leptin concentration is directly related to cow's BCS and may be indicative of the adipose tissue amount available for lipomobilization (Wathes et al., 2007) and for eventual ketoacids production.

NEB is able to negatively change ovarian activity also through other metabolic pathways. The plasmatic insulin, IGF-1 (Insulin-Like Growth Factor 1) and leptin decrease has direct effects (lower ovarian sensibility to pituitary gonadotropins) and indirect effects on follicular development, such as on lower pulse secretion frequency of pituitary LH. Insulin has a positive effect on ovarian sensibility to gonadotropins, resulting in best recruitment of small follicles and development of the dominant follicle.

During post-calving, follicle capacity to produce required oestrogens for next ovulation is directly influenced by insulin and IGF-1 plasmatic concentration (Butler, 2003); in particular the latter can result in 40-50% lower levels during negative energy balance in the first two weeks post-calving, and can reflect the same molecular concentration in follicular fluid (Fenwick et al., 2008; Fitzpatrick et al., 2007).

Therefore, there is an high decrease of IGF-1 plasmatic levels in the first post-calving week and this fall directly influences several reproductive parameters (calving to first ovulation and to conception intervals). IGF-1 is essential to improve gonadotropins activity on ovarian structures and to stimulate cell proliferation and follicular steroidogenesis (Wathes et al., 2007). Unlike IGF-1, lower insulin plasmatic levels have not shown a strong influence on follicular cell proliferation, but can participate to the regulation of oestrogen production in case of serious energy decrease (Wathes et al., 2007; Figure 6).

Glucose concentration in blood during NEB period is significantly reduced. However, cow hematic glucose is physiologically low because of reduced ruminal absorption of glucose after almost complete fermentation to propionic acid (47,2-55,8 mg/dl) and it directly influences follicular fluid glucose concentration (Lopez et

al., 2003; Martin et al., 2008). During NEB and ketosis, a delayed oocyte development is observed, due to glucose shortage, fundamental molecule for energy (ATP), RNA and DNA production by follicular and oocyte cells.

Another consequence of the post-calving lower energy is a lack of follicular ovulation, which continues its development to follicular cyst (Boland et al., 2001). This pathogenesis is supported by the lack of pituitary LH secretion that does not allow follicular ovulation and a major oestrogen production. These are responsible of continue cysts development and of the characteristic behavior of cows affected by this pathology, (increase of walking activity and mount other animals).

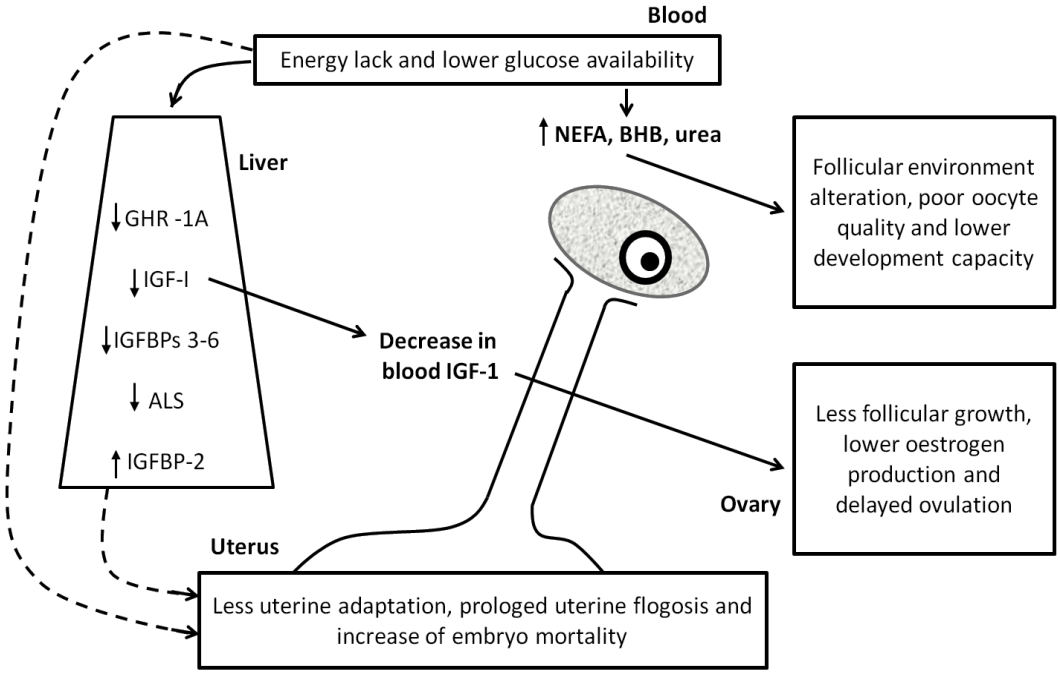


Figure 6. Direct and indirect negative Energy balance's effects on the reproductive apparatus function, mediated by metabolic-hormonal change that involve bloodstream and liver: decrease og GH receptors (GHR-1A) and IGF-1, IGF binding protein (IGF-BP), acid-labile subunit (ALS), non-esterified fatty acids (NEFA) and β-hydrossibuthyrate (BHB) (Whates at al., 2007 modified).

Between NEB effects, some authors (Van Knegsel et al., 2007) mention the effect on progesterone (P4) plasmatic concentration; during the first post-calving days, NEB impacts corpus luteum activity (lower

energy availability for lutheal cells), and consequently progesterone production, especially during second and third oestrus cycles.

This condition does not allow changes in endometrial morpho-functional characteristics at the time of post-calving first service, and negatively affects intrauterine environment, necessary for embryo implantation.

The uterine effects of negative energy balance are identified as a delayed uterine involution after post-calving (30-40 days) and a major incidence of herd endometritis.

These facts, responsible for strong delay in resume of normal reproductive functions after calving, are due to alteration of some genes expression connected with NEB, that regulate the immune response of the reproductive tract (IL 1β and IL 8 encoding) and the recondition of normal uterine size (Boland et al., 2001).

The latter problem derived from alteration that regulate protein change and collagen catabolism of uterine wall (matrix metalloproteinases MMPs-1), that resulte poorly tonic, unable to expel the fetal membranes and virtually defenseless towards pathogens because of lack of mononuclear cells (Wathes et al., 2007).

Retained placenta, is a serious disease connected with the two previous illness, resulting from problem in separation of placental cotyledons from endometrial caruncles and hence lack expulsion of fetal membranes within 24 hours after calving.

NEB can influence differently this aspect: for example reducing the intensity of uterine contractions essential for expulsion of pregnancy residuals and immune response decrement (Van Knegsel et al., 2007).

The main problem in management of reproductive disorders through negative energy balance control (in post-calving dairy cows) is that this phenomena has become almost impossible to avoid in current modern dairy herds, because of the genetic potential.

One of the most used method for indirectly estimate the energy balance, is the control of body condition score (BCS); it was observed that a decrease of 0.5 BCS points between dry period and first weeks of lactation (transition period), coincide with 10% conception rate (CR) loss (Butler, 2003).

In several studies (Butler, 2006; Schneider, 2004; Wathes et al., 2007) it was concluded that the decrease of one point BCS at the moment of post-calving first insemination, can reduce cow reproductive efficiency by 17-38%. Consequently interval from calving to conception can increases over 120-130 days (usually if cow

exceed 180-200 days after calving, becomes to high culling risk).

1.7 Organization and aim of the thesis

The study reported in this thesis is refered to the Italian National Interest's Research Project 2008 (PRIN08; n. 1407) titled “follicular dynamic and oxidative stress markers in the ovarian follicles’ microenvironment of dairy cow”, which has been developed with the collaboration of the University of Udine, Department of Agricultural and Environmental Sciences, and the University of Milan, Faculty of Veterinary and Medicine.

As mentioned above in the introduction, during the past 50 years, intensive genetic selection for increased milk production, coupled with technological improvements in nutrition, has led to significant increases in milk yield in cows. However, this increase in milk output per cow has been accompanied by worldwide decline in cow fertility. High-yielding dairy cows are typically in a state of negative energy balance (NEB) postopartum because the amount of energy required for maintenance of metabolic function end milk production exceeds the amount of energy cow consume. The NEB cause a “pollution” due to the side products of this heavy energy traffic from the digestive tract and body reserve towards the udder and the considerable increase of oxygen requirements results in augmented production of reactive oxygen species (ROS). This peculiar metabolic condition results in poor reproductive performance, which includes a delay in onset of estrus cycles postpartum and a reduction of oocyte quality, resulting in low conception rates and a high rate of early embryonic death. Health problem such as mastitis could also increase oxidative and inflammatory side products in the blood, and further augment energy requirements. The typical metabolic adaptations which can be found in serum of high-yielding dairy cow shortly post-partum, are reflected in follicular fluid and, therefore, my affect the quality of both the oocyte and the granulose cells.

The aim of this study, was to investigate the different events, in terms of gene expression and molecular/cellular function, that occurs in the ovarian preovulatory follicles’ microenvironment during the progressive recovery from negative energy balance (BEN) condition in dairy cow. To conduct this study,

dairy cow's preovulatory follicles have been collected at different times from the parturition: 30, 60, 90 and 120 days in milk (DIM). These researches have been realized also in collaboration with the University of Laval, Quebèc, Canada.

2 Materials and Methods

2.1 Animals and experimental groups

Thirty-four (34) Holstein-Friesian cows were selected on the basis of general clinical examination and normal ovarian cyclicity during the 30 d, 60 d, 90 d and 120 d post partum periods. Three different farms, in Friuli Venezia Giulia region, Udine's city and Udine's city provinces, in Italy, have contributed to providing animals which were housed in the same conditions, fed with silage and concentrate as well as water *ad libitum*. The use of animals originating from different locations and field conditions ensured that the results were not specific to a given environment and allowed for broader conclusions even if it may have reduced the number of differences found.

2.2 Synchronization protocol

The estrous cycles were synchronized by administration of one dose of PGF2α injection (15 mg Luprostiol, Prosolvin) in the first treatment day, day 0, following anultrasonographic scan (Medison Sonovet 600, 7.5-MHz linear probe, Medison, Seul, Korea) which had confirmed the presence of corpus luteum and growing follicles in the ovaries. The next day, day 1, additional scanning was performed to ensure the regression of the corpus luteum and an increase of the follicles' size in all cows treated with $PGF_{2\alpha}$. The third day (day 3), yet within 42h after $PGF_{2\alpha}$ treatment granulosa cells were collected.

2.3 Follicular aspiration and collection of granulosa cells

Prior to the granulosa cell collection, cows were sedated with 10 mg/mL detomidine hydrochloride (Domosedan, Orion Corporation, Janssen Cilag Spa) to simplify handling of the animals and their ovaries. Caudal epidural anesthesia was induced using 5 mL of Lidocaine to prevent abdominal and anal strain. Follicles were aspirated using an ultrasonic scanner and a 7.5 MHz vaginal sector transducer equipped with

a needle guide attached to a 19-gauge needle that was connected to a 5 ml sterile syringe. The pipeline and attached syringe were filled with an exact volume of 2 ml sterile saline solution BSA supplemented with heparin (5000 U/ml). For each cow, in each time postpartum, the content of the preovulatory follicle (> 10 mm in diameter) was aspirated while repumping the aspirated volume 2 or 3 times in order to detach the granulosa cells from the follicle wall. At the end of the procedure, the pipette was flushed with an additional 1 ml of saline solution to recover the dead volume content. After that it was examined under stereo microscope for to remove the presence of the oocyte, if it was present in the follicular liquid previously aspirated. Granulosa cells were separated from follicular fluid by centrifugation, they were washed with 500 µl of sterile phosphate-buffered saline, snap frozen in liquid nitrogen and stored at -80 °C.

2.4 Total RNA extraction

Total RNA extraction of each pool of GC was performed using the PicoPure™ RNA isolation kit (Arcturus, Molecular Devices) and RNA was recovered into a 30 µl elution volume using the provided buffer. The RNA extraction procedure included an on-column DNase I treatment to remove genomic DNA. The RNA concentration and quality, as measured by the RNA integrity number (RIN), were assessed using Agilent 2100-Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA NanoLab Chip (Agilent Technologies).

2.5 RNA amplification, labeling, hybridization and microarray scanning

For each treatment groups, 30 d, 60 d, 90 d and 120 d postpartum, the three GC samples with the highest RIN were selected for microarray hybridization. Five nanograms of the total RNA was amplified using T7 RNA polymerase (RiboAmp®HS^{Plus} RNA Amplification kit; Molecolar Devices, Sunnyvale, CA, USA) according to the manufacturer's indications. After purification, the amplification product was eluted in 30 µl of RNA-

eluted buffer and 1 µl was used to quantify the RNA amplification yield using NanoDrop (Wilmington, DE, USA).

Universal Linkage system (ULS) aRNA Fluorescent Labelling Kits (KREATECH Biotechnology, Amsterdam, The Netherlands) were used to label the samples in a dye-swap design as shown in Figure 7. Briefly, 4 ug of aRNA from the three biological replicates of the 30 d, 60 d, 90 d and 120 d post partum treatment groups was labeled with 2 µl of either Cy5/DY647-ULS or Cy3/DY547/ULS . Labelling was performed by incubating the total volume of 20 µl at 85 °C for 15 min. Unbound dye was removed using the PicoPure™ RNA Isolation kit. Labeled RNA was quantified by NanoDrop as described above.

The three biological replicates for each of the 30 d, 60 d and 90 d treatment groups were hybridized against the three biological replicates of the 120 d treatment group, and the hybridization were then repeated, however inversing the dye colours, to give a dye-swap design (Figure 7). The EmbryoGENE Bovine Microarray slides, as descripted by Robert C. et al, 2011, are in a 4X44K array format, contain a total of 42,242 probes, including 21,139 known reference genes; 9,322 probes for novel transcribed regions (NTRs); 3,677b alternatively spliced exons; 3,353 3'-tiling probes; and 3,723 controls. Hybridization was performed in SlideHyb buffer # 1 (Ambion, Austin, TX, USA) at 65 °C for 17 h in the SlideBooster hybridization station (Advalytix, San Francisco, CA, USA). Slides were washed twice with 2X SSC-0.5% SDS at 42 °C for 15 min and twice with 0.5X SSC-0.5% SDS at 42 °C for 15 min. They were then dipped three times in 1X SSC and three times in H2O. Scanning was done using the VersArray ChipReader system (Bio-Rad) and visualized with the ChipReader software (Media Cybernetics, San Diego, CA, USA).

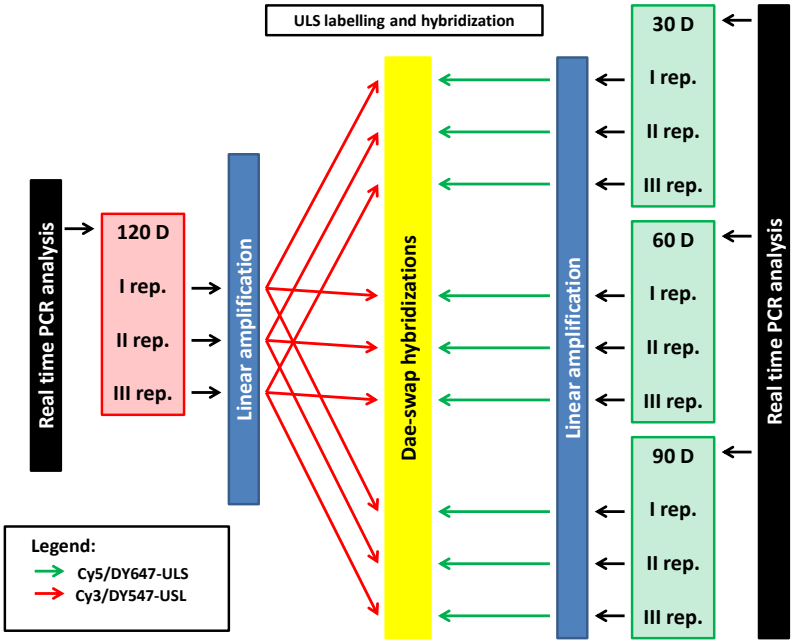


Figure 7. Experimental design of linear amplification, dye-swap hybridization and real-time PCR validation.

2.6 Microarray and data analysis

Signal intensity data files were analyzed using ArrayPro software (Media Cybernetics). In the preprocessing step, the background of the intensity files was removed using Minimum Background Subtraction. Data analysis of our dye-swap contrasts was subsequently achieved through the EmbryoGENE LIMS database (http://elma.embryogene.ca). Once they had passed all quality control criteria, microarray datasets were downloaded into the FlexArray microarray analysis software (http://genomequebec.mcgill.ca/FlexArray/) while datasets that failed the quality control analysis were rejected. With FlexArray, a Bioconductor R based software, up-regulation or down-regulation of genes expression was evaluated following transformation of the hybridization data in log2, normalized for dye bias using a within-array Loess and between-array Quantile normalization in order to obtain a similar distribution across all arrays.

Differential gene expression between treatment groups was determined using an e-Bayes moderated *t*-test (LIMMA), included in the FlexArray software. Transcripts were considered as differentially expressed if their respective probes were associated to an *M* value (log ratio) > ± 1 (fold change of 2.0) and a *P* value < 0.01.

Data processing, including hierarchical clustering (Figure 8), Venn diagram (Figure 9), was also performed using the FlexArray program.

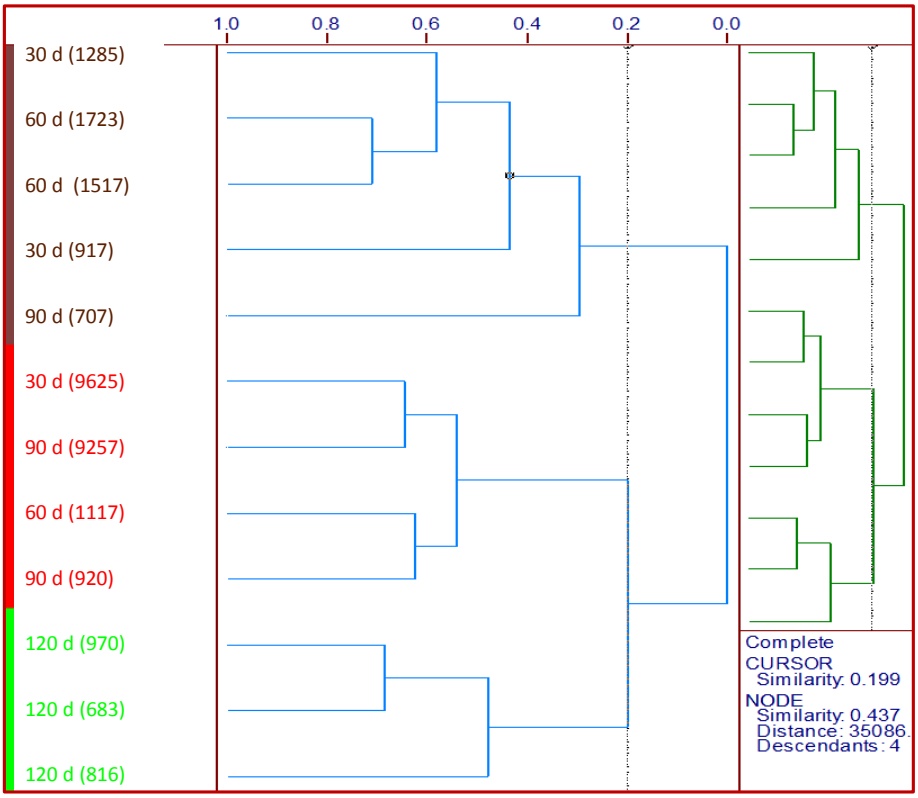


Figure 8. Hierarchical clustering of GC sample biological replicates collected at each of the postpartum periods, 30 d, 60 d, 90 d and 120 d postpartum. The serial numbers in brackets correspond to the animal from which the animal originated. The color in the leftmost section (brown, red and green) depict the three distinct sample clusters. Nodes associated to a greater value in the horizontal axis correspond to a greater similarity between linked samples.

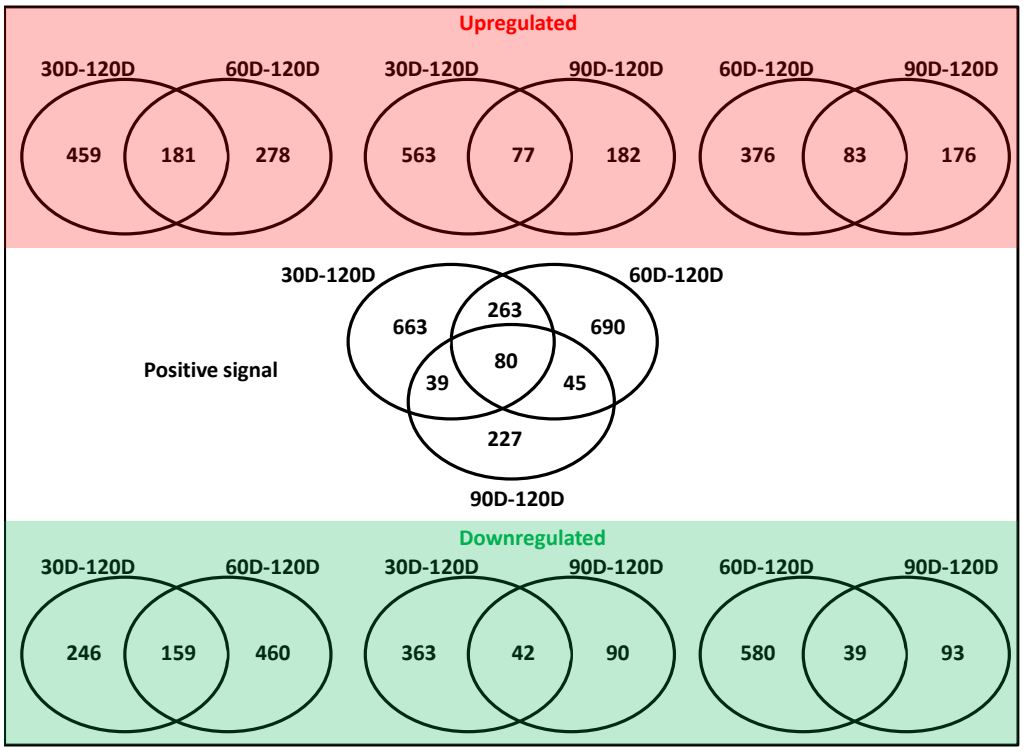


Figure 9 Microarray analysis results in Venn diagram between 30 d-120 d, 60 d-120 d and 90 d-120 d groups combination. Venn diagram representing all positive signal (white area), all upregulated (red area) and downregulated transcripts (green area) shared and non-shared between the samples using a threshold cut-off of 2 fold change and *P* value < 0.01.

Determination of the positive signals was performed on normalized data using the procedure described by Gilbert et al. (2007). Uninformative data was removed from the analysis by establishing a significant threshold cutoff based on a degree of confidence associated with the variability of the negative controls. This cutoff threshold was calculated as follows: T= M + 2 X S.D., where T is the calculated cutoff threshold, M is the average of negative controls present on the slides and S.D. is the standard deviation. All the data equal or lower to the cutoff threshold were not considered in the analysis. Moreover, a transcript was included in the analysis only if its signal was higher than the background noise and if it was present in all biological replicates as well both of their technical replicates.

Processed FlexArray data were downloaded into a commercial software Pirouette (Version 4.0; Infometrix Inc., Bothell, WA, USA) for to generate a dendrogram of hierarchical cluster analysis (HCA) (Figure 8), where distances between the biological replicates of each group at 30 d, 60 d, 90 d and 120 d were calculated and compared. Relatively small distances imply that the samples are similar, while dissimilar samples are separated by relatively large distances. The dendrogram classification was employed using

Euclidean distance and complete-link clustering algorithm. The HCA dendrogram of all biological replicates groups produced three clusters when the similarity values (between 0 and 1) was set at 0.199.

Functional analysis of the gene evaluated as being upregulated or downregulated at the transcript level in the 30 d, 60 d, 90 d postpartum treatment groups compared to 120 d treatment group was carried out through the Ingenuity Pathway Analysis software (Ingenuity Pathways Analysis , v 8.0; Ingenuity Systems, Redwood City, CA, USA). Briefly, genes' official name and fold change were uploaded into IPA. Using a database of previously published results, the software established potential functional relationships between the genes of the inputted datasets. Gene networks are generated and scored according to the negative exponent of the right-tailed Fisher's exact test result, where a higher score indicates a lesser likelihood that the functionally related genes appeared in the network by chance. In addition, network analysis provide the most significant functions associated to a given network. A broader functional analysis is also executed by the IPA software in order to assess the functions significantly modulate between treatment groups. Using the genes' documented impact on a given function and integrating the fold change reported in the dataset, a z-score is calculated whereby a value > 2 indicates a significant activation of a function, and a value < -2 indicates a significant inhibition of a function. The p-value, calculated by Fisher's Exact Test, measures the likelihood that the genes in a dataset could be randomly associated to a given function. P-values < 0.05 (-log (p-value) > 1.3) were considered statistically significant, non-random associations. The aim of this analysis was to discover some gene networks and pathways that were modulated in GCs at different times following calving in dairy cows.

2.7 Real-time PCR validation

Real-time PCR validation of selected candidate genes was carried out for the four treatment groups, 30 d, 60 d, 90 d and 120 d (Figure 7). Equal amounts of total RNA were taken from the three GC biological replicate samples of each group. Fifty nanograms of total RNA was reverse transcribed using the reverse transcriptase qScript™ Flex cDNA Sythesis Kit (Quanta Biosciences) with a mix of oligo-dT primers and decamers according to the manufacturer's instructions. Real-time PCR was performed on the twenty three

selected candidates (fourteen upregulated and nine downregulated) in LightCycler capillaries (Roche Applied Science) using the LightCycler FastStar DNA Master SYBR Green I (Roche). The primers for each gene were designed using IdtDNA web interface (http://www.idtdna.com/analyzer/Application/oligoAnalyzer/). Sequences, size of amplified product, GenBank accession numbers, and annealing temperatures are shown in Table 1. Normalization was achieved using three housekeeping genes, the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein L13A (RPL13A) transcripts (Table 1) using Geometric averaging normalization (GeNorm, http://medgen.ugent.be/~jvdesomp/genorm/). Following the GeNorm software analysis, RPL13A was determined to be the most stable control genes (*M* value= 1.2; *P*> 0.05), followed by HPRT1 and YWHAZ (*M* value= 1.3; *P*> 0.05). These three housekeeping genes were subsequently used for qPCR data normalization. Specificity of the primer pairs for each candidate gene was confirmed by analyzing the LightCycler melting curve (Roche) and by sequencing the PCR product. For genes: ATG16L1, CALM1, CREB3L2, CYP19A1 and HSD3B7 a one-way ANOVA and Tukey's multiple comparison tests were performed using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) to determine the statistically significant differences in GC mRNA levels among all four postpartum periods. For CALM1, CREB3L2, CYP19A1 and HSD3B7, a paired t-test analysis was statistically significant only between the 60 d and 120 d condition, as well as for CYP11A1, GPX4, GSTA1, PCYOX1, TDG and VNN2 (See Table 2 for gene names).

Tabele 1. qPCR primer sequences, annealing temperature, fluorescence acquisition temperature, product size and accession number.

[a] Indicates the housekeeping primers used for the real-time PCR normalizzation.

*** Indicates the test primers used in real-time PCR selection samples: TIMP1 (TIMP metallopeptidase inhibitor 1), TIMP2 (TIMP metallopeptidase inhibitor 1); TNFAIP6 (tumor necrosis factor alpha-induced protein 6).**

Gene	Primer sequence 5' -3'	Annealing temperature (°C)	Fluorescence acquisition temperature (°C)	Product size (bp)	Accession number
ALDH16A1	F 5'-tcgtgaatgtggtgacaggaga-3' R 5'-agtttattggagggcacgggaa-3'	58	84	363	NM_001101869
ANKRD1	F 5'-gcttggatgtcgtgacacgata-3' R 5'-tgaatattggacgccacttcga-3'	57	80	280	NM_001034378
ATG16L1	F 5'-agtgtgtgatgagcgggcatt-3' R 5'-tggacgagctgtgatgctttga-3'	59	84	360	NM_001191389

CALM1	F 5’-acgggcaggtcaactacgaagaat-3’ R 5’-acaaacgcctgactgtgctcaa-3’	59	78	414	NM_001242572
CREB3L2	F 5’-tgctgtgctttgccgtagcatt-3’ R 5’-aaggtggcgttcactcttctgt-3’	59	83	409	NM_001102533
CYP11A1	F 5’-atccagtgtctcaggacttcgt-3’ R 5’-gaacatcttgtagacggcatca-3’	59	84	219	NM_1766442
CYP19A1	F 5’-gcgttgtctaagcaaactctccca-3’ R 5’-cagcttccaacggcatttcccat-3’	60	77	389	NM_1743051
GATM	F 5’-aatgcctgtgttccaccgttca-3’ R 5’-tccctgagcagccaatttgtgt-3’	59	83	474	NM_001045878
GPX4	F 5’-tcggcaaagtcgacgtcaat-3’ R 5’-gcagtcgctccttcaggtactta-3’	59	83	289	NM_174770
GSTA1	F 5’-tccagcaagtgccaatggttga-3’ R 5’-atttcacccaaatctgccacaccc-3’	59	79	230	NM_001097694
HPRT1[a]	F 5’-ggctcgagatgtgatgaagg-3’ R 5’-gcaaagtctgcattgtcttcc-3’	57	79	293	NM_001034035
HSD17B11	F 5’-aacagccaccgaatgcaagaga-3’ R 5’-ctgctgaagccacagtgacaat-3’	59	78	296	NM_001046286
HSD3B7	F 5’-acacaagcagcatggaagtcgt-3’ R 5’-agggcgagttgtcatagcagaa-3’	59	84	417	NM_001034696
MAFG	F 5’-tgccaccagcgtcatcacaata-3’ R 5’-tgtttactcccacaggctgact-3’	59	84	360	NM_001098980
MTPN	F 5’-actgcctttgaagccactgaca-3’ R 5’-tttcgtcacagcagccactact-3’	59	78	479	NM_203362
NTS	F 5’-avggcaattgaacccattgaaa-3’ R 5’-attcgccagggcattcaccgct-3’	57	74	198	NM_173945
PCYOX1	F 5’-agctggaattggtggcacttca-3’ R 5’-agcctgctttctgcaaggtttc-3’	59	81	500	NM_001105474
RPL13A[a]	F 5’-tgaggttggctggaagtacc-3’ R 5’-tgaggacctctgtgaatttgc-3’	57	80	161	NM_001076998
SERPINE1	F 5’-tgtgcctttgccgtaactcaca-3’ R 5’-taacgtcacagtacccactcgt-3’	59	81	357	NM_174137
SLC16A3	F 5’-ttgtgctggtgctgggtaactt-3’ R 5’-ttgtctccaaagcgtcgggttt-3’	59	84	492	NM_001109980
TDG	F 5’-agaaatgccagccgaagttcct-3’ R 5’-ttgctgcctggtgttgtccttt-3’	59	81	492	NM_001083696
TIMP1*	F 5’-gacatccggttcatctacacc-3’ R 5’-accagcagcataggtcttgg-3’	57	82	201	NM_174471
TIMP2*	F 5’-ctcggcaacgacatctacg-3’ R 5’-gcacgatgaagtcacagagg-3’	57	80	227	NM_174472
TNFAIP6*	F 5’-caagggcagagttggatacc-3’ R 5’-tgtgccagtagcagatttgg-3’	57	78	230	BC151789
TRIB2	F 5’-agctggtgtgcaaggtgtttga-3’ R 5’-agcttgacccgagtcctttctt-3’	59	84	322	NM_178317
UBR1	F 5’-tgccaggagccaggaaactaat-3’ R 5’-atgctcgccaactgcttgatgt-3’	59	78	423	NM_001205897
VNN2	F 5’-tgctgtgaccctggtgaaagat-3’ R 5’-tggcgtaggcactccaattcat-3’	59	80	317	NM_001163920
VCAN	F 5’-attggatgccgttggtgcagaa-3’ R 5’-ttgtcgggtttcagcgtggaat-3’	59	82	312	NM_181035
YWHAZ[a]	F 5’-ccagtcacagcaagcatacc-3’ R 5’-cttcagcttcgtctccttgg-3’	57	78	287	NM_174814

Table 2. Gene names of qPCR validated candidates.

Gene Symbol	Genes name
ALDH16A1	Aldehyde Dehydrogenase Family
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)
ATG16L1	ATG16 autophagy related 16-like 1
CALM1	Calmodulin 1
CREB3L2	c-AMP Responsive Element Binding Protein3-Like2
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
GATM	glycine amidinotransferase
GPX4	Glutatione peroxidase4
GSTA1	Glutatione S Transferase alpha1
HSD17B11	hydroxysteroid (17-beta) dehydrogenase 11
HSD3B7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7
MAFG	musculoaponeurotic fibrosarcoma oncogene
MTPN	Myotrophin
NTS	Neurotensin
PCYOX1	prenylcysteine oxidase 1
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator
SLC16A3	solute carrier family 16, member 3 (monocarboxylic acid transporter 4)
TDG	thymine-DNA Glycosylase
TRIB2	tribbles homolog 2 (Drosophila)
UBR1	Ubiquitin protein Ligase E3 Component N-Recognin1
VNN2	vanin 2
VCAN	Versican

3 Results

3.1 Hierarchical clustering analysis: relationship between samples at 30 d, 60 d, 90 d and 120 d

Hierarchical clustering analysis (HCA) (Figure 8) represents the distribution of microarray hybridizations for each of the GC group: 30 d, 60 d, 90 d and 120 d. Results from that analysis support the hypothesis advanced at the onset of experimental whereby the 120 days (d) postpartum group could be considered as the positive control in the microarray contrasts with other postpartum periods. Indeed, the HCA assembled all three 120 d samples in a cluster (green in Figure 8) distinct from any samples originating from the other postpartum periods. The remaining two clusters are each composed of samples originating from all three of the other postpartum periods. The cluster most similar to the 120 d cluster (red) is composed of two samples from the 90 d group and one sample from each of the 60 d and 30 d groups. The last cluster (brown) contains the remaining two samples from each of the 60 d and 30 d group and one sample from the 90 d group. These HCA analysis results reveal similarities in GC gene expression profiles between the 30 d, 60 d and 90 d pospartum groups while indicating that gene expression is distinct once the 120d period is reached.

3.2 Differential gene expression and transcription profile of positive signals from microarray analysis

The data generated by the microarray analysis is summarized in (Figure 9). Hybridization of the GCs samples has revealed 2007 positive signals after the cut-off threshold was applied (Figure 9), which accounts for 5% of the probes spotted on the array. Among those probes, 80 (4%) were common between to all three microarray contrasts, indicating that relatively few transcripts were present in every group studied. Despite the small amount of mRNA shared between the three GC groups contrast, some transcripts were expressed in a specific manner for each group. To identify all downregulated and upregulated transcripts direct comparisons between the three contrast were performed in the Venn

diagram (Figure 9). In addition to the number of probes found to have a positive signal, the modulation of the genes' expression also provides valuable information regarding the transcriptomic differences between the follicles collected at different postpartum periods. The transcript levels of 259 genes were significantly upregulated between the 90 d and 120 d condition, relative to 459 for the 60 d and 640 for the 30 d condition when hybridized against 120 d, indicating that the number of modulated genes is progressively lower as the postpartum period approaches the 120 d mark. For the downregulated genes, the pattern is slightly different whereby 132, 619 and 405 transcripts are significantly modulated when the 90 d, 60 d and 30 d samples are hybridized against the 120 d, respectively. Therefore, although the 60 d condition would be expected to have a number of downregulated that is intermediate between 132 and 405 genes, for 90 d and 30 d respectively, there seems to be a greater emphasis on downregulation at that intermediate period.

Most of the genes associated to the probes giving a positive signal presented in the Venn diagrams (Figure 9) are known to encode a protein or correspond to a sequence containing an opern reading frame (ORF) region. For each contrast, short lists of genes with the highest fold-change values are presented in Table 3 and 4 for upregulated and downregulated transcripts, respectively.

Table 3. Top of downregulated transcripts in each granulosa cell groups comparison in IPA software.

30 d vs 120 d			
Gene symbol	**Description**	**Fold Change**	**p-value**
TRIB2	Tribbles homolog 2 (Drosophila)	-18,615	0,00549
GATM	Glycine amidinotransferase (L arginine:glycine amidinotransferase)	-13,468	0,00023
ANKRD1	Ankyrin repeat domain 1 (cardiac muscle)	-12,674	0,0021
NTS	Neurotensin	-10,986	0,00094
GSTA1	Glutathione S-transferase alpha 1	-8,852	0,00203
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	-6,768	0,00215
60 d vs 120 d			
Gene symbol	**Description**	**Fold Change**	**p-value**
VNN2	Vanin 2	-73,717	0,0000704
UBD	Ubiquitin D	-37,548	0,000897
GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	-15,298	0,000161
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-13,260	0,000381
TIMP1	TIMP metallopeptidase inhibitor 1	-13,245	0,002764
90 d vs 120 d			
Gene symbol	**Description**	**Fold Change**	**p-value**
STAR	Steroidogenic acute regulatory protein	-4,185	0,007132
TUBA4A	Tubulin, alpha 4a	-3,661	0,000055
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	-3,390	0,000366
HSD17B11	Hydroxysteroid (17-beta) dehydrogenase 11	-2,391	0,004164

Table 4. Top of upregulated transcripts in each granulosa cell groups comparison in IPA software.

30 d vs 120 d			
Gene symbol	**Description**	**Fold Change**	**p-value**
SLC16A3	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	10,053	0,00056
MTPN	myotrophin	7,160	0,00030
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	6,844	0,00003
TDG	Thymine-DNA glycosylase	4,096	0,00001
PCYOX1	prenylcysteine oxidase 1	3,371	0,00064
60 d vs 120 d			
Gene symbol	**Description**	**Fold Change**	**p-value**
SEMA6D	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	13,509	0,003270
SLC16A3	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	6,267	0,002464
CREB3L2	cAMP responsive element binding protein 3-like 2	5,454	0,000104
VCAN	Versican	4,609	0,000009
UBR1	ubiquitin protein ligase E3 component n-recognin 1	3,583	0,000039
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	2,997	0,008070
90 d vs 120 d			
Gene symbol	**Description**	**Fold Change**	**p-value**
ARPC1B	Actin related protein 2/3 complex, subunit 1B, 41kDa	5,781	0,006584
SLC16A3	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	5,333	0,004255
CREB3L2	cAMP responsive element binding protein 3-like 2	3,709	0,000658
CALM1	Calmodulin 1 (phosphorylase kinase, delta)	2,785	0,002168

The Functions analysis in the IPA software calculates which biological functions show significant association with uploaded experimental dataset. In the 30 d vs 120 d function, the functions showing the most significant association with the examined gene list were: small molecule biochemistry (Heme Degradation, Arginine Degradation I, G Protein Signaling Mediated By Tubby), DNA replication, recombination and repair (Thrombin Signaling, CREB Signaling in Neurons, RAN Signaling) and cellular assembly and organization (Telomerase Extension by Telomerase, CCR3 Signaling in Eosinophils, Signaling by Rho Family GTPases) (Figure 10).

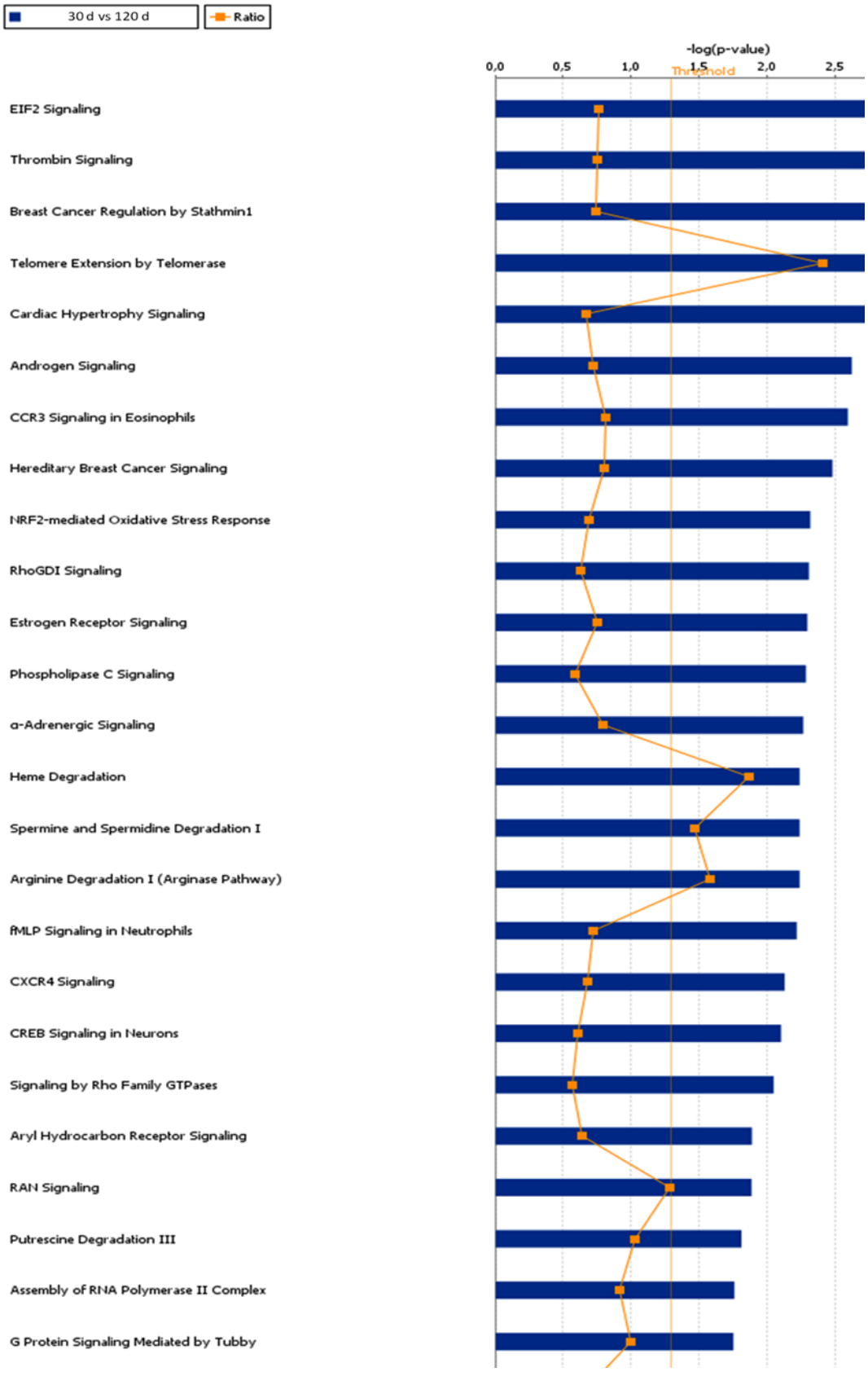


Figure 10. Most significant molecular and cellular functions showing a significant association (P < 0.05) to the 30 d vs 120 d contrast of GC samples as generated by Ingenuity Pathway Analysis (IPA).

The IPA functional analysis indicated that cell death and survival (IL-6 Signaling, NRF2-mediated Oxidative Stress Response, Role in Tissue Factor Cancer, Prolactin Signaling), cellular growth and proliferation (EIF2 Signaling, Cdc42 Signaling, TGF-β Signaling) and cell cycle (Phospholipase C Signaling, ERK/MAPK Signaling, Regulation eIF4 and p70S6K Signaling) were the cellular functions showing the greatest association with the 60 d vs 120 d dataset (Figure 11).

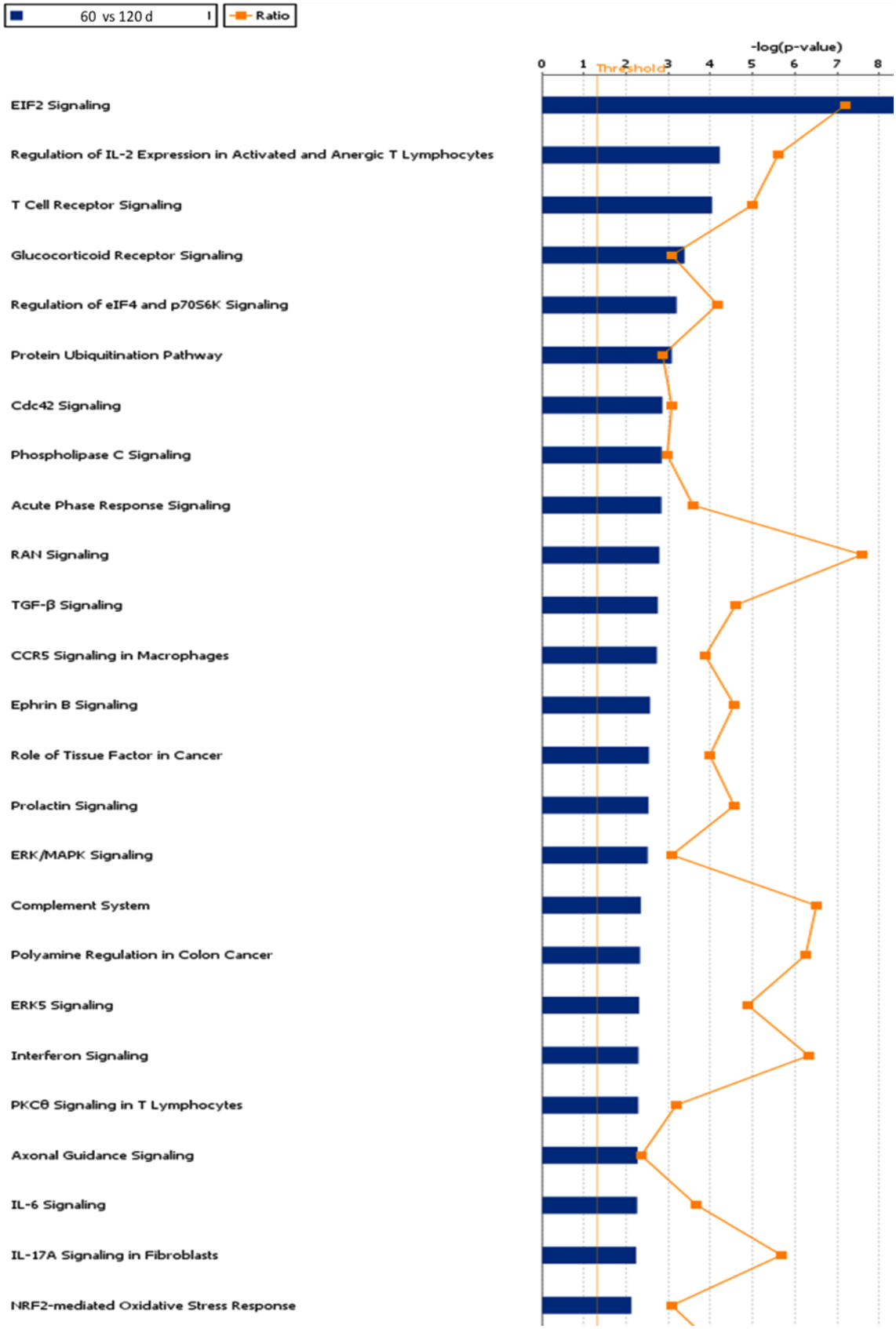


Figure 11. Most significant molecular and cellular functions showing a significant association ($P < 0.05$) to the 60 d vs 120 d contrast of GC samples as generated by Ingenuity Pathway Analysis (IPA).

In 90 d vs 120 d microarray comparison, the functions showing the greatest association were: gene expression (Spermine and spermidine degradation I, BMP signaling pathway, Androgen Signaling, EIF2 Signaling, Estrogen Receptor Signaling), cell-To-cell signaling and interaction (Insulin Receptor Signaling, Rac Signaling, Synaptic Long Term Potentiation, Glucocorticoid Receptor Signaling, α-Andrenergic Signaling) and cell cycle (CDK5 signaling, Ovarian Cancer Signaling, ERK\MAPK Signaling) (Figure 12).

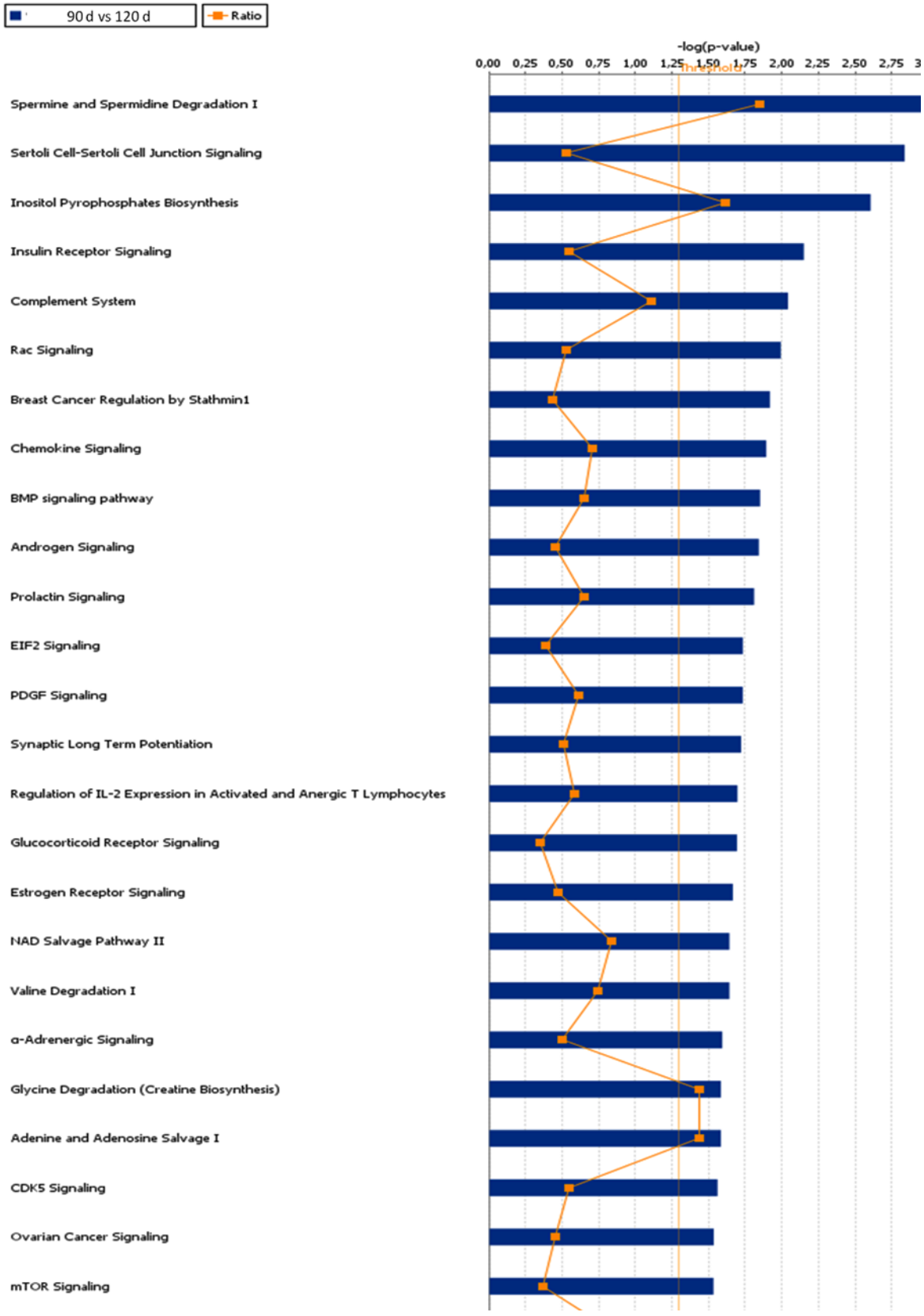


Figure 12. Most significant molecular and cellular functions showing a significant association (P < 0.05) to the 90 d vs 120 d contrast of GC samples as generated by Ingenuity Pathway Analysis (IPA).

Particular genes modulated between the 30 d and 120 d postpartum periods were characteristic of notable cellular functions including cellular organization and proliferation (KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; and MRAS, muscle RAS oncogene homolog; both upregulated in the cellular function), genome replication end cellular growth (up and downregulated POLR, Polymerase RNA II, family; GNAI, Guanine Nucleotide-binding Protein, Alpha-inhibiting Activity polypeptide 1; upergulated in cellular growth function) and molecule accumulation and biosynthesis (APOE, apolipoprotein E; upergulated in the molecule accumulation function and CYP11A1, cytochrome P450, family 11, subfamily A, polypeptide 1; down regulated in molecule biosynthesis function). Expression of gene markers associated to apoptosis (IL6, Interleukin 6 Receptor), steroidogenesis (NR3C1, Glucocorticoid Receptor; CYP19A1, cytochrome P450), ovarian cancer (VCP, valosin containing protein; MAF, v-maf musculoaponeurotic fibrosarcoma oncogene homolog) and the dowregulation of genes involved follicular development (MAPK, mitogen-activated protein kinase1; Cdc42, cell division cycle 42 and AP-1) characterize the main biological function in the 60 d vs 120 d contrast. In addition, the functions associated to the gene list resulting from the comparison of GC samples collected at 90 d postpartum were: cell cycle progression (JUN, jun proto-oncogene; KAT5, K(lysine) acetyltransferase 5; both upregulated in the function), cell proliferation (CDK11A/CDK11B, cyclin-dependent kinase 11B; NCOA1, nuclear receptor coactivator 1; both genes upregulated in the function) and cell interaction (PCYOX1, prenylcysteine oxidase 1; upregulated in this function).

Finally, Figure 13, represents for each main molecular and cellular function category the specific downstream effect functions in terms of predicted activation state (increased or decreased by the z-score value) related to each contrast by IPA. At 30 d relative to 120 d, decreased reduction of lipid function (z-score of -2) corresponds to the third hierarchical level, p value 1.41 E-02, of the first molecular and cellular function category: small molecular biochemistry; while in the second and third main molecular and cellular function category, respectively to DNA replication, recombination and repair, and cellular assembly and organization, the increased quantity of centrosome function (z-score of 2.19) was the fifth hierarchical level of downstream quantity function, p value 5.85 E-03, while related to the third function category

(cellular assembly and organization) it was represented in the second hierarchical level, p value 5.85 E-03, of downstream quantity effect.

At 60 d vs 120 d contrast the increased apoptosis of cancer cell (z-score of 2.337) shown in the second IPA hierarchical level (p value of 2.73 E-03), and the increased of cell death of cancer cell (z-score of 2.224) at the first IPA hierarchical level (p value 4.44 E-5), they were the main downstream effects related to the first main biological function category in the IPA samples contrast: cell death and survival (Figure 13). In addition, the second greatest molecular and cellular function category, cell growth and proliferation in 60d vs 120 d, was found related to the decreased downstream function: proliferation of cell. With a z-score of -2.575, this function was represented in the first hierarchical level (p value 1.8 E-06) in IPA software. Another greatest decreased downstream functions in 60 d relative to 120 d were: differentiation of cell (z-score of -2.332) and function of leucocytes (z-score of -2.190). These two functions were shown in IPA in the second hierarchical level for the first downstream function (3.02 E-03) related to the main forth molecular and cellular function category: cellular development; and in the first hierarchical level for function of leucocytes in relation to the last main category function, cell cycle, in 60 d vs 120 d contrast (Figure 13). No downstream function has been found particularly increased or decreased in the main molecular and cellular function related to 90 d vs 120 d.

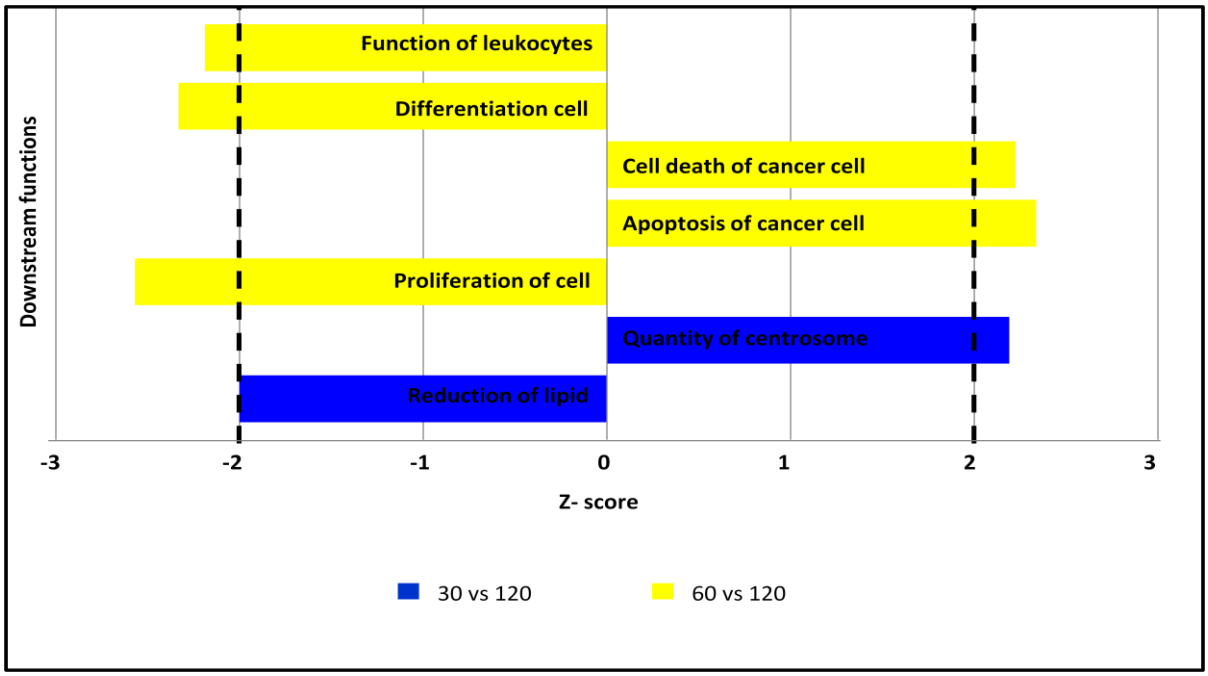


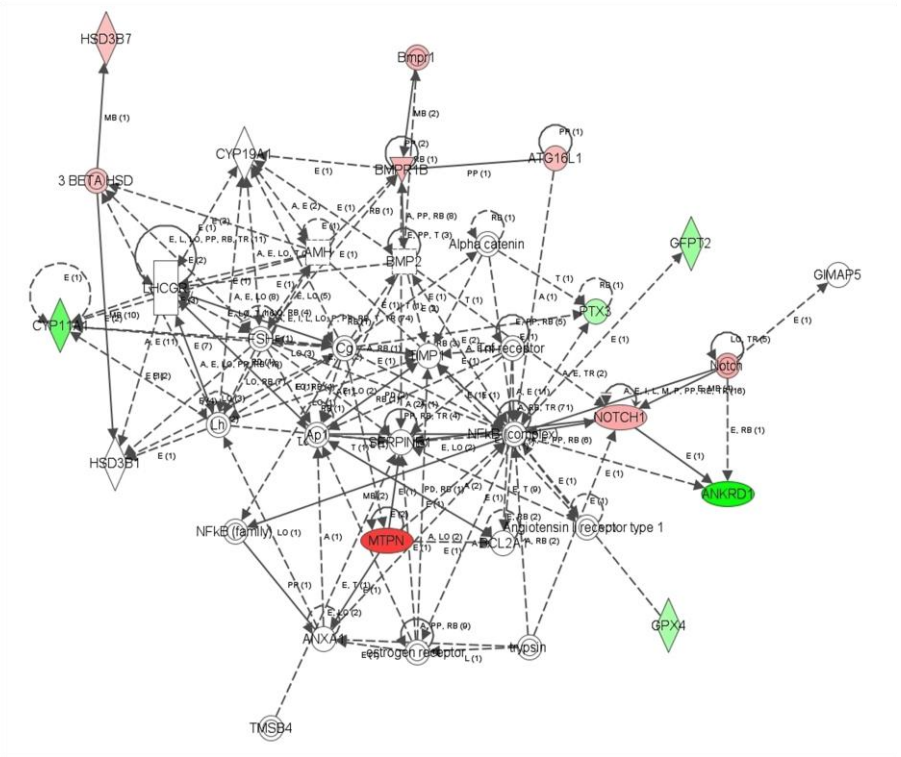
Figure 13. Graphical representation of downstream functions from IPA software's Molecular and Cellular Functions analysis in contrasts: 30 d vs 120 d and 60 d vs 120 d. Dashed black lines indicate the z-score threshold for statistical significance (-2.00 and +2.00). Only downstream functions with a z-score value > +2.0 were considered as being significantly increased in the GC samples, while values < -2 considered as decreased.

3.3 Transcriptor factors interactions: networks and pathways analysis

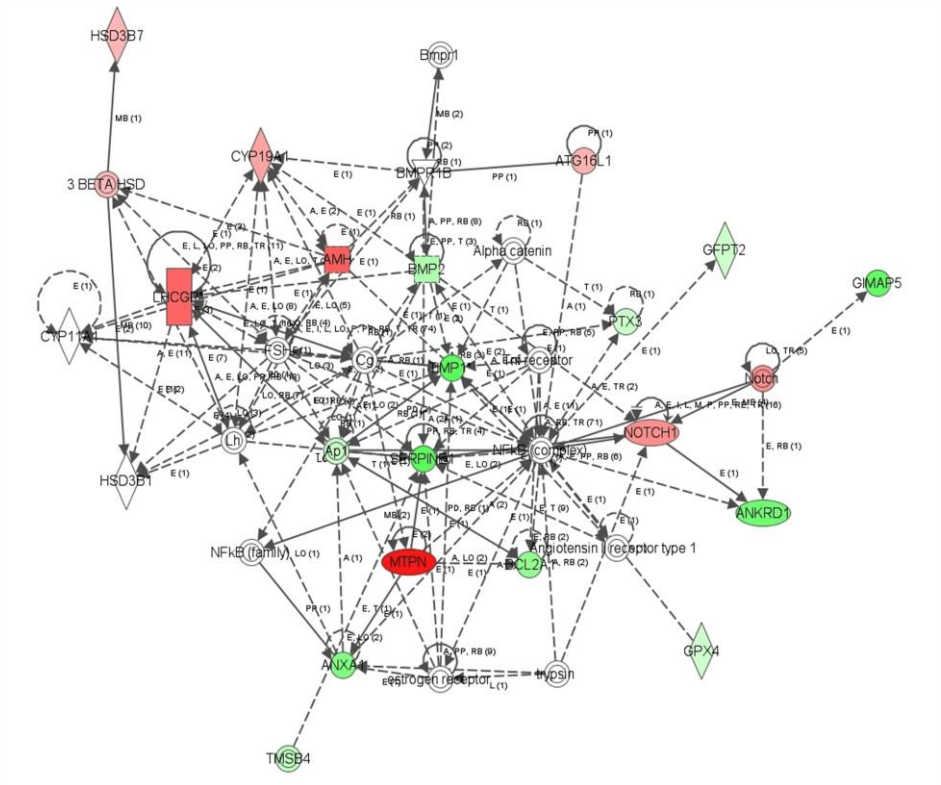
IPA-generated gene interaction networks showing the greatest association with the microarray results of all data contrasts combined together (30-60-90 vs 120) are shown in Figures 14, 15 and 16 and the gene modulation specific to single contrast overlap to the others combined together has been depicted in the A, B and C figures for the 30 d vs 120 d, 60 d vs 120 d and 90 d vs 120 d contrasts, respectively.

The first Network (Figure 14), with the highest score value at 48, assembled 21 molecules whose functions were involved in embryonic development, organ development and organismal development. The Gonadotropins LH and FSH, as well as the LH receptor LHCGR, hold central positions in the network, indicating they may play an important role in these observed genes interactions.

A


HSD3B7
Bmpr1
3 BETA HSD
CYP19A1
BMPR1B
ATG16L1
Alpha catenin
BMP2
GFPT2
GIMAP5
PTX3
CYP11A1
FSHR
Cg
BMP1
Notch
Lh
Ap1
NFkB complex
NOTCH1
HSD3B1
ANKRD1
NFkB (family)
MTPN
Angiotensin II receptor type 1
BCL2A1
GPX4
ANXA4
estrogen receptor
trypsin
TMSB4

B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Bmpr1
3 BETA HSD
CYP19A1
BMPR1B
ATG16L1
Alpha catenin
BMP2
GFPT2
GIMAP5
PTX3
CYP11A1
FSHR
Cg
BMP1
Notch
Lh
Ap1
NFkB complex
NOTCH1
HSD3B1
ANKRD1
NFkB (family)
MTPN
Angiotensin II receptor type 1
BCL2A1
GPX4
ANXA4
estrogen receptor
trypsin
TMSB4

C

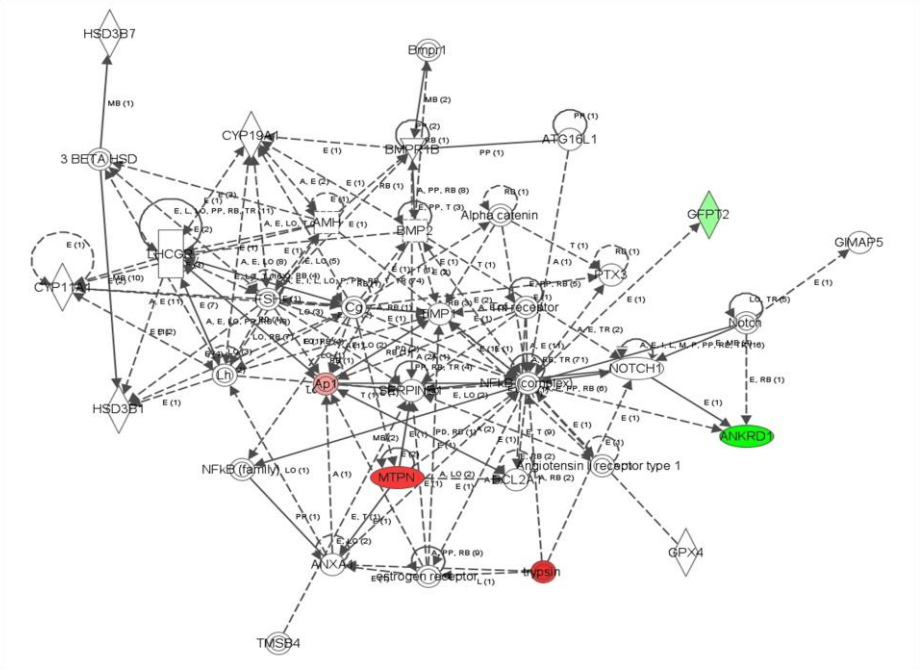
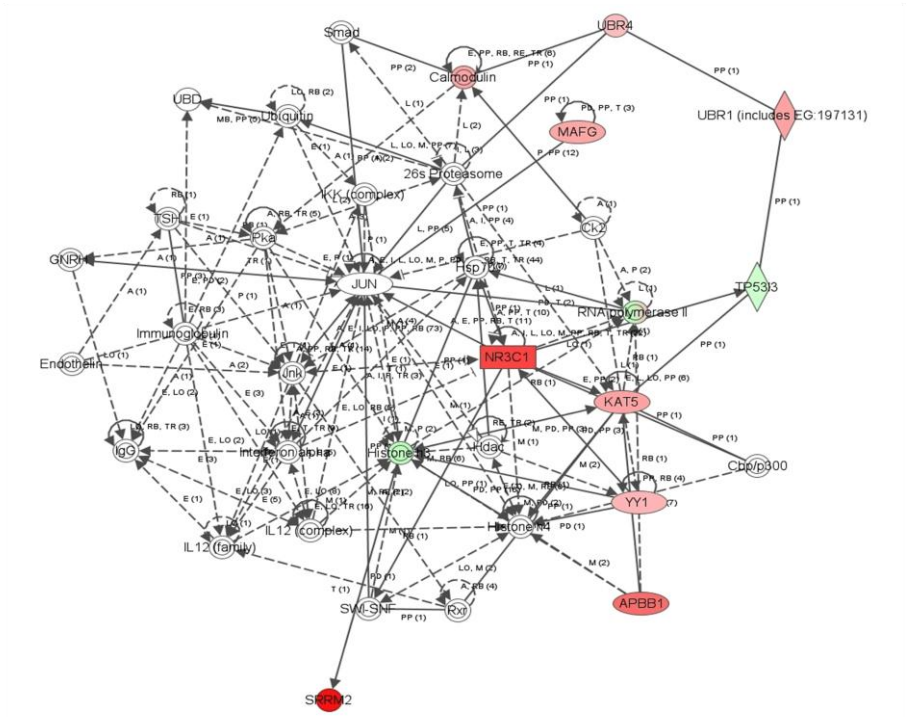


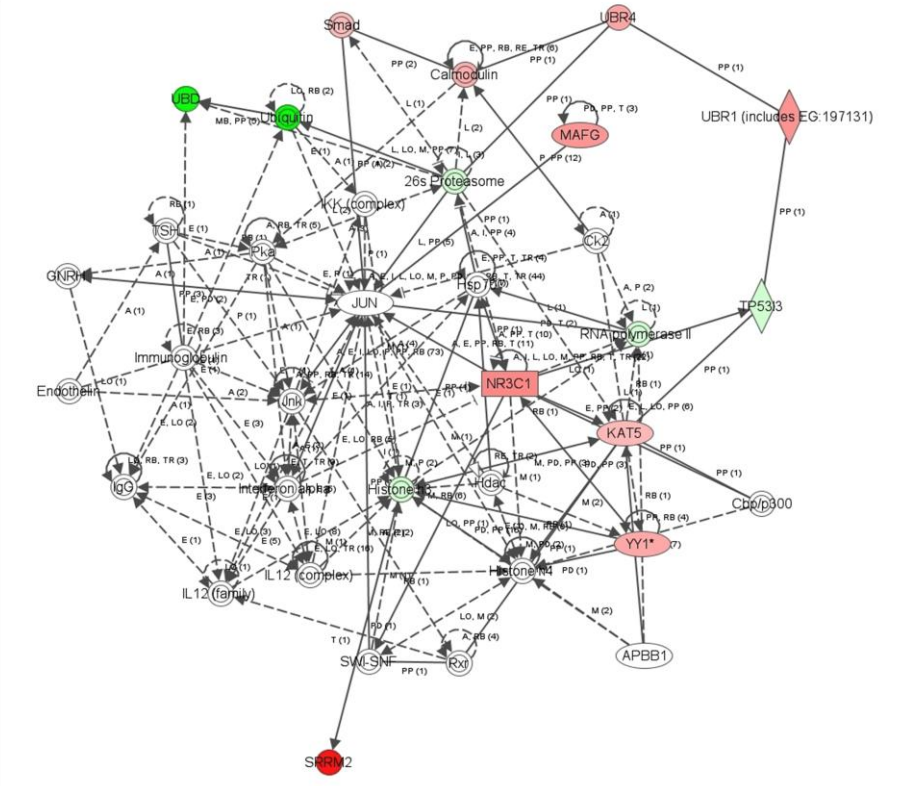
Figure 14. IPA First Network (Score value = 48). Differentially regulated genes in preovulatory follicles in dairy cows at 30 d, 60 d, 90 d and 120 d post partum. The node (gene/gene product) color intensity indicates the expression of genes: red is upregulated, green is downregulated in 30 d vs 120 d (A), 60 d vs 120 d (B) and 90 d vs 120 d (C). The networks are displayed graphically as nodes and lines (the biological relationship between nodes).

The second and third networks (Figures 15 and 16), both with a network score of 21, each contain 11 molecules. The central genes in the second networkwere JUN, KAT5, YY1(ubiquitous transcription factor Yin Yang 1) and NR3C1 (Glucocorticoid receptor) were the main genes involved in IPA pathway, and the top function associated to the network were: gene expression, infectious disease, gastrointestinal disease. Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function and maintenance were the functions associated to the third network (Figure 16) in which ERK1/2 (mitogen-activated protein kinase 3/1, MAPK3/1) plays a central role.

A


Smad
UBR4
Calmodulin
UBD
Ubiquitin
UBR1 (includes EG:197131)
MAFG
26s Proteasome
TSH
Pka
Ck2
GNRH
JUN
Hsp70
TP53I3
RNA polymerase II
Immunoglobulin
NR3C1
Endothelin
Jnk
KAT5
IgG
Histone H3
Hdac
Cbp/p300
YY1
IL12 (complex)
Histone H4
IL12 (family)
APBB1
SWI-SNF
Rxt
SRRM2

B


Smad
UBR4
Calmodulin
UBD
Ubiquitin
UBR1 (includes EG:197131)
MAFG
26s Proteasome
TSH
Pka
Ck2
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JUN
Hsp70
TP53I3
RNA polymerase II
Immunoglobulin
NR3C1
Endothelin
Jnk
KAT5
IgG
Histone H3
Hdac
Cbp/p300
YY1*
IL12 (complex)
Histone H4
IL12 (family)
APBB1
SWI-SNF
Rxt
SRRM2

C

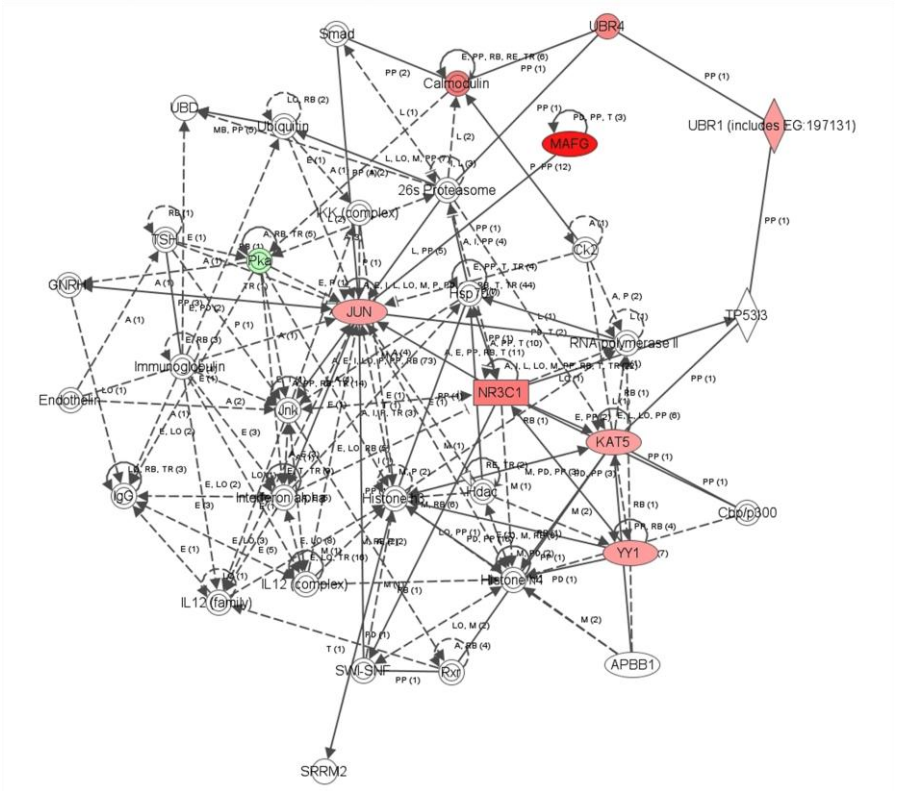
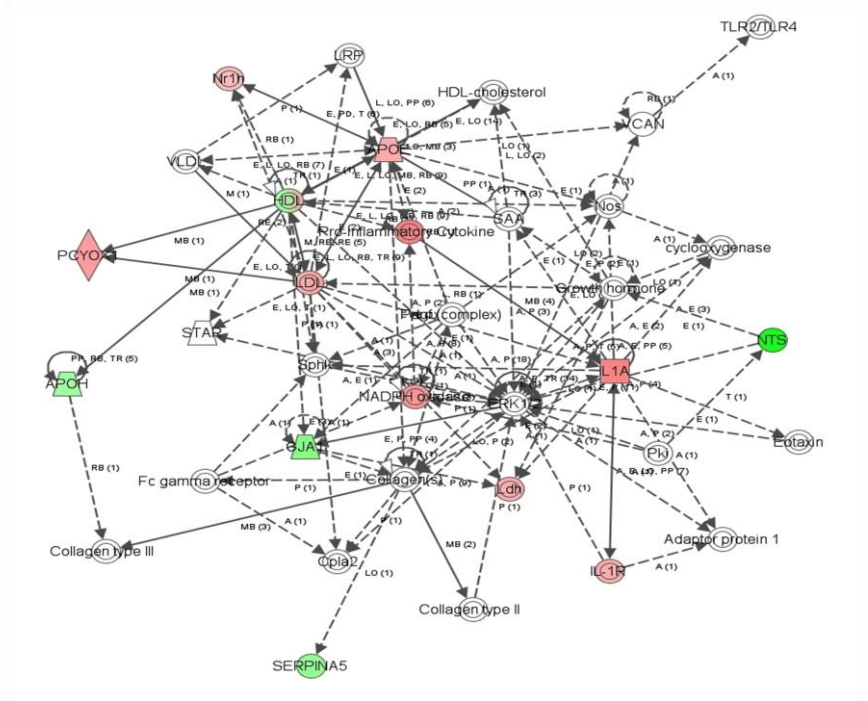
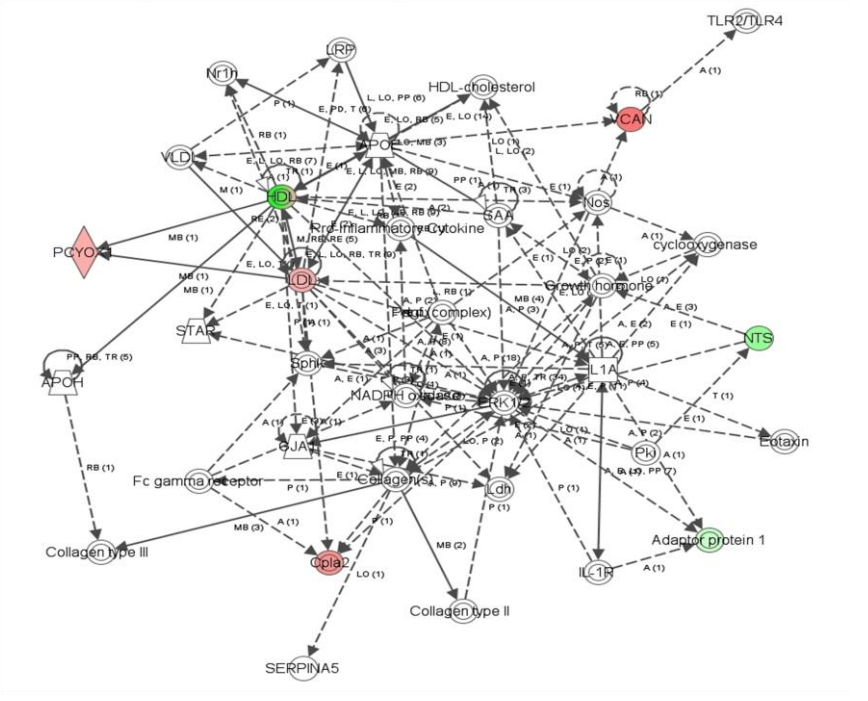


Figure 15. IPA Second Network (Score value = 21). Differentially regulated genes in preovulatory follicles in dairy cows at 30 d, 60 d, 90 d and 120 d post partum. The node (gene/gene product) color intensity indicates the expression of genes: red is upregulated, green is downregulated in 30 d vs 120 d (A), 60 d vs 120 d (B) and 90 d vs 120 d (C). The networks are displayed graphically as nodes and lines (the biological relationship between nodes).

A


TLR2/TLR4
LRP
Nr1h
HDL-cholesterol
VCAN
APOE
VLDL
HDL
Nos
SAA
Pro-inflammatory Cytokine
cyclooxygenase
PCYOX1
LDL
Growth hormone
Prdx1 (complex)
STAR
NTS
Sphk1
L1A
APOH
NADPH oxidase
ERK1/2
GJA1
Eotaxin
Pla2
Fc gamma receptor
Collagen(s)
Ldh
Adaptor protein 1
Collagen type III
Cpla2
IL-1R
Collagen type II
SERPINA5

B


TLR2/TLR4
LRP
Nr1h
HDL-cholesterol
VCAN
APOE
VLDL
HDL
Nos
SAA
Pro-inflammatory Cytokine
cyclooxygenase
PCYOX1
LDL
Growth hormone
Prdx1 (complex)
STAR
NTS
Sphk1
L1A
APOH
NADPH oxidase
ERK1/2
GJA1
Eotaxin
Pla2
Fc gamma receptor
Collagen(s)
Ldh
Adaptor protein 1
Collagen type III
Cpla2
IL-1R
Collagen type II
SERPINA5

C

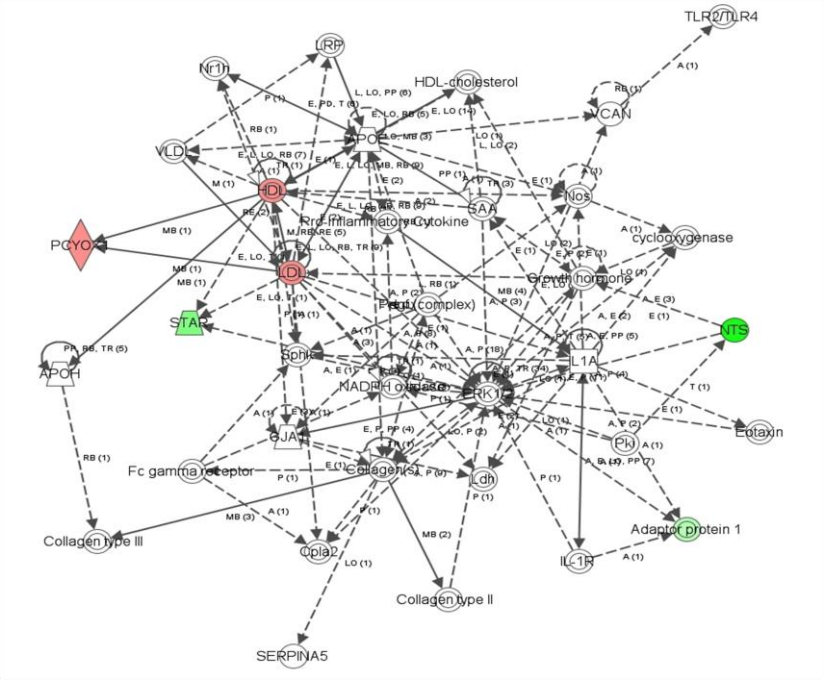


Figure 16. IPA Third Network (Score value = 21). Differentially regulated genes in preovulatory follicles in dairy cows at 30 d, 60 d, 90 d and 120 d post partum. The node (gene/gene product) color intensity indicates the expression of genes: red is upregulated, green is down regulated in 30 d vs 120 d (A), 60 d vs 120 d (B) and 90 d vs 120 d (C). The networks are displayed graphically as nodes and lines (the biological relationship between nodes).

3.4 qPCR analysis validation

Twentythree transcripts were chosen for qPCR validation in order to confirm their expression modulation observed in the microarray results (Table 1). The samples used for microarray hybridization were also used for qPCR validation as none of the few other samples collected in this study had acceptable RNA quality.

Of the 23 genes validated by qPCR, the qPCR results confirmed the gene expression modulation measured by microarray in less than 50% of the 23 genes validated. One-way ANOVA analysis performed between all microarray samples (30 d, 60 d, 90 d, 120 d) (Figure 17) and paired t-test analysis performed between the 60 d and 120 d conditions (Figure 18) show that the 60 d condition was usually the postpartum period in which GC of preovulatory follicle had significantly higher transcript levels relative to the other postpartum periods.

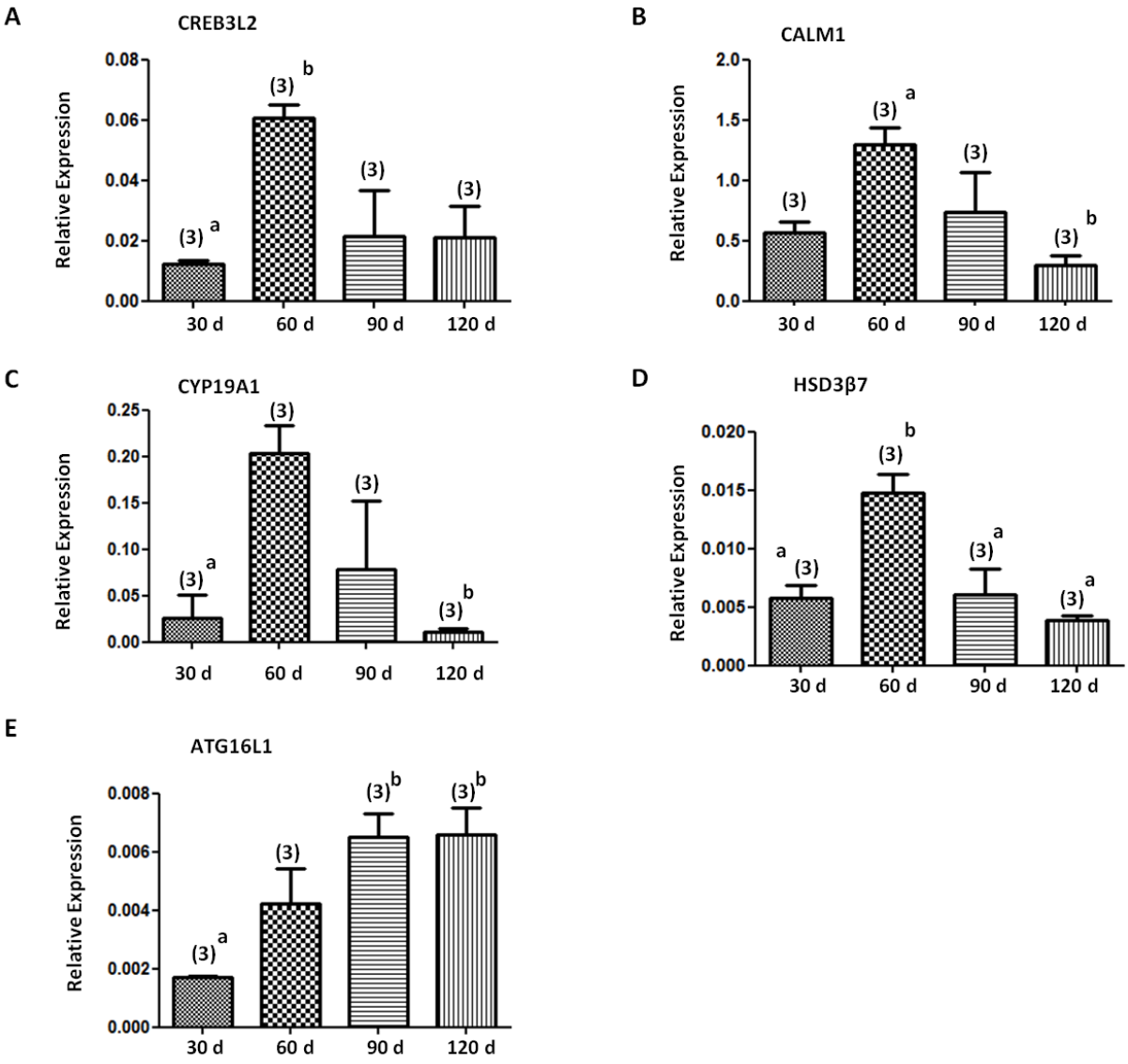


Figure 17. One-way ANOVA analysis of qPCR relative transcript expression of candidate genesTotal RNA was extracted from GC of preovulatory follicles at 30 d, 60 d, 90 d and 120 d post-partum in dairy cow. Expression of CREB3L2 (A), CALM1 (B), CYP19A1 (C), HSD3β7 (D) was higher in GC of sample collected at 60 d post-partum, while ATG16L1 (E) expression was higher and similar in GC samples at 90 d and 120 d post-partum. Different letters above bars denote samples that were significantly different ($P < 0.05$) when Turkey-Kramer multiple comparison test was performed. Data are presented as last-square means ± SEM, and the number of biological replicates per group is indicated in parentheses. Graphs genenarated with GraphPad Prism 5.02.

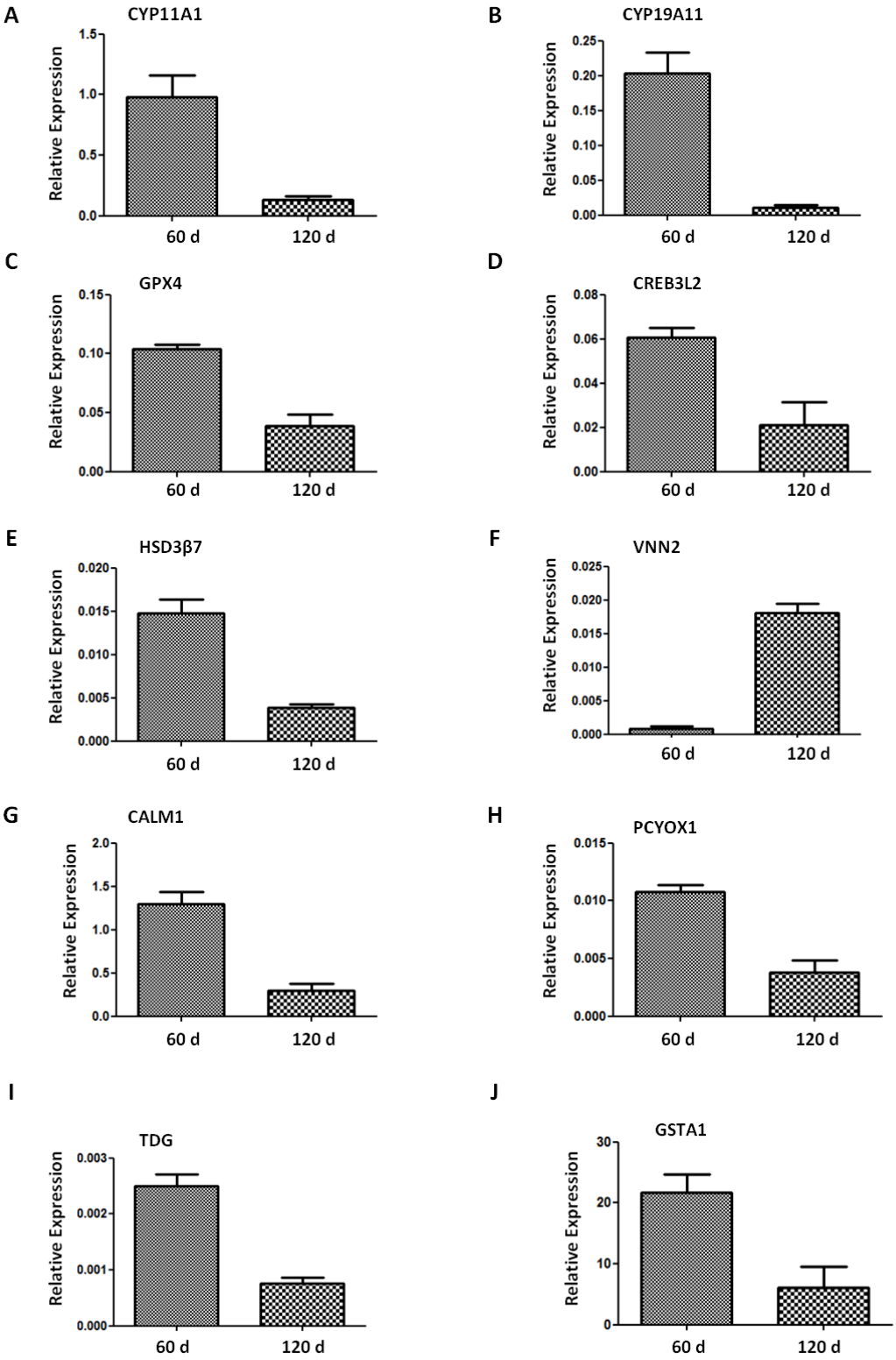


Figure 18. t-test analysis in qPCR relative transcript expression of candidate genes. Total RNA was extracted from GC of preovulatory follicles at 30 d, 60 d, 90 d and 120 d post-partum in dairy cow. Comparison of samples between 60 d and 120 d have shown statistically significant differences in transcript expressionfor around 50 % of total candidates selected (see Table 1) from microarray results. Expression of CYP11A1 (A), CYP19A1 (B), GPX4 (C), CREB3L2 (D), HSD3β7 (E), CALM1 (G), PCYOX1 (H), TDG (I) and GSTA1 (J) transcripts was higher in GC samples at 60 d than in GC samples collected at 120 days postpartum. An expression profile contrary to microarray results was detected for the VNN2 transcript (G). Graphs generated with GraphPad Prism 5.02.

The following 23 genes were selected for quantification by qPCR: ALDH16A1, ANKRD1, ATG16L1, CALM1, CREB3L2, CYP11A1, CYP19A1, GATM, GPX4, GSTA1, HSD3B7, HSD17B11, MAFG, MTPN, NTS, PCYOX1, SERPINE1, SLC16A3, TDG, TRIB2, UBR1, VCAN and VNN2 (Table 2). In 11 of the 23 genes: ATG16L1 , CALM1, CREB3L2, CYP11A1, CYP19A1, GPX4, GSTA1, HSD3B7, PCYOX1, TDG and VNN2 a significant difference found in at least one of the microarray contrast was corroborated by qPCR validation (Table 5).

Table 5. Representation of Microarray and qPCR results compared. FC: Fold Change; All comparisons: One-way ANOVA results with all qPCR samples (p < 0.05); ND: non-detected transcript in microarray analysis (FC= 2 and p value < 0.01); TNS: No significant difference in the relative expression in both the one-way ANOVA comparing all post partum periods or in the paired t-test comparing individual postpartum periods to the 120 d period (p value < 0.05).

Gene Symbol	Microarray results						qPCR Results				Concordance (C) or discordance (D) in microarray and qPCR results confront
	30 d vs 120 d		60 d vs 120 d		90 d vs 120 d		All comparisons	Paired t-test 30 d vs 120 d	Paired t-test 60 d vs 120 d	Paired t-test 90 d vs 120 d	
	FC	P value	FC	P value	FC	P value					
ALDH16A1	2,1	0,004	3,0	0,0003	ND	ND	TNS	TNS	TNS	TNS	
ANKRD1	-12,6	0,002	-11,2	0,0028	-9,4	0,004	TNS	TNS	TNS	TNS	
ATG16L1	2,2	0,0023	2,4	0,0014	ND	ND	30 d < 120 d 30 d < 90 d	TNS	TNS	TNS	C
CALM1	3,2	0,0009	2,7	0,0025	2,7	0,002	60 d > 120 d	TNS	60 d > 120 d	TNS	C
CREB3L2	2,3	0,0087	5,4	0,0001	3,70	0,00065	30 d < 60 d	TNS	60 d > 120 d	TNS	C
CYP11A1	-6,7	0,002	ND	ND	ND	ND	TNS	TNS	60 d > 120 d	TNS	D
CYP19A1	ND	ND	2,9	0,0080	ND	ND	30 d > 120 d	TNS	60 d > 120 d	TNS	C
GATM	-13,4	0,0002	-15,2	0,0001	-9,4	0,0006	TNS	TNS	TNS	TNS	
GPX4	-3,6	0,0007	-3,3	0,0012	ND	ND	TNS	TNS	60 d > 120 d	TNS	D
GSTA1	-8,8	0,002	ND	ND	ND	ND	TNS	TNS	60 d > 120 d	TNS	D
HSD17B11	ND	ND	-4,1	0,000168	-2,3	0,0041	TNS	TNS	TNS	TNS	
HSD3B7	2	0,001	2,2	0,0006	ND	ND	30 d < 60 d 60 d > 90 d 60 d > 120 d	TNS	60 d > 120 d	TNS	C
MAFG	3	0,0002	3,5	0,0001	5	0,00001	TNS	TNS	TNS	TNS	
MTPN	7,1	0,0003	8,4	0,0001	4,2	0,002	TNS	TNS	TNS	TNS	
NTS	-10,9	0,0009	-8,01	0,0023	-8,2	0,002	TNS	TNS	TNS	TNS	
PCYOX1	3,3	0,0006	2,6	0,002	2,3	0,006	TNS	TNS	60 d > 120 d	TNS	C
SERPINE1	ND	ND	-13,2	0,0003	ND	ND	TNS	TNS	TNS	TNS	
SLC16A3	10	0,0005	6,2	0,0024	5,3	0,004	TNS	TNS	TNS	TNS	
TDG	4,09	10E-5	3,1	0,00007	2,06	0,001	TNS	TNS	60 d > 120 d	TNS	C
TRIB2	-18,6	0,005	ND	ND	ND	ND	TNS	TNS	TNS	TNS	
UBR1	3,2	0,0001	3,5	0,00003	2,06	0,002	TNS	TNS	TNS	TNS	
VNN2	ND	ND	-73,7	7,04E-05	ND	ND	TNS	TNS	60 d < 120 d	TNS	C
VCAN	ND	ND	4,60	9,76E-06	ND	ND	TNS	TNS	TNS	TNS	

qPCR validation also provided additional information by allowing a comparison of transcript expression levels between conditions whose samples had not been hybridized against one another on the microarrays. For example, ATG16L1 was found to be more highly expressed in the 120 d condition relative to the 60 d, as were observed on the array (Table 5), but in addition, it was found to also be more highly expressed in the 90 d condition relative to the 30 d condition (Figure 17 E). For the CYP19A1 gene, array results indicated that transcript levels were significantly higher in the 60 d condition when compared to 120 d (Table 5) and

qPCR validation confirmed that result in a paired t-test between those two condition, however, the one-way ANOVA comparing all conditions only found a significant different between 30 d and 120 d. See the results for the other genes in Figures 17 C, 18 B and table 5.

For 3 genes: GSTA1, GPX4 and CYP11A1, the significant differences measured by microarray were in discordance (Table 5) with significant differences found in the qPCR analysis (Figure 18 L, C and A). While the array results indicate that the transcript levels for these increased with a longer postpartum period, as indicated by lower levels in 30 d relative to 120 d for all three genes, in addition to lower levels in 60 d relative to 120 d for GPX4 (Table 5), the qPCR analysis showed that in fact transcript levels were higher in the 60 d period compared to the 120 d period post calving. 7 other genes also had higher transcript levels in 60 d relative to 120 d when compared by one-way ANOVA or paired t-test: ATG16L1 (Figure 17 E), CALM1 (Figure 17 B and Figure 18 G), CREB3L2 (Figure 17 A and Figure 18 D), CYP19A1 (Figure 17 C and Figure 18 B), HSD3B7 (Figure 17 D and Figure 18 E), PCYOX1 (Figure 18 H), TDG (Figure 18 I).

Discussion

NEB may begin prepartum in association with declining feed intake. During the first 3 weeks of lactation, NEB delays early ovulation, recovery of postpartum reproductive function and provides the major nutritional link to low fertility in lactating dairy cows (Harrison et al., 1990).

This study aimed to understand what happens in the preovulatory follicle during the progressive recovery from the NEB condition in dairy cows, and to determine the approximate period at which the animals had recovered normal ovarian function. This information could be useful for to reduce the prolonged postpartum anoestrus period, conditioning the cow to be ready for the breeder's requirements.

The use of samples collected at 120 d postpartum as a positive control in the microarray analysis appears to have been warranted as demonstrated by their separate clustering in the HCA. This indicates a potential recovery at 120 d for all animals, while one of the 90 d sample from one animal is clustering with two of the 30 d samples, suggesting that its gene expression profile, even at 90 days postpartum, still corresponds to impaired ovarian function (Figure 8). The Venn diagrams of positive signals and of significantly up- and down-regulated genes (Figure 9) give additional details as to the modulation of gene expression between respective conditions. Next analysis on these Venn results should be addressed knowing whose are the positive signals common in each group condition in such a manner to evaluate the similarity in each samples condition.

According to the IPA analysis, various cellular and molecular functions were statistically significant in their association with the modulation of gene expression observed between 120 d and the other periods post calving (Figure 10, Figure 11 and Figure 12). Within the Small Molecule Biochemistry function category, the first of the main molecular and cellular function category affected in the 30 d vs 120 d contrast, the specific reduction of lipid downstream reduction function (Figure 13), was evaluated as being decreased (z-score of -2) when the fold change and effect of modulated genes (Glutathione peroxidase 4, GPX4, Glutathione S-transferase alpha 4, GSTA4, Peroxiredoxin 2, PRDX2, Prostaglandin reductase 1, PTGR1, all downregulated) on that function were integrated by IPA. Consequently, this could be associated with an increase in the accumulation of mitochondrial lipid drops in GCs at 30 d, derived from enzymatic activity involved in

progesterone biosynthesis, in part linked to the higher transcript levels of HSD3β7 at 30 d relative to 120 d (Figure 14 A). Progesterone biosynthesis normaly starts in the preovulatory follicle of the bovine ovary prior to ovulation (Smith *et al*., 1994), exactly that happened in our sample collected at 30 d.

In the 30 d vs 120 d contrast, quantity of centrosome, was predicted to be increased with a z-score of 2.19 (Figure 13). It is a function within the second and third most significant molecular and cellular function categories associated to the IPA molecular and cellular function for 30 d relative to 120 d: DNA replication, recombination and repair and cellular assembly and organization. Some of the gene in the dataset reported to have an effect on the quantity of centrosome function were: Charge multivesicular body protein 3 (CHMP3), Growth arrest and DNA-damage-inducible alpha (GADD45A), Nucleophosmin (NPM1) and Vacuolar protein sortin 4 homolog B (VPS4B), which were all found to be downregulated by microarray, while Homeobox A7 (HOXA7) was upregulated, according to microarray. Microtubules of interphase cells are composed of helical arrays of repeating heterodimers of tubulin subunits that arise from an organizing center: the centrosome. These structures are involved in the activity of steroidogenesis, mainly to deliver the cholesterol (e.g., contained within lipid droplets) to mitochondria (Murdoch W.J., 1996). Thus the increase in centrosome quantity could also be related to the activation of progesterone biosynthesis.

The modulation of biological functions between the 60 d and 120 d periods (Figure 11), suggest that the cells are probably affected by some abnormalities such as the inhibition of cell proliferation and differentiation, the occurrence of events linked to apoptosis and the presence of certain disease markers and functions. How just mentioned in the results, decreased proliferation of cell (z-score = -2.57), increased apoptosis of cancer cell (z-score = 2.33) and cell death of cancer cells (z-score = 2.224) (Figure 13), were the downstream effect functions from the cellular growth and proliferatin and cell death and survival general function categories significantly associated to the 60 d vs 120 d dataset. Among the gene list transcript associated to the regulation of these functions are : v-myc myelocytomatosis viral oncogene homolog (MYC), mitogen-activated protein kinase 13 (MAPK13), activator protein-1 (Ap-1) and JUN, which were all downregulated in 60 d relative to 120 d, as per microarray results. In particular, MYC regulates cell cycle progression and cell proliferation and tumor cell invasion functions. c-Myc can stimulate cyclin D2, which was also downregulated at 60 d relative to 120 d on the microarray (data not shown), and cyclin-dependent

kinase 4 expression, both of which promote cell cycle progression (Pelengaris et al., 2002). Moreover MYC regulates the expression of MAPK and activation of AP-1 dependent transcription (Wang et al., 2012). MAPK and phosphoinositide 3'-OH kinase (PI3K) pathways, are important in the protection of various cell types from apoptosis. IL-6 Signaling in IPA canonical pathway at 60 d relative to 120 d (Figure 11), shows (pathway not reported) the expression of p38 MAPK downregulated per microarray results. Ricardo et al., (2000), reported that p38MAPK played a critical role in the activation of p53, wich was the first tumor suppressor gene linked to apoptosis and it was clearly established as a checkpoint protein involved in cell-cycle arrest and maintaining genomic integrity following DNA damage (Yonish-Rouach et al., 1991). The lacking detection of p53 expression in our microarray IPA analysis at 60 d relative to 120 d, have seen to delays and inhibits the mechanisms regulating cell cycle progression in GCs of preovulatory follicles (Sherr and Roberts, 1999). Always at 60 d vs 120 d contrast, cellular development and cellular function and maintenance contain specific downstream functions which have been evaluated as decreased by IPA , such as differentiation of cells (z-score= -2.332) and function of leukocytes (z-score = -2.19) (Figure 13). These functions are necessary the GC of the preovulatory follicle as they are related to differentiation occurring in the corpus luteum cells and the protection against the invasion of inflammatory cells in preovulatory follicle (Espey and Richards, 2006; Richards et al., 2008). The functions of genes like: AMH, ATG16L1, BCL2, BMP2, CYP19A1, YY1, KAT5, IL1A, NR3C1 and VNN2, associated to these downstream effects are better explained in the IPA networks (Figure 14, 15 and 16) discussion below.

As presented in the IPA network with the highest score, HSD3B7 is upregulated at 30 d relative to 120 d (Figure 14 A) (according to microarray results, this difference is not found to be significant in qPCR validation) (Figure 17 D), which is associated to progesterone synthesis (Zelieann et al., 2009), and has an inhibitory action on the expression of the LH receptors (LHCGR) (Williams et al., 1982 ; Azzazi et al., 1983; Troxel et al., 1980; Troxel and Kesler, 1984c). Indeed, LHCGR expression was not detected on the 30 d vs 120 d microarray (Figure 14 A). At 60 d however, both HSD3B7 (confirmed by qPCR) and LHCGR (microarray, not confirmed by qPCR) (Figure 14 B) are upregulated relative to transcript levels at 120 d. In addition, transcripts of CYP19A1 and CYP11A1 are also shown to be higher in 60 d GC relative to 120 d GC

according to qPCR validation. (Figure 18). This reflects the favorable effect of CREB3L2 activity (Figure 17 A , Figure 18) and presence of LH surge (Rhodes et al., 2001), which increase the LHCGR mRNA (Luo et al., 2003) in the granulose cell and consecutively the synthesis of pregnanolone (CYP11A1), progesterone (HSD3β7) (Zelieann et al., 2009) and estradiol (CYP19A1) (Jones et al., 2005). Peters *et al.*, (1983), have described these conditions in the cow's corpus luteum, whereas Bao et al., (1997) and Xu et al., (1995), showed and attributed the high level of LHCGR mRNA expression in early stages of follicle development. However, the steroidogenesis mechanism in preovulatory GCs has a gene expression profile that differs from the one found in dominant follicles and in the luteal stage. Studies recently conduced in this field suggest that these transcripts of HSD3β7, CYP11A1, CYP19A1, LHCGR and Steroidogenic acute regulatory protein (STAR) are dowregulated in preovulatory follicles and highly expressed in luteal cell (Conley *et al.*, 1995; Lenz *et al.*, 2004). These results are in accordance with part of the data obtained in the current study. In the comparison of the 90 d vs 120 d periods, steroidogenic activity appeared to be low in 90 d GC based on the downregulation of STAR (Figure 16 C) and the absence of other steroidogenesis-related transcript on the microarray (Figure 14 C) or their low expression in qPCR ANOVA analysis (Figure 17 C and D).

In GC samples collected at 60 d compared to 120 d, the expression of steroidogenic genes was upregulated in microarray analysis (Figure 14 B) and more highly expressed in qPCR ANOVA analysis (Figure 17 A, C, D), especially in qPCR t-test statistical analysis (Figure 18 A, B, D and E). In experiments conducted by Guraya *et al.*, (1994), pycnotic granulosa cells, a carectiristic of apoptosis and follicular atresia stage, have a high expression of the HSD3β7 transcript, suggesting an increased steroidogenic activity. This concords with what is observed in the follicles collected at 60 d postpartum in the current study.

Calmodulin (CALM) was found to be involved in the regulation of granulosa cell steroidogenesis by both LH and FSH in early follicular development (Carnagie et al., 1984). Contrary, in our qPCR validation, the highest levels of CALM1 transcripts have been found in preovulatory follicles collected at 60 d from the partum in dairy cow (Figure 17 B, Figure 18 G).

Anti-Müllerian hormone (AMH) is an endocrine marker of a growing follicles population in all studied species. Its expression has been shown to be highest in granulosa cells of preantral and small antral follicles

and to decrease during terminal follicular growth as well as during atresia in rat (Baarends et al., 1995) and cow (Rico et al., 2009). AMH expression was recently shown to be upregulated by bone morphogenetic protein 6 (BMP6), an oocyte-derived factor, in cultured human granulosa/luteal cells (Voutilainen et al., 1987), suggesting that factors of the BMP family may participate in the regulation of *AMH* expression in follicles. In vitro, neither luteinizing hormone (LH) nor FSH have an effect on AMH expression by human granulosa cells from normal ovaries, but LH enhances whereas FSH inhibits AMH production by cells from polycystic ovaries (Thomas et al., 2007). These similar gene interactions were represented in the IPA network with the highest network score (Figure 14 B). Interpreting this information in light of other gene interaction appearing in network (Figure 14 B), such as downregulation of the BCL2A1 proteins, an antiapoptotic factors (Yuan et al.,2000), downregulation of SERPINE1 and TIMP1, ovulatory follicular markers (Rao JU et al., 2011), and the upregulation of versican transcripts (Figure 16 B), VCAN, mainly express in dominant cow follicles and not in preovulatory cow follicles (Fayad et al., 2004), suggests that that the 60 d follicles are not characteristic of the normal preovulatory follicular stage. However, it is important to note, that these are not absolute RNA levels, but levels relative to the 120 d post partum period.

Ovulation is characterized by the recruitment and invasion of inflammatory cells, such as leukocytes (neutrophils and macrophages) that can be regulated by cytokines which are secreted by and act on immune cells and ovarian cells (Norman and Brannstrom, 1994). In Figure 16 A IPA network indicates gene modulation between 30 d and 120 d, is the most characteristic of the preovulatory inflammation stage relative to other postpartum periods studied. Cytokines were upregulated and regulate interleukin 1A, IL1A, which synthesizes interleukin-8 (IL-8), a potent neutrophil chemoattractant or chemokine (Arici et al., 1996) that would lead neutrophils and macrophages to the ovulatory follicles. Furthermore, NR3C1 (Nuclear Receptor Subfamily 3, group C, member 1) more highly expressed at 30 d relative to 120 d (Figure 15 A) than at 60 d or 90 d (Figure 15 B and C). NR3C1 has been reported to be expressed in presence of a low number of macrophages cells and high number of cytokines cells in the ovary (Penny et.al., 1999). But, autophagy related 16-like 1, ATG16L1, in qPCR analysis (Figure 17 E) contrary to microarray data (Figure 14

A), had a higher transcript expression of in the 90 d vs 120 d group comparison, even if NR3C1 was found to be upregulated in the IPA network in Figure 14 B. Overexpression of NR3C1 has been suggested to be involved in the regulation of inflammatory responses (Gougat et al., 2002). More functions were evaluated to be regulated by NR3C1 in Figure 15 C in GCs at 90 d vs 120 d. The interaction with the Activator protein (Ap-1) transcript (upregulated only at 90 d vs 120 d), specifically with its protein members JUN (Jun oncogene) and MAFG (v-maf musculoaponeurotic fibrosarcoma oncogene homolog G), regulates the expression and function of NR3C1 that were directly involved in the control of YY1 (Yin Yang 1) and KAT5 (histone deacetylase) transcript expression. Upregulation of the ubiquitous transcription factor Yin Yang 1 (YY1) activity was increased by its expression and its interaction with the histone deacetylase KAT5 (Figure 15 C) in normal biologic processes such as embryogenesis, differentiation, replication and cell proliferation (Shi et al., 1997). Some studies have confirmed these effects of YY1 expression. Gordon et al., (2006), have performed comprehensive experiments evaluating the putative role of YY1 in regulation of tumorigenesis, inflammatory response, cell death, and apoptosis by the interaction with cell cycle signaling (Cdc), which was found to be upreguleted in the GC of follicles collected at 90 days post partum and not in samples collected at 60 d and 30 d (Figure 12), as mentioned earlier in the discussion.

Vanin2 (VNN2), function may participate in leukocyte trafficking in human (Suzuki et al., 1999). The lower expression of VNN2 in qPCR analysis at 60 d relative to GCs sample collected at 120 d (Figure 18 F) and the absence of some transcripts involved in inflammatory response (Figure 16 B) may indicate these samples were not yet in the preovulatory follicular stage.

Negative energy balance (NEB) in the dairy cow's postpartum period, the inflammatory reaction during ovulation and steroidogenis exposes the animal to an increased level of reactive oxygen species (ROS) inducing oxidative stress in the body. These negative effects in the cells are countered by detoxification systems such as the superoxide dismutase (SOD), an aldo-keto reductase with an enzymatic activity using NADPH to reduce lipid carbonyl (HDL and LDL) steroidogenic groups to alcohol (Figure 16 A). Glutathione peroxidase 4 (GPX4) and prenylcysteine oxidase 1 (PCYOX1) transcripts involved in detoxification activities have been downregulated (GPX4) (Figure 14 A and B) and upregulated (PCYOX1) in the collected GC of various periods according to the microarray analysis (Figure 16 A, B and C). qPCR analysis have confirmed

(Table 5) the highest expression of the PCYOX1 (Figure 18 H) transcript only in the sample collected at 60 d and contrary (Table 5) to the microarray analysis, qPCR validation of GPX4 and glutathione s-transferase A1 (GSTA1) showed a higher expression in samples collected at 60 d than in samples collected at 120 d (Figure 18 C and J). The presence of the transcripts of these detoxification factors (GPX4, GSTA1 an PCYOX) in GC sample collected at 60 d from partum in dairy cow, may be associated with a high steroid metabolism in the follicle (Figure 14 B) which has not been detected in the studies conducted by Conley *et al.*, 1995 and Lenz *et al.*, 2004.

The Thymine-DNA glycosylase (TDG) forms a physical and functional complex with transcriptional coactivators CBP and p300 (CBP/p300), directly linking chromatin modifying activity and DNA repair (Marc Tini et al. 2002). Severe NEB in dairy cow reduced the expression of TDG in liver cells during the early postpartum period (McCarthy S.D., 2010), instead in our case, in 60 d vs 120 d contrast the high TDG expression (Figure 18 I) could be due to the attenuate NEB condition from the first day from the partum or due to the presence of ROS produced during an increased steroidogenic activity which may damage nucleic acids (Junichi et al., 2005) .

Miotrophin (MTPN) is upregulated in all three conditions evaluated by microarray (Figure 14 A, B and C) and even more so at 60 d. This indicates the same functional roles of MTPN protein in follicular development and corpo luteum formation as in rat (Taoka et al., 1992). Asakai et al., 1993 have explained that upregulation of MTPN protein expression during CL formation in rat was due to an increased progesterone synthesis as seen in the developing CL. This could be explein the strong upregulation of this transcript in the samples collected at 60 d.

Taken together, the results and finding of the current study lead to the conclusion that microarray analysis was a useful and valid method for the study of the different gene expression profiles in preovulatory follicles collected in dairy cow at different time post partum. In addition, the validation of microarray results by qPCR, and the analysis of the different gene pathways and molecular and cellular function by IPA software, have contributed to characterizing and distinguishing the samples collected at 30 d, 60 d, 90 d and 120 d postpartum. IPA has revealed different gene pathways and molecular and cellular functions

modulated in samples collected at 30 d and 90 d relative to sample collected at 120 d, characterizing these two periods as having a GC gene expression profile characteristic of preovulatory follicles. However, that same analysis also accentuated that samples from the 90 d group were most similar to samples from the 120 d group, supporting the HCA (Figure 8). Follicles from the 60 d period, however, contained GC which had a gene expression profile characteristics of cells that are dying, diseased and in which there is an arrest of cellular proliferation, distinguishing the 60 d group from the other postpartum periods.

Subsequent studies should aim to understand which molecular mechanisms or external factors negatively influence ovarian development during the time interval between the 30 d and 60 d postpartum periods in dairy cow. These experiments could be useful in reducing and avoiding these negative effects in the animals' body during the anoestrus period and consequently increase the reproductive success of dairy cows.

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