ANA PAULA MATTOSO MISKULIN CARDOSO

Mecanismo de ação do 17β-estradiol no corpo lúteo de cadelas não prenhes

São Paulo

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Tese apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

Departamento:

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Orientador:

Profa. Dra. Paula de Carvalho Papa

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CERTIFICADO DA COMISSÃO DE ÉTICA

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "Efeitos do 17β-estradiol no processo de luteólise e resistência insulínica em cadelas não prenhes durante o diestro", protocolado sob o nº 2719/2012, utilizando 16 (dezesseis) cães, sob a responsabilidade da Profa. Dra. Paula de Carvalho Papa, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 15/8/2012.

We certify that the Research "Effects of 17β-estradiol in the luteolysis process and insulin resistance in non-pregnant bitches during diestrus", protocol number 2719/2012, utilizing 16 (sixteen) dogs, under the responsibility Profa. Dra. Paula de Carvalho Papa, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 8/15/2012.

São Paulo, 5 de outubro de 2012.

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DEDICATÓRIA

À Deus por ter me concedido paciência e força durante esses anos

Aos meus filhos, Guilherme e Maria Laura, por sempre me apoiarem e me encorajarem

Aos meus pais por sempre me incentivarem

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"Perder tempo em aprender coisas que não interessam, priva-nos de descobrir coisas interessantes"

(Carlos Drummond de Andrade)

RESUMO

CARDOSO, A. P. M. M. **Mecanismo de ação do 17β-estradiol no corpo lúteo de cadelas não prenhes.** [Action mechanism of 17β-estradiol in the corpus luteum of non-pregnant bitches]. 2016. 84 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

O corpo lúteo (CL) canino é responsável pela síntese de E2 durante o diestro. O E2 atua de forma autócrina e/ou parácrina sobre essa glândula. O mecanismo de atuação do E2 depende da razão entre receptor alfa (ERα) e receptor beta (ERβ). A ligação ao ERα tem efeito proliferativo e ao ERβ antiproliferativo. O objetivo deste trabalho foi entender a sinalização mediada pelo ERα e ERβ no processo de formação e regressão do CL. CLs foram obtidos de cadelas não prenhes (n=30) nos dias 10, 20, 30, 40, 50 e 60 (n=5/grupo) após a ovulação (po). No dia da ovariossalpingohisterectomia foi colhido sangue para mensuração das concentrações de P4 e E2. Dezoito CLs (n=3/grupo) foram submetidos ao sequenciamento de RNA (RNAseq). Os genes diferencialmente expressos (DE) identificados pelo RNAseq foram submetidos ao software oPOSSUM3 para identificação das regiões de ligação mais representadas nos fatores de transcrição relacionados aos genes do ERα (ESR1) e ERβ (ESR2). A análise de expressão temporal revelou a presença de 5.116 diferencialmente expressos (DE) em pelo menos uma comparação e 1.106 não foram anotados ainda no genoma canino, destes genes DE 295 apresentavam regiões de ligação de fatores de transcrição em comum com ESR1 e ESR2. Dez genes DE foram selecionados para validação dos resultados de RNAseq através do qPCR; usando o GAPDH como gene de referência. Desses genes, quatro apresentaram regiões em comum com ESR2 (LEF-1; PAPPA; NDGR2*;* ATP1A1*)* e um com ESR1 *(*CAV1) e os demais controlam proliferação celular (CTNNB1; CCND1; IGFBP 3, 4 e 5). A expressão proteíca de PAPPA e IGFBP 3, 4 e 5 (componentes do sistema IGF) foi avaliada também por imuno-histoquímica. Durante a primeira metade do diestro, nossos resultados indicam que a sinalização mediada pelo E2 ocorre via interação do ERα com CAV-1 (sinalização não genômica), ativando as vias de sinalização IGF e WNT/βcatenina, regulando o processo de proliferação celular. Enquanto na segunda metade, o ERβ regularia a expressão gênica de NDGR2 e ATP1A1, contribuindo para a regressão do CL. Assim nos resultados sugerem que o E2 atue tanto como um fator luteotrófico e quanto regulador da regressão do CL canino.

Palavras-chave: Estradiol. Corpo lúteo. RNAseq. ESR1. ESR2

ABSTRACT

CARDOSO, A. P. M. M. **Action mechanism of 17β-estradiol in the corpus luteum of non-pregnant bitches.** [Mecanismo de ação do 17β-estradiol no corpo lúteo de cadelas não prenhes]. 2016. 84 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

The canine corpus luteum (CL) is responsible for E2 synthesis during diestrus, which acts in an autocrine and / or paracrine manner in this gland. The E2 mechanism of action depends on the ratio between alpha (ERα) and beta (ERβ) receptor The binding to ERα has a proliferative effect whereas to ERβ an antiproliferative. The objective of this study was to understand the signaling mediated by ERα and ERβ in the formation and regression of the CL. CLs were obtained from non-pregnant bitches (n = 30) on days 10, 20, 30, 40, 50 and 60 (n = 5 / group) post-ovulation (po). On the day of ovariohysterectomy blood was collected for measurement of P4 and E2 concentrations. Eighteen CLs (n = 3 / group) were subjected to RNA sequencing (RNA-Seq). Genes differentially expressed (DE) identified by RNA-Seq were submitted to oPOSSUM3 software for detection of over-represented transcription factors binding sites (TFBS) related to the ERα (ESR1) and ERβ (ESR2) genes. The temporal expression analysis revealed the presence of 5116 differentially expressed (DE) genes in at least one comparison, and 1106 genes, which have not been recorded to the canine genome yet From these, 295 genes showed TFBS in common with ESR1 and ESR2. Ten DE genes were selected to validate the results of RNA-Seq by qPCR; using GAPDH as reference gene. Of these genes, four had TFBS in common with ESR2 (LEF-1; PAPPA; NDGR2; ATP1A1) and one with ESR1 (CAV1) and others genes were chosen because they control cell proliferation (CTNNB1; CCND1, IGFBP 3, 4 and 5). Protein expression of the IGF related genes (PAPPA and IGFBP 3, 4 and 5) was also evaluated by immunohistochemistry. During the first half of diestrus, it appears that E2 mediated signaling occurs via interaction of ERα with CAV-1 (non-genomic signaling), activating signaling pathways of IGF and WNT / βcatenin, then regulating the cell proliferation process. Whereas in the second half, ERβ appears to regulate NDGR2 and ATP1A1 gene expression, contributing to the regression of the CL. Thus our results suggest that E2 may act as a luteotrophic and also a luteolytic factor in the canine CL.

Keywords: Estradiol. Corpus luteum. RNAseq. ESR1. ESR2.

SUMÁRIO

1 INTRODUCTION

The corpus luteum (CL) is a temporary endocrine gland responsible for the production of progestrone (P4) and estrogens in the case of canine species (PAPA; HOFFMANN, 2011). CL develops from theca and granulosa cells, remnants of the ovarian follicle (DAVIS; RUEDA, 2002). It comprises luteal steroidogenic and non-steroidogenic cells such as fibroblasts, endothelial cells and immune system cells (WEBB;WOAD; ARMSTRONG, 2002).

The CL undergoes a cyclic development process, which comprises development, maintenance and regression. It reaches full secretory capacity when its formation is complete (NISWENDER et al, 2000). . The CL gene expression varies constantly after ovulation and its life span differs among species (SUGINO; OKUDA, 2007). In dogs, CL steroidogenic profile differs along the diestrus, and there are a regency of P4 in the initial phase of development of CL, decreasing values of P4 and increasing of 17β-estradiol (E2) in the maintenance phase (PAPA et al., 2014).

In contrast to other species, in dogs the duration of functional CL is almost identical in pregnant and non-pregnant animals, except that in non-pregnant dogs regression phase is slower and may last for 1 to 3 months (median 70 days) until P4 reaches baseline levels <1 ng / ml (KOWALEWSKI, 2012).

There are three main mammalian estrogens: E2, estrone and estriol. In the reproductive tract, estrogens regulate many biological processes such as cell growth, development and gene regulation in a tissue-specific manner (MATTHEWS; GUSTAFSSON, 2003). These biological actions are mediated by binding to estrogen receptors (ERs): receptor alpha (ERα) or receptor beta (ERβ) (MATTHEWS; GUSTAFSSON, 2003; DRUMMOND; FULLER, 2012).

These receptors belong to the superfamily of nuclear receptors, and exert their effects through two signaling pathways: genomic and non-genomic. In genomic signaling E2 binding to its ERs present in the nucleus or cytosol, forming a complex that interacts with elements responsives to estrogens (EREs) in the DNA in the promoter and regulatory region of the target gene or by receptor interactions with another transcription factor that mediates the association between ER and DNA, thereby regulating gene expression and cell function (SILVA; KABIL;

KORTENKAMP, 2010; PROSSNITZ; BARTON, 2014).On the contrary, non-genomic signaling is mediated by ERs present in the plasma membrane that promote signaling cascade activation as ERK / MAPK and PI3K / AKT (MARINO; ASCENZI, 2008; VIVAR et al., 2010; ZHAO; DAHLMAN-WRIGHT; GUSTAFSSON, 2010; WALL et al., 2014). While ERα and ERβ share similar mechanisms of action, several differences in transcriptional skills have been identified (MATTHEWS; GUSTAFSSON, 2003). Studies show that when both ERs are co-expressed, the ERβ exhibits an inhibitory effect on gene expression mediated by ERα (HALL; MCDONNELL, 1999; MATTHEWS; GUSTAFSSON, 2003; GAO; DAHLMAN-WRIGHT, 2011), as seen for example in ovarian epithelial cells and breast cancer cells, in which the ERβ decreases gene expression of cyclin D1, a target gene of proliferative signaling mediated by ERα activation (HÄRING et al., 2012). In general, the ERα has proliferative action, whereas ERβ is considered an antagonist of ERα and the ratio between them is important for the action of E2 in the target cells (HALL; MCDONNELL, 1999; DRUMMOND; FULLER, 2012).

E2 regulates function of the corpus luteum in non-pregnant bitches in an autocrine / paracrine manner (PAPA; HOFFMANN, 2011) and during diestrus the concentration of E2 is highest on day 40 post-ovulation (PAPA et al., 2014), whereas the expression of its receptors (ERα and ERβ) varies along diestrus: in the early phase ERα gene expression is higher, and near to regression, ERβ expression increases (BONFIM NETO, 2014).

Thus based on reported evidences, we hypothesized that E2 regulates CL function mediated by its receptor (ERβ and ERrα) signaling along diestrus.

2 GLOBAL GENE EXPRESSION OF TRANSCRIPTOMES IN CORPUS LUTEUM OF NON-PREGNANT BITCHES

ABSTRACT

The mechanisms regulating corpus luteum (CL) function and life span in non-pregnant bitches are not completely understood, although many inputs have been added in the last 4 decades. Under these circumstances new generation high-performance sequencing technologies appear as an effective tool for studying large-scale transcriptome and scanning different molecular pathways that might be activated at the same time. In the present study, we performed RNAseq technology to compare the difference in CL mRNA expression during diestrus. The corpora lutea were submitted to RNA sequencing strategy (RNA-seq). Illumina HiScanSeq generated a total of 771.208.718 reads (approximately 42 million per sample), which showed an average lengh of 100bp. The software Cufflinks revealed that the reads corresponded to 34.408 genes, from which 9000 were not annotated to the canine genome and 29.011 showed some level of expression in at least one of the studied time points. Additionally, the analyses showed that 5116 genes were differentially expressed, although 1106 among them were not annotated yet to the canine genome. A total of 5116 genes were differentially expressed (DE), although 1106 among them were not annotated yet to the canine genome. Gene ontology (GO) and KEEG analysis showed that the plenty of genes were related to cell proliferation, cell survival and angiogenesis and the immune system regulated luteal formation and regression. This study provides mega data that could drive researches reproduction related to the regulation of CL lifespan.

2.1 INTRODUCTION

The domestic dog (*Canis lupus familiaris*) is classified as a monoestrus, i.e., non-seasonal breeder with a long period of luteal activity (diestrus) and it is known that the canine reproductive function is controlled by specific mechanisms (KOWALEWSKI, 2012). So, the corpus luteum (CL) seems to be a key gland in the regulation of the reproductive cycle in dogs

(KOWALEWSKI, 2014). This gland is responsible to produce progesterone (P4) and 17β-estradiol (E2) in pregnant and non-pregnant bitches.

In contrast to other species, the duration of functional CL in dogs is almost identical in pregnant and non-pregnant animals, therefore the physiological luteal lifespan is analogous in both (KOWALEWSKI, 2014). However, the regression phase in non-pregnant is slower than in pregnant bitches. Also unlike to other species, the canine CL is independent of gonadotropic support during the first third of diestrus and luteal regression/luteolysis occurs in spite of an increased gonadotropic support.

The prostaglandin E2 (PGE2) has been proposed as one of the most important luteotrophic factors during CL formation, i.e., during the first third of diestrus and it acts in an autocrine and/or paracrine manner (KOWALEWSKI et al., 2008; KOWALEWSKI et al., 2011). During the second half of diestrus, prolactin (PRL) is the main luteotrophic factor and its role is to sustain CL function and/or slowing down luteal regression (KOWALEWSKI, 2014). But CL function suffers regulation by others factors like: components of the immune system through a set of cytokines produced by them may be involved in luteal formation and regression (HOFFMANN et al., 2004b), E2 can exert luteotrophic or luteolytic action through activation of its receptors, respectively, estrogen receptors-alfa (ERα) and estrogen receptors-beta (ERβ) (BONFIM NETO, 2014) and many others, as vascular endothelial growth factor (VEGF) (MARIANI ET AL., 2006) , insulin-like growth factor (IGF) (KOWALEWSKI, 2012) and glucose transporter member 1 (SLC2A1) (PAPA et al., 2014) which are currently under discussion and / or research.

In pregnant dogs, P4 declines during prepartum luteolysis and prostaglandin F2α (PGF2α) levels increases in maternal blood at the same time. These events are associated with strong apoptotic signals in those animals (KOWALEWSKI, 2014). The endogenous prostaglandin F2α (PGF2α), as opposed to what occurs in pregnant bitches, does not present a crucial role in the luteal regression in non-pregnant bitches (KOWALEWSKI et al., 2006). So far, studies were not able to decipher the mechanism of action that leads to CL regression in non-pregnant dogs. Thus, the characterization of molecular mechanism involved in the control of CL lifespan appears to be essential for a better understanding of female dog's reproductive physiology.

The use of techniques of high-performance sequencing can be an effective tool for large-scale study of transcriptome, (LAN et al., 2014). Global gene expression patterns may vary in

space (within different cell types or tissues), and the time, for example, during development (MARTÍNEZ-LÓPEZ; OCHOA-ALEJO; MARTÍNEZ,2014).

The RNA sequencing (RNAseq) may identify: rare transcripts and not yet known, new splice variants, events of gene fusion and chimeras, single nucleotide polymorphisms (SNP) and differentially expressed genes (LING et al., 2014). The primary goal of whole-transcriptome analysis is to identify, characterize, and catalog all the transcripts expressed within a specific cell or multiple tissues, either at a static given stage or across dynamic time-varying stages (OH et al., 2014).

In reproduction, data obtained using this technology already exist. The ovarian follicle transcriptome of sheep and women (BONNET et al., 2013; YERUSHALMI et al., 2014) and somerelated to ovarian tissue in primates, cattle and goats (BABBITT et al., 2012; CÁNOVAS et al., 2014; LAN et al., 2014; LING et al., 2014) have been published, but there is not data about CL development, maintenance and regression to the knowledge of the authors. Thus, we used the transcriptoma sequencing to characterize the CL function during diestrus in non-pregnant dogs since this species presents a long diestrus (about 60 days) and some particularities, which would be of interest to comparative reproduction. The aim of this study was therefore to identify genes and pathways in which they are involved, which are differentially expressed during diestrus to obtain a broader insight of CL function and control.

2.2 MATERIAL E METHODS

2.2.1 Animals and experimental design

Eighteen healthy mongrel bitches were included in this study. This study was approved by the Committee of Ethics in the Use of Animals of the Faculty of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil (protocol number 2719/2012). After the onset of pro-oestrous bleeding, blood samples were collected every two days to determine the progesterone (P4) concentrations. The day of ovulation was considered, the day when P4 plasma concentrations reached 5ng/ml (CONCANNON et al., 1989). Six groups (n=3 animals per group) were established. The corpora lutea (CLs) were collected via

ovariosalpingohysterectomy on days 10, 20, 30, 40, 50 and 60 post- ovulation (p.o.). After collection, the CLs were dissected from the surrounding ovarian tissue and immediately frozen in liquid nitrogen for total RNA extraction.

2.2.2 RNA extraction

Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. The quantification and determination of A260/A280 ratio were determined using a Biophotometer (Eppendorf, Hamburg, Germany). Subsequently, the samples were diluted to a concentration of 150 ng/µL. Integrity of RNA was assessed by analysis in Agilent 2100 Bioanalyser (Agilent Technologies).

2.2.3 cDNA library construction and RNA sequencing

All RNAseq libraries were constructed from 150 ng of RNA using Truseq RNA sample Prep (Ilumina, San Diego, CA, USA) in accordance with the manufacter's standard procedure. The quality of the produced libraries was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) using a DNA chip 1000. Samples were considered valid when presented fragments next to 260 bp. The quantification of the individual libraries was performed using the PCR real time through the kit KAPA Library Quantification (KAPA Biosystems, Willmigton, MA, USA).

Sequencing was performed on an Ilumina HiqSeq 200 using the pair-end reads protocol and the Ilumina TruSeq PE cluster kit v3-cBotHS (Ilumina, San Diego, CA, USA), in accordance with the manufacter's instructions. In each lane, 6 samples were run sequenced and generated 10 million reads per library.

2.2.4 Data Analysis

The CASAVA 8.2 software (Ilumina, San Diego, CA, USA) was used to obtain the raw data. The reads were mapped against the reference genome (Canis_familiaris. CanFam 3.1.75.dna.toplevel.fa) using TopHat v2.0.9 and the transcripts were assembled using Cufflinks. The relative abundance of transcripts of RNAseq fragments was measured by Cufflinks in FPKM (Fragments per kilobase of exon per million fragments mapped). For each gene, we compared the expression levels pairwise among groups. Cufflinks first computes the logarithm of the ratio of FPKM between the subjects within each group, so it uses delta method to estimative the variance of log ratio. The statistical method is log ratio of FPKM divided by the standard deviation of log ratio (TRAPNELL et al., 2012). A gene was considered differentially expressed if the FDR adjusted p value was < 0.05.

The differentially expressed (DE) genes detected by Cufflinks were converted in their human orthologs using the Inparanoid eukaryotic ortholog database (http://inparanoid.cgb.ki.se/) which is a collection of pairwise ortholog groups between 17 whole genomes (O'BRIEN;REMM; SONNHAMMER, 2005).

Clustering of gene expression profiles for different post ovulation days was performed with the self-organizing tree algorithm (SOTA; Pearson correlation, cell variability, P-value 0.0001) of Multi Experiment Viewer (MeV) v4.8.1 based on mean-centered normalized log2 transformed read counts (normalized value of a sample minus mean of all normalized values). Co-regulated orthologous genes pooled in co-expression clusters and DE genes found in the comparison 20x40 and 40x60 were used to extract Gene Ontology (GO) information from public databases using Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/).

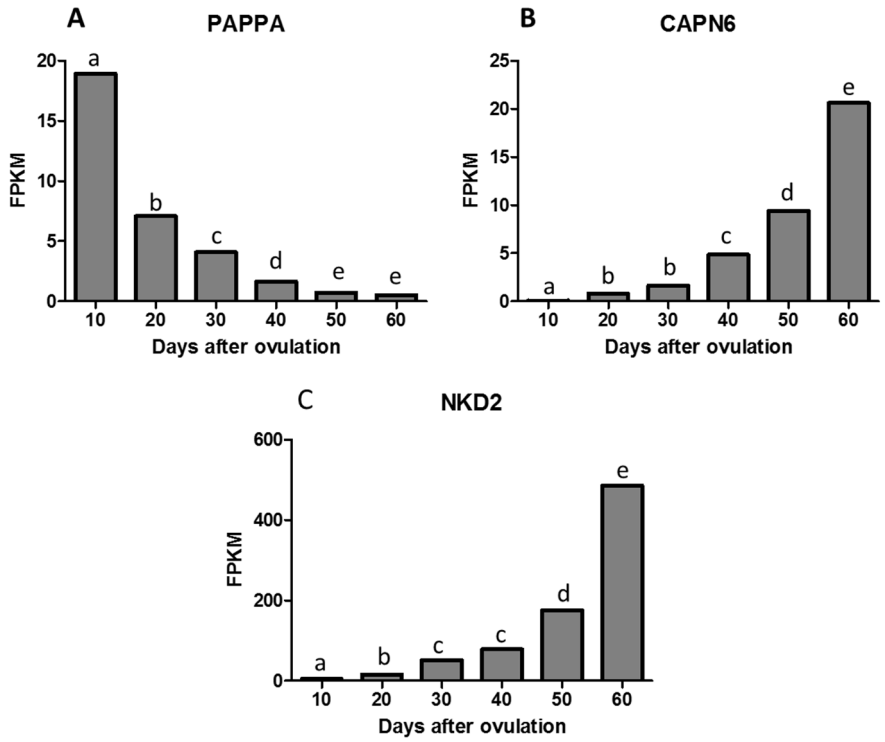
In order to identify the significantly enriched biological pathways, all DE genes were mapped to KEGG database using the Functional Annotation Charts tools of DAVID. Pathways with p-value (adjusted) $\leq 0{,}05$ were considered significant.

Venn diagram was constructed using all differentially expressed genes using day 10 p.o. as reference.

2.3 RESULTS

RNA integrity number ranged from 6,3 to 9,70. A total of 771 208 718 reads were generated by Illumina HiScanSeq with an average size of 100 bp. Each sample produced an average of 42 million reads. Alignment analysis showed that 673.577.446 (87,3%) of total reads were mapped to the canine genome. The mapped reads were aggregated within 34.408 genes, and of this total 9.000 were not annotated in the canine genome. Our study showed that 29.011 genes had some level of gene expression in some point during diestrus. The temporal gene expression analysis revealed the presence of 5.116 genes differentially expressed (DE) at least one comparison and 1106 genes s had not yet been annotated in the canine genome. This analysis showed that 1141 genes were DE in one of 15 possible comparisons and three were DE in almost all diestrus (PAPPA, CAPN6 and NKD2). The gene PAPPA was more expressed at the beginning of diestrus (Figure 1) and the NKD2 and as well as CAPN6 genes were more expressed at the end of diestrus (60 p.o.), but their expression increased gradually (Figure 1). In relation to isoforms, the analysis dectected 1215 DE isoforms and of this a total of 499 did not show a corresponding described gene.

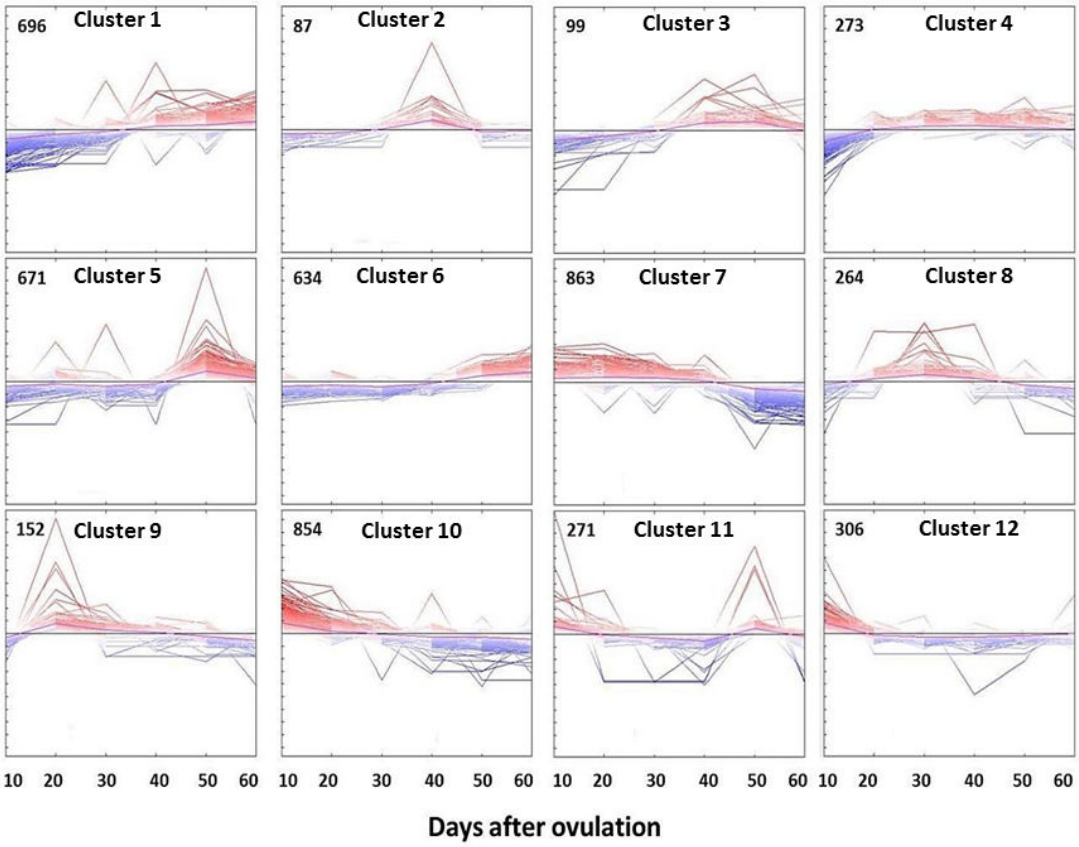
Figure 1 - Genes differentially expressed in almost all diestrus



Source: (CARDOSO, A. P. M. M., 2016).

Those transcripts found to be differentially expressed at least in one of days p.o. were subject to SOTA clustering. Cluster methods are frequently used for grouping genes by their expressions patterns. Expression profiles were visualized as 12 clusters of similar expression profiles during diestrus (Figure 2). The clusters 10, 11 and 12 contained genes up regulated in the beginning of diestrus during the CL formation (day 10 p.o.) The cluster 9 contained genes that had the highest expression on day 20 p.o. The clusters 1,3 and 4 included the genes that their expression began to increase on day 35, 40 and 20 p.o., respectively until the end of diestrus. The cluster 6 showed genes which were upregulated at the end of diestrus (day 50 p.o.) when started the structural regression. The cluster 7 added the genes up regulated until the second third of luteal phase (day 40 p.o.). The genes upregulated during the midle of luteal phase were showed in the cluster 8. The clusters 2 and 5 contained the genes with highest expression on day 40 and 50 p.o., respectively.

Figure 2- Clustering of genes expression profiles during diestrus.SOTA of MeV software (version 4.7.1) was used to obtain group of genes with similar expression profiles. Clusters 1 to 12 from left to right and top to down. Numbers of genes for each cluster are shown at top left



Source: (CARDOSO, A. P. M. M., 2016).

A Functional Annotation Clustering analysis to GO terms was performed using DAVID for the individual SOTA clusters (Table 1). GO categories are organized into three groups: biological process (BP), cellular compartiment (CC) and molecular function (MF). The clusters 3 (up- regulated on days 40 and 50 p.o.) did not show enrichment for any above described categories. The cluster 1 (up-regulated on days 40, 50 and 60 p.o.) exhibited enrichment of functions related to extracellular region and negative regulation of biological process. Genes with highest expression on day 40 p.o (cluster 2) were enriched with functional terms as plasma membrane and process that modulates a qualitative or quantitative trait of biological quality. Genes down-regulated on the beginning of diestrus (Cluster 4) presented functional terms related to membrane, such as endoplasmatic reticulum membrane and plasma membranes, as well as anatomical structure development. Cluster 5 corresponded to genes up-regulated on 50 p.o. and the enriched functional categories were structural constituent of ribosome, intracellular organelle and cellular metabolic process. Cluster 6 contained genes with highest expression at the end of diestrus (days 50 and 60 p.o.), the most enriched terms were similar to the cluster 5, except the immune response. The genes down-regulated at the end of diestrus (days 50 and 60 p.o.) were represented in cluster 7 and their overrepresented functional terms were cell motility, development process and regulation of biological process. Cluster 8 exhibited genes up-regulated on day 30, but with average lower levels on days 20 and 40. Enriched terms were, for example, anatomical structure morphogenesis, plasma membrane part and cell motility. Only one annotation cluster was found to cluster 9 (up-regulated on day 20 and less on day 40) and this was related to extracellular membrane vesicle. The genes up-regulated at the first half of diestrus (days 10, 20 and 30) were represented at cluster 10 and overrepresented functional terms were related to cell motility, development process, plasma membrane and positive regulation of cellular process. Strong enrichment of functions related to immune system was found for genes up-regulated on day 10 and 50 p.o. (Cluster 11). Only one enriched functional category, reproductive process, was found to cluster 12 (genes up-regulated on day 10 p.o.).

Table 1 - Overrepresented DAVID annotation clusters for the obtained SOTA expression clusters

(to be continued)

Expression cluster	Representative enriched functional terms	Enrichment score	No. of genes
Cluster 1	Extracellular region part (110,1.45); extracellular organelle (82,1.47), vesicle (99,1.34)	3.78	347
Up-regulated on days 40, 50 and 60	Negative regulation of biological process (123,1.34); negative regulation of cellular process (136,1.20); Negative regulation of metabolic process (68,1.27);	3.16	320
	Anatomical structure development (132,1.27); single-organism developmental process (136,1.19); single-multicellular organism process (150,1.12)	2.25	320
Cluster 2	Plasma membrane (15, 1.88); cell periphery (15,1.84), plasma membrane part (10, 2.46); membrane part (16,1.50); intrinsic component of membrane (14, 1.58)	1.71	28
Up-regulated on day 40 po	Regulation of biological quality (12, 2.03), regulation of localization (9, 2.27); response chemical (12.1.67)	1.52	27
Cluster 3	…….....	0	0
Up-regulated on days 40 and 50 po			
Cluster 4	Endoplasmic reticulum membrane (19,2.11); nuclear outer membrane-endoplasmic reticulum membrane network (19,2.07); organelle membrane (36,1.24)	1.89	165
Down-regulated on day 10 po	Extracellular vesicle (39,1.47); extracellular organelle (39,1.47); extracellular region part (49,1.35); vesicle (47,1.34)	1.85	165
	Plasma membrane part (36,1.50); plasma membrane (60,1.27); cell periphery (61,1.26)	1.74	165
	Single-organism developmental process (69,1.25); anatomical structure development (61,1.21); single-multicellular organism process (76,1.17)	1.46	155

(continuation)

Expression cluster	Representative enriched functional terms	Enrichment score	No. of genes
Cluster 5 Up-regulated on day 50 po	Structural constituent of ribosome (29,7.96); multi-organism metabolic process (23,6.14); ribonucleoprotein complex (41,3.14); interspecies interaction between organisms (35,2.34); multi-organism cellular process (33,2.28)	9.31	319
	Intracellular organelle part (186,1.29); intracellular organelle part (189,1.28); intracellular organelle (250,1.16)	5.58	317
	Membrane-bounded organelle (255,1.18); intracellular organelle (250,1.17); intracellular part (275,1.11); intracellular (279,1.10); cell part (296,1.02)	4.09	289
	Nitrogen compound metabolic process (162,1.35); cellular metabolic process (211,1.15); biosynthetic process (139,1.23); primary metabolic process (206.1,12); organic substance metabolic process (208,1.10)	3.42	289
	Cellular localization (75,1.41); establishment of localization (104,1.26); single-organism localization (93,1.28); macromolecule localization (65,1.36)	2.44	289
	Extracellular organelle (72,1.41); extracellular vesicle (72,1.41); vesicle (89,1.32); extracelular region part (79,1.14)	2.25	317
Cluster 6 Up-regulated on days 50 and 60 po	Structural constituent of ribosome (20,4.94); multi-organism metabolic process (20,4,72); ribonucleoprotein complex (35,2.51); multi-organism cellular process (35,2.14); interspecies interaction between organisms (35,2,07)	5.84	340
	Extracellular region part (116,1.56); extracellular organelle (88,1.61); extracellular vesicle (88,1.61); vesicle (99,1.37)	5.27	340
	Macromolecule localization (82,1,51); establishment of localization (118,1.27); cellular localization (81,1.35); single-organism localization (103,1.25)	2.88	327
	Activation of immune response (22,2.02); positive regulation of immune system process (31,1.63); immune response (47,1,39); regulation of immune system process (41,1.31)	1.87	327

(continuation)

Expression cluster	Representative enriched functional terms	Enrichment score	No. of genes
Cluster 7 Down-regulated on days 50 and 60 po	Localization of cell (99,2.07); cell motility (99,2.07); regulation of locomotion (67,2.40)	11.05	596
	Anatomical structure morphogenesis (175,1.70); single-organism developmental process (293,1.38); Anatomical structure development (267,1.39); single-organism multicellular process (319,1.27)	10.61	596
	Extracellular matrix component (20,4.38); basement membrane (17,4.94); proteinaceous extracellular matrix (31,2.46)	6.12	608
	Single organism signaling (305,1.34); cellular response to stimulus (316,1.21); regulation of cellular process (404; 1.07); regulation of biological process (421;1.06)	4.85	596
	Positive regulation of biological process (273,1.34); positive regulation of cellular process (229,1.30); positive regulation of metabolic process (179,1.31); regulation of metabolic process (255,1.03)	4.85	596
	Cell projection (100,1.57); neuron part (68,1.57); cell projection part (55,1.65)	4.15	608
	Postsynaptic density (22,3.21); synapse part (39,1.84); postsynapse (28,2.05)	3.98	608
	Vesicle (169,1.31); extracellular vesicle (127,1.30); extracellular organelle (127,1.30); extracellular region part (165,1.24)	3.24	608
	Negative regulation of cellular process (200,1.28); negative regulation of biological process (210,1.24); negative regulation of metabolic process (111,1.12)	3.02	596
	Establishment of localization (209,1.23); single-organism localization (185,1.24); cellular localization (128,1.17)	2.74	596

(continuation)

Expression cluster	Representative enriched functional terms	Enrichment score	No. of genes
Cluster 8 Up-regulated on day 30 po and less pronounced on days 20 and 40 po	Anatomical structure morphogenesis (59,2.18); single-multicellular organism process (102,1.56); anatomical structure development (86,1.71); single-organism developmental process (87,1.57)	7.99	156
	Cell periphery (81,1.66); plasma membrane part (50,2.06); plasma membrane (78,1.64)	6.41	167
	Localization of cell (36,2.88); cell motility (36,2.88); regulation of locomotion (23,3.15); regulation of localization (43,1.87)	6.38	156
	Single-organism cellular process (143,1.20); single organism signaling (90,1.51); cellular response to stimulus (98,1.43); regulation of cellular process (122,1.23); regulation of biological process (124,1.19)	5.18	156
	Regulation of multicellular organism process (51,2.06); regulation of developmental process (43,2.0); positive of multicellular organism (3 2,2.33); positive regulation of developmental process (26,2.34)	5.15	156
	Postsynapse (10,2.66); synapse part (13,2.24); synapitc membrane (7,2.80)	1.72	167
	Basement membrane (5,5.28); proteinaceous extracellular matrix (9,2.58); extracellular matrix component (5,3.99)	1.63	167
Cluster 9 Up-regulated on day 20 po and less pronounced on day 40 po	Extracellular organelle (25,1.75); extracellular vesicle (25,1.75); vesicle (30,1.59); extracellular region part (30,1.54)	2.18	89

(continuation)

Expression cluster	Representative enriched functional terms	Enrichment score	No. of genes
Cluster 10 Up-regulated on day 10 po and less pronounced on days 20 and 30 po	Cell motility (90,1.88); localization of cell (90,1.88); regulation of locomotion (61,2.19)	8.06	597
	Single-organism developmental process (275,1.30); anatomical structure development (250,1.30); single-multicellular organism process (307,1.22)	6.42	597
	Cell periphery (238,1.31), plasma membrane (230,1.30); plasma membrane part (131,1.45)	5.65	238
	Extracellular region part (189,1.39); vesicle (181,1.37); extracellular organelle (134,1.34); extracellular vesicle (134,1.34)	4.86	622
	Positive regulation of cellular process (237,1.35); positive regulation of biological process (265,1.30); positive regulation of metabolic process (169,1.24); regulation of metabolic process (250,1.00)	4.34	597
	Establishment of localization (209,1.23); macromolecule localization (131,1.33); single-organism localization (186,1.24); cellular localization (141,1.28)	3.32	597
	Negative regulation of cellular process (199,1.27); negative regulation of biological process (213,1.25); negative regulation of metabolic process (115,1.16)	3.19	597
	Single organism signaling (281,1.23); cellular response to stimulus (297,1.14); regulation of biological process (409,1.03); regulation of cellular process (390,1.03)	2.38	597
	Developmental process involved in reproduction (44,1.86); single organism reproductive process (60,1.46); multicellular organism reproduction (38,1,29); multicellular organismal reproductive process (37,1.28); multi-organism reproductive process (43,1.24); sexual reproduction (28,0.99)	1.60	597
	Intracellular organelle part (310,1.10); organelle part (315,1.09); intracellular organelle (427,1.02	1.33	622

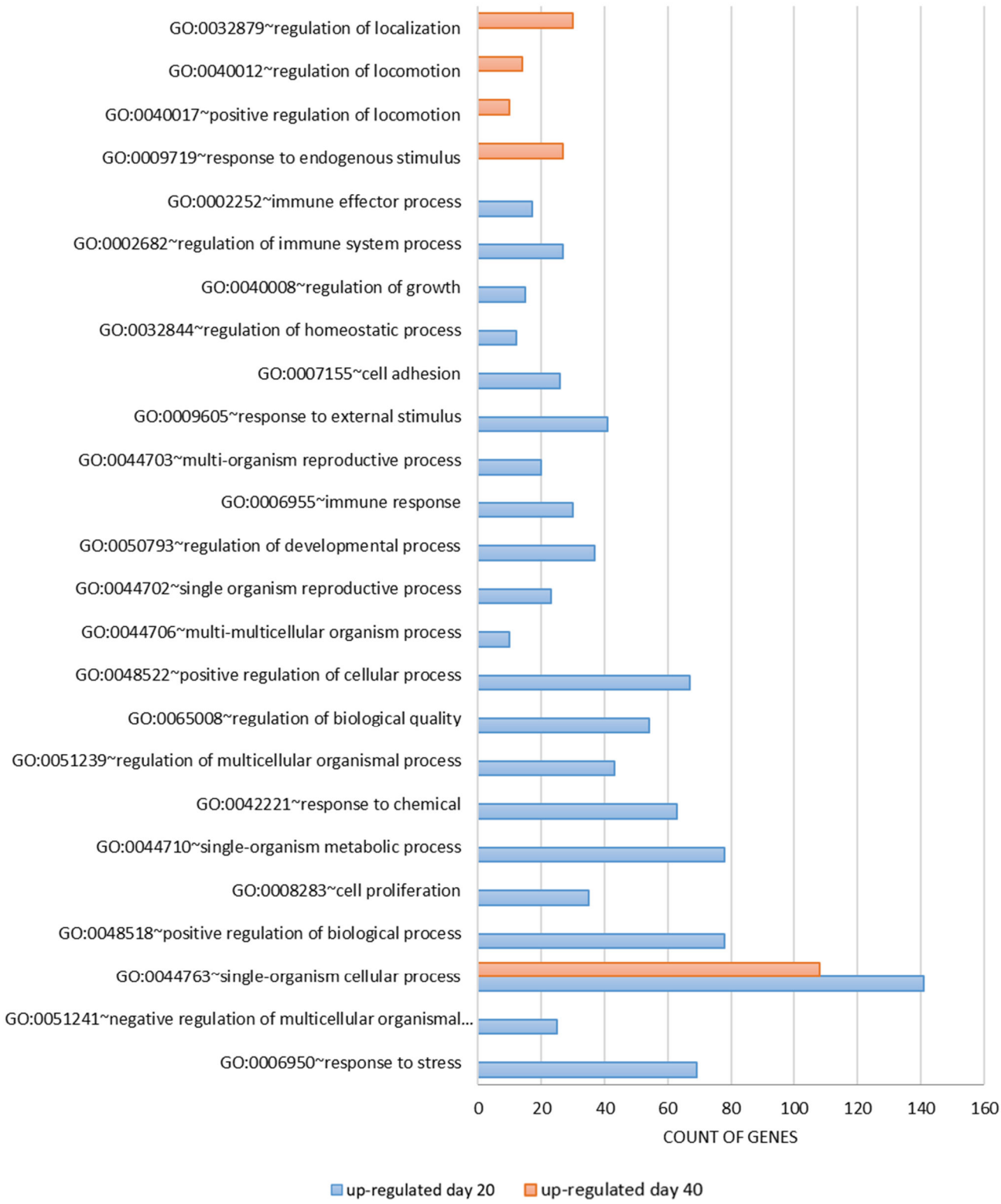
(conclusion)

Expression cluster	Representative enriched functional terms	Enrichment score	No. of genes
Cluster 11 Up-regulated on days 10 and 50 po	Immune response (66,3.89); regulation of immune system process (54,3.43); immune effector process (37,4.58); positive regulation of immune system process (40,4.18); activation of immune response (25,4.58); regulation of response to stimulus (73,1.94); positive regulation of response to stimulus (48,2.21)	12.89	164
	Leukocyte activation (37,4.92); cell adhesion (40,2.77); single organism cell adhesion (26,3.35)	9.88	164
	Response to other organism (28,2.98); response to biotic stimulus (28,2.86); response to external stimulus (46,1.72)	5.29	164
	Leukocyte migration (20,5.56); cell motility (31,2.36); localization of the cell (31,2.36); taxis (22,2.54); regulation of locomotion (16,2.09)	4.82	164
	Cellular response to stimulus (100,1.40); single organism signaling (90,1.43); regulation of biological process (128,1.17); regulation of cellular process (122,1.17)	3.90	164
	Vesicle (53,1.48); extracellular region part (54,1.46); extracellular organelle (40,1.47); extracellular vesicle (169,1.47)	2.35	169
	Single- organism develpmental process (73,1.25); anatomical structure development (67,1.27); single-multicellular organism process (81,1.17)	1.68	164
	Establishment of localization (61,1.31); single-organism localization (52,1.26); cellular localization (36,1.19)	1.33	164
	Regulation of molecular function (42,1.47); positive regulation of metabolic process (50,1.33); regulation of metabolic process (65,0.95)	1.32	164
Cluster 12 Up-regulated on day 10	Single-organism reproductive process (29,2.06); multi-organism reproductive process (25,2.11); multicellular organismal reproductive process (17,1.73); multicellular organism reproduction (17,1.68); sexual reproduction (13,1.35)	1.96	204

Source: (CARDOSO, A. P. M. M., 2016).

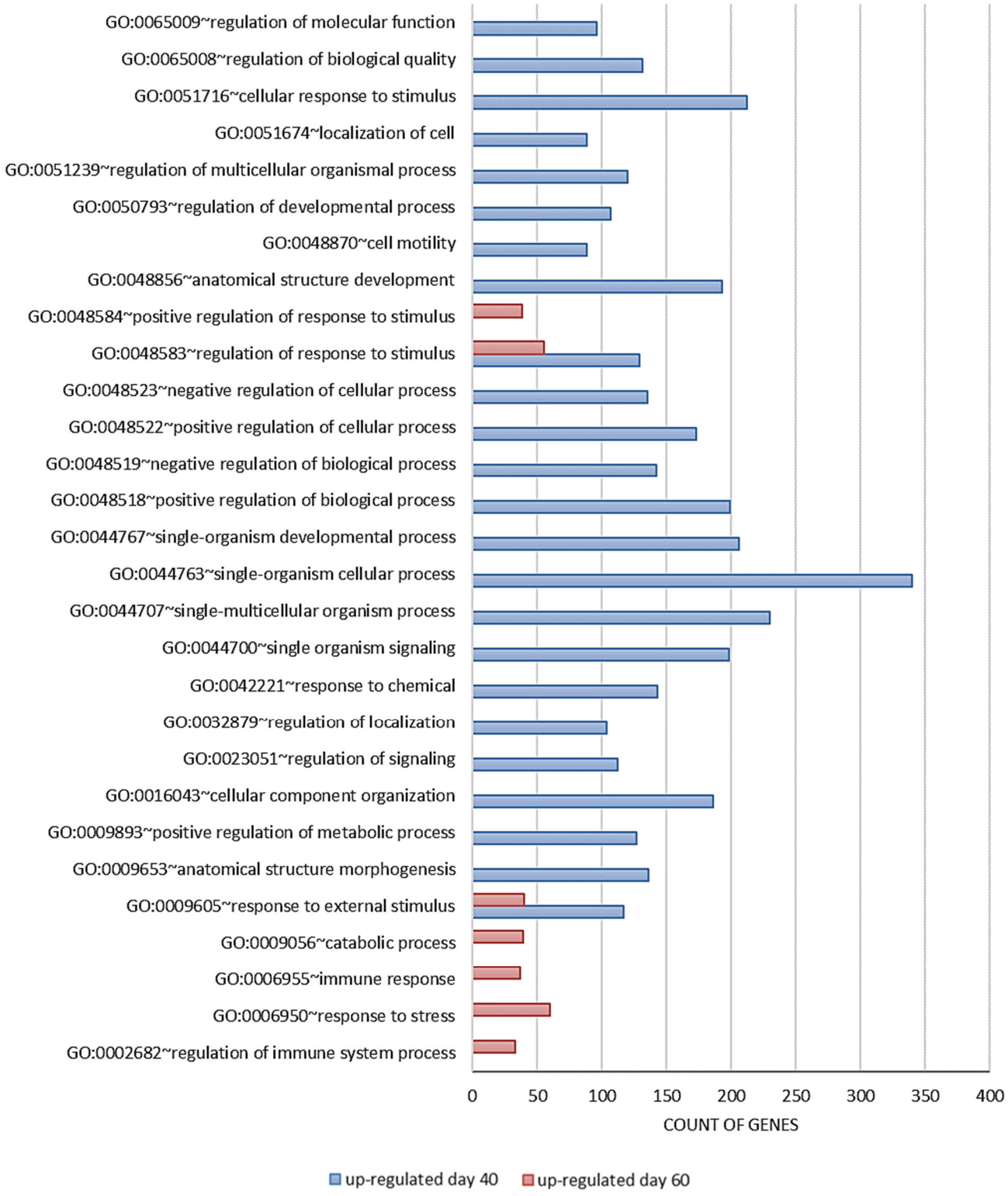
In addition to understand better the CL dynamic during proliferative phases, luteal physiological regression and structural, we performed GO analysis between groups 20x40 and 40x60. In both comparisons, the majority of enriched genes was up-regulated at the first group of comparison (on days 20 and 40 p.o, respectively). At the comparison 20x40, the majority of genes enriched to this category were up-regulated on day 20 p.o. (Figure 3) and various terms were related to immune process. The term, single-organism cellular process (process that is carried out at the cellular level), was the most enriched term and could be observed in both moments. The other enriched terms were: positive regulation of biological process, metabolic process and change in state or activity of a cell. In relation to 40x60 comparison (Figure 4), the term single-organism cellular process was again the most enriched, followed by single-organism developmental process (likewise a progression of an anatomical structure) and positive regulation of biological process and some terms enriched on day 60 p.o. were related to immune process and response to stress.

Figure 3 – GO analysis of differentially expressed genes in comparisons 20x40. The differentially expressed genes are classified in biological process



Source: (CARDOSO, A. P. M. M., 2016).

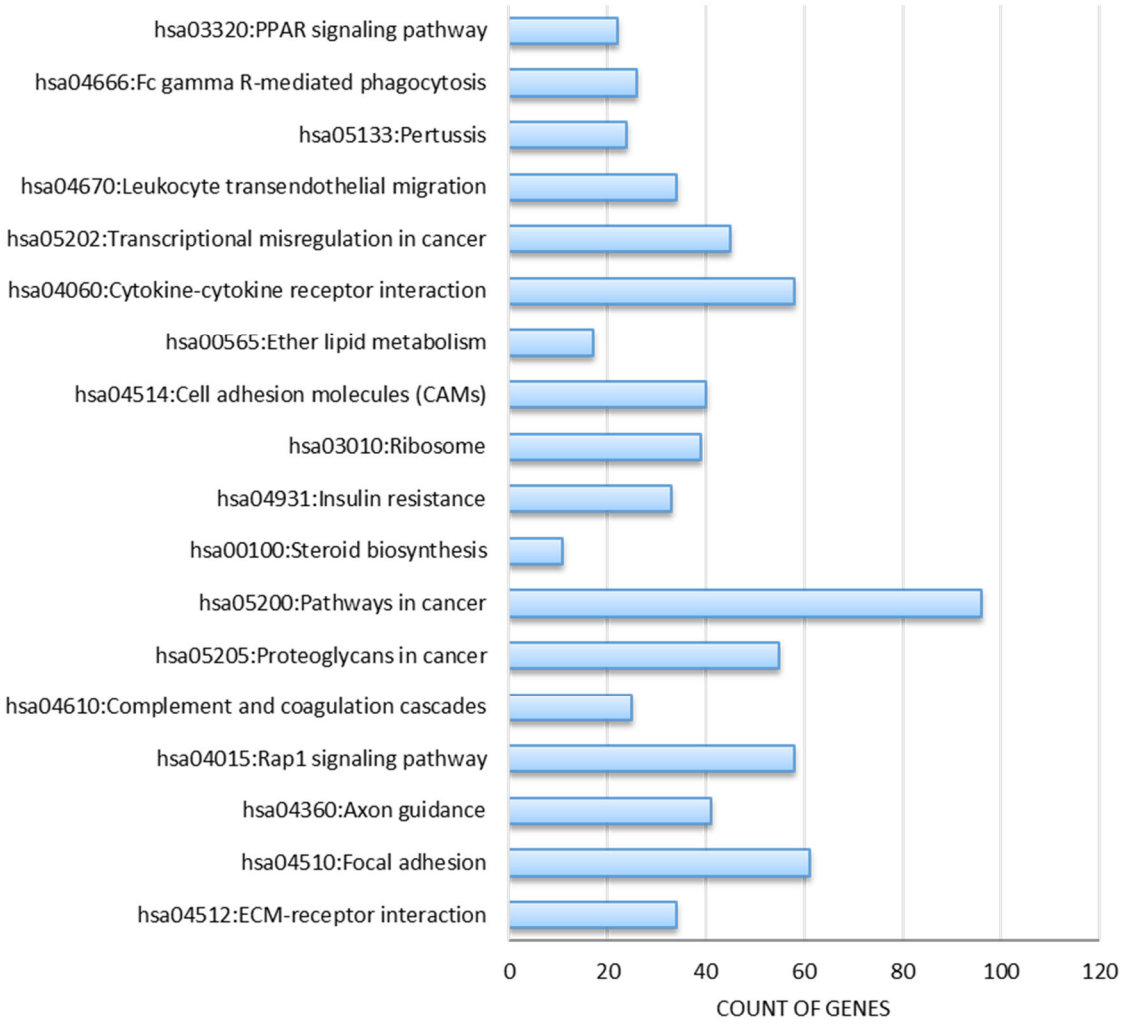
Figure 4 – GO analysis of differentially expressed genes in comparisons 40x60. The differentially expressed genes are classified in biological process



Source: (CARDOSO, A. P. M. M., 2016).

The pathways analysis was performed with all DE genes to predict the significantly enriched metabolic pathways and signal transduction based on Kyoto Encyclopedia of Genes and Genomes (KEEG) pathway. The significantly enriched pathways are showed in Figure 5. In total, 1229 DE genes had KEGG pathways annotations. The results showed that the significant signaling pathways included 18 pathways, for example, pathways in cancer (as Wnt signaling, VEGF signaling, PI3K-AKT signaling, MAPK signaling), focal adhesion (that regulates cell motility, cell proliferation and cell survival), Rap1 signaling pathways (regulate cell proliferation and survival), proteoglycans in cancer (regulate angiogenesis, proliferation and survival), cytokine-cytokine receptor interactions (as IGF-1/IGFR, TGFα/EGFR, IL/ILR).

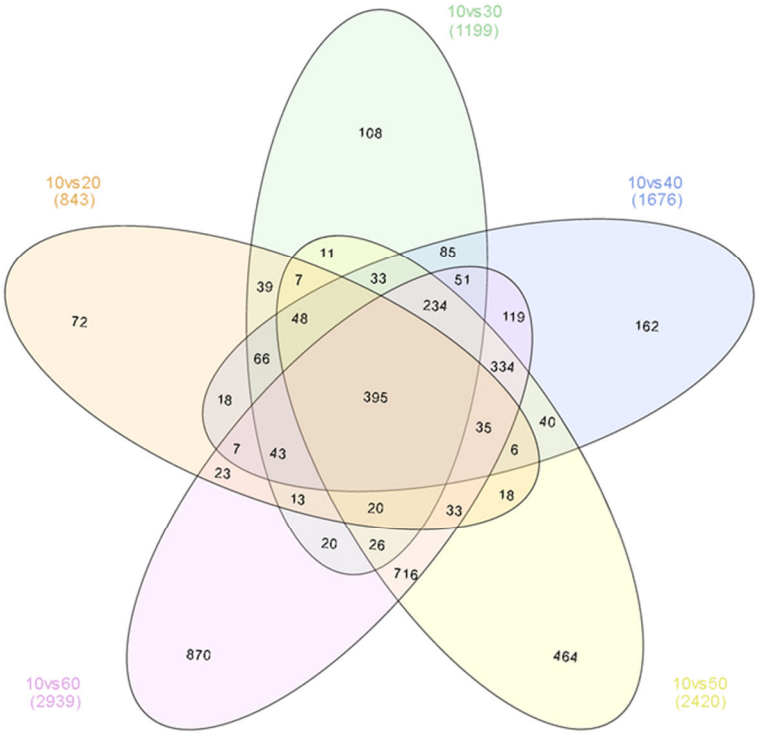
Figure 5 - The significant signaling pathways based on KEGG pathways annotations



Source: (CARDOSO, A. P. M. M., 2016).

Using day 10 as reference, the Venn diagram showed the possible intersection among DE genes (Figure 6). The results show that 395 genes occur in all comparisons which have day 10 p.o. as reference. Through comparisons between different days studied: 10vs20, 10vs30, 10vs40, 10vs50 and 10vs60 showed 72, 108, 162, 464 and 870 different genes in each type of comparison, respectively and the number of the DE genes was increased when comparison time points were more distant from each other.

Figure 6 - Venn diagram comparing significant differentially expressed genes between days 20, 30, 40, 50 and 60 p.o. compared to 10 p.o



Source: (CARDOSO, A. P. M. M., 2016).

2.4 DISCUSSION

To the knowledge of the authors, there are no studies related to the transcriptome of the corpus luteum in other domestic species using the new generation sequencing technology. Differential gene expression analysis during the luteal phase showed the presence of 5116

differentially expressed genes, which is a higher number than that found by Bogan and cols (BOGAN et al., 2008) when assessing the gene expression of the primate corpus luteum through microarray. The largest amount of differentially expressed genes obtained in our experiments must be due to the capacity of RNAseq to detect low abundance transcripts (GUO et al., 2013; ZHAO et al., 2014). However, likewise the data obtained by Bogan and cols (BOGAN et al., 2008), the larger amount of differentially expressed transcripts were observed when CL from the early luteal phase was compared with CL from the end of luteal phase.

Our data indicated that plenty of genes related to cell proliferation, cell survival and angiogenesis were differentially regulated during diestrus, mainly during the first third of diestrus (days 10 and 20 p.o.). The PAPPA gene, one of three DE genes in almost all comparisons in diestrus, was an example. This gene encodes the protein denominated as pregnancy-associated plasm protein-A (PAPP-A) in human and domestic animals (NYEGAARD et al., 2010), which is a secreted metalloproteinase responsible for cleavage of the insulin-like growth factor binding protein 4 (IGFBP4), the most abundant IGFBP in the ovary (HOURVITZ et al., 2000; BROGAN et al., 2010; NYEGAARD *et al.*, 2010). The PAPP-A is a marker of corpus luteum formation (HOURVITZ et al., 2000; CONOVER et al., 2001; CONOVER, 2012), which could be corroborated by our experiments. The highest expression of this gene was found on day 10 p.o., which points towards its role in luteal steroidogenesis, survival and proliferation (BROGAN et al., 2010; LODDO et al., 2014). Furthermore, insulin-like growth factor 1 (IGF1) and some IGFBPs (as 3, 4 and 7) were DE genes in the early diestrus too, relating the modulator role of IGF system in cellular differentiation and steroidogenesis (NYEGAARD et al., 2010; BØTKJÆR et al., 2015). Another different signaling proliferative and angiogenic pathway molecules were also DE, such as Wnt-5 and β-catenin (SAITO-DIAZ et al., 2013; ROSENBLUH et al., 2014) regulating Wnt pathway, MAP2K1 in MAPK pathway (NADEAU et al., 2009), EGFR in EGF signaling (SESHACHARYULU et al., 2012),VEGFA and VEGFC in VEGF signaling (MARIANI ET AL., 2006; KOCH; CLAESSON-WELSH, 2012; LAURENZANA ET AL., 2015). In addition, proteins required for transport, such as iron uptake TFRC1, amino acid transporter SLC36A1 and monocarboxylate transporter SLC16A7, are also DE at beginning of diestrus. Most of these genes was up-regulated on day 10 p.o, emphasizing data of high proliferative activity found in early luteal phase (HOFFMANN et al., 2004a; PAPA; HOFFMANN, 2011). Indeed we observed that the majority of regulatory mechanisms controling CL lifespan is expressed during the period of increased cell growth and tissue modeling.

Some enriched terms up-regulated on days 40, 50 and 60 p.o. were related to negative regulation of biological processes. These periods correspond to observed structural regression and degeneration (KOWALEWSKI, 2014). Two of three DE genes in almost all studied comparisons were clustered here. The Naked 2 gene (NKD2) encodes a protein, which is a member of Naked family protein and a negative regulator of canonical Wnt signaling (CAO et al., 2013), a proliferative pathway. The NKD2 expression gradually increased during diestrus, being highest on day 60 p.o. Axin 2 and SFRP4, other inhibitors of Wnt signaling (GÖTZE et al., 2010; SAITO-DIAZ et al., 2013), were found DE at this moment as well; thus these increases seem to be important to reduce cell proliferation. The calpain 6 (CAPN6) is a protein encoded by the CAPN6 gene. This protein is a member of the calpains family, which comprises intracellular cysteine proteins, whose activity is highly dependent upon Ca^{2+} ions (SORIMACHI et al., 2011; SMITH; SCHNELLMANN, 2012). It is known that the calpains regulate various biological processes like cell migration and apoptosis (TONAMI et al., 2007; SORIMACHI et al., 2011; SMITH; SCHNELLMANN, 2012; TONAMI et al., 2013), through the limited proteolytic activity, which transforms their substrates into new functional states (TOMANI et al., 2013; SORIMACHI et al., 2011). In cultured cells, CAPN6 regulates microtubule dynamics and actin reorganization (TONAMI et al., 2007), but its *in vivo* function is still unclear (TONAMI et al., 2013). CAPN6 is a suppressive modulator for differentiation and growth in skeletal muscle (TONAMI et al., 2013) and perhaps this gene can suppress the growth in CL, because its expression increased gradually during the luteal phase, being highest on day 60 p.o., but more studies are necessary to understand the involvement of these calpains in CL physiology. On day 60 p.o., moment for observation of the first apoptotic signals (HOFFMANN et al., 2004a), some apoptosis related genes were DE. Likewise NOXA1, which encodes the enzyme NADPH oxidase, responsible to generate superoxide anion, a reactive oxygen species (ROS), leading to ROS-mediated apoptotic cell death (PARK et al., 2012) was upregulated, as well as IGFBP6, a glycoprotein inhibitor of IGFII actions, although it may act independently of IGF action, promoting apoptosis and inhibition of angiogenesis (BACH, 2015b).

DE genes related to immune system were found during early and regression luteal phases: CD44, CD209, IL10 and TNFRSF18, and the enriched pathway related to cytokine-cytokine interaction confirm that the immune system regulates luteal formation and regression (HOFFMANN, BÜSGES; BAUMGÄRTNER, 2004; HOFFMANN et al., 2004a).

Our transcriptome results also confirmed the role of prostaglandins as a luteotrophic factors (KOWALEWSKI et al., 2006; KOWALEWSKI et al., 2008), since COX-2 gene was up-regulated during early diestrus. The expression of CYP1B1 and KIAA1324 genes suggests the importance of E2 controlling CL lifespan. The CYP1B1 codes a cytochrome P450 enzyme responsible of producing a cathecol estrogen, which is a strong agonist of estrogen receptors (PICCINATO et al., 2016) and its expression was higher on day 10 p.o, decreased afterwards, but increased again during regression (days 50 and 60 p.o). Whereas KIAA1324, an estrogen regulated gene (ESTRELLA et al., 2014), was more expressed during regression. The encoded protein has been characterized as a transmembrane protein, which is related to autophagic vacuole formation (DENG et al., 2010) and in pancreatic tumor cells its expression may be regulated by ERβ (ESTRELLA et al., 2014). Thus, these results suggest a role of E2 during CL formation and regression.

The number of reads obtained in our sequencing, approximately 770 million, was larger than reported for other animal reproduction experiments, or even the transcriptome of ovarian follicles in women (BONNET et al., 2013; YERUSHALMI et al., 2014), and the ovarian tissue of primates, cattle, goat and sheep (BABBITT et al., 2012; CÁNOVAS et al., 2014; LAN et al., 2014; LING et al., 2014; CHEN et al., 2015). The only study able to obtain a larger number of reads was the one from Bonnet and cols. (BONNET et al., 2013) , who reported a total of 2 billion reads, when sequencing the ovarian follicle during folliculogenesis in sheep. Ours results demonstrated also a great depth in this sequencing.

On the average, 80% of reads generated from each sample were mapped in the canine genome, similar to results obtained in the sequencing of ovarian tissue in goat, cattle and sheep (CÁNOVAS et al., 2014; LAN et al., 2014; LING et al., 2014; CHEN et al., 2015) and larger than that observed by Yerushalmi and cols (YERUSHALMI et al., 2014), when they analyzed humans oocytes, from which the average alignment of each sample relative to the reference genome was 70%.

In summary, the results provide an overview of the distribution of transcriptome along the canine diestrus, bringing valuable data that will be explored in the future for a better understanding of the physiological functions of this gland along canine diestrus.

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3 NEW INSIGHTS ON THE ROLE OF 17B-ESTRADIOL IN CORPUS LUTEUM LIFESPAN OF NON-PREGNANT BITCHES

ABSTRACT

Canine corpus luteum (CL) is responsible for E2 synthesis during diestrus, which acts in an autocrine and/or paracrine manner within this temporary endocrine gland. The mechanism of action of E2 depends on the expression ratio of ERα and ERβ. Binding to ERα has a proliferative and to ERβ an antiproliferative effect. The aim of this study was to better understand the signaling mediated by ESR1 and ESR2 in the formation and regression of canine corpus luteum (CL). For this purpose, we applied RNA sequencing (RNAseq) to identify differentially expressed (DE) genes during diestrus and used these genes to detect over-represented conserved transcription binding sites (TFBS) related to ESR1 and ESR2. We found that during the first half of diestrus, E2 signaling appears to be mediated by ERα via interaction with caveolin-1 (non-genomic pathways): IGF system and Wnt/β-catenin signaling were identified as one of the cascades activated by this interaction, with a major role in the proliferative process. During the second half of diestrus, ERβ appears to regulate NDGR2 and ATP1A1 gene expression, contributing to the regression of the CL. Thus our results suggest that E2 might activate both luteotrofic and regression-related factors in canine CL.

3.1 INTRODUCTION

The canine corpus luteum (CL) produces progesterone (P4) and 17β-estradiol (E2) and these hormones regulate CL lifespan in an autocrine and/or paracrine manner (HOFFMANN et al., 2004a; PAPA; HOFFMANN, 2011). During its lifespan, CL undergoes development, maintenance and regression processes, which are regulated by local factors controlled to induce both morphological and physiological changes (KOWALEWSKI, 2014).

Studies in different species reported E2 as a luteotrofic factor for example, in rabbits and rats, whereas in humans and bovines it acts as a luteolytic factor (SHIBAYA et al., 2007; KOHEN et al., 2013; TRIPATHY et al., 2016). The effects mediated by E2 depend on the binding of E2 to estrogen receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), are encoded by two distinct genes, ESR1 and ESR2, respectively. These receptors belong to the nuclear receptor family and show similar structure, although their proteins have showed distinct regulatory function. In general, ERα promotes cell proliferation and ERβ appears to have an anti-proliferation role (VIVAR et al., 2010), as in cells where both receptors are expressed, ERβ appears to inhibit ERα transcriptional activity; therefore the E2 response signaling depends on the ERα/ERβ ratio (MATTHEWS; GUSTAFSSON, 2003).

Upon ligand activation, ERs produce genomic and non-genomic effects. The genomic effects regulate gene transcription and occur through direct binding of ERs to estrogen responsive elements (EREs) in the regulatory regions of E2 target genes; alternatively, ERs can interact with other transcription factors such as AP-1 (activating protein-1) and SP-1 (stimulating protein 1) to indirectly influence gene expression (VIVAR et al., 2010; ZHAO; DAHLMAN-WRIGT; GUSTAFSSON, 2010; WALL et al., 2014). Non-genomic effects regulate ion channel and signal transduction, and are mediated by ERs located in the plasma membrane within caveolar rafts (MARINO; ASCENZI, 2008). Their actions regulate the majority of known estrogen extranuclear responses and are mediated by activation of downstream cascades such as ERK/MAPK and PI3K/AKT (MARINO; ASCENZI, 2008; (VIVAR et al., 2010; ZHAO; DAHLMAN-WRIGT; GUSTAFSSON, 2010; WALL et al., 2014).

Canine CL expresses ESR1 and ESR2. ESR1 expression is highest at the beginning of diestrus (PAPA; HOFFMANN, 2011), whereas ESR2 expression is highest at the end of diestrus (BONFIM NETO, 2014) and the ratio ERα/ERβ varies throughout diestrus (PAPA; HOFFMANN, 2011). However, E2 signaling mechanisms in the canine luteal tissue are still poorly understood.

The evaluation of global transcriptome sequencing using high-throughput sequencing (RNA-seq) is a key step to understand the complex biological process of several conditions or tissues, since this method is very sensitive, quantifies RNAs expressed at very low levels and is able to detect splicing variants/isoforms, even if previously unknown. Thus, this tool is a good method to determine differentially expressed genes (DE) and to characterize their temporal spectrum within a biological process. Gene expression is controlled by regulating

elements denominated cis-regulatory elements (CRES) (KWON et al., 2012; LI et al., 2015) such as promoters, enhancers and silencers. CRES are represented by DNA motifs and they determine which genes are specifically active in a cell (LOVÉN et al., 2013; LI et al., 2015). Therefore, the identification of CRES that control gene expression helps understanding the network of gene interactions in a biological process identifying the sequence motifs that are over-represented in the cis-regulatory regions, i.e., identifying over-represented transcription factor binding sites (TFBS) (KIM; JUNG, 2006; WANG et al., 2009; KWON et al., 2012; SURYAMOHAN; HALFON, 2015).

In the present study, deep-sequencing technology (RNA-seq), was used to determine the CL transcriptome of non-pregnant bitches during diestrus and to identify over-represented TFBS related to ESR1 and ESR2 through enrichment analyses of TFBS among DE genes. The aim of the study was to gain further insights on the signaling mediated by ESR1 and ESR2 in the formation and regression of canine CL.

3.2 MATERIAL AND METHODS

3.2.1 Animals and experimental design

Thirty healthy mongrel bitches were included in this study after approval by the Committee of Ethics in the Use of Animals of the School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil (protocol number 2719/2012). After the onset of pro-oestrous bleeding, blood samples were collected on alternate days to determine plasma progesterone (P4) concentrations. In the day of ovulation, P4 plasma concentrations reached 5ng/ml (CONCANNON et al., 1989). Six groups (n=5 animals per group) were established. The CLs were collected via ovariosalpingohysterectomy on days 10, 20, 30, 40, 50 and 60 post -ovulation (p.o.). After collection, CLs were dissected from the surrounding ovarian tissue and immediately frozen in liquid nitrogen for total RNA extraction and further RNA sequencing (RNAseq) or fixed in 4% buffered formalin for 24h for immunohistochemistry. Blood samples were also collected just before anesthesia to determine the hormonal profile of P4 and E2during diestrus.

3.2.2 Library construction and sequencing

Eighteen healthy mongrel bitches were included in the RNA sequencing (RNAseq) experiment (n=3 per group). Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Samples were diluted to a concentration of 150 ng/µL. Integrity of the RNA was assessed through analysis in an Agilent 2100 Bioanalyser (Agilent Technologies). All RNAseq libraries were constructed using Truseq RNA sample Prep (Ilumina, San Diego, CA, USA) in accordance with the manufacter's standard procedure. The quality of the produced libraries was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) with a DNA chip 1000. Samples were considered appropriate when fragments presented close to 260 bp. The quantification of the individual libraries was performed using PCR real time through the kit KAPA Library Quantification (KAPA Biosystems, Willmigton, MA, USA).

Sequencing was performed on an Ilumina HiqSeq 200 using the pair-end reads protocol and the Ilumina TruSeq PE cluster kit v3-cBotHS (Ilumina, San Diego, CA, USA), in accordance with the manufacturer's instructions. Six samples were ranged per lane, sequenced and generated 10 million reads per library.

3.2.3 RNAseq analysis

CASAVA 8.2 software (Ilumina, San Diego, CA, USA) was used to obtain the raw data. The reads were mapped against the reference genome (Canis_familiaris. CanFam 3.1.75.dna.toplevel.fa) using TopHat v2.0.9 and the transcripts were assembled using Cufflinks. The relative abundance of transcripts of RNAseq fragments was measured by Cufflinks in FPKM (Fragments per kilobase of exon per million fragments mapped). For each gene, expression levels were compared among groups. The differential expression of the genes is assayed by log ratio of FPKMm divided by the standard deviation of log ratio. A gene was considered differentially expressed if the FDR adjusted p value was < 0.05.

The differentially expressed (DE) genes detected by Cufflinks were converted into their human orthologs using the Inparanoid eukaryotic ortholog database

(http://inparanoid.cgb.ki.se/), which is a collection of pairwise ortholog groups among 17 whole genomes. Finally, we used oPOSSUM3 (http://opossum.cisreg.ca.) to identify over-represented conserved transcription factor binding sites (TFBS) related to ERs.

3.2.4 Quantitative Real-Time RT-PCR

We performed qRT-PCR for validation of RNAseq results The relative transcripts levels of 10 target genes were measured, five presented over-represented TFBS with ERs, three were related to the IGF system and two with Wnt/beta catenin signaling. RNA samples (n=5 per group) were used to measure the relative gene expression by quantitative real-time RT-PCR (qPCR). Concentration and quality of RNA were determined using a Biophotometer (Eppendorf, Hamburg, Germany) and integrity was analysed by electrophoresis through 2% agarose gel. The samples (1 µg of total RNA) were transcribed to cDNA (complementary DNA) using Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA) according to the manufactures instructions. PCR reactions were performed using an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems) and 96-well optical plates. Each sample (25 ng of total RNA) was analyzed at least in duplicate. Gene-specific primers used are listed in table 2. GAPDH was selected as reference gene based on Normfinder (ANDERSEN; JENSEN; ØRNTOFT, 2004) results. Relative gene expression of lymphoid enhancer-binding factor 1 (LEF1), betacatenin (CTNNB1), cyclin D1 (CNND1), pregnancy-associated plasma protein A (PAPPA), insulin-like growth factor-binding protein 3 (IGFBP3), insulin-like growth factor-binding protein 4 (IGFBP4), insulin-like growth factor-binding protein 5 (IGFBP5), caveolin 1 (CAV1), N-myc downstream-regulated gene 2 (NDGR2) and ATPase Na^+/K^+ (ATP1A1) was calculated by the Pfafll method (PFAFFL, 2001) followed by Linear Regression (LingRegPCR 7.0) fluorescent analysis (RAMAKERS et al., 2003).

Table 2- List of primers for q PCR

Gene	Primer	Sequence	Probe	Genbank number
LEF1		cf02686726_mh		FJ374770.1
CTNNB1	Forward	5' ACTGAGCCTGCCATCTGTGC3'	TTCGTCATCTGACCAGCCGACAC	FJ268743.1
	Reverse	5'TCCATAGTGAAGGCGAACAGC3'	CA	
CCND1		cf02626707-m1		AY620434.1
PAPPA		cf02700554_m1		XM_538813.2
IGFBP3		Cf02655026_g1		XM_548740.2
IGFBP4		Cf02656701_m1		XM_845091.1
IGFBP5		Cf02691124_s1		XM_847792.1
NDGR2		Cf02722935_m1		XM_858273.1
ATP1A1		Cf02627969_m1		L42173.1
CAV-1		Cf02628396_m1		U47060.1
GAPDH		ID cf04419463_gH		AB038240.1

Source: (CARDOSO, A. P. M. M., 2016).

3.2.5 Immunohistochemistry

The immunoperoxidase immunohistochemistry protocol was applied on tissue sections prepared from four CLs per dog, after a microwave pre-incubation in TRIS-EDTA buffer (10mM Tris base, 1mM EDTA, pH 9.0). The primary antibodies used were monoclonal antimouse for PAPPA, IGFBP3, IGFBP4 and IGFBP5 (table 3). Negative controls were prepared using IGg isotype control antibody (normal mouse IGg1, Santa Cruz Biotechinologies, Dallas, TX, USA) for IGFBP4 and IGFBP5, IGg2a isotype control antibody (normal mouse IgG2a, Abcam, Cambridge, UK) for IGFBP3 and IGg2b isotype control antibody (normal mouse IGg2b, Abcam, Cambridge, UK) for PAPP-A. Positive controls were human placenta sections for PAPP-A and IGFBP5 (BAXTER, 2014; OXVIG, 2015) and human liver sections for IGBP3 and IGFBP4 (BACH, 2015a; BAXTER, 2015).

Table 3- List of antibodies for immunohistochemistry

Antibody	Isotype	Immunogen	Diluition	Catolog number
PAPPA	IgG2b	Full length native protein	1:80	Abcam (Ab52030)
IGFBP3	IgG2a	Recombinant full length	1:40	Abcam (Ab 89331)
IGFBP4	IgG1	Human recombinant IGF binding protein 4	1:80	Abcam (Ab4252)
IGFBP5	IgG1	Human IGFBP5	1:80	Novus Biological (NBP2-12366)

Source: (CARDOSO, A. P. M. M., 2016).

3.2.6 Hormone assay

Serum steroid hormones (P4 and E2) were determined by validated chemiluminescence immune assay. The analytical sensitivity of P4 and E2 assay were 0.10ng/mL and 15pg/mL respectively. The inter-assay coefficient of variation (CV) for P4 and E2 was 7.51% and 9.3% respectively. The intra- assay CV was 6.11% and 9.5% respectively.

3.2.7 Statistical Analysis

Data were tested for homogeneity and normality using the Kolmogorov- Smirnov test. Data are presented as mean ±SEM.

The qPCR data were compared by the one-way ANOVA test, followed by the Bonferroni test, for normal distributed data. The correlations between the RNAseq and qPCR results were verified by Pearson's correlation. The difference was considered significant when $p<0.05$. All statistical analyses were performed with GraphPadPrism 5 (GraphPad Software, Inc, San Diego, CA, USA).

3.3 RESULTS

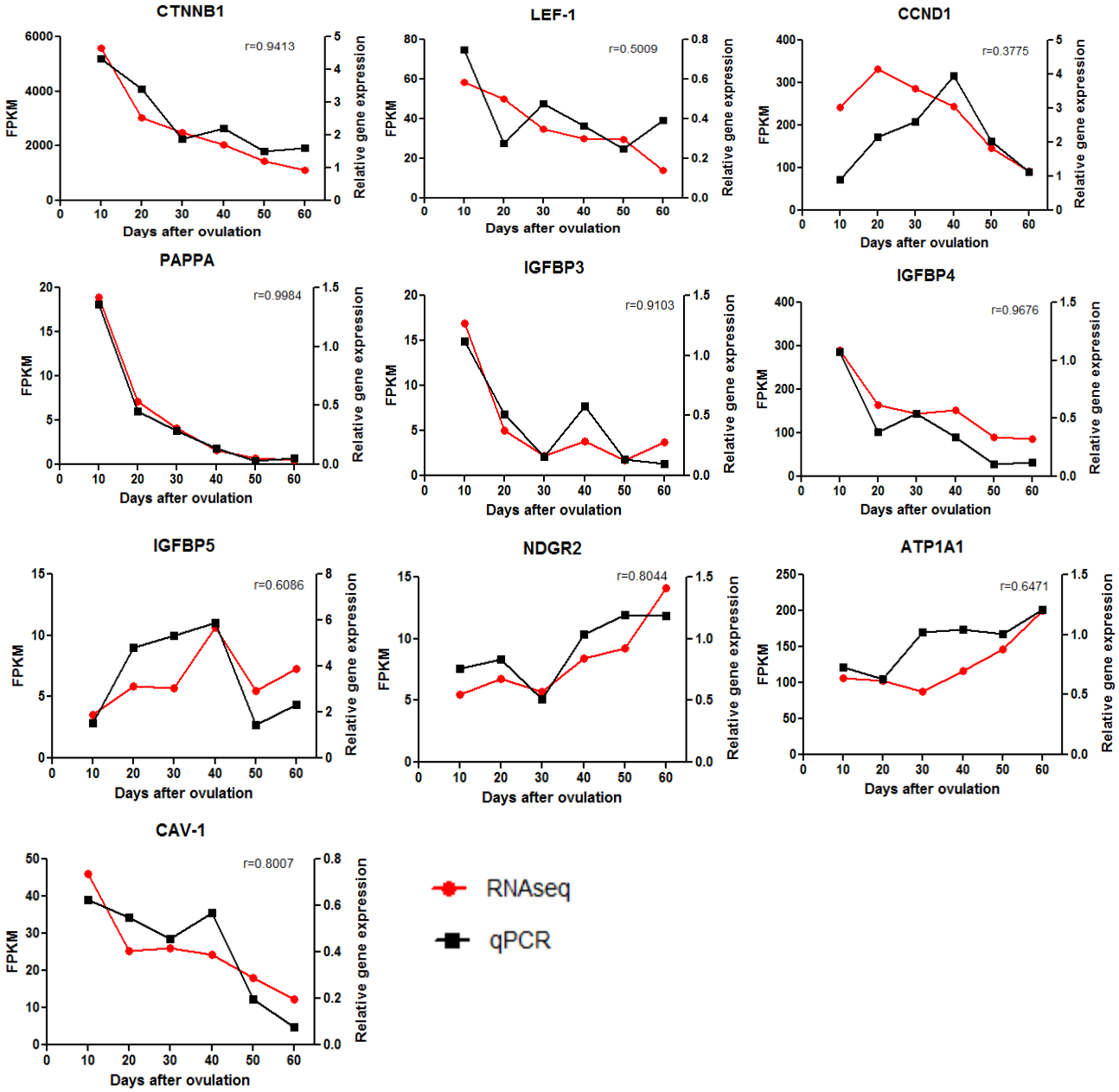
3.3.1Transcripton factor binding sites related with estradiol receptor

The temporal gene expression analysis revealed the presence of 5.116 differentially expressed genes in at least one comparison, and 1106 genes which have not been annotated in the canine genome yet. We converted 4010 genes into their human orthologs in order to identify the over-represented TFBS related to ERs. In our ortholog approach to TF analysis, we assumed that the TF binding sites are conserved evolutionarily, as demonstrated elsewhere (WANG et al, 2012). Fifty genes showed TFBS with ESR1 and 293 with ESR2 (supplementary data S1 and S2); ESR1 and ESR2 shared the same TFBS (in 48 genes).

3.3.2 Validation of selected mRNA levels as estimated by qPCR and RNAseq

We used qPCR analysis to validate the results obtained by RNAseq. Genes were selected after being identified as DE by RNAseq analysis and shared TFBS with ERs or genes associated with the proliferation process. The selected genes included IGF system (PAPPA, IGFBP3, IGFBP4 and IFGBP5), Wnt/betacatenin signaling (CTNNB1, LEF-1 and CCND1), genes regulated by estrogen hormones (NDRG2 and ATP1A1) and plasma membrane ER (CAV1). Results showed that the expression levels detected by qPCR were consistent with the results from RNAseq analysis. Ralues of correlation|(r) from all genes ranged from 0.3775 (for CCND1) to a maximum of 0.9984 (for PAPPA) with a mean value of 0.756 ($p<0.05$) (Figure 7).

Figure 7 - Validation of differentially expressed genes identified by RNAseq, using qRT-PCR. Genes are indicated by their official gene symbols. Data are presented as mean of FPKM (RNAseq) and of relative gene expression (qPCR). The correlation information in each analysis is indicated by Pearson (r)

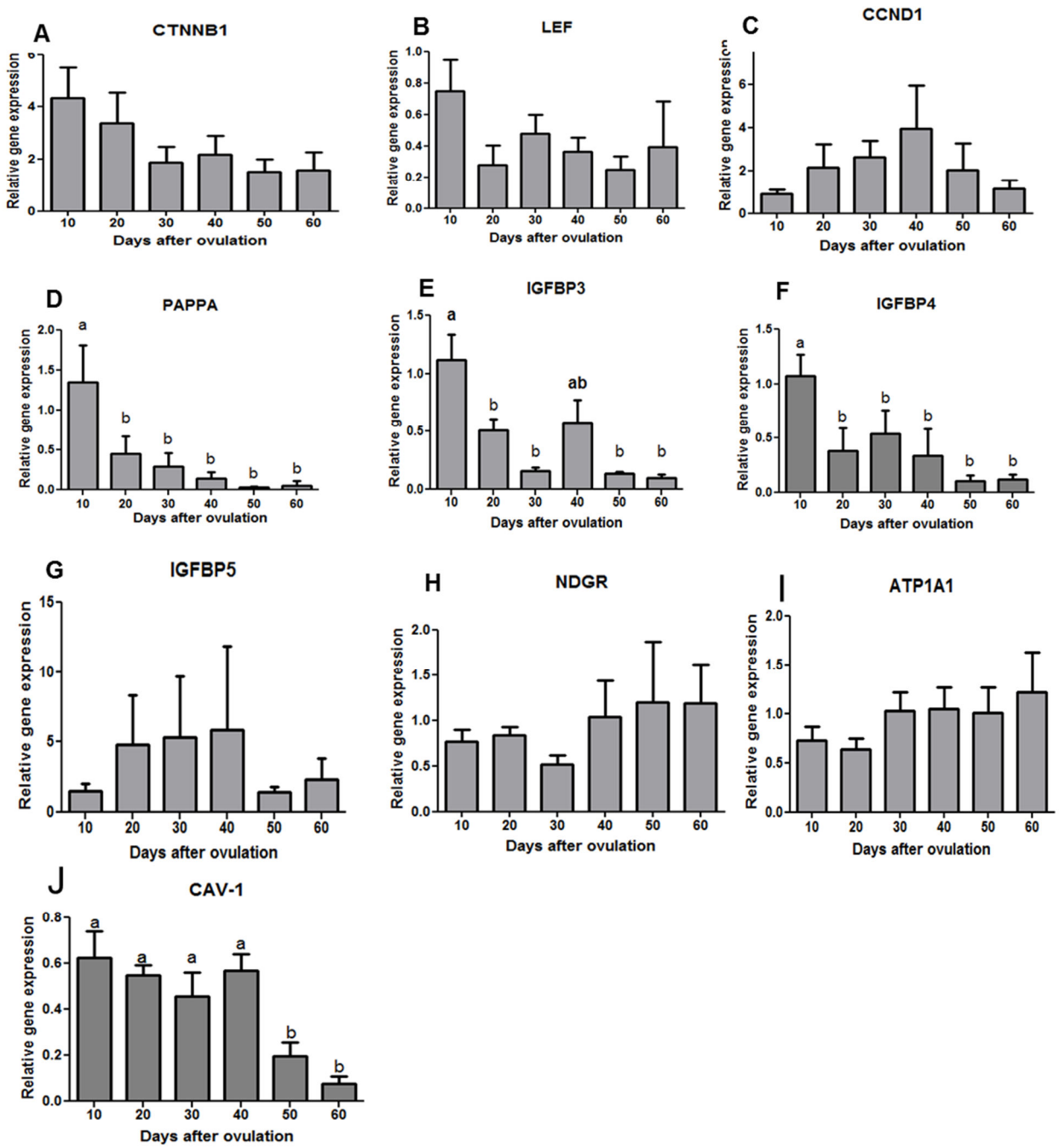


Source: (CARDOSO, A. P. M. M., 2016).

3.3.3 CTNNB1, LEF-1, CCND1, PAPPA, IGFBP3, IGFBP4, IGFBP5, NDGR2, ATP1A1 and CAV1 gene expression in CL during diestrus

No significant differences were observed for CTNNB1, LEF-1, CCND1, IGFBP5, NGDR2 or ATP1A1 mRNA expression (Figures 8A, 8B, 8C, 8G, 8H and 8I) during diestrus. PAPPA, IGFBP3 and IGFBP4 mRNA expression changed significantly (Figures 8D, 8E and 8F) with the highest expression on day 10 p.o ($p<0.001$) and a decreased thereafter. Caveolin-1 gene expression showed no variation from days 10 to 40 p.o (Figure 2J), and then decreased after day 50 p.o ($p<0.001$).

Figure 8 - Gene expression of CTNNB1(A), LEF-1(B), CCND1(C), PAPPA(D), IGFBP3(E), IGFBP4(F), IGFBP5(G), NGDR2(H), ATP1A1(I) AND CAV-1(J) in canine CL during diestrus (10 – 60 days p.o.). Data are presented as mean ± standard error of relative gene expression (n=5 animals group). Bar with different letters indicate significant differences between groups

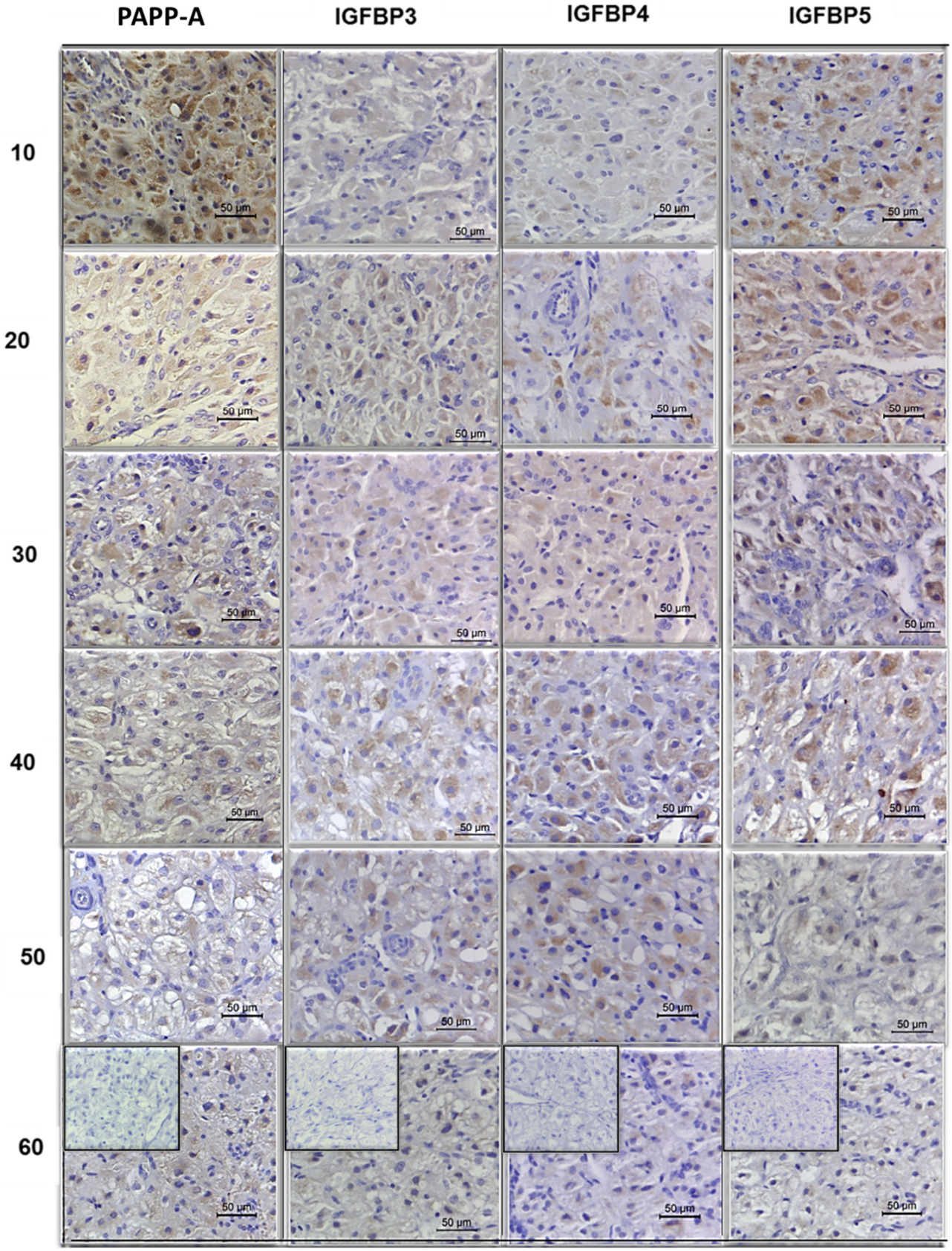


Source: (CARDOSO, A. P. M. M., 2016).

3.3.4 Localization of PAPPA and IGF binding proteins

To get further insights on the role of IGF system over CL lifespan, we verified the localization of PAPPA and IGF binding proteins (IGFBP3, IGFBP4 and IGFBP5). All proteins presented immunopositive staining, which could only be observed in the cytoplasm of luteal and endothelial cells in all studied phases (days 10-60p.o., Fig 4). PAPPA exhibited a stronger positive signal on day 10 p.o.; whereas IGFBP3 and IGFBP4 on days 40 and 50 p.o.(Fig. 4). IGFBP5 showed a stronger staining in the beginning of diestrus and a qualuitative decrease on days 50 and 60 p.o. (Figure 9).

Figure 9 - Immunolocalization of PAPPA, IGFBP3, IGFBP4 and IGFBP5 in CL during diestrus (days 10-60 p.o.).NC=negative control. Scale bars = 50µm

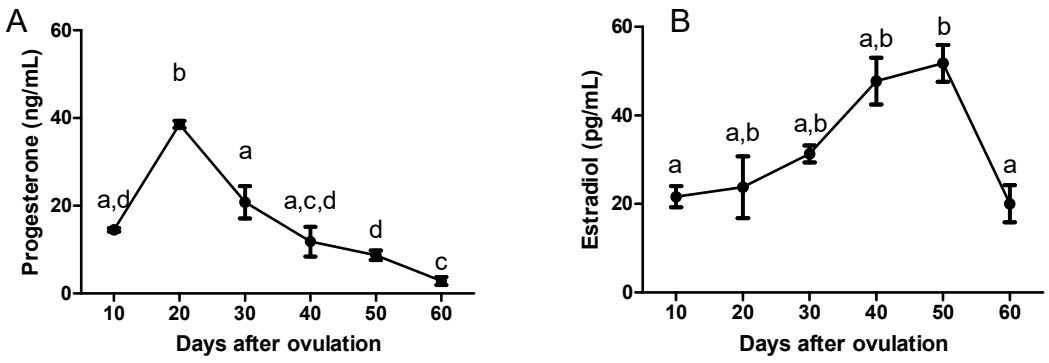


Source: (CARDOSO, A. P. M. M., 2016).

3.3.5 Progesterone (P4) and 17β- estradiol (E2) concentrations

The steroidogenic plasma profile of bitches variated over diestrus. P4 is abundant during the developing phase and it presented the highest plasma concentration on day 20 p.o. ($p<0.0001$) decreasing gradually thereafter (Figure 10A). E2 concentrations reached highest plasma concentrations on day 50 p.o., which differed significantly from days 10 and 60 p.o. (Figure 10B).

Figura 10 - Mean ±SEM concentrations of serum progesterone (A) and estradiol17-β (B) in periferical plasma of 30 bitches during diestrus.Different letters indicate a significant difference ($p<0,05$)



Source: (CARDOSO, A. P. M. M., 2016).

3.4 DISCUSSION

This is the first study that used a high sequencing technology to focus on the continuous changes of cellular transcriptome probably mediated by E2 in cyclic canine CL. Our results suggest an action of E2 during proliferative and regression phases of canine CL mediated by binding of E2 to ERα and ERβ, respectively.

An essential step to identify the regulatory network is the analysis of the promoter region (HO SUI et al., 2007; KWON et al., 2012; LI et al., 2015) and this can be perfomed by the identification of over-represented TFBS. This region is modeled using position specific scoring matrices (PSSMs), which are constructed from alignments of binding sites sequences characterized experimentally or identified in high-throughput protein-DNA binding assays (HO

SUI et al., 2005, LEE; HUANG, 2013). The use of PSSMs to detect TFBS is well established (HO SUI et al., 2005, LEE; HUANG, 2013) and studies indicate that oPPOSSUM produces few false positive results (MENG; MOSIG; VINGRON, 2010; KWON et al., 2012). Furthermore, the presence of gene and protein were demonstrated in canine CL by Bonfim Neto (2014) and Papa and Hoffmann (2011), confirming that this analysis may be a complement to understand deeper the role of E2 in canine lifespan. We found 295 genes showing TFBS related to ERs, from which 48 were shared between ESR1 and ESR2. This finding is not surprising, since it has also been observed by other researchers in cancer cells (CHANG et al., 2006; ZHAO; DAHLMAN-WRIGHT; GUSTAFSSON, 2010; GROBER et al., 2011), although in these studies each receptor had distinct effects on target genes (and this possibility cannot be rouled out in canine CL). Some ERs target genes were related to different signaling pathways, showing that E2 signaling in CL is, probably, mediated by cross-talk. For example, our results showed that LEF-1 and PAPPA had TFBS with ESR2 and they are key elements in different signaling pathways (Wnt/β-catenin signaling and IGF system, respectively).

Our P4 results were similar to those reported previously by Hoffmann et al (1992) and our research group Papa et al (2014). E2 results were similar to those published previously (PAPA et al., 2014), confirming our hypothesis that E2 plasma concentrations may influence CL dynamic, especially its regression, as the first signs of regression (HOFFMANN et al., 2004a) matches highest E2.

The Wnt/ β-catenin signaling is a pathway related to cell differentiation, cell proliferation and apoptosis (MACDONALD; TAMAI; HE, 2009; BAARSMA; KÖNIGSHOFF; GOSENS, 2013; SHY et al., 2013). The activity of this pathway is controlled by β-catenin protein stability (MACDONALD; TAMAI; HE, 2009), which depends upon the action of the β-catenin destruction complex. Glycogen synthase kinase (GSK3) is the main protein of this complex; when GSK3 is inhibited, β-catenin stabilizes and translocates to the nucleus (CLEVERS; NUSSE, 2012; BODNAR et al., 2014). In the nucleus, β-catenin interacts with DNA binding transcriptional regulators, such as LEF-1. When β-catenin and LEF-1 are bound, they act as transcriptional co-activator and activate the transcription of WNT signaling of specific genes such as CCND1, VEGF, c-Myc and Mmp7 (RIDER et al., 2006; RAY et al., 2008; YANG et al., 2010; GUPTA et al., 2011).

In the absence of nuclear β-catenin, LEF-1 acts as a transcriptional repressor (MACDONALD; TAMAI; HE, 2009; CLEVERS; NUSSE, 2012). As described by Holmes and cols. (2008), LEF-1 binds to several ER-cis regulatory elements and acts as transcriptional

a repressor of estrogen, since LEF-1 competes with ERs to bind to DNA. Our results showed that LEF-1 had over-represented TFBS with ESR2 and LEF-1 expression is higher at the beginning of diestrus. Thus, probably, LEF-1 was a repressor of ERβ signaling in canine CL during early luteal phase or can active the proliferation process via binding to β-catenin, because the CTNNB1 and LEF-1 gene expression showed a similar behavior pattern during diestrus: although no significant difference was observed, their qualitative expression was highest on day 10 p.o. and decreased over the studied period, pointing towards a relation with CL proliferation. In mouse uterine stromal and epithelial cells, neural cells, colon and breast cancer cells, E2 stimulated β-catenin translocation to the nucleus through ERα signaling and increased the amount of this protein binding to LEF-1 (KOUZMENKO et al., 2004; RIDER et al., 2006; RAY et al., 2008; VAREA et al., 2010; WANDOSELL et al., 2012). As ERS1 expression is highest at the beginning of diestrus (PAPA; HOFFMANN, 2011), probably, a similar signaling could be observed in CL.

In neural and hepatoma cells, IGF-I estimulates β-catenin translocation to the nucleus via activation of PI3K/AKT signaling, which inhibits GS3K and stabilizes β-catenin (DESBOIS-MOUTHON et al., 2001; VAREA et al., 2010; WANDOSELL et al., 2012). In neural cells, Varea and cols. (2010) and Wandosell and cols. (2012) observed that E2 induced the association between ERα and IGF-IR and this complex activated PI3K/AKT signaling. Our results bring some members of IGF system to the factors related with CL lifespan in bitches, and the observed alterations in gene and protein expression are probably homologous to that described for other species, as bovines (BROWN; BRADEN, 2001; UNIYAL et al., 2015), ovines (HASTIE; HARESIGN, 2006), porcines (GE et al., 2003; GADSBY et al., 2006), humans (SUGINO et al., 1999; FRASER et al., 2000; IÑIGUEZ et al., 2001), rats (SUGINO et al., 1999) and non-humans primates (BROGAN et al., 2010).

The IGFBPs are a group of six proteins that bind IGF with high affinity and block their interaction with IGF receptors. IGFBPs cleavage by enzymes such as matrix metalloproteinases (MMPs) and PAPPA can increase IGF bioavailability and activate its cell surface receptor (BUNN; FOWLKES, 2003; BAXTER, 2014; BACH, 2015; BRAHMKHATRI; PRASANA; ATREYA, 2015; DENDULURI et al., 2015). As IGFBPs modulate the effects of IGF, they are probably involved in luteal function (BROWN; BRADEN, 2001) and their presence in the CL has been reported also for bovines (BROWN; BRADEN, 2001; NEUVIANS et al., 2003; UNIYAL et al., 2015), humans (FRASER et al., 2000; IÑIGUEZ et al., 2001), porcines (GE et al., 2003) and ovines (HASTIE; HARESIGN, 2006).

In our study, we demonstrated IGFBPs 3-5 mRNA and protein expression in CL of non-pregnant bitches. IGFBP3 and IGFBP4 mRNA expression was highest at the beginning of diestrus, but staining was stronger when luteal regression started, whereas IGFBP5 mRNA and protein expression did not vary significantly during diestrus. These protein results could be explained by IGFBP protease, such as PAPP-A. This enzyme is a marker of corpus luteum formation (HOURVITZ et al., 2000; CONOVER et al., 2001; CONOVER, 2012); which was corroborated by our experiment, in which it exhibited a time-dependent gene and protein expression, with highest values at the beginning of diestrus. Although its gene expression decreased during CL maintenance phase, its protein signals could still be observed. PAPP-A cleaves IGFBP4 and IGFBP5, but cleavage of IGFBP4 is enhanced by the presence of IGF, whereas the IGFBP5 cleavage is slightly reduced (SØE et al., 2002). PAPP-A is an important regulator of IGF bioavailability and this function is important to determinate the follicular fate, since its expression is restricted to dominant follicles and CL (HOURVITZ et al., 2000; CONOVER et al., 2001; CONOVER, 2012). This protease is a key regulator of ovarian steroidogenesis in mice and humans (NYEGAARD et al., 2010; BØTKJÆR et al., 2015). IGFBP3 is cleaved by other proteinases such as MMPs, mainly MMP1, MMP2 and ADAM12 (OXVIG, 2015) and these proteinases were also found among our DE genes, showing highest expression on day 10 p.o. (data not shown). Thus, our data suggest strong presence of IGFs during CL proliferation and maintenance phases.

The increased protein expression of IGFBP3 and IGFBP4 could be related to CL structural regression in our study, as they are growth-inhibitory in many systems *in vitro* and *in vivo* (BACH, 2015; BAXTER, 2015). This inhibitory role for IGFBP3 has been described in porcine and bovine CL (BROWN; BRADEN, 2001; GE et al., 2003), whereas IGFBP4 just in bovines (SCHAMS et al., 2002). Some IGFBP3 inhibitory effects are mediated by induction of apoptosis (GRIMBERG, 2000; JOHNSON; FIRTH, 2014; BAXTER, 2015; RANKE, 2015) and inhibition of angiogenesis (KIM et al., 2011), whereas IGFBP4 inhibits steroidogenesis (BACH, 2015), angiogenesis (RYAN et al., 2009; MORENO et al., 2013; BACH, 2015) and is a β-catenin signaling inhibitor (BAXTER, 2015). Therefore, our data suggest a wide and complex regulation of the IGF system in canine CL.

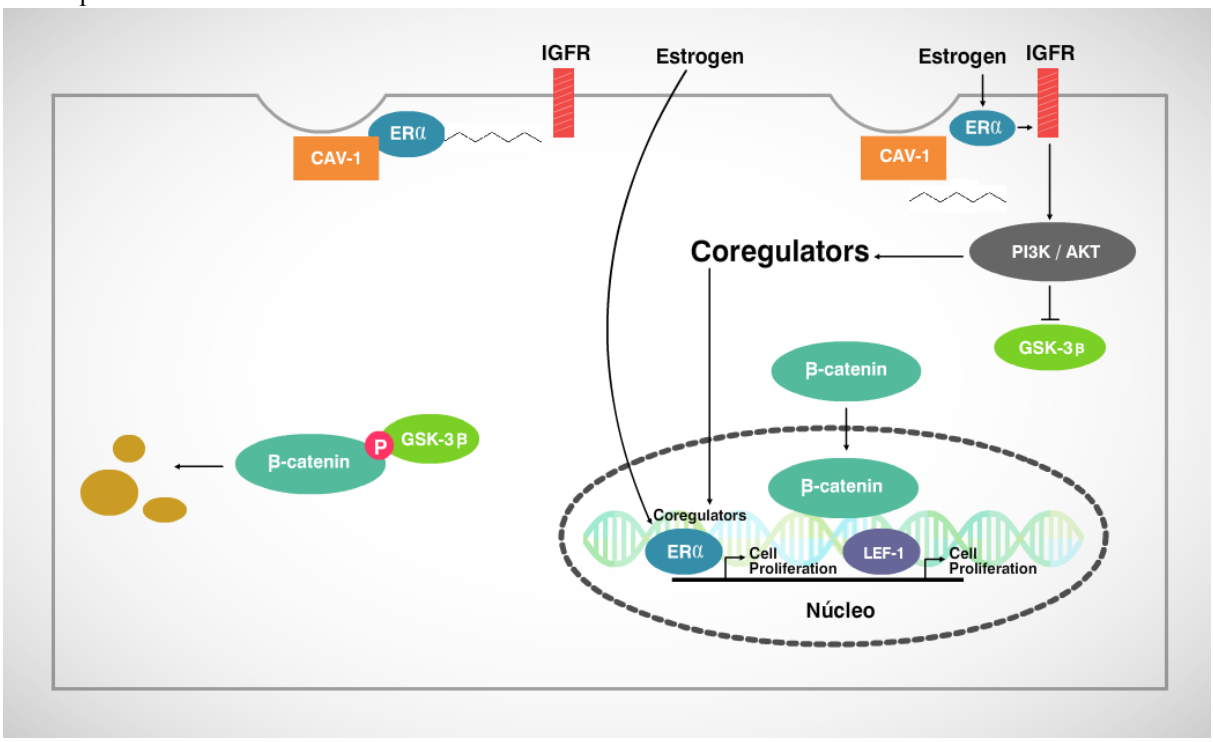
Caveolin-1 (CAV-1) is a membrane protein present in caveolae, a unique type of lipid raft that is responsible for signal transduction. The presence of caveolae was observed in early CL of bitches and humans (CRISP; DESSOUKY; DENYS, 1970; ABEL et al., 1975).Our results showed highest CAV1 expression at the beginning of diestrus, but its expression did not

change from day 20 to 40 p.o. when it started decreasing. This suggests that CAV-1 is necessary during CL formation and maintenance, due to its importance during angiogenesis. CAV-1 is considered an excellent marker for vascularization (LUPIÁÑEZ et al., 2012; LAURENZANA et al., 2015) and is necessary for optimal neovascularization (SOWA, 2012). However, CAV-1 can bind to ERα and potentiates ERα activity (SCHLEGEL et al., 2001; ANWAR et al., 2015). This engagement with ERα occurs at the cell membrane and activates many signal transduction cascades (non-genomic pathways) related to proliferation and differentiation (MARINO; ASCENZI, 2008; LA ROSA et al., 2012; PEDRAM et al., 2012; HAMMES; DAVIS, 2015), confirming our TFBS results, which emphasized a co-regulation between CAV1 and ERS1. The presence of ERα in plasma membrane was first observed in endometrial cell trough intact cell binding to estradiol immobilized on nylon fibers (PIETRAS; SZEGO, 1977) and it was supported by other studies that have also demonstrated its presence by enzyme-linked immunocytochemistry, confocal microscopy, flow cytometric analysis and affinity chromatography in pituitary tumor cells, endothelial cells and breast cancer cells lines, (NORFLEET et al., 1999; RUSSELL et al, 2000,PEDRAM; RAZANDI; LEVIN, 2006).

In order to be located in the plasma membrane and associated with CAV-1, ERα undergoes a post-translational modification denominated palmitoylation, which is mediated by palmitoyltransferase (PAT) (MARINO; ASCENZI, 2008; LA ROSA et al., 2012; PEDRAM et al., 2012).Palmitoylation promotes the physical association of ER with caveolin-1, thus facilitating ER translocation to the plasma membrane. We found some differentially expressed PATs during diestrus, with highest expression during the proliferation and maintenance phases (10-30 p.o.) as demonstrated by our RNAseq results (data not shown). These results lead to speculation of plasma membrane-localized ERα in canine CL. Scientific community has come to a consensus model that E2 exerts its proliferative effects exclusively mediated by binding to membrane ERα (LA ROSA et al., 2012; HAMMES; DAVIS, 2015). This E2-ERα membrane complex promotes rapid activation of growth factors, such as epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR), which results in the activation of several kinases and enhancement of ERα-mediated transcription as seen in CCND1 transcription, a key regulator of cell cycle progression (MARINO; ASCENZI, 2008; LA ROSA et al., 2012; HAMMES; DAVIS, 2015). Thus, this signaling could be responsible for CCND1 expression (depicted from our RNAseq results), which increased during the proliferation phase with no changes until the start of structural regression, when this expression decreased. Therefore, our results suggest that genomic and non-genomic ERα pathways are linked to the

control of CL proliferation and these processes may also depend on cross-talk mediated by the IGF system and Wnt/β catenin signaling (Figure 11). Therefore, we intend to deeper the study of these complicated cross-talk pathways.

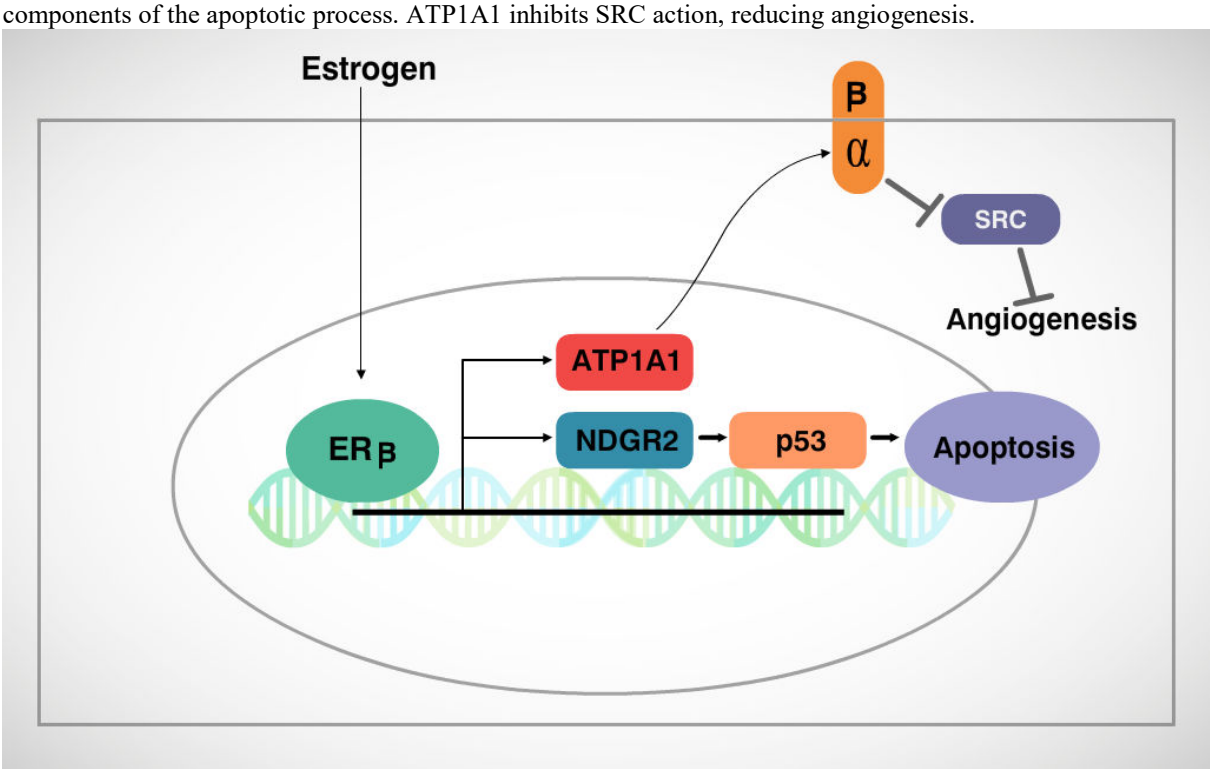
Figure 11- Proposed model for the interaction between ERα, the IGF system and WNT/β-catenin signaling in CL of non-pregnant bitches. When E2 activates ERα present at the plasma membrane, this receptor separates from CAV-1 and interacts with IGFR, activating PI3/AKT, which stabilizes β-catenin and improves ERα control of transcription.



NDRG2 is a cytoplasmic protein and member of the NDRG family that belongs to the α/β hydrolase family and is known as a tumor suppressor gene (MA et al., 2012; OBRADOVIC et al., 2013; SHEN; YAO; ZHANG, 2015). NDRG2 is a target gene of E2 through binding to ERβ, which in turn regulates its expression via transcriptional activation (LI et al., 2011), reinforcing our TFBS data: on day 60 p.o. its expression was highest, which coincides with increased ESR2 mRNA (BONFIM NETO, 2014). In many malignant tumors, this protein is able to suppress endothelial cell proliferation and enhance apoptosis by increasing p53 expression (MA et al., 2012; SHEN; YAO; ZHANG, 2015). The highest p53 gene expression was found on day 60 p.o. in our RNAseq results, suggesting a contribution of E2 in CL regression mediated by ERβ involving also the apoptotic mechanism (Figure 12).

The ATP1A1 gene encodes the catalytic subunit of Na^+/K^+ ATPase protein (NKA). NKA is responsible for creating an electrochemical gradient across the plasma membrane, but exerts other functions like cell adhesion, motility and signal transduction (OBRADOVIC Et al., 2013; CHEN et al., 2014; LI; LANGHANS, 2015). In porcine preovulatory luteinized follicles, ATP1A1 was functionally classified as a cell growth inhibitor (AGCA et al., 2006). A decrease in ATP1A1 expression has also been observed in several human cancers such as prostate, kidney and bladder (ESPINEDA et al., 2003; SELIGSON et al., 2008; LI et al., 2011). These data corroborate our study, as ATP1A1 expression increased when the structural regression started and its expression showed a positive correlation with NDGR2 protein and gene expression (LI et al., 2011). These genes had TFBS with ESR2, indicating that their activity is necessary to reduce CL proliferation. Li and cols (2011) demonstrated that the NKA α1 subunit regulates cellular kinases activity through its interaction with c-Src (Proto-oncogene tyrosine-protein kinase) inhibiting Src cellular action, through reduction of VEGF production and angiogenesis. To act as a signal transduction factor, the NKA α1 subunit must be located in tight junctions (RAJASEKARAN; RAJASEKARAN, 2009), enabling us to further comprehend the role of ATP1A1 in CL regression (Figure 12).

Figure 12- Proposed model for ERβ signaling in CL of non-pregnant bitches. E2 signaling activates ERβ, which increases the genic transcription of NDGR2 and ATP1A1. NDGR2 increases p53 expression, triggering components of the apoptotic process. ATP1A1 inhibits SRC action, reducing angiogenesis.



The correlation analysis between the RNAseq and qPCR results was satisfactory, showing that RNAseq may be a good tool for finding DE genes, as demonstrated already by others researches (NAGALAKSHMI ET AL., 2008; SAMBORSKI ET AL., 2013; MARTÍNEZ-LÓPEZ; OCHOA-ALEJO; MARTÍNEZ, 2014). Transcriptome evaluation is a key step to understanding the complex tissue formation process, especially when weakly expressed genes are involved. Although, no significant difference has been observed for the majority of the qPCR validated genes, these could be explained by the low level of expression of some genes (NAGALAKSHMI et al., 2008).

In summary, our study suggests a possible role of E2 in the regulation of the CL lifespan. During the first half of diestrus, E2 seems to act as a luteotrofic factor throughout, via non-genomic signaling mediated by ERα and interacting with the IGF system and Wnt/β catenin signaling. In the second half of diestrus, one pro-luteolytic action appears to be mediated by the ERβ pathway, regulating the expression of NDGR2 and ATP1A1.

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ANEXOS

ANEXO A – Differentially expressed genes (DEGs) with overrepresented transcription binding sites (TFBS) with ESR1

Ensembl ID(s)	Official gene symbol	Chr	TFBS Rel. Start	TFBS Rel. End	Score	%Score	TFBS Sequence
ENSG00000003137	CYP26B1	2	1670	1689	16,48	86,40%	CCCTCAGGTCCCCATGACCT
ENSG00000011347	syt17	11	1752	1771	15,78	85,30%	ATGCCTGGGCACCATGACAT
ENSG00000033327	GAB2	11	-2790	-2771	15,7	85,20%	TCAGCTGGTCACACTGACCT
ENSG00000059915	psd	10	173	192	17,78	88,40%	GTTCCAGGGCCCCCTGACCT
ENSG00000079432	Cic	19	941	960	15,96	85,60%	GATCCGGATCAACCTGACCT
ENSG00000080573	Col5a3	19	-4178	-4159	19,65	91,20%	AGTCCAGGCCACTCTGACCT
ENSG00000086730	LAT2	7	-2990	-2971	17,95	88,60%	GCCCCAGGCCACCCTGGCCC
ENSG00000105612	DNASE2	19	-4591	-4572	16,64	86,60%	GGCCCGGGTCCTCCTGACCC
ENSG00000105974	cav1	7	1109	1128	18,05	88,80%	AGCTCAGGGCAGGCTGACCC
ENSG00000108515	ENO3	17	-2580	-2561	15,63	85,10%	TGGCCAGGTGGCCCTGCCCC
ENSG00000108771	dhx58	17	-4991	-4972	15,93	85,60%	AGTGATGGTCAGCATGACCT
ENSG00000108846	Abcc3	17	-4007	-3988	18,35	89,20%	GGCACAGGGCACCCTCACCT
ENSG00000113070	HBEGF	5	-1057	-1038	16,1	85,80%	GGGCAGGGCCAGACTGACCC
ENSG00000116191	ralgps2	1	648	667	18,4	89,30%	GCAACAGGTCACCCTGACCG
ENSG00000121769	fabp3	1	634	653	16,21	86%	GAGTGAGGTCATTGTGACCT
ENSG00000125967	NECAB3	20	-221	-202	16,57	86,50%	CACTTGTGTCACCCTGACCT
ENSG00000129116	palld	4	1593	1612	15,79	85,30%	TTGTCAGGTCATGCTGACTT
ENSG00000130052	Stard8	X	-1031	-1012	15,66	85,20%	CAGCCATGGCACCCTGCCTT
ENSG00000130299	GTPBP3	19	-4820	-4801	15,95	85,60%	AAGCAGGGCCAGCATGACCT
ENSG00000131435	PDLIM4	5	-2826	-2807	15,84	85,40%	AGGCCTGGGCATCGTGCCCT
ENSG00000131620	ANO1	11	-1926	-1907	18,78	89,90%	TGACCAGGCCACCCTGCCCT
ENSG00000131771	PPP1R1B	17	-2639	-2620	16,31	86,10%	AATGCAGGGCACCCTGGCCC

ENSG00000132639	SNAP25	20	-1272	-1253	16,26	86,10%	ATCCCAGGGGACCCTGCCTT
ENSG00000133424	LARGE	22	1423	1442	16,2	86%	TGCTCAAGTCACTGTGACCC
ENSG00000138061	CYP1B1	2	-37	-18	17,03	87,20%	CTGCCAGGTCGCGCTGCCCT
ENSG00000139531	SUOX	12	-3181	-3162	19,69	91,30%	ATCCTAGGTCACTGTGACCT
ENSG00000141295	SCRN2	17	-4148	-4129	16,75	86,80%	CGGGAAGGTCATCCTGTCCC
ENSG00000143365	Rorc	1	-547	-528	16,77	86,80%	TTGCCAGGACTCCCTGACCC
ENSG00000148339	Slc25a25	9	-1607	-1588	17,99	88,70%	AGCCACGGTCACCCTGACAC
ENSG00000156453	PCDH1	5	-784	-765	15,77	85,30%	TGCCCCGGTCTCCCTGCCCC
ENSG00000157193	LRP8	1	-4543	-4524	16,14	85,90%	CTCCCTGGTCCCCCTGCCCT
ENSG00000158710	TAGLN2	1	-270	-251	16,59	86,60%	GGCCCTGGGCAGAGTGACCT
ENSG00000161203	AP2M1	3	179	198	16,52	86,50%	GACTTGGGCCACCCTGACCC
ENSG00000161328	LRRC56	11	-4562	-4543	19,96	91,70%	GCCCCGGGCCACCCTGACCT
ENSG00000162496	Dhrs3	1	-4249	-4230	17,22	87,50%	CATGAAGGTCACGCTGCCCT
ENSG00000166272	C10orf26	10	-1739	-1720	17,07	87,30%	GGTCAAGGTCACATTGACTT
ENSG00000168453	TAGLN2	8	684	703	17,66	88,20%	GGGCTAGGGCAGCTTGACCC
ENSG00000170345	AP2M1	14	-3362	-3343	18,35	89,20%	TCTCAAAGTCACCCTGACCT
ENSG00000172009	LRRC56	19	-2390	-2371	16,82	86,90%	GGCCAGGGTCACCGCGACCC
ENSG00000172819	Dhrs3	12	1316	1335	16,38	86,20%	ACGCCAGGTCACCAAGCCCC
ENSG00000175445	C10orf26	8	499	518	16,89	87%	AGGCGAGGTCACCTTGGCCC
ENSG00000179104	TMTC2	12	-933	-914	15,58	85%	GACCCAGGGCTGCCTGACAC
ENSG00000182199	SHMT2	12	-2059	-2040	15,61	85,10%	CCCAGTGGGCACCCTGACCC
ENSG00000196208	GREB1	2	-1583	-1564	15,87	85,50%	CTTCTAGGTCAGAATGACCC
ENSG00000196517	SLC6A9	1	-4790	-4771	17,48	87,90%	CTGCCAGGACTCCCTGACCC
ENSG00000196535	Myo18a	17	-1131	-1112	16,59	86,60%	ACCCCAAGTCACCCTGATCT
ENSG00000197905	tead4	12	-1149	-1130	16,98	87,20%	GGCCAAGGCCAAGCTGACCC
ENSG00000198133	TMEM229B	14	823	842	16,93	87,10%	GGCCAATGTCTCCCTGCCCT

ENSG00000213246	SUPT4H1	17	-1702	-1683	18,15	88,90%	ACCCCAGGGCAGCCTGACCA

ANEXO B – Differentially expressed genes (DEGs) with overrepresented transcription binding sites (TFBS) with ESR2

Ensembl ID(s)	Official gene symbol	Chr	TFBS Rel. Start	TFBS Rel. End	Score	%Score	TFBS Sequence
ENSG00000001617	sema3f	3	1448	1465	12,516	85,70%	CAAGGTCACCTGAGCCTG
ENSG00000003137	CYP26B1	2	1674	1691	17,477	92,80%	AAAGGTCATGGGGACCTG
ENSG00000004866	ST7	7	197	214	12,759	86%	GAAGATCAGAATGCCCTA
ENSG00000005020	skap2	7	-1347	-1330	13,152	86,60%	CAAGGTCACAATGAATTC
ENSG00000005249	prkar2b	7	-680	-663	14,279	88,20%	AAAGGTCACTGTCATCTT
ENSG00000006468	etv1	7	1255	1272	12,529	85,70%	AAGGGTCAAGATGTCTTA
ENSG00000009335	UBE3C	7	-1185	-1168	17,114	92,30%	TAAGGTCACGTTGTCCCC
ENSG00000011347	Syt7	11	1756	1773	12,488	85,70%	TGATGTCATGGTGCCCAG
ENSG00000012171	SEMA3B	3	-2316	-2299	15,266	89,60%	CCAGGTCATGCTGCCCAG
ENSG00000017427	Igf1	12	-2737	-2720	12,669	85,90%	AGGTGTCATGGTGAACTT
ENSG00000027075	PRKCH	14	-482	-465	12,388	85,50%	CCAGGGCACGGCGCCCCC
ENSG00000030419	ikzf2	2	815	832	13,046	86,50%	AAAGCTCATTGTCACCTG
ENSG00000033327	GAB2	11	-2786	-2769	17,32	92,60%	GAAGGTCAGTGTGACCAG
ENSG00000033867	Slc4a7	3	-30	-13	13,146	86,60%	TTAGGTCACTGTGCTTTG
ENSG00000048707	VPS13D	1	-1684	-1667	12,901	86,20%	GAAGGGCACGCTGCACTC
ENSG00000049449	RCN1	11	-683	-666	14,27	88,20%	NNAGGCCACGCTGTCCTG
ENSG00000059915	psd	10	177	194	15,732	90,30%	ACAGGTCAGGGGGCCCTG
ENSG00000063241	isoc2	19	-2113	-2096	12,699	86%	CTGAGTCTCGCTGCCCTG
ENSG00000064205	Wisp2	20	-54	-37	12,739	86%	CTGGGTCACACCCACCTC
ENSG00000065882	TBC1D1	4	-4166	-4149	13,482	87,10%	TAATGTCATTTTCACCTG
ENSG00000066136	nfyc	1	-1954	-1937	12,112	85,10%	ATTGGTCATTTTGACCCC
ENSG00000067842	Atp2b3	X	1283	1300	12,097	85,10%	TTGGGCCACTCTGTCCTT
ENSG00000069188	sdk2	17	-1156	-1139	13,092	86,50%	CCGGCTCACGCTGCCCCC
ENSG00000069399	Bcl3	19	1871	1888	12,255	85,30%	CTAGGCCACTTTCTCCTG
ENSG00000070961	ATP2B1	12	627	644	12,104	85,10%	GCCGGGCACGGTGCCCTC
ENSG00000071537	SEL1L	14	-25	-8	13,191	86,70%	CTGGGTCAGGGAAGCCTG
ENSG00000073350	llgl2	17	-1358	-1341	12,807	86,10%	GTGGATCATGGTGACATC
ENSG00000073792	Igf2bp2	3	47	64	15,317	89,70%	AAATGTCACAGTACCCTG
ENSG00000073861	tbx21	17	-1678	-1661	13,165	86,60%	CTGGGTCATGCCCACCTC
ENSG00000074527	NTN4	12	-80	-63	13,448	87%	AGGGGTCATGATGTCCCT

ENSG00000076356	Plxna2	1	-1061	-1044	12,025	85%	CTGGGTCAGGCCAGCCTC
ENSG00000078114	Nebl	10	-333	-316	14,345	88,30%	CACTGTCAGATTGCCCTG
ENSG00000079308	TNS1	2	-127	-110	12,328	85,40%	CCAGCTCAGCTTGCTCTG
ENSG00000079337	Rapgef3	12	-2414	-2397	12,904	86,30%	CCGGGTCATGGGGCCCAG
ENSG00000079432	Cic	19	939	956	15,196	89,50%	TCAGGTCAGGTTGATCCG
ENSG00000079691	Lrrc16a	6	906	923	12,369	85,50%	ATGGGTCACCTTGCACAG
ENSG00000080573	Col5a3	19	-4174	-4157	15,461	89,90%	AGAGGTCAGAGTGGCCTG
ENSG00000081189	MEF2C	5	1212	1229	12,82	86,10%	GAAGGTCAGGGTGAGCAG
ENSG00000084734	Gckr	2	-2601	-2584	14,887	89,10%	CTGGCTCAGGCTGCCCTT
ENSG00000086015	MAST2	1	-696	-679	12,599	85,80%	GCAGGTCAGGCTACCCAG
ENSG00000086730	LAT2	7	-4753	-4736	12,451	85,60%	GGAGGGCATCTTGTCCTG
ENSG00000088298	EDEM2	20	855	872	13,272	86,80%	GAGGGTCACTGTGGCACG
ENSG00000089558	Kcnh4	17	-4364	-4347	13,387	86,90%	CAGGGGCACAGTGATCCA
ENSG00000090006	LTBP4	19	-2250	-2233	12,661	85,90%	CTGGGCCACTCTGTCCCC
ENSG00000090924	Plekhg2	19	-2943	-2926	13,234	86,70%	CTGGGTCAAGGAGCCCAG
ENSG00000093072	CECR1	22	-266	-249	12,114	85,10%	CTAGGTCTTAATGCCCCC
ENSG00000100116	gcat	22	-2369	-2352	15,022	89,30%	CTCGGTCATGGTGGCCTG
ENSG00000100351	GRAP2	22	-4574	-4557	12,949	86,30%	CAAGGTCAGCTTAATCAC
ENSG00000100360	rabl4	22	-104	-87	14,126	88%	AAAGGTCATTCTGCACTT
ENSG00000101040	Zmynd8	20	-112	-95	13,918	87,70%	CAAGGACACTGTCACCTC
ENSG00000101871	MID1	X	-1568	-1551	15,769	90,30%	AAGGGTCATTTTGAACTC
ENSG00000101935	AMMECR1	X	300	317	12,923	86,30%	AAAGCGCAGGGTGCCCTT
ENSG00000102362	SYTL4	X	-2591	-2574	11,995	85%	GAAGCCCAGCTTGACCTG
ENSG00000102935	ZNF423	16	-1821	-1804	14,165	88,10%	CAATGTCAGTTTATCCTA
ENSG00000104783	KCNN4	19	-3352	-3335	12,572	85,80%	CTGAGTCACCTCGCCCTC
ENSG00000104833	Tubb4	19	382	399	12,714	86%	CTGGGCCACTGTCCCCTC
ENSG00000104915	STX10	19	-2987	-2970	13,94	87,70%	CAGGGTCATGATGCGCTG
ENSG00000105612	DNASE2	19	-4587	-4570	12,739	86%	TGGGGTCAGGAGGACCCG
ENSG00000105642	KCNN1	19	250	267	13,947	87,70%	GAAGGTCACTCTGCCACC
ENSG00000105767	cadm4	19	618	635	16,306	91,10%	CTGGGTCACTCTGTCCCC
ENSG00000105855	ITGB8	7	-1020	-1003	13,253	86,80%	CCAGGTCAAGGTGAAATA
ENSG00000106003	LFNG	7	-1279	-1296	14,153	88%	CCAGGTCAGAAGGCCCTG
ENSG00000106066	cpvl	7	-572	-555	13,014	86,40%	GAAGGTCATGAAGTCCTA

ENSG00000106069	Chn2	7	1065	1082	13,014	86,40%	GAAGGTCATGAAGTCCTA
ENSG00000108515	ENO3	17	-1453	-1436	12,199	85,20%	CAATGTCACTGTCACCAA
ENSG00000108771	dhx58	17	-4993	-4976	13,353	86,90%	TGAGGTCATGCTGACCAT
ENSG00000108823	SGCA	17	-104	-87	14,433	88,40%	GTGGGTCACAGTGGCCCC
ENSG00000108830	RND2	17	823	840	14,258	88,20%	GGAAGTCAGGGTGACCCC
ENSG00000108846	Abcc3	17	-4003	-3986	13,779	87,50%	CCAGGTGAGGGTGCCCTG
ENSG00000110400	pvrl1	11	1902	1919	13,157	86,60%	CAGGGCCAGCCTGGCCTG
ENSG00000110436	Slc1a2	11	1219	1236	14,09	87,90%	CAAAGTCAGAGTGTCATG
ENSG00000110514	MADD	11	-260	-243	13,572	87,20%	GAAGATCATGCTGACCCC
ENSG00000110880	CORO1C	12	1776	1793	12,402	85,50%	CTAGGTCATACTGAAATA
ENSG00000111087	GLI1	12	-4534	-4517	13,628	87,30%	CAGGGTCTGTGTGTCCCT
ENSG00000112561	TFEB	6	-3154	-3137	12,724	86%	GAAGGTCATGAGGACATC
ENSG00000112584	Fam120b	6	-1058	-1041	12,672	85,90%	CCAGGCCAGCGTGTCCCC
ENSG00000113070	HBEGF	5	-1054	-1037	14,242	88,20%	CAGGGCCAGACTGACCCC
ENSG00000113391	fam172a	5	-4355	-4338	12,401	85,50%	TTAGGTCAGAGAGATCTA
ENSG00000113594	Lifr	5	-1604	-1587	12,753	86%	CTGGGACAGGATGTCCCC
ENSG00000113657	Dpysl3	5	875	892	13,092	86,50%	TTATGTCACACTGAACTG
ENSG00000116191	ralgps2	1	646	663	17,296	92,50%	CGCGGTCAGGGTGACCTG
ENSG00000116285	ERRFI1	1	1226	1243	13,881	87,70%	AAAGGGCATTTTAACCTC
ENSG00000116396	KCNC4	1	-1486	-1469	14,477	88,50%	GAGGATCAGGCTGCCCTC
ENSG00000116679	IVNS1ABP	1	-1635	-1618	13,127	86,60%	TAGTGTCTTTGTGACCTG
ENSG00000117114	LPHN2	1	-1398	-1381	12,782	86,10%	TAAGGTCATGCTGAAATT
ENSG00000117676	RPS6KA1	1	-105	-88	12,025	85%	ATGGGTCAGGCTCCTCTA
ENSG00000118257	NRP2	2	-3764	-3747	12,326	85,40%	CAGGGTCAGAGGACCATG
ENSG00000119715	esrrb	14	-68	-51	12,044	85%	CGCGCTCACTGTGCCCTG
ENSG00000120725	SIL1	5	1146	1163	13,226	86,70%	GAGGGCCATGATGCCCTA
ENSG00000120868	Apaf1	12	-1725	-1708	14,874	89,10%	CAAGGTCAAATTCTCCTG
ENSG00000121769	fabp3	1	637	654	17,126	92,30%	TGAGGTCATTGTGACCTC
ENSG00000121931	C1orf103	1	1684	1701	14,598	88,70%	CCATGTCAGGGTGCTCTT
ENSG00000122971	Acads	12	-2525	-2508	12,714	86%	TGAGGACAGTGTGTCCTG
ENSG00000123999	inhA	2	-1665	-1648	12,033	85%	CACGGTCCTGTTGACCTG
ENSG00000124479	ndp	X	-2017	-2000	15,954	90,60%	AAAGGTCACTGTGCCACG
ENSG00000125454	SLC25A19	17	491	508	12,369	85,50%	TGGGGTCACGTTGCTCAG
ENSG00000125743	snrpd2	19	-3196	-3179	13,83	87,60%	TCAGGTCACGTGGCCCTT

ENSG00000125967	NECAB3	20	211	228	13,15	86,60%	TGGGCTCATTGTGACCTC
ENSG00000128016	ZFP36	19	-3207	-3190	13,89	87,70%	CTGAGTCAGGCTGTCCCC
ENSG00000129116	palld	4	-692	-675	12,655	85,90%	CTGAGTCACTATGACATA
ENSG00000129422	MTUS1	8	242	259	12,473	85,60%	GAAGGTCAAGCTGCCAAG
ENSG00000129451	Klk10	19	-2920	-2903	14,316	88,30%	CCAGGTCAGTGTGGTCTC
ENSG00000129521	egln3	14	-54	-37	12,782	86,10%	CTGGGTCAGCATGATTTC
ENSG00000130234	ACE2	X	-140	-123	13,234	86,70%	CAATGTCACCTGAACCTG
ENSG00000130299	GTPBP3	19	-4817	-4800	14,917	89,10%	CAGGGCCAGCATGACCTT
ENSG00000130595	TNNT3	11	-122	-105	13,413	87%	GAGGGCCACGCTGCCCCC
ENSG00000131069	ACSS2	20	1235	1252	15,162	89,50%	AAGGGTCAGTGTGTCATA
ENSG00000131459	gfpt2	5	526	543	14,463	88,50%	AAAGGTCACTGTGCTCNN
ENSG00000131620	ANO1	11	-1922	-1905	14,935	89,20%	ATAGGGCAGGGTGGCCTG
ENSG00000131711	MAP1B	5	-3461	-3444	13,038	86,40%	GGAGGCCATAGTGCCCTG
ENSG00000131771	PPP1R1B	17	-2635	-2618	14,841	89%	AGGGGCCAGGGTGCCCTG
ENSG00000132361	kiaa0664	17	-394	-377	14,334	88,30%	CAAGGTCACAGTGCCCGC
ENSG00000132563	reep2	5	819	836	13,62	87,30%	CAGGGGCAGGAGGCCCTG
ENSG00000132849	INADL	1	-3343	-3326	12,744	86%	GAGGGTCAAATTGCCACC
ENSG00000133424	LARGE	22	1421	1438	14,616	88,70%	TCGGGTCACAGTGACTTG
ENSG00000134250	Notch2	1	-2382	-2365	13,911	87,70%	CTGGGTCATTATGTACTC
ENSG00000134954	ETS1	11	-3984	-3967	13,752	87,50%	AAAGGTCACTGAGACCAC
ENSG00000135127	CCDC64	12	-32	-15	15,6	90,10%	CAATGTCACGGTACCCCC
ENSG00000135426	KIAA0748	12	-3838	-3821	12,102	85,10%	CCAGGTCTGGGCAACCTG
ENSG00000135503	ACVR1B	12	995	1012	13,031	86,40%	GAATGTCATGTTCTCCTG
ENSG00000136153	Lmo7	13	-550	-533	12,097	85,10%	AAAGGTCTTGGTGTTCTA
ENSG00000136634	il10	1	-351	-334	14,856	89%	CTAGGTCACAGTGACGTG
ENSG00000137070	IL11RA	9	-1893	-1876	12,06	85%	TAGGGCCATGTTGCCCAG
ENSG00000137801	Thbs1	15	2244	2261	12,046	85%	AAGTGTCATTATATCCTC
ENSG00000138061	CYP1B1	2	-34	-17	12,261	85,30%	CCAGGTCGCGCTGCCCTC
ENSG00000138131	Loxl4	10	-1207	-1190	15,793	90,40%	CAGGGTCATTAAGACCTG
ENSG00000138316	ADAMTS14	10	-2328	-2311	12,218	85,30%	TGGGGCCACCTTGCCCTC
ENSG00000138411	Hecw2	2	915	932	12,992	86,40%	AAGGGTCATTGTATTCTT
ENSG00000138430	Ola1	2	1060	1077	13,813	87,60%	CAATGTCAGTTTAACCAG
ENSG00000138795	Lef1	4	-2034	-2017	12,107	85,10%	GAGGGCCAGGGGGCCCTT
ENSG00000139044	B4GALNT3	12	-938	-921	13,061	86,50%	CCAGGCCAGTGTGCCCCA
ENSG00000139263	LRIG3	12	870	887	12,058	85%	GGAGGTCATGCTGCCTTA
ENSG00000139318	dusp6	12	-3659	-3642	12,145	85,20%	AAAGGTCATGCTAGCCCA

ENSG00000139531	SUOX	12	-3182	-3165	20,161	96,60%	CTAGGTCACTGTGACCTT
ENSG00000139734	diaph3	13	388	405	12,267	85,30%	AAGGGTCATTTTATTCTA
ENSG00000139970	RTN1	14	155	172	12,038	85%	AAAAGTCAGGGTATTCTC
ENSG00000141295	SCRN2	17	-4150	-4133	14,677	88,80%	AAGGGACAGGATGACCTT
ENSG00000141429	GALNT1	18	1166	1183	12,278	85,40%	GAGGGTCAGGCTGGCACC
ENSG00000141756	Fkbp10	17	-2931	-2914	12,687	85,90%	GGGGGTCAAATTGGCCTC
ENSG00000142784	WDTC1	1	-4038	-4021	14,069	87,90%	TGGTGTCAGGCTGCCCTC
ENSG00000143365	Rorc	1	-4983	-4966	13,06	86,50%	CAAGGTCTTGGTGACACT
ENSG00000143850	PLEKHA6	1	-72	-55	13,124	86,60%	GAGAGTCACGTGGCCCTG
ENSG00000144648	CCBP2	3	418	435	13,719	87,40%	CCAAGTCATTCTGCCCTC
ENSG00000144724	PTPRG	3	-707	-690	13,639	87,30%	CAGTGTCACTGGGATCTG
ENSG00000145332	Klhl8	4	1106	1123	13,573	87,20%	CTGGGTCAGTTTGACAAA
ENSG00000145391	setd7	4	-4025	-4008	12,897	86,20%	CAGATTCAGGGTGACCTG
ENSG00000146376	arhgap18	6	-3766	-3749	12,833	86,20%	CAAGGTCATGAGGTTCTC
ENSG00000146776	Atxn7l1	7	-3661	-3644	14,11	88%	ACAGGTCACTTTATCCTT
ENSG00000147604	RPL7P20	8	-2591	-2574	12,084	85,10%	ATAGGTCACATTAAACCT
ENSG00000147677	eIF3h	8	-4672	-4655	12,049	85%	CTGAGACAAGCTGACCTG
ENSG00000148339	Slc25a25	9	-1609	-1592	12,699	86%	CTGTGTCAGGGTGACCGT
ENSG00000148655	C10orf11	10	-3184	-3167	12,201	85,20%	CTGTGTCAGGCTGACGTG
ENSG00000149090	PAMR1	11	-4378	-4361	12,617	85,80%	CCAGATCATTGTGGCCTG
ENSG00000149289	ZC3H12C	11	2380	2397	12,961	86,30%	CAATGTCATCGTGTACTA
ENSG00000149577	SIDT2	11	-68	-51	13,948	87,70%	CTGTGTCAGTTTCCCCTG
ENSG00000149654	Cdh22	20	-256	-239	13,146	86,60%	GAGGGGCACTGTCCCCTG
ENSG00000149781	fermt3	11	-190	-173	12,974	86,40%	CTGAGTCACCGTGACACC
ENSG00000149972	Cntn5	11	-1056	-1039	17,361	92,60%	CAGGGTCAGGCTGCTCTC
ENSG00000151148	UBE3B	12	-2980	-2963	12,64	85,90%	CCAGGCCACGGTGTTCTT
ENSG00000151276	MAGI1	3	1439	1456	14,467	88,50%	AAAAGTCACAATGCCCTC
ENSG00000153113	cast	5	519	536	14,384	88,40%	AAAGGTCAGAGTGAGCTC
ENSG00000154380	Enah	1	-1939	-1922	12,424	85,60%	GAGGATCAAGGTGGCCTC
ENSG00000155252	DHDPSL	10	-827	-810	12,998	86,40%	CAATGTCAGGTTACACTT
ENSG00000155324	GRAMD3	5	-66	-49	12,341	85,40%	CTGTGTCACACTGATATG
ENSG00000156110	adk	10	-3942	-3925	14,401	88,40%	CAGGGGCACAGTGTTCTG
ENSG00000156398	SFXN2	10	-3657	-3640	14,586	88,70%	CAGAGGCATGGTGCCCTG

ENSG00000156453	PCDH1	5	-4535	-4518	13,233	86,70%	AGGGGTCAAGGTGCTCCC
ENSG00000156687	UNC5D	8	316	333	13,454	87%	CCGGGTCACAGCCACCTG
ENSG00000156709	AIFM1	X	-326	-309	12,852	86,20%	CAGGCTCAGTTTCACCTT
ENSG00000157193	LRP8	1	-4545	-4528	13,174	86,60%	CAAGGGCAGGGGGACCAG
ENSG00000157216	SSBP3	1	-816	-799	12,491	85,70%	AAATGTCAAGTTCCCCTT
ENSG00000157404	KIT	4	-1615	-1598	13,768	87,50%	AAAGGTCTCTGTGCTCTG
ENSG00000157554	ERG	21	-489	-472	12,337	85,40%	CAGGGTGAGTGTGCCCCC
ENSG00000157985	AGAP1	2	-4978	-4961	12,479	85,60%	CTGTATCACGTTGTCCTA
ENSG00000158710	TAGLN2	1	-266	-249	13,401	87%	TGAGGTCACTCTGCCCAG
ENSG00000158711	ELK4	1	-395	-378	13,23	86,70%	CAATGTCATTCTGCCCAC
ENSG00000159216	Runx1	21	-867	-850	13,189	86,70%	CAGGGGCAGAGTAAACTG
ENSG00000159403	ALDH4A1	12	-3892	-3875	13,217	86,70%	CACGGGCATTGTGTCCTG
ENSG00000159423	AP2M1	1	-3093	-3076	12,677	85,90%	CAGTGTCATATTGACACT
ENSG00000161203	LRRC56	3	-4630	-4613	13,34	86,90%	AGGGGCCAGTGTGTCCTG
ENSG00000161328	ITGA5	11	-4564	-4547	17,084	92,20%	AAAGGTCAGGGTGGCCCG
ENSG00000161638	RACGAP1P	12	1115	1132	12,07	85,10%	CTAAATCAGGGTGAACTG
ENSG00000161800	racgap1	12	-3528	-3511	15,546	90%	CTAGGTCATAATAACCTA
ENSG00000161921	CXCL16	17	-3812	-3795	13,488	87,10%	TGGGGTCAGGGAGACCCC
ENSG00000162373	BEND5	1	875	892	12,144	85,20%	CTGGGTCAGAATAACACT
ENSG00000162413	klhl21	1	-799	-782	13,104	86,50%	CTGGGTCAGCTTACCCAT
ENSG00000162496	Dhrs3	1	-4250	-4233	18,231	93,90%	GAAGGTCACGCTGCCCTT
ENSG00000163399	ATP1A1	1	-113	-96	13,074	86,50%	CGGTGTCAGGTTGCTCCG
ENSG00000163463	KRTCAP2	1	-1588	-1571	13,057	86,50%	CAGGGGCAAGCTGCCCAG
ENSG00000163486	srgap2	1	-667	-650	14,474	88,50%	AAAGGTCAGCATGCCATT
ENSG00000163531	Nfasc	1	-2902	-2885	14,186	88,10%	AAAGGTCATACTGTTCTC
ENSG00000163618	CADPS	3	-148	-131	12,372	85,50%	CAGGGACAGGGTGATCAC
ENSG00000163659	TIPARP	3	-85	-68	14,459	88,50%	GAGGGTCACTTTGTTCCG
ENSG00000163931	tkt	3	-4131	-4114	14,879	89,10%	AGAGGCCACAGTGACCTG
ENSG00000163947	ARHGEF3	3	-20	-3	13,492	87,10%	GAAGGGCATTGTCACCTG
ENSG00000164031	Dnajb14	4	-788	-771	12,185	85,20%	AACTGTCAAAATGACCTG
ENSG00000164056	SPRY1	4	-104	-87	12,264	85,30%	TGAGCTCATGGTAACCTC
ENSG00000164081	Tex264	3	-3300	-3283	13,298	86,80%	CTATGTCAAATAGACCTG
ENSG00000164330	EBF1	5	-1605	-1588	13,267	86,80%	AGGGGTCATTTTCCCCTG
ENSG00000164398	Acsl6	5	-1825	-1808	12,576	85,80%	GTGGGTCAGTGTGAACAA

ENSG00000164574	GALNT10	5	1912	1929	12,212	85,30%	GAAGGTCAAGGTTAACTG
ENSG00000164591	MYOZ3	5	-3120	-3103	12,047	85%	CCAGCTCAAGGTGTCCCA
ENSG00000164970	C9orf25	9	1255	1272	14,409	88,40%	CGAGGTCAGGGAGCCCCC
ENSG00000165795	Ndrg2	14	-1214	-1197	14,226	88,10%	CTGGGTCAGGATGACTCA
ENSG00000165995	CACNB2	10	-2708	-2691	13,028	86,40%	CTGGGTCATCGTGCCTCA
ENSG00000166086	Jam3	11	1548	1565	14,998	89,20%	CAGTGTCAGTGTGACCAA
ENSG00000166272	C10orf26	10	-1740	-1723	16,35	91,20%	CAAGGTCACATTGACTTT
ENSG00000166446	CDYL2	16	-915	-898	12,74	86%	CAGGGACACTGTGCACTT
ENSG00000167123	CERCAM	9	-2253	-2236	12,483	85,70%	ACGGGTCACTGCATCCTG
ENSG00000167281	**RBFOX3**	17	-3064	-3047	12,136	85,20%	GAAGGGCAGGCAGCCCTC
ENSG00000167703	Slc43a2	17	-2382	-2365	12,984	86,40%	AAGTGCCATTGTGCCCTG
ENSG00000167889	Mgat5b	17	-1194	-1177	14,078	87,90%	CACGGTCAGCATGTCCTT
ENSG00000167992	VWCE	11	-4710	-4693	12,963	86,30%	CAAGGGCAGGGGGCCCCT
ENSG00000168453	HR	8	688	705	16,315	91,10%	ATGGGTCAAGCTGCCCTA
ENSG00000168476	REEP4	8	800	817	13,551	87,20%	GTGGGGCATGGTGCCCCT
ENSG00000168487	BMP1	8	-744	-727	13,732	87,40%	TGAGGTCATTCTGCCCCC
ENSG00000169047	IRS1	2	-478	-461	13,303	86,80%	GTGGGTCAGTCTTACCTG
ENSG00000169169	Cpt1c	19	-4464	-4447	13,101	86,50%	CACGGTCAGGTAGTCCTC
ENSG00000169291	She	1	-145	-128	12,191	85,20%	GAGAGCCACGGTGTCCTC
ENSG00000169436	Col22a1	8	-41	-24	13,365	86,90%	CAGGGGCAAGCTGGCCTC
ENSG00000169554	Zeb2	2	-142	-125	12,203	85,30%	CTGTGTCACTTTGAAATC
ENSG00000170044	ZPLD1	3	-2978	-2961	12,73	86%	AAAGGTCACTTTCAACCG
ENSG00000170345	FOS	14	-3359	-3342	16,989	92,10%	CAAAGTCACCCTGACCTG
ENSG00000170471	RALGAPB	20	546	563	12,014	85%	AAGTGTCACTGTGCAATG
ENSG00000170743	SYT9	11	-3847	-3830	14,835	89%	CTGGGTCAGGATGACTCC
ENSG00000171219	CDC42BPG	11	-2745	-2728	13,294	86,80%	AAGAGTCACTGAGACCTC
ENSG00000171223	junb	19	-2588	-2571	12,613	85,80%	AGGGGTCACAGGGACCCT
ENSG00000171241	SHCBP1	16	-3364	-3347	13,892	87,70%	CAGTGCCACGGTGACCCA
ENSG00000171246	NPTX1	17	-88	-71	14,098	88%	CAGGGTCACGTGGCCTTG
ENSG00000171843	MLLT3	9	-311	-294	13,324	86,90%	TAAAGTCAGTTTGACATC
ENSG00000172009	THOP1	19	-2391	-2374	16,791	91,80%	CAGGGTCACCGCGACCCG
ENSG00000172053	QARS	3	-2782	-2765	12,393	85,50%	ATGGGTCATGAAGCCCCC
ENSG00000172159	frmd3	9	-4192	-4175	12,832	86,20%	AAATGTCATTTTCACCTA

ENSG00000172819	rarg	12	1320	1337	12,863	86,20%	CGGGGGCTTGGTGACCTG
ENSG00000173599	Pc	11	3494	3511	12,008	85%	TGAGGTCACGGGGACACG
ENSG00000174059	CD34	1	-62	-45	12,422	85,60%	CAAGGCCACAGGGTCCTC
ENSG00000175445	Lpl	8	498	515	14,613	88,70%	CGAGGTCACCTTGGCCCG
ENSG00000175505	CLCF1	11	905	922	13,013	86,40%	CCAGGCCACGGTGGCCCG
ENSG00000175600	C7orf10	7	-2819	-2802	14,425	88,40%	CAAGGTCACAGAATCCTG
ENSG00000176490	DIRAS1	19	-1850	-1833	12,875	86,20%	ATGAGTCAGCCTGCCCCG
ENSG00000176871	Wsb2	12	-4252	-4235	13,473	87,10%	CAATGTCACTTTGCTCCT
ENSG00000176927	EFCAB5	17	-278	-261	12,742	86%	GAGGCTCACGGTCCCCTC
ENSG00000177098	SCN4B	11	-3528	-3511	12,199	85,20%	CAGGGTCTCGATGCCTTC
ENSG00000177666	PNPLA2	11	-2673	-2656	13,213	86,70%	CTGGGTCAGGCTGTTTTG
ENSG00000177685	EFCAB4A	11	-4618	-4601	12,675	85,90%	CAGGGTCTGTATGCCATG
ENSG00000178038	ALS2CL	3	800	817	14,189	88,10%	CAGGGTCACTGGGGCCTT
ENSG00000178772	CPN2	3	-132	-115	12,763	86,10%	CAGGGTCATTTTGTAATC
ENSG00000178860	MSC	8	-1484	-1467	12,338	85,40%	CCAGGCCACGCTACCCTT
ENSG00000179218	CALR	19	-52	-35	12,373	85,50%	CTGGGTCAGGTTGGTTTG
ENSG00000179761	PIPOX	17	-390	-373	12,144	85,20%	AAGAGTCACTCTGGCCTT
ENSG00000180694	TMEM64	8	-346	-329	12,108	85,10%	CAAGGGCATGTCGATCTT
ENSG00000182175	rgma	15	411	428	12,451	85,60%	CTCGGGCAGGGTGCCCCT
ENSG00000182199	SHMT2	12	-2061	-2044	16,029	90,70%	CGGGGTCAGGGTGCCCAC
ENSG00000182718	ANXA2	15	-519	-502	13,36	86,90%	ACATGTCAGCTTAACCTC
ENSG00000182718	PAPPA	15	-519	-502	13,36	86,90%	ACATGTCAGCTTAACCTC
ENSG00000182752	PAPPAS	9	1851	1868	13,723	87,40%	CTAGGTCACAGTGCATTG
ENSG00000182901	rgs7	1	-73	-56	12,539	85,70%	AAGGGTCAGAGAGACTTC
ENSG00000183111	flj41603	5	-4741	-4724	13,159	86,60%	GAAAGTCAGGTTAATCTG
ENSG00000183785	Tuba8	22	-4989	-4972	14,863	89,10%	CTGGGTCAGCTTCCCCTT
ENSG00000183963	smtn	22	-192	-175	12,527	85,70%	ATGGGCCAGGCTGATCTT
ENSG00000184005	ST6GALNAC3	1	-1881	-1864	13,561	87,20%	AAATGTCACTGTGCCCAA
ENSG00000184154	lrtomt	11	413	430	13,668	87,30%	AAAGGTCAGTGTTCCCTA
ENSG00000184347	SLIT3	5	-4126	-4109	13,626	87,30%	CTGGGCCAGGGTGCCATT
ENSG00000184381	pla2g6	22	-653	-636	12,441	85,60%	ACAGGGCAAGGGGACCTG
ENSG00000184922	FMNL1	17	974	991	12,326	85,40%	CAGGGTCTTGGAGACCCC
ENSG00000184937	wt1	11	-3876	-3859	13,011	86,40%	AAAAGTCATGGTGTCCNN
ENSG00000185338	Socs1	16	-1276	-1259	13,949	87,70%	CTGGGACACACTGCCCTC
ENSG00000185909	KLHDC8B	3	-623	-606	13,175	86,60%	AAAGGCCAAGCTGATCTG

ENSG00000185989	Rasa3	13	-2231	-2214	12,602	85,80%	GAGGAGCATGGTGCCCTG
ENSG00000186174	BCL9L	11	84	101	12,935	86,30%	CAGGGGCACACAGACCTC
ENSG00000186998	EMID1	22	1188	1205	12,51	85,70%	CAGGGGCATGCTGGCCCC
ENSG00000187091	Plcd1	3	-1737	-1720	12,184	85,20%	AGGAGTCAGAGTGAACTG
ENSG00000187098	MITF	3	319	336	12,949	86,30%	CAAGGGCACTGGGTCCTT
ENSG00000188706	Zdhhc9	X	-2085	-2068	12,722	86%	ATGTGTCATGGTGACACA
ENSG00000188747	NOXA1	9	-1142	-1125	16,328	91,10%	GAGGGTCAGAGTGTCCCC
ENSG00000188766	SPRED3	19	-4987	-4970	14,593	88,70%	CTGGGCCAGGGTGTCCCC
ENSG00000188848	BEND4	4	-1644	-1627	13,061	86,50%	AAAGGACATGCTGTCCTT
ENSG00000189337	kaz	1	-3807	-3790	15,873	90,50%	CAGGGTCATGGGGACCCT
ENSG00000196208	GREB1	2	-1585	-1568	17,806	93,30%	CTGGGTCATTCTGACCTA
ENSG00000196482	esrrg	1	-4833	-4816	12,922	86,30%	CAAGGTCAATGTCCCATT
ENSG00000196517	SLC6A9	1	-4786	-4769	17,198	92,40%	CAGGGTCAGGGAGTCCTG
ENSG00000196535	Myo18a	17	-1133	-1116	13,638	87,30%	TAAGATCAGGGTGACTTG
ENSG00000197905	tead4	12	-1150	-1133	14,537	88,60%	CAAGGCCAAGCTGACCCC
ENSG00000197956	S100A6	1	-3719	-3702	12,147	85,20%	CAGGGGCAGGGTGGCCAA
ENSG00000198133	TMEM229B	14	821	838	12,456	85,60%	CAAGGGCAGGGAGACATT
ENSG00000198719	DLL1	6	-1360	-1343	12,232	85,30%	GAAGGCCAGGTTCTCCTG
ENSG00000198755	RPL10AP9	6	-2167	-2150	14,461	88,50%	CTGGGTCTCAGTGATCTG
ENSG00000198792	TMEM184B	22	196	213	12,668	85,90%	CCAGGTCACTGCCACCCG
ENSG00000198898	Capza2	7	-611	-594	12,929	86,30%	TTAGGTCAGC
ENSG00000213246	SUPT4H1	17	-1698	-1681	12,717	86%	AGTGGTCAGGCTGCCCTG
ENSG00000213901	SLC23A3	2	-2198	-2181	14,126	88%	CAAGGTCAGGCGGCCCAG

4 CONCLUSIONS

The majority of regulatory mechanisms, which control CL lifespan, is expressed during the period of increased cell growth and tissue modeling and these regulatory mechanisms involve different signaling pathways such as Wnt / βcatenin, IGF system and MAPK pathway.

During the structural regression of CL, there is an increase in the gene expression related to inhibition of proliferative processes and apoptosis.

Estradiol (E2) participates in regulating both proliferative activity, as well as CL regression processes, through signaling mediated by its receptors (ERα and ERβ).

The proliferative signaling mediated by ERα appears to be related to the activation of these receptors present in the membrane, resulting in activation of the IGF system and Wnt/βcatenin signaling.

Whereas ERβ regulates corpus luteum regression controlling gene expression related to inhibition of angiogenesis and apoptotic processes.

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