

**RNA-SEQUENCING BASED ANALYSIS OF BOVINE ENDOMETRIUM DURING THE  
MATERNAL RECOGNITION OF PREGNANCY**

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## ABSTRACT

**Background:** Reproductive efficiency is crucial to the production of food animals and overall profitability of the farm. The majority of pregnancy losses occur in the first month, especially around Day-19 of gestation, mainly due to the inability of the uterus to support conceptus growth or abnormal development of conceptus. Since the incidence of pregnancy failure does not occur in a single day of Day-19, then days 15-17 is a critical period for the maternal recognition and establishment of pregnancy. We hypothesize that RNA-Sequencing based analysis of bovine endometrial tissues during the critical period of maternal recognition of pregnancy will reveal important genes and biological pathways required for the conceptus growth and development.

**Objectives:** Hence, the objectives of the current study are:

- 1) To identify the important differentially expressed genes (DEGs) and biological pathways in the bovine caruncular endometrium among the groups (Pregnant vs. Cyclic) and (Pregnant vs. Non-Pregnant), and
- 2) To validate the most highly up-regulated DEGs using quantitative polymerase chain reaction (qPCR).

**Methods:** Grass-fed Angus heifers (2-3 years old) were used for sampling. The estrous cycles of heifers (n=21) were synchronized using the intramuscular injection of a Prostaglandin F2 alpha (on Day-0 and -11). Fifteen heifers were bred by natural mating at estrus. Endometrial samples were collected at Day 15-17 of gestation (pregnant), of estrous cycle (cyclic), and absence of conceptus (nonpregnant) heifers. Total RNAs were isolated and were subjected to high throughput RNA-sequencing (n=4/group). The genes with at least two-fold change, and Benjamini and Hochberg q-value  $\leq 0.05$  were considered as differentially expressed. The mRNA expression of selected candidate genes in the bovine endometrium was also validated using qPCR.

**Results:** A total of 107 genes (pregnant vs. cyclic), and 98 genes (pregnant vs. Nonpregnant) were differentially expressed (FDR <0.05) in the pregnant endometrium. The most highly up-regulated genes in the pregnant endometrium were *MRS2*, *CST6*, *FOS*, *VLDLR*, *ISG15*, *IFI6*, *MX2*, *C15H11ORF34*, *EIF3M*, *PENK*, *PRSS22*, *MS4A8*, *CLDN4*, *TINAGL1*, and *R3HDM1*. Gene ontology analysis revealed that the biological process related to Type-1 interferon signaling (*MX1*, *MX2*, *IFI6*, *IRF1*, and *ISG15*), immune response (*IL23A*, and *RSAD2*), extracellular matrix organization (*COL1A1*, *COL1A2*, *COL3A1*, and *TIMP2*) and ion transporters (*SLC34A2*, *SLC2A1*, *SLC16A11*, *SLC16A4* and *ATP1B1*) were significantly enriched in the pregnant endometrium. The qPCR results confirmed the significantly higher (P <0.05) mRNA expression of *MRS2*, *CST6*, *FOS*, *VLDLR*, *ISG15*, *IFI6*, *MX2*, *C15H11ORF34*, *PRSS22*, *TINAGL1*, and *MS4A8* in the presence of conceptus in the bovine endometrium.

**Conclusions:** Both the RNA-Seq and qPCR confirmed the differential expression of several pre-discovered and novel genes, and their biological pathways during the maternal recognition of pregnancy (Day 15-17 of gestation) compared to cyclic and non-pregnant endometrium. Interferon signaling, immune response, nutrient transporter, synthesis, and secretion of proteins are crucial pathways during the maternal recognition of pregnancy. Overall, this study identified the differentially expressed genes and their pathways in the pregnant caruncular endometrium compared to cyclic and non-pregnant. In this study, using RNA-sequencing, we found some novel genes (*MRS2*, *C15H11ORF34*, and *PRSS22*). The study demonstrated that the presence of conceptus on day 15-17 of gestation could actively affect the endometrial gene expression during the maternal recognition of pregnancy. In summary, this study provides a comprehensive dataset of transcripts associated with maternal recognition of pregnancy.

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## **LIST OF ABBREVIATIONS**

ADCY6: Adenylate Cyclase 6

ALDH2: Aldehyde Dehydrogenase 2 Family Member

AMMECR1L: AMMECR1 Like

ANOVA: Analysis of variance

ANTKMT: Adenine Nucleotide Translocase Lysine Methyltransferase

ARL2BP: ADP Ribosylation Factor Like GTPase 2 Binding Protein

ATP1B1: ATPase Na<sup>+</sup>/K<sup>+</sup> Transporting Subunit Beta 1

BCAM: Basal Cell Adhesion Molecule (Lutheran Blood Group)

BIVM: Basic, Immunoglobulin-Like Variable Motif Containing

BPIFB1: BPI Fold Containing Family B Member 1

bta-mir-6518: Adrenomedullin

C15H11orf34: Placenta Expressed Transcript 1

C1R: Complement C1r

C1R: Complement C1r

CAR: Caruncle

CCDC125: Coiled-Coil Domain Containing 125

CCDC152: Coiled-Coil Domain Containing 152

CEBPD: CCAAT Enhancer Binding Protein Delta

CHRNA2: Cholinergic Receptor Nicotinic Alpha 2 Subunit

CL: Corpus Leuteum

CLDN4: Claudin 4

COL: Collagen

COL1A1: Collagen Type I Alpha 1 Chain

COL1A2: Collagen Type I Alpha 2 Chain

COL3A1: Collagen Type III Alpha 1 Chain

COL6A3: Collagen Type VI Alpha 3 Chain

COLQ: Collagen Like Tail Subunit of Asymmetric Acetylcholinesterase

CST6: Cystatin E/M

CST7: Cystatin F

Ct: Cycle threshold

CYP21: Cytochrome P450 Family 21

CYP2W1: Cytochrome P450 Family 2 Subfamily W Member 1

CYTB: Mitochondrially Encoded Cytochrome B

DAAM2: Dishevelled Associated Activator of Morphogenesis 2

DE: Differentially expressed

DEG: Differentially expressed gene

DENND1C: DENN Domain Containing 1C

DIMT1: DIMT1 RRNA Methyltransferase and Ribosome Maturation Factor

DNA: Deoxyribo nucleic acid

DNAH11: Dynein Axonemal Heavy Chain 11

DNAJB13: DnaJ Heat Shock Protein Family (Hsp40) Member B13

DTX1: Deltex E3 Ubiquitin Ligase 1

ECM: Extracellular matrix

EHD2: EH Domain Containing 2

EIF3M: Eukaryotic Translation Initiation Factor 3 Subunit M

EtBr: Ethidium bromide

EVL: Enah/Vasp-Like

F: Forward

FC: Fold change

FIGNL2: Fidgetin Like 2

FKBP11: FKBP Prolyl Isomerase 11

FKBP4: FKBP Prolyl Isomerase 4

FLI1: Fli-1 Proto-Oncogene, ETS Transcription Factor

FLVCR2: Feline Leukemia Virus Subgroup C Cellular Receptor Family Mem

FLVCR2: FLVCR Heme Transporter 2

FOS: Fos Proto-Oncogene, AP-1 Transcription Factor Subunit

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GNL2: G Protein Nucleolar 2

GNL2: G Protein Nucleolar 2

GNPTG: N-Acetylglucosamine-1-Phosphate Transferase Subunit Gamma

GO: Gene ontology

GPT2: Glutamic--Pyruvic Transaminase 2

HMBOX1: Homeobox Containing 1

HNF1B: HNF1 Homeobox B

HOXB9: Homeobox B9

ICAR: Intercaruncle

ICM: Inter cell mass

IFI6: Interferon Alpha Inducible Protein 6

IFNT: Interferon tau

IGFBP2: Insulin Like Growth Factor Binding Protein 2

IGSF10: Immunoglobulin Superfamily Member 10

IHH: Indian Hedgehog Signaling Molecule

IPA: Ingenuity pathway analysis

IRF1: Interferon Regulatory Factor 1

IRF9: Interferon Regulatory Factor 9

ISG15: ISG15 Ubiquitin Like Modifier

ITGB5: Integrin Subunit Beta 5

JAK: Janus activated kinase

KEGG: Kyoto encyclopedia of genes and genomes

KLK11: Kallikrein Related Peptidase 11

LINGO2: Leucine Rich Repeat and Ig Domain Containing 2

LIPA: Lipase A, Lysosomal Acid Type

LRATD1: LRAT Domain Containing 1

LRFN4: Leucine Rich Repeat and Fibronectin Type III Domain Containing

LRRC41: Leucine Rich Repeat Containing 41

LRRC8C: Leucine Rich Repeat Containing 8 VRAC Subunit C

LRWD1: Leucine Rich Repeats and WD Repeat Domain Containing 1

MAP3K15: Mitogen-Activated Protein Kinase Kinase Kinase 15

MAPK7: Mitogen-Activated Protein Kinase 7

MCC: MCC Regulator of WNT Signaling Pathway

MFSD14A: Major Facilitator Superfamily Domain Containing 14A

MMP: Matrix extracellular phosphor glycoprotein

MPST: Mercaptopyruvate Sulfurtransferase

MRS2: Magnesium Transporter MRS2

MS4A8: Membrane Spanning 4-Domains A8

MX1: MX Dynamin Like GTPase 1

MX2: MX Dynamin Like GTPase 2

NBEA: Neurobeachin

NDE1: NudE Neurodevelopment Protein 1

NDRG2: NDRG Family Member 2

NGS: Next Generation Sequencing

NME7: NME/NM23 Family Member 7

NOC2L: NOC2 Like Nucleolar Associated Transcriptional Repressor

NP: Non-pregnant

NTN3: Netrin 3

NTN3: Netrin 3

OAS1Y: 2'-5'-Oligoadenylate Synthetase 1

OXA1L: OXA1L Mitochondrial Inner Membrane Protein

P2RX6: Purinergic Receptor P2X 6

PACSIN1: Protein Kinase C and Casein Kinase Substrate in Neurons 1

PAIP2: Poly(A) Binding Protein Interacting Protein 2

PAIP2B: Poly(A) Binding Protein Interacting Protein 2B

PCIF1: PDX1 C-Terminal Inhibiting Factor 1

PCIF1: PDX1 C-Terminal Inhibiting Factor 1

PCR: Polymerase chain reaction

PDCD1: Programmed Cell Death 1

PDE3B: Phosphodiesterase 3B

PDZD9: PDZ Domain Containing 9

PENK: Proenkephalin

PERM1: PARGC1 And ESRR Induced Regulator, Muscle 1

PGR: Progesterone receptor

PGS1: Phosphatidylglycerophosphate Synthase 1

PHF21A: PHD Finger Protein 21A

PHGDH: Phosphoglycerate Dehydrogenase

PIGY: PIGY Upstream Reading Frame

PKD1L3: Polycystin 1 Like 3, Transient Receptor Potential Channel Interacting

PKMYT1: Protein Kinase, Membrane Associated Tyrosine/Threonine 1

PKN1: Protein Kinase N1

PPP1R12C: Protein Phosphatase 1 Regulatory Subunit 12C

PRPF40B: Pre-mRNA Processing Factor 40 Homolog B

PRSS22: Serine Protease 22

PXDN: Peroxidasin

PXDN: Peroxidasin

qPCR: Quantitative polymerase chain reaction

R: Reverse

R3HDM1: R3H Domain Containing 1

RASSF4: Ras Association Domain Family Member 4

RNA: Ribonucleic acid

RNASE13: Ribonuclease A Family Member 1, Pancreatic

RNA-Seq: RNA-Sequencing

RSAD2: Radical S-Adenosyl Methionine Domain Containing 2

SELENOP: Selenoprotein P

SLC: Solute carrier

SLC16A11: Solute Carrier Family 16 Member 11

SLC16A4: Solute Carrier Family 16 Member 4

SLC24A5: Solute Carrier Family 24 Member 5

SLC2A1: Solute Carrier Family 2 Member 1

SLC34A2: Solute Carrier Family 34 Member 2

SLC46A1: Solute Carrier Family 46 Member 1

SLC7A4: Solute Carrier Family 7 Member 4

SMG6: SMG6 Nonsense Mediated mRNA Decay Factor

SNX20: Sorting Nexin 20

SPAG8: Sperm Associated Antigen 8

SPARC: Secreted Protein Acidic and Cysteine Rich

SPARCL1: SPARC Like 1

SPSB2: SplA/Ryanodine Receptor Domain and SOCS Box Containing 2

ST3GAL6: ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 6

SYNE2: Spectrin Repeat Containing Nuclear Envelope Protein 2

TAF3: TATA-Box Binding Protein Associated Factor 3

TBC1D10B: TBC1 Domain Family Member 10B

TBE: TRIS borate EDTA

TE: Trophectoderm

TERF1: Telomeric Repeat Binding Factor 1

TINAGL1: Tubulointerstitial Nephritis Antigen Like 1

TMEM151B: Transmembrane Protein 151B

TMPRSS2: Transmembrane Serine Protease 2

TMPRSS6: Transmembrane Serine Protease 2

TNC: Tenascin C

TPCN1: Two Pore Segment Channel 1

TPT1: Tumor Protein, Translationally-Controlled 1

TRADD: TNFRSF1A Associated Via Death Domain

TRANK1: Tetratricopeptide Repeat and Ankyrin Repeat Containing 1

TRIM34: Tripartite Motif Containing 34

TRPV6: Transient Receptor Potential Cation Channel Subfamily V Member 6

TSEN54: TRNA Splicing Endonuclease Subunit 54

TTC28: Tetratricopeptide Repeat Domain 28

TUBD1: Tubulin Delta 1

TUBE1: Tubulin Epsilon 1

TYK2: Tyrosine kinase 2

VAR2: Valyl-TRNA Synthetase 2, Mitochondrial

VEPH1: Ventricular Zone Expressed PH Domain Containing 1

VLDLR: Very Low-Density Lipoprotein Receptor

VPS37B: VPS37B Subunit Of ESCRT-I

WNT5B: Wnt Family Member 5B

ZNF1: Zinc Finger NFX1-Type Containing 1

ZNF2: Zinc Finger HIT-Type Containing 2

## **CHAPTER 1: LITERATURE REVIEW**

### **1.1 Significance of the study**

#### **1.1.1 Global population growth and significance of reproduction for food animal production**

The human population is increasing exponentially and is estimated to be 9.15 billion in 2050 (U.S Census Bureau, 2011), necessitating more animal source foods to feed the growing population (Steinfeld et al., 2006, Dangour et al., 2012). According to The United Nations Food and Agriculture Organization (UN-FAO), it is estimated that the global per capita demand for beef meat will increase annually (approximately 10-15 kg) by 2050 (Ebi, 2009). The reports from FAO and the world bank revealed a rapid increase in cattle populations with a prediction to increase from 1.5 billion to 2.6 billion animals between 2000 and 2050 (Pica-Ciamarra et al., 2013). The challenge is to increase animal productivity rather than increasing numbers that create additional pressure on the natural resources and environment (Turk, 2016). Therefore, improving the animals' reproductive efficiencies will be instrumental in solving some of the future challenges.

#### **1.1.2 Potential challenges in the beef production**

Reproduction ensures that a farm has an abundant stock of animals to be sold. Reproductive rates are a major determinant of the farms' profitability, implying that the faster the rate at which an animal can reproduce, the greater its productivity and farm profitability. However, despite the proper feeding and body score, there are still several problems associated with early pregnancy failure in cattle (De Vries, 2006). Some reasons for reproductive failure include poor oocyte quality, poor embryo quality, and utero-embryonic asynchrony (Diskin & Shreenan, 2000). This is a huge problem for farmers or the industries as if the cattle miss only one estrus cycle;

farmers/producers have to feed them for an additional 21 days, with all additional feed, labor, and technical costs for artificial insemination. The majority of pregnancy losses (25-41%) occur in the first month of pregnancy, especially around Day-19 of gestation, mainly due to the uterus's inability to support conceptus growth or abnormal development of conceptus or luteal insufficiency (Diskin et al., 2016). A successful pregnancy establishment requires synchronous interactions of the conceptus with the endometrium (Spencer et al., 2004).

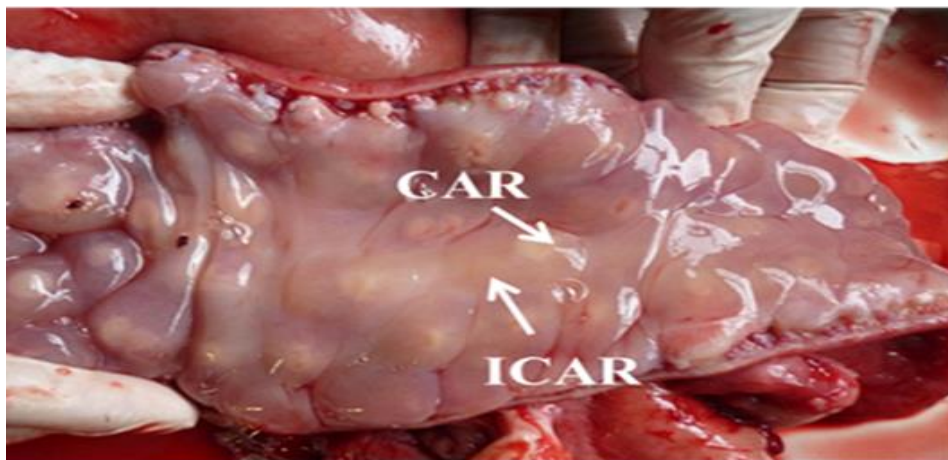
## **1.2 The uterus**

Understanding uterine changes during pregnancy provide critical insight into reproductive success. The uterus is a female reproductive organ that provides the biological environment for fetal growth and development. In the bovine, the uterus consists of a uterine body and two uterine horns. Horns are bipartite and are 17 to 20 centimeters in length. The fusion between two horns forms the body of the uterus, and is the common opening for both horns. During artificial insemination, the uterine body is the site of the semen deposition (Senger et al., 2003).

The uterine wall primarily consists of three layers: serosa, myometrium, and endometrium. Serosa or the perimetrium is the smooth outermost protective layer of the uterus. It protects the uterus from friction by secreting watery serous fluid to lubricate its surface. Myometrium is the middle thick and muscular layer. Myometrium is made up of smooth muscle. The myometrium is stimulated by oxytocin and estradiol to contract the muscle to facilitate sperm transport. Myometrial contraction also promotes parturition. During pregnancy, myometrium is one of the main components of the enlargement of the uterus (Lonergan et al., 2018). The endometrial lining is the inner mucosal layer of the uterus that helps maintain the uterine lumen's patency. The endometrium lines the uterus and comprises a single layer of columnar luminal and glandular

epithelial cells. This epithelial layer is supported by a base of stromal connective tissue (Aplin, 2012). The endometrium provides the biological environment for embryo implantation and placentation (Aplin, 2012). The endometrium also provides nutrition to the fetus and supports the removal of waste products.

The bovine endometrium consists of two distinct regions; caruncle (aglandular) and intercaruncle (glandular). Caruncles are benign fleshy round outgrowth in the uterine wall. They are formed from the proliferation of subepithelial connective tissues. In the mature bovine endometrium, four irregular rows of oval caruncles are found (Senger, 2003). During gestation, the caruncles grow rapidly and increase the contact's surface area by developing the crypts. The caruncle develops the vascular bed and is an important site for the metabolic exchange. Intercaruncle forms from the connective tissues that are located between the two caruncular regions. Gland orifices and their associated ciliated cells are found in the intercaruncle region. Intercaruncle acts as the supply of large molecules (amino acids) to the embryo (Senger, 2003).



**Figure 1:** Representative image of the bovine caruncle (CAR) and intercaruncle (ICAR) endometrium (Mishra et al., unpublished data).

### **1.2.1 Regulation of endometrial function**

Reproductive function in cattle is primarily regulated by two ovarian steroid hormones, estrogen, and progesterone. Estrogen levels are highly elevated around ovulation and facilitate sperm transport for successful fertilization (Senger, 2003). In contrast, progesterone increases after ovulation and peaks during the luteal phase of the estrus cycle and pregnancy (Senger, 2003). Progesterone, also known as the hormones of pregnancy, prepares the endometrium for pregnancy after ovulation. Progesterone inhibits the muscle contractions in the uterus to prevent embryo rejection. In the presence of estrogen, progesterone's physiological effects get amplified. Estrogen receptors induce progesterone receptors in the endometrial stroma & epithelium (Ellmann et al., 2009). The modifications occur when progesterone downregulates the nuclear progesterone receptor (PGR) in the luminal and then glandular epithelium, allowing the gene expression, protein secretions, and active molecule transportation are necessary for conceptus elongation (Spencer et al., 2016). Progesterone transforms the endometrium to its secretory stage to prepare the uterus for implantation. Progesterone level declines if pregnancy does not occur. It is unequivocal to say that progesterone is required for maternal support of conceptus survival and development in the uterus that regulates endometrial functions, pregnancy recognition signaling, and conceptus-uterine interactions (Spencer et al., 2004; Bauersachs et al., 2009; Zakar & Mesiano, 2011). Progesterone induces both temporal and spatial changes in the endometrial transcriptome during the establishment of a pregnancy. Progesterone is a crucial component of uterine physiology during the estrous cycle and pregnancy in all mammals (Spencer et al., 2004). Therefore, progesterone plays a pivotal and undisputable role in maintaining pregnancy in mammals.

### **1.3 Early development of embryo**

In the ampulla of the oviduct, a few hours after ovulation of the mature oocyte, gamete interaction takes place (Hyttel et al., 1988). The spermatozoon undergoes the acrosome reaction and penetrates the zona pellucida of the ova. As a result, the oocyte releases the cortical granules that elicit zona hardening and avoid polyspermic penetration. After fertilization of oocytes in the oviduct, fertilized oocytes undergo a series of morphological and biochemical changes. Around 15-19 h after ovulation, while the S-phase of the first mitotic cell cycle occurs, the pronuclei develops in an ovoid shape (Laurincik et al., 1994). Both maternal and paternal chromosomes align during the formation of the prophase and metaphase of the first mitotic division at around 20 h after ovulation.

The cleavage formation takes place to the 2-cell stage, followed by 8-cell stage formation at around 24 h. At the fourth cell stage, the embryonic genome activates that leads to the activation of the blastomere's nucleoli to initiate transcription and ribosome production (King et al., 1988; Laurincik et al., 2000). The Morula stage (16-32 cells) takes place between day 5-8 days. During the morula stage, the embryo travels from the oviduct to the uterus, where implantation occurs. When the embryonic genome activates, the embryo forms different cell lineages, resulting in the morula's compaction. Tight junctions and desmosomes develop water transporting capacity resulting in fluid-filled blastocyst formation at around day 8. The inner cell mass (ICM) is formed. At Day 9-11 of gestation, the blastocyst hatches from the zona pellucida and starts elongation at Day 12 of gestation. The hatched blastocyst comprises inner cell mass (ICM), and a single outer layer of cells called the trophoctoderm (TE), which surrounds the fluid-filled blastocoel cavity.

The ICM comprises of two structures; an upper epiblast and a lower epithelium, the hypoblast (Maddox et al., 2003). The hypoblast develops inside the blastocyst to form an inner lining of both

the epiblast and the TE. The embryonic disc of pluripotent cells, which later initiates gastrulation, is formed by epiblast to give rise to the embryo proper. The primitive streak is formed through which endoderm and mesoderm formation initiates.

The endoderm becomes united in the hypoblast. On the other hand, loose mesenchyme is developed from the mesoderm between the epiblast and the hypoblast (Maddox et al., 2003). The epiblast will develop into the neural ectoderm (located near to notochord) and surface ectoderm (peripheral to notochord). Between 12-14 days in the cattle, conceptus elongation occurs due to the rapid proliferation of trophectoderm cells (Mansouri-Attia et al., 2009). By day 15-17, a period of embryonic-maternal signaling, conceptus develops into filamentous form. After the embryo elongation, the peri-implantation process begins on day 16-18, and placentation starts around day 22 at the caruncular region of the endometrium. For the proper implantation, cell adhesion molecules such as integrins are necessary. Integrins lie on the surface of the endometrium, which is important for embryo attachment and invasion (Mansouri-Attia et al., 2009). The endometrial secretions composed of uterine lumen fluid, or histotroph, helps in the conceptus elongation process (Spencer et al., 2016). The endometrial secretions that support conceptus elongation are produced from the uterus' luminal and glandular epithelium (Bazer et al., 2012). Successful convergence of the hatched blastocyst and endometrium at implantation is required for the subsequent survival and development of the bovine embryo (Spencer et al., 2007).

**Table 1:** Early embryonic development with respect to day and location (adapted from Senger, 2003)

<b>Location</b>	<b>Day</b>	<b>Development</b>
Ampullary Isthmic Junction	0-1	One cell
Ampullary Isthmic Junction	1-3	Two cell
Isthmus	2-3	Four cell
Isthmus	3-5	Eight cell
Uterus	4-5	Sixteen cell
Uterus	5-8	Morula
Uterus	6-7	Tight morula
Uterus	7-8	Early blastocyst
Uterus	7-9	Blastocyst
Uterus	8-10	Expanded blastocyst
Uterus	9-11	Hatching blastocyst

### **1.3.1 Maternal recognition of pregnancy**

Maternal recognition of pregnancy is the period when the mother becomes aware of the presence of an embryo within her and responds accordingly within her reproductive tract (Roberts et al., 1996). Initially, the mother recognizes the conceptus as an intruder that manages to survive using the pregnancy-associated glycoproteins, multiple isoforms released at the trophoblast-endometrial interface pregnancy, thereby serving an immunoprotective role. Soon after, the mother senses the biochemical information, and the conceptus attempts to gain some measure of control over corpus luteum (CL) function, uterine blood supply, the mother's immune system, and other aspects of maternal physiology. For the maternal recognition of pregnancy, the conceptus requires producing a hormone that acts on the uterus and/or CL to ensure the maintenance of a functional CL for progesterone production; the hormone required for pregnancy (Bazer et al., 2012). In primates, the

chorionic gonadotrophin acts directly on the CL via luteinizing hormone receptors to ensure the maintenance of functional CL during pregnancy and is known for providing the maternal signal. In contrast, interferon tau (IFNT) acts as maternal recognition of pregnancy in ruminants (Bazer et al., 2012). IFNT was discovered while culturing the conceptuses inside the sheep endometrium. Earlier it was given the name protein X, and then later, it was called ovine trophoblast protein (Bazer et al., 2012; Godkin et al., 1982) and trophoblastin (Martal et al., 1979). When the trophoblastin was cloned and sequenced, it was termed as Type 1 IFNT (Kazuhiko et al., 1987; Roberts, 1993). The molecular weight of IFNT is 19 to 24 kDa and consists of 172 amino acids (Bazer et al., 1997). Bovine IFNT is N- glycosylated, and is very stable to pH as low as 2 to 3.

IFNT, Type I IFNs cytokines, shows antiviral, antiproliferative and immunomodulatory biological effects critical to immune responses (Leonidas, 2005). Different types of IFNT with a high degree of structural homology include interferons alpha (IFNA1-IFNA10, IFNA13, IFNA14, IFNA16, IFNA17, and IFNA21), interferon-beta (IFNB), interferon delta (IFND), interferon epsilon (IFNE), interferon kappa (IFNK), interferon tau (IFNT) and interferon omega (IFNW1, IFNW3). Among them, IFNT has a unique feature that provides a signal for the maternal recognition of pregnancy in cattle. Interestingly, the IFNT family of proteins resemble each other structurally and functionally. It is estimated that IFNT type I is likely to arise from duplication of an IFNW gene when IFNT came to be expressed in the trophectoderm under control of an Ets-2/AP-1 enhancer element (Roberts et al., 2003). IFNT is produced by mononuclear cells of trophectoderm as conceptus modifies from spherical to tubular to the filamentous structure during the peri-implantation period (around day 16) of pregnancy in cattle. Type I IFNs binds to a common receptor consisting of IFNAR1, and IFNAR2 and it induces cell signaling through the Janus activated kinases (JAKs), and tyrosine kinase 2 (TYK2) pathway (Darnell et al., 1994; Der et al.,

1998; Leonidas, 2005). Expression of different genes such as *IRF2*, *Wnt7A*, *ISG12*, *GBP2*, *IFIH1*, *IFIT1*, *IRF1*, *B2M*, *DDX58*, *IRF9*, *MIC*, *NMI*, *OAS*, *PLSCR1*, *RSAD2*, *STAT1*, and *STAT2* that plays an important role in the antiviral response, growth factor, cell signaling, and apoptosis, are recorded under the action of interferon signaling (Bazer et al., 2012). Some additional type I interferon signaling genes include *MX2*, *IFI6*, *IFIT1*, *IRF1*, *IRF9*, *ISG15*, *MX1*, *STAT1*, and *TAP1* found in our current research.

In order to establish and maintain the pregnancy, Interferon tau exerts multiple effects on the uterus (Spencer et al., 2016). One of the important ways to maintain the pregnancy is to prevent luteolysis by maintaining the progesterone's concentration and the endometrial function to facilitate implantation and fetal development. The functional lifespan of the corpora lutea, if sustained, will increase the production of progesterone. Progesterone is essential for proper uterine functions required for successful pregnancy outcomes (Bazer et al., 2012). Also, to establish and maintain the pregnancy, proper communication between the developing and maternal endometrium is required (Forde et al., 2011). For successful implantation, spatial and temporal changes in the endometrial transcriptome and histotroph composition are required that lead to a successful pregnancy of the cattle. These changes are monitored by corpus luteum derived progesterone and conceptus-derived INFT in cattle (Spencer et al., 2007).

#### **1.4. Measuring gene expression**

RNA-Sequencing (RNA-Seq) is a powerful technique to detect the presence of RNA transcripts in any given biological sample (Zhong et al., 2009). It is based on the principle of high throughput sequencing, which is exclusively used to determine the expression profiles of genes in any cell or tissue at a particular time point. It can be used to study the dynamics of transcriptomes in a tissue. While there is another common and cost-effective method of

studying the transcript profiles, such as microarray, the RNA-Seq technique has gained popularity in recent years because of its breakthrough discoveries. Microarray relies on the existing genomic knowledge of the species of interest; therefore, it is limited to the analysis of pre-discovered genes only. However, the RNA-Seq can detect and discover any novel transcripts present in the sample. Moreover, it has advantages of higher accuracy, less background noise, requires less amount of RNA, able to determine gene isoforms, and quantifies gene expression with over 8,000- fold changes.

### **1.5 Rationale of the study**

Early embryo mortality in cattle is one of the major factors affecting fertility and the farm's profitability. The majority of pregnancy losses occur in the first month, especially around Day-19 of gestation, mainly due to the inability of the uterus to support conceptus growth or abnormal development of conceptus (Diskin et al., 2016). The other reason could be the insufficient production of IFNT which is responsible for the maternal recognition of pregnancy in cattle. IFNT is produced by the embryo around day-16 of gestation in cattle (Spencer et al., 2004), leading to the persistence of CL and establishing the gestation in the cow. Successful pregnancy establishment requires synchronous interactions of the conceptus with the endometrium and maternal nutritional support (Spencer et al., 2004). The endometrium provides the biologic environment for the growth and development of growing conceptus. As Day 15-17 of gestation is a critical period for the maternal recognition and establishment of pregnancy, we hypothesized that RNA-Seq based analysis of bovine caruncular endometrial tissues during the maternal recognition of pregnancy (Day-16 of gestation) would reveal important genes and biological pathways required for the maternal recognition of pregnancy. Most of the previous studies have investigated the conceptus induced gene expression in ICAR and compared with cyclic cows. However,

caruncular endometrial transcriptomes involved in the maternal recognition of pregnancy are not clearly understood. To further improve the conception rate in cattle, the knowledge of specific genes, proteins, and biological pathways during the maternal recognition of pregnancy is required.

### **1.6 Hypothesis and objectives**

A few transcriptomic studies and some gene-specific studies have highlighted the importance of several intercaruncular endometrial genes and proteins involved during the maternal recognition of pregnancy. However, the underlying molecular mechanisms and involvement of biological processes in the caruncular endometrium are still obscure. We hypothesize that RNA-Sequencing based analysis of bovine caruncular endometrial tissues during the maternal recognition of pregnancy (Day 15-17 of gestation) will reveal important genes and biological pathways required for the maternal recognition of pregnancy. We tested this hypothesis under the following objectives:

1. To identify the genes and biological pathways in the caruncular endometrium during the maternal recognition of pregnancy among the groups (Pregnant vs. Cyclic) and (Pregnant vs. Non-Pregnant), and
2. To validate the most highly up-regulated differentially expressed genes using qPCR.

Overall, this study aimed to provide greater insight into the genetic mechanisms related to pregnancy success and failure.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 Animal handling and sampling**

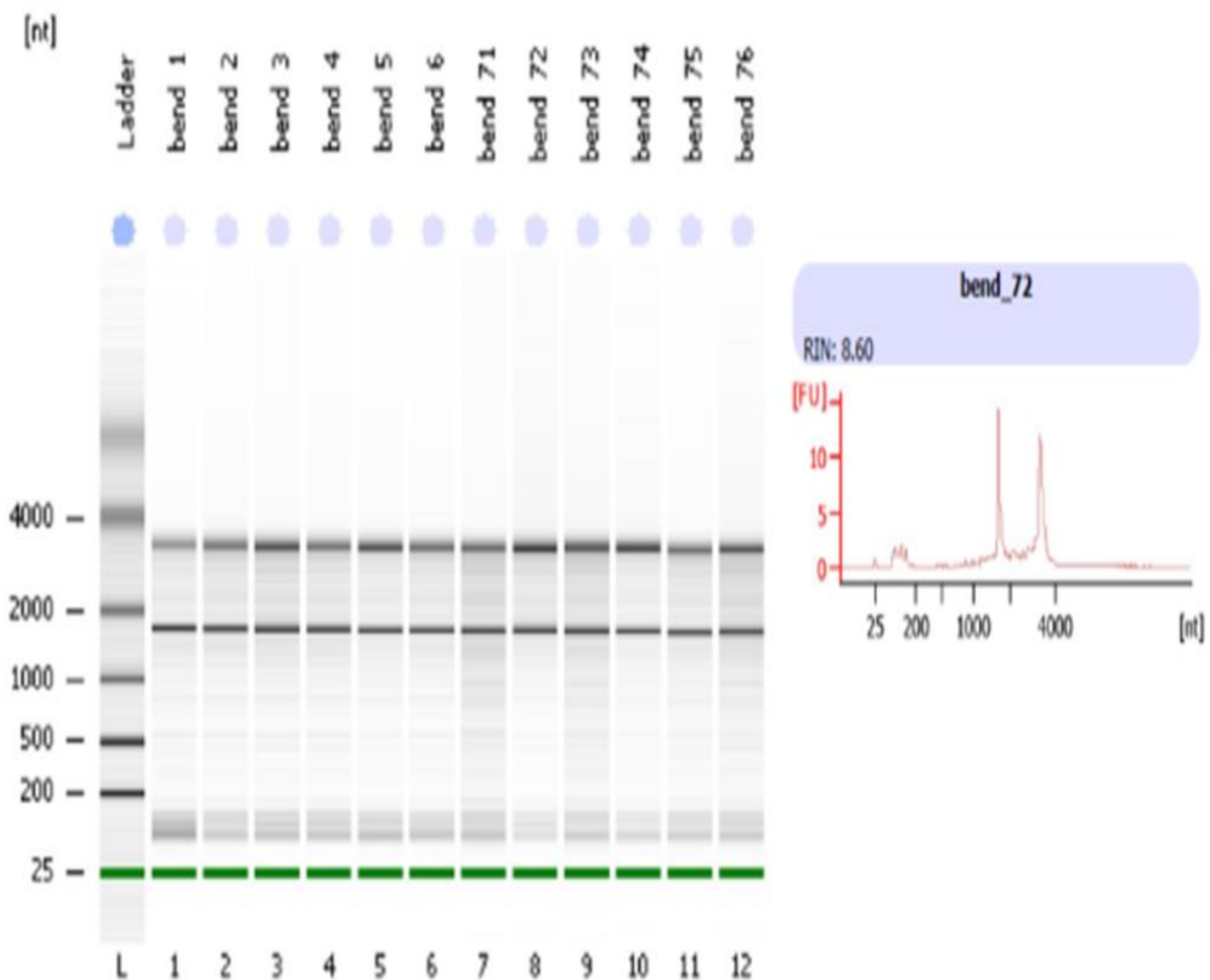
All animal experiments were carried out in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the University of Hawai'i at Manoa (Approval no. 17-2605). Angus heifers aged 2-3 years (n=21) were used for sampling. The estrous cycles of heifers were synchronized using the intramuscular injection of a Prostaglandin F2 alpha (On Day-0 and -11). Control heifers (n=6) were not bred and were referred to as Cyclic heifers on day 15-17 of the estrous cycle. Fifteen heifers were bred naturally after detecting the estrus. Following slaughter at a commercial abattoir, uteri were examined for the presence or absence of conceptus, and samplings were done immediately after the animals were slaughtered. Out of fifteen heifers, conceptus was only present in seven heifers and was referred to as Pregnant (n=7), and uteri without conceptus were referred to as Non-pregnant (n=8). Caruncular endometrial tissues were collected at Day 15-17 of gestation (pregnant), of the estrous cycle (cyclic), and absence of conceptus (nonpregnant) heifers in a well-labeled tube containing RNAlater (RNAlater™ solution, Invitrogen, Thermofisher Scientific, USA). Further, the RNAlater was removed before storing the sample at -80°C until analysis.

### **2.2 Total RNA isolation and quality control**

Total RNA was isolated from frozen tissues (60-100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For the RNA isolation protocol, the steps include: tissue homogenization using bullet blender, phase separation, total RNA precipitation, RNA wash and RNA extraction using chloroform, RNA drying, and resuspension.

Firstly, 100 mg of 1 mm size beads were added in a sterile Eppendorf tube, followed by adding 500  $\mu$ L of TRIzol in that sterile tube. Approximately 100 mg of endometrial tissue was then added to the tube containing TRIzol and beads. Tissue samples were homogenized using Bullet Blender Cr (Next Advance, Inc., Troy, NY), setting the speed at 8 for 3 minutes. The homogenized tissue was centrifuged at 10,000 rpm for 1 min at 4°C. The supernatant was transferred into a new sterile tube where 500  $\mu$ L of TRIzol was added and allowed to stand at room temperature for 5 minutes. Then, 0.2 mL of chloroform was added to each sample, shaken vigorously for 15 seconds, and allowed to incubate at room temperature for 5 minutes. The resultant mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C, which resulted in the separation of the homogenate into three distinct layers: an upper aqueous layer containing the RNA, the bottom organic phase containing protein, and the middle interface with DNA. Avoiding the interphase, the upper clear liquid was pipetted and transferred into a new Eppendorf tube. Then, 0.5 mL of isopropanol was added, mixed, incubated for 5 minutes, and finally centrifuged at 10,000 rpm for 10 minutes at 4°C for precipitating the total RNA. The RNA pellet was washed by adding 1 mL of 75% ethanol after removing the supernatant, centrifuged at 14,000 rpm at 4°C for 5 minutes. The supernatant ethanol was then removed and allowed to air dry by inverting on the clean Kim wipe for 10 minutes. The pellet was resuspended in nuclease-free water (Thermo Scientific, Waltham, MA) and incubated on a heating plate at 60°C for 10 minutes. The total RNA concentration was determined using NanoDrop one (Thermo Fisher Scientific, Madison, WI). The quality of the RNA was determined by running samples on 2 % agarose gel. The RNA samples were stored at -80°C until further analysis. RNA quality was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). The samples with an RNA integrity number (RIN) > 7 (Figure 2) were further

used for RNA-Sequencing, and quantitative real-time PCR. The RNA was stored at  $-80^{\circ}\text{C}$  until further use.



**Figure 2:** A representative image showing the electrophoresis run of a good quality RNA sample measured using 2100 Agilent Bioanalyzer.

### 2.3 Library preparation and sequencing

RNA-Seq libraries were prepared and sequenced at the University of Hawai'i Cancer Center Genomics and Bioinformatics Shared Resource (UHCC GBSR) facility. A TruSeq

Stranded mRNA kit (Illumina, San Diego, CA) was used to prepare the RNA-Seq libraries from total RNA samples extracted from bovine endometrium, including Pregnant (n=4), Non-pregnant (n=4), and cyclic (n=4). Libraries were prepared according to the manufacturer's protocol. Briefly, poly-A RNA fraction, containing mRNAs and certain non-coding RNAs, was enriched from 500 ng of total RNA in two rounds of purification using Poly-T oligo magnetic beads. RNA was eluted using Elute, Prime, Fragment Mix, followed by 8-minute fragmentation at 94°C. Fragmented RNA samples were subsequently used for cDNA first- and second-strand synthesis. The blunt-ended dsDNAs were separated using AMPure XP beads (Beckman Coulter, Brea, CA) from the unincorporated nucleotides and enzymes. cDNAs were then adenylated at their 3' ends followed by ligation of indexing adapters, specific for each sample. The DNA fragments with adapter molecules on both ends were enriched with 15 cycles of PCR followed by two rounds of purification on AMPure XP beads. The libraries' size and quality were assessed in a High Sensitivity DNA Bioanalyzer assay (Agilent Technologies, Massy, France). Next, libraries were quantified by qPCR using KAPA Library Quantification Kit (KAPA Biosystems, Boston, Massachusetts) and were normalized to the concentration of 4 nM. Libraries were pooled and denatured using freshly prepared 0.2N NaOH followed by further dilution with HT1 buffer to obtain a final concentration of 1.8 pM. As a sequencing control, the library pool was spiked-in with 1% (v/v) of 1.8 pM denatured PhiX library and loaded onto the reagent cartridge. The sequencing run was performed with NextSeq 500 (Illumina, San Diego, CA), in single-end mode with a read length of 1×76bp. Illumina BaseSpace-created FASTQ files were used for further analysis.

## **2.4 RNA sequence analysis**

Data analysis of the RNA sequences were done at the University of Hawaii John A. Burns School of Medicine Bioinformatics core. Single-end reads in the FASTQ format were explored using FastQC (Babraham Institute, Cambridge, UK) and cleaned using Prinseq, a Perl script (Schmieder & Edwards, 2011). The cleaning procedure included trimming low quality reads from both 3' and 5' ends until a base pair of Phred quality score of 30 (99.9% accurate) or greater was found and filtering out reads having a mean quality score less than 30 and length below 30 nucleotides. Cleaned reads were aligned against the bovine reference genome (Bos\_taurus.ARS-UCD1.2) using HiSAT2. The resulting SAM files were sorted, converted to BAM files using SAMtools. Read counts mapped to bovine gene models were generated using htseq-count script from HTSeq package. Finally, bioconductor DESeq2 was used to get the differentially expressed genes among Pregnant vs. Non-Pregnant (P vs. NP), Pregnant vs. Cyclic (P vs. C), and Non-Pregnant vs. Cyclic (NP vs. C) groups.

The genes with at least two-fold change (FC) and Benjamini and Hochberg q-value < 0.05 were considered as differentially expressed (DE) in the bovine endometrium. The DE genes with higher expression were referred to as up-regulated genes in bovine endometrium while comparing different groups, i.e., (P vs. NP), (P vs. C), and (NP vs. C). Likewise, the DE genes with lower expression were referred to as down-regulated genes.

## **2.5 Pathways analysis (Pathways and Network Analysis)**

Additional analyses determined whether DEGs were significantly enriched in specific pathways and expression networks.

### **2.5.1 Functional Annotation and Gene Ontology enrichment analysis**

Functional and pathway analysis was carried out using an open web source named Enrichr (<https://maayanlab.cloud/Enrichr/>) to gain insight into the various Gene Ontology (GO) terms of the genes in bovine endometrium. The official gene symbol of the up-regulated genes was uploaded to the functional annotation tool in the Enrichr system, and the *Bos taurus* was selected as the reference genome. The genes that matched up with the genes in Enrichr were annotated into three GO terms; biological process, cellular component, and molecular function. All the GO terms were considered enriched at a modified P-value  $< 0.05$  and a threshold gene count of 2.

### **2.5.2 Kyoto Encyclopedia of Genes and Genomes (KEGG)**

The pathways enrichment for the up-regulated genes in the bovine endometrium using the Kyoto Encyclopedia of Genes and Genomes were analyzed. The official gene symbol of the up-regulated genes was uploaded to the functional annotation tool in the Enrichr system, and the bovine was selected as the reference genome. The enrichment parameters were set to a threshold gene count of 2 and a modified Fisher Exact P-value  $< 0.05$ . The over-represented KEGG pathways terms were considered as enriched KEGG pathways.

### **2.5.3 Ingenuity Pathway Analysis (IPA) and Networks**

The ingenuity pathway analysis (IPA) is a human genome-based powerful search tool with several advanced functions that allows insightful data analysis and interpretation. The differentially expressed genes (DEGs) were subjected to the Ingenuity Pathway Analysis (IPA; QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>; (Kraemer et al., 2014) to gain insights into the canonical pathways and network discovery.

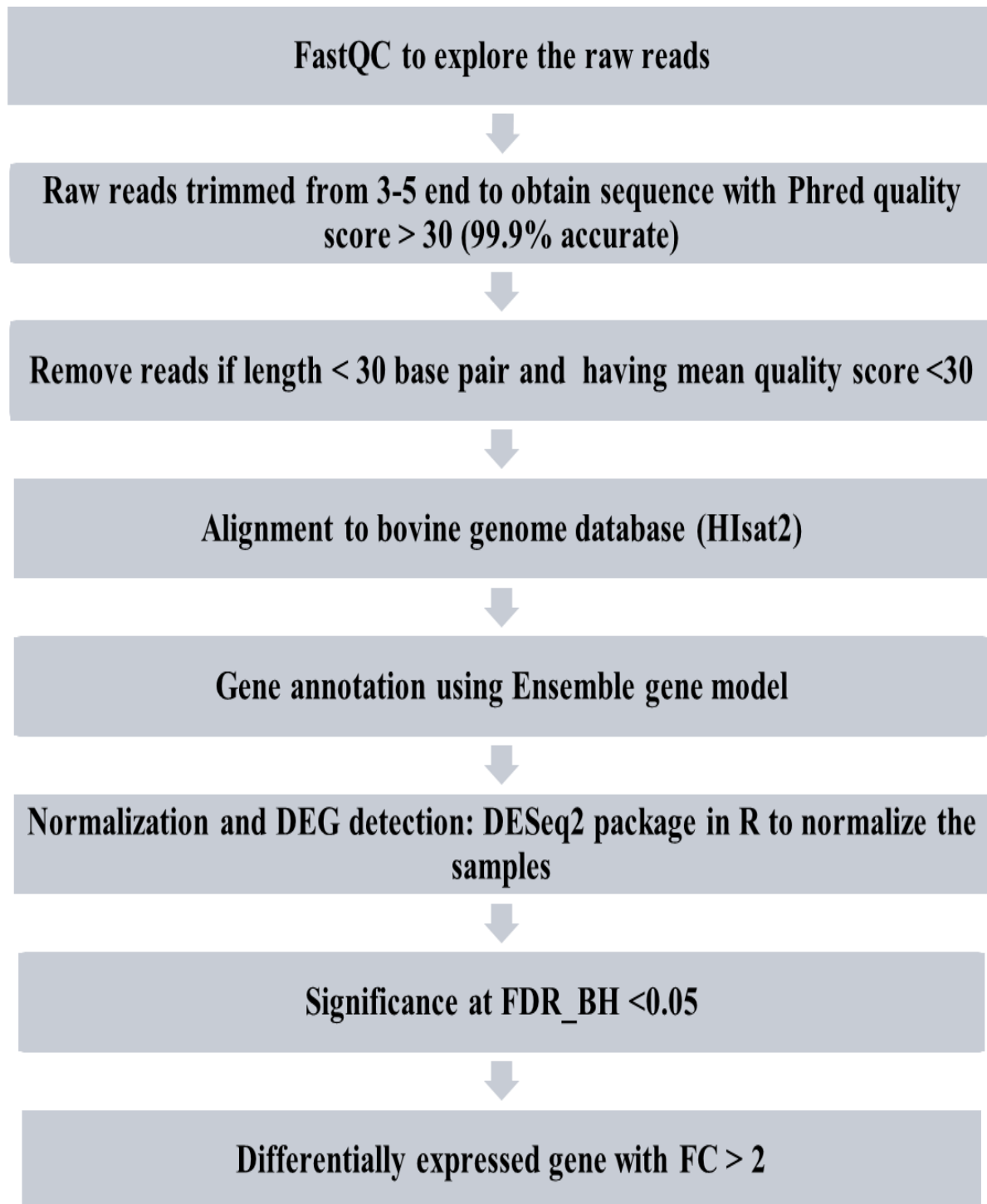
## **2.6 Complementary DNA synthesis (cDNA)**

By the method of reverse transcription of 1 µg total RNA (20 µL reaction of RT mixture) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), the first-strand cDNA was synthesized. Firstly, the RNA samples were diluted with nuclease-free water to standardize them to a concentration of 1000 ng per 10 µL. Then, 10 µL of reverse transcriptase (RT) mix was prepared by using 2 µL of 10X RT Buffer, 0.8 µL of 25X dNTP (100nM), 2 µL of 10X RT Random Primer, and 1 µL of Multiscribe Reverse Transcriptase enzyme (Applied Biosystems, Foster City, CA). The RNA sample (10 µL) and RT mix (10 µL) were mixed in a microcentrifuge tube and incubated into a thermal cycler to synthesize single-stranded cDNA. The thermal cycler's run condition was 25<sup>0</sup>C for 10 minutes, 37<sup>0</sup>C for 120 minutes, 85<sup>0</sup>C for 5 minutes, and 4<sup>0</sup>C for infinity. The newly synthesized cDNA (20 µL) was diluted (20X) with 480 µL of nuclease-free water. Finally, the cDNAs were stored at -20<sup>0</sup>C until qPCR assay.

## **2.7 Validation of RNA-Seq data using Quantitative real-time PCR (qPCR)**

The qPCR assay was performed in a 10 µL reaction mixture containing 3 µL of cDNA and 7 µL of PCR mix using QuantStudio™ 3 System (Applied Biosystems, Foster City, CA). The PCR mixture was prepared by adding 5 µL of PowerUp SYBR Green Master Mix (Applied Biosystems) and 1 µL each of forward and reverse primers specific to the target gene. Primer pairs (forward and reverse) specific to each candidate gene were designed using the NCBI primer blast tool (Table 2). The PCR mix and cDNA samples were loaded into a 96-well optical plate and were sealed with clear optical adhesive films (Applied Biosystems). A PCR run template file was designed using QuantStudio™ 3 software (Applied Biosystems, Foster City, CA) following the reagents used and the samples loaded. Then the PCR plate was inserted into the machine. The amplification conditions were 50<sup>0</sup>C for 2 minutes (hold), 95<sup>0</sup>C for 2 minutes (hold), followed by 40 repeat cycles

of 95<sup>0</sup>C for 15 seconds (denaturation), 60<sup>0</sup>C for 15 seconds (annealing), and 72<sup>0</sup>C for 1 minutes (extension). After the run was completed, the data was downloaded, and the PCR plates were stored at 4<sup>0</sup>C until validated for target specificity by gel electrophoresis. The specificity of primers was validated by running the melting curve and qPCR products were assessed using gel electrophoresis. To determine the most stable housekeeping gene in the endometrial tissues, the expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Beta-actin ( $\beta$ -actin), and TATA-Box Binding Protein (TBP) were analyzed in triplicates across the samples.  $\beta$ -actin was the most uniform housekeeping genes. The target genes were analyzed in triplicates, and the expression level was determined using the cycle threshold (Ct) values following the standard curve method after normalization with  $\beta$ -actin. The fold change for each gene was calculated using the  $2^{-\Delta\Delta Ct}$  method. Data for fold change were presented as a mean  $\pm$  standard error on the bar diagram.



**Figure 3:** Processing of RNA-Sequencing data to obtain high-quality non-redundant transcriptomes.

**Table 2:** Primers used to quantify the expression of target genes by qPCR.

S.N.	Gene	Accession no.	Primer Sequence	Amplicon (bp)
1	<i>MRS2</i>	NM_001101903.1	F: GGGATTGACCATGCAGAGGA R: CACATTACGGTGGCTGTCCA	150
2	<i>CST6</i>	NM_001012764.3	F: CAAGTACTACCTGACCGTGGAC R: CACAGCGCAGCTTCTCCT	125
3	<i>FOS</i>	NM_182786.2	F: AAAGGCGAATCCGAAGGGAA R: AGTTGGTCTGTCTCCGCTTG	100
4	<i>VLDLR</i>	NM_174489.2	F: TGTGCAAGGCAGTAGGCAAA R: CAGCGATGTCAGCATCGAGA	141
5	<i>IFI6</i>	XM_002685877.4	F:GTCAAGGATACACCTGTGAAGAAAA R: GGAGTCTGAAGAAGGCCCTTAG	140
6	<i>MX2</i>	NM_173941.2	F: GCCCGCCATTGCCGTTA R: CCGGGTGATGATTCCGCTG	103
7	<i>C15H11ORF34</i>	NM_001113538.1	F: AGCACGCTCTTCAAGGCAAA R: GACGGTCACAGTCCCAACTT	141
8	<i>EIFM3</i>	XM_010812722.2	F: TGGGAATGGCCGTGGAAAAT R: TCCGATGTGTGCTATGACATCAA	113
9	<i>TINAGL1</i>	XM_015459968.2	F: CACGGCAGCTGTGGCA R: CACCAGGCACCATCCAGTC	141
10	<i>ISG15</i>	NM_174366.1	F: GCAGACCAGTTCTGGCTGTCT R: CCAGCGGGTGCTCATCAT	140
11	<i>PENK</i>	NM_174141.2	F: GAACAGCGGCAACCCCAT R: GCAGTCCTGGCTGCATTCT	149
12	<i>PRSS22</i>	XM_002697927.5	F: CTATCAAGACAGCCGGCCC R: TCTTCCGGATGCTCACAACC	114
13	<i>MS4A8</i>	NM_001034056.2	F: GGGGAGGCATCTGGTTCATC R: GACGATGTAAAGCCCACGC	144
14	<i>R3DHM1</i>	XM_024980920.1	F: GTCATTCCACCTGGCCAACA R: GTGGAGGTGGCGCTGC	148
15	$\beta$ -ACTIN	NM_173979.3	F: GAAGATCAAGATCATCGCGCC R: GTGTAACGCAGCTAACAGTC	177

F= Forward, R= Reverse

## **2.8 DNA gel electrophoresis**

The specificity of PCR products was verified using 1% agarose gel electrophoresis. First, 1 gm of agarose was weighed out in a volumetric flask and 100 mL of 1X Tris-borate-EDTA (TBE) buffer. The solution was then microwaved briefly for 1-2 minutes but did not overboil the solution, as some of the buffers will evaporate, that alter the final percentage of agarose in the gel. The solution needs to be boiled until the complete dissolution of agarose and the clear solution's visibility. About 2-3  $\mu\text{L}$  of 1% ethidium bromide (EtBr) was added to the melted agarose solution. EtBr binds to the DNA and allows visualization of the DNA under ultraviolet (UV) light. The agarose solution was let to cool down to about 50°C for 5 mins and poured into a gel tray. A comb was inserted to create the wells and allowed to solidify for 20-30 minutes. Meanwhile, PCR products were prepared for electrophoresis.

Loading samples were prepared in the ratio of 5:1 by adding 5  $\mu\text{L}$  of PCR product and 1 $\mu\text{L}$  of loading buffer (Omega Bio-tek Inc., Norcross, GA). The samples' molecular weight (MW) was compared with a DNA molecular weight marker (100bp, VWR, Radnor, PA) to determine the specific size of the PCR products. After casting the gel, it was transferred to the gel box, and 1X TBE was poured into submerging the gel. Next, 5  $\mu\text{L}$  each of the samples and the MW marker was loaded in the gel wells. After loading the gel, the run was initiated for 1 hour at 100 volts, and the results were viewed under the UV rays' light.

## **2.9 Statistical analysis**

In RNA-seq, genes having fold change (FC) greater than 2 in the endometrial sample and Benjamini and Hochberg q-value < 0.05 were considered differentially expressed. The fold-change values for qPCR results were calculated and analyzed using the  $2^{-\Delta\Delta\text{Ct}}$  method to calculate the fold change. The design was completely random. Values were subjected to a one-way analysis of

variance (ANOVA) followed by Tukey HSD test for mean separation and comparison to determine differences between the treatments on a R studio considering P-value  $<0.05$  for significant differences.

## CHAPTER 3: RESULTS

### 3.1 RNA-Seq results and identification of DEGs

#### 3.1.1 Analysis of differentially expressed genes in the bovine endometrium during the maternal recognition of pregnancy

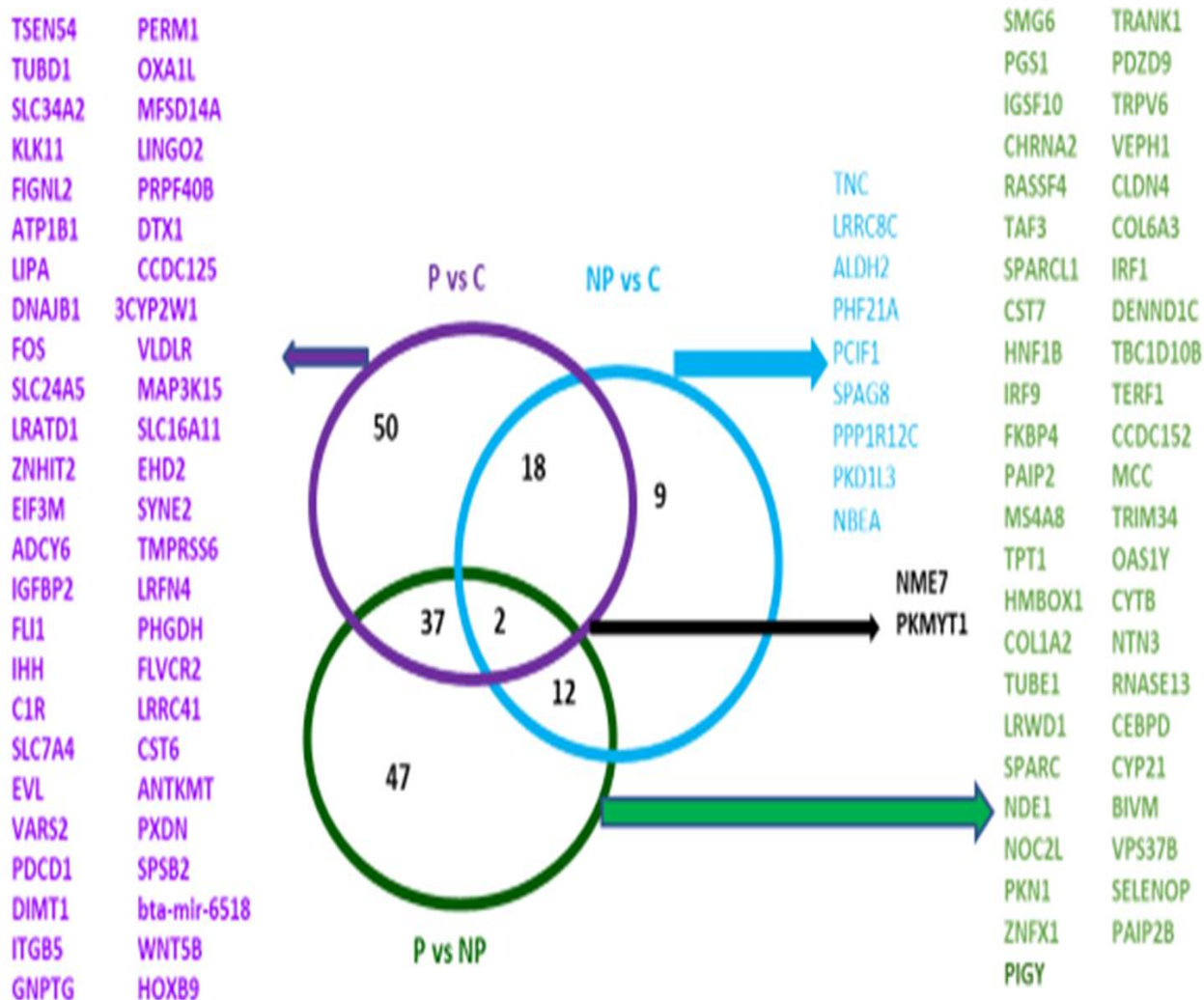
Maternal recognition of pregnancy is critical for the establishment of pregnancy. Using RNA-Seq, the transcriptomics profile was analyzed in pregnant endometrium (Day 15-17 of gestation) compared to non-pregnant and cyclic cows. Raw sequencing reads in the FASTQ format was obtained from the replicated RNA-Seq libraries and evaluated their qualities using FastQC. There was an average of 19.5M, 23.6M, and 20.6 M original raw reads in pregnant (P), non-pregnant (NP), and cyclic (C) cows, respectively. All the groups (P, NP, and C) had excellent quality sequences (>96%) (Table 3). Mapping results to the bovine genome database showed that an average of 93.3% of the retained reads from pregnant, 94.3% from non-pregnant, and 94.2% from cyclic was uniquely mapped (Table 4). A total of 27,270 gene transcripts were annotated using the Ensemble alignment of the bovine genome assembly (Wu et al., 2014). Differential expression analysis between pregnant, non-pregnant, and cyclic cows was conducted (DESeq2). A total of 98 genes were differentially expressed between pregnant and non-pregnant cows, 107 genes were differentially expressed between pregnant and cyclic, and 41 genes were differentially expressed between cyclic and non-pregnant (Figure 4). Of the 98 genes found in non-pregnant endometrium, 47.9% (47) were uniquely expressed in pregnant cattle, and of the 107 DE genes in pregnant cattle, 46% (50) were uniquely expressed in pregnant cattle. Only 23% of DE genes were shared between pregnant and nonpregnant cattle.

**Table 3:** Filtration summary of RNA-Seq raw reads in the bovine endometrium (P vs. NP vs. C).

Observations	Cyclic			Non-Pregnant			Pregnant		
	Library 1	Library 2	Library 3	Library 1	Library 2	Library 3	Library 1	Library 2	Library 3
Good sequences	20,031,490 (95.32%)	19,305,938 (95.82%)	18,698,448 (96.42%)	21,606,901 (96.62%)	25,832,748 (96.58%)	24,544,606 (96.36%)	18,490,951 (96.42%)	20,845,914 (96.14%)	16,026,899 (96.33%)
Input sequences	21,014,070	20,148,391	19,393,059	22,363,908	26,746,189	25,472,782	19,177,870	21,683,228	16,637,239
Input bases	1,586,803,660	1,521,449,961	1,464,413,922	1,688,532,305	2,019,640,603	1,922,265,638	1,448,210,205	1,637,233,649	1,256,021,663
Input mean length	75.51	75.51	75.51	75.5	75.51	75.46	75.51	75.51	75.49
Good sequences	20,031,490 (95.32%)	19,305,938 (95.82%)	18,698,448 (96.42%)	21,606,901 (96.62%)	25,832,748 (96.58%)	24,544,606 (96.36%)	18,490,951 (96.42%)	20,845,914 (96.14%)	16,026,899 (96.33%)
Good bases	1,509,437,200	1,454,831,651	1,408,996,043	1,627,917,494	1,946,591,795	1,849,390,260	1,393,400,235	1,570,794,366	1,207,431,115
Good mean length	75.35	75.36	75.35	75.34	75.35	75.35	75.36	75.35	75.34
Bad sequences	982,580 (4.68%)	842,453 (4.18%)	694,611 (3.58%)	757,007 (3.38%)	913,441 (3.42%)	928,176 (3.64%)	686,919 (3.58%)	837,314 (3.86%)	610,340 (3.67%)
Bad bases	74,243,336	63,628,269	52,471,381	57,174,266	69,001,811	69,021,111	51,897,264	63,125,062	46,059,946
Bad mean length	75.56	75.53	75.54	75.53	75.54	74.36	75.55	75.39	75.47

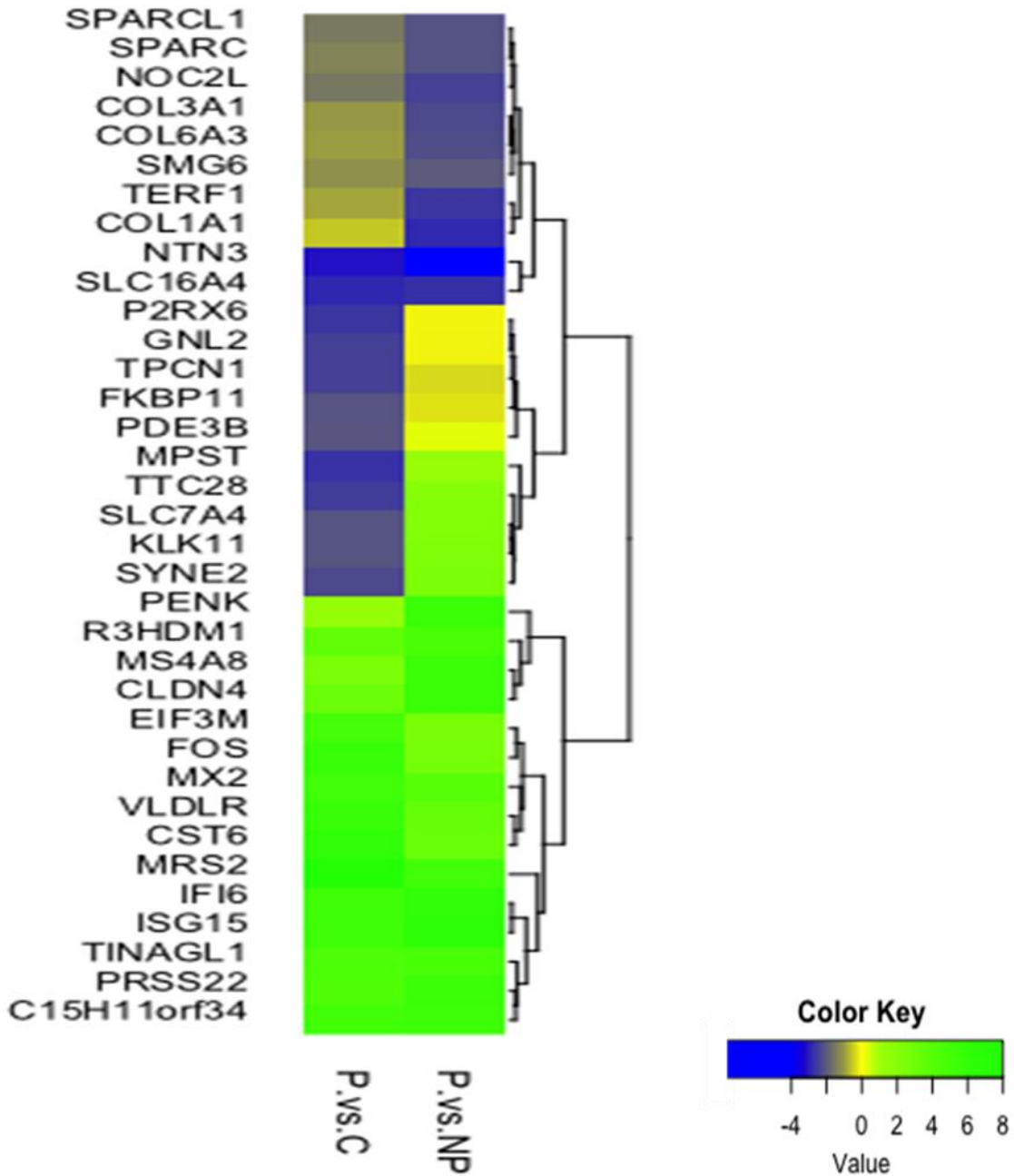
**Table 4:** Summary of Bovine endometrium RNA-Seq data mapping to the cattle genome.

cDNA Library	Reads (pre- filter)	Reads (post-filter)	Uniquely aligned reads
Cyclic 1	21014070	20031490	18877475(94.24%)
Cyclic2	20148391	19305938	18134703(93.93%)
Cyclic 3	19393059	18698448	17614417(94.20%)
Non-Pregnant 1	22363908	21606901	20415168(94.48%)
Non-Pregnant 2	26746189	25832748	24347122(94.25%)
Non-Pregnant 3	25472782	24544606	23101017(94.12%)
Pregnant 1	19177870	18490951	17261750(93.35%)
Pregnant2	21683228	20845914	19569704(93.88%)
Pregnant 3	16637239	16026899	14879028(92.84%)



**Figure 4:** A Venn diagram comparing the number of identified genes in the endometrium of Pregnant (P) vs. Cyclic (C), Pregnant (P) vs. Non-Pregnant (NP) and Non-Pregnant (NP) vs. Cyclic (C) along with the list of the name of the genes.

Number of differentially expressed genes (DEGs) in P vs. C (n=107), P vs. Np (n= 98), and NP vs. C (n=48).



**Figure 5:** Heat-map of the 35 up-regulated genes in the bovine endometrium compared with pregnant versus non-pregnant (P vs. NP) and pregnant versus cyclic (P vs. C). Extremes of green color indicate the gene's higher expression, while blue color indicates a lesser expression of genes.

### 3.1.1.1. Highly up-regulated genes in the bovine endometrium (P vs. C)

The highly up-regulated genes in the pregnant endometrium (vs. cyclic) are *MRS2*, *CST6*, *FOS*, *VLDLR*, *ISG15*, *IFI6*, *MX1*, *MX2*, *C15H11ORF34*, *EIF3M*, and *TINAGL1* (Table 5). Among many genes, *MRS2* was highly expressed with log2foldchange 7.72, followed by genes *CST6*, *FOS*, *VLDLR*, and *ISG15* with log2foldchange 6.306, 5.760, 5.691, and 5.314, respectively.

**Table 5:** Up-regulated genes in the bovine pregnant endometrium (P vs. C).

	Gene	Gene Description	Fold Change (log2)	padj
1	<i>MRS2</i>	Magnesium Transporter <i>MRS2</i>	7.727	0.000
2	<i>CST6</i>	Cystatin E/M	6.306	0.001
3	<i>FOS</i>	FOS Proto-Oncogene, AP-1 Transcription Factor Subunit	5.760	0.045
4	<i>VLDLR</i>	Very Low-Density Lipoprotein Receptor	5.691	0.008
5	<i>ISG15</i>	<i>ISG15</i> Ubiquitin Like Modifier	5.314	0.012
6	<i>IFI6</i>	Interferon Alpha Inducible Protein 6	5.215	0.000
7	<i>MX2</i>	MX Dynamin Like GTPase 2	5.189	0.018
8	<i>C15H11ORF34</i>	Placenta Expressed Transcript 1	5.107	0.006
9	<i>EIF3M</i>	Eukaryotic Translation Initiation Factor 3 Subunit M	4.973	0.003
10	<i>TINAGL1</i>	Tubulointerstitial Nephritis Antigen Like 1	4.489	0.000
11	<i>PXDN</i>	Peroxidasin	4.489	0.045
12	<i>PRSS22</i>	Serine Protease 22	4.442	0.039
13	<i>TUBD1</i>	Tubulin Delta 1	4.376	0.049
14	<i>TMPRSS2</i>	Transmembrane Serine Protease 2	4.348	0.000
15	<i>C1R</i>	Complement C1r	3.698	0.006
16	<i>FLVCR2</i>	Feline Leukemia Virus Subgroup C Cellular Receptor Family	3.692	0.013
17	<i>SLC2A1</i>	Solute Carrier Family 2 Member 1	3.688	0.002
18	<i>R3HDM1</i>	R3H Domain Containing 1	3.490	0.045
19	<i>MX1</i>	MX Dynamin Like GTPase 1	3.481	0.000
20	<i>BCAM</i>	Basal Cell Adhesion Molecule (Lutheran Blood Group)	3.468	0.001

### 3.1.1.2. Highly down-regulated genes in the bovine endometrium (P vs. C)

The highly down-regulated genes in the pregnant endometrium (vs. cyclic) are *LRFN4*, *PKMYT1*, *ZNHIT2*, *VAR2*, *ARL2BP*, *LINGO2*, *SLC16A11*, *SLC16A4*, *TMEM151B*, and *NME7* (Table 6). Among many genes, *LRFN4* was highly down-regulated with log2foldchange -3.276, followed by genes *PKMYT1*, *ZNHIT2*, *VAR2*, and *ARL2BP* with log2foldchange -3.202, -3.182, -3.167, and -3.141, respectively.

**Table 6:** Down-regulated genes in the bovine pregnant endometrium (P vs. C).

S.N.	Gene	Gene description	Fold Change (log2)	padj
1	<i>LRFN4</i>	Leucine-Rich Repeat and Fibronectin Type III Domain Containing	-3.276	0.000
2	<i>PKMYT1</i>	Protein Kinase, Membrane Associated Tyrosine/Threonine 1	-3.202	0.000
3	<i>ZNHIT2</i>	Zinc Finger HIT-Type Containing 2	-3.182	0.000
4	<i>VAR2</i>	Valyl-TRNA Synthetase 2, Mitochondrial	-3.167	0.001
5	<i>ARL2BP</i>	ADP Ribosylation Factor Like GTPase 2 Binding Protein	-3.141	0.000
6	<i>LINGO2</i>	Leucine-Rich Repeat and Ig Domain Containing 2	-3.104	0.000
7	<i>SLC16A11</i>	Solute Carrier Family 16 Member 11	-3.005	0.001
8	<i>SLC16A4</i>	Solute Carrier Family 16 Member 4	-2.861	0.001
9	<i>TMEM151B</i>	Transmembrane Protein 151B	-2.795	0.000
10	<i>NME7</i>	NME/NM23 Family Member 7	-2.743	0.000
11	<i>MPST</i>	Mercaptopyruvate Sulfur transferase	-2.672	0.000
12	<i>P2RX6</i>	Purinergic Receptor P2X 6	-2.636	0.000
13	<i>TTC28</i>	Tetratricopeptide Repeat Domain 28	-2.490	0.000
14	<i>GNL2</i>	G Protein Nucleolar 2	-2.454	0.000
15	<i>TPCNI</i>	Two Pore Segment Channel 1	-2.444	0.000
16	<i>SYNE2</i>	Spectrin Repeat Containing Nuclear Envelope Protein 2	-2.292	0.000
17	<i>FKBP11</i>	FKBP Prolyl Isomerase 11	-2.106	0.000
18	<i>SLC7A4</i>	Solute Carrier Family 7 Member 4	-2.096	0.000
19	<i>KLK11</i>	Kallikrein Related Peptidase 11	-2.095	0.001
20	<i>PDE3B</i>	Phosphodiesterase 3B	-2.076	0.000

### 3.1.1.3. Highly up-regulated genes in the bovine endometrium (P vs. NP)

The highly up-regulated genes in the pregnant endometrium (vs. nonpregnant) are *ISG15*, *IFI6*, *PENK*, *PRSS22*, *MS4A8*, *CLDN4*, *C15H11ORF34*, *MRS2*, *TINAGL1*, and *R3HDM1* (Table 7). Among many genes, *ISG15* was highly expressed with log2foldchange 6.520, followed by genes *IFI6*, *PENK*, *PRSS22*, and *MS4A8* with log2foldchange 6.300, 6.300 5.578 5.565, and 5.560, respectively.

**Table 7:** Up-regulated genes in the bovine pregnant endometrium (P vs. NP).

S.N.	Gene	Gene Description	Fold Change (log2)	padj
1	<i>ISG15</i>	<i>ISG15</i> Ubiquitin Like Modifier	6.520	0.001
2	<i>IFI6</i>	Interferon Alpha Inducible Protein 6	6.300	0.000
3	<i>PENK</i>	Proenkephalin	5.578	0.000
4	<i>PRSS22</i>	Serine Protease 22	5.565	0.010
5	<i>MS4A8</i>	Membrane Spanning 4-Domains A8	5.560	0.012
6	<i>CLDN4</i>	Claudin 4	5.526	0.006
7	<i>C15H11ORF34</i>	Placenta Expressed Transcript 1	5.379	0.003
8	<i>MRS2</i>	Magnesium Transporter MRS2	5.096	0.000
9	<i>TINAGL1</i>	Tubulointerstitial Nephritis Antigen Like 1	4.738	0.000
10	<i>R3HDM1</i>	R3H Domain Containing 1	4.711	0.013
11	<i>MX1</i>	MX Dynamin Like GTPase 1	4.468	0.000
12	<i>GPT2</i>	Glutamic--Pyruvic Transaminase 2	4.427	0.000
13	<i>OASIY</i>	2'-5'-Oligoadenylate Synthetase 1	4.209	0.043
14	<i>LRWD1</i>	Leucine-Rich Repeats and WD Repeat Domain Containing 1	4.004	0.006
15	<i>MX2</i>	MX Dynamin Like GTPase 2	3.981	0.039
16	<i>TRIM34</i>	Tripartite Motif Containing 34	3.880	0.002
17	<i>IRF9</i>	Interferon Regulatory Factor 9	3.790	0.042
18	<i>NDRG2</i>	NDRG Family Member 2	3.693	0.019
19	<i>SLC2A1</i>	Solute Carrier Family 2 Member 1	3.621	0.001
20	<i>TRANK1</i>	Tetratricopeptide Repeat and Ankyrin Repeat Containing 1	3.611	0.037

### 3.1.1.4. Highly down-regulated genes in the bovine endometrium (P vs. NP)

The highly down-regulated genes in the pregnant endometrium (vs. non-pregnant) are *SNX20*, *PACSINI*, *NTN3*, *COL1A1*, *SLC16A4*, *TERF1*, *NOC2L*, *COL3A1*, *COL6A3* and *SPARCL1* (Table 8). Among many genes, *SNX20* was highly down-regulated with log2foldchange -5.182, followed by genes *PACSINI*, *NTN3*, *COL1A1*, and *SLC16A4* with log2foldchange -5.086, -3.975, -2.834, and -2.705, respectively.

**Table 8:** Down-regulated genes in the bovine pregnant endometrium (P vs. NP).

S.N.	Gene	Gene Description	Fold Change (log2)	padj
1	<i>SNX20</i>	Sorting Nexin 20	-5.182	0.014
2	<i>PACSINI</i>	Protein Kinase C and Casein Kinase Substrate in Neurons 1	-5.086	0.022
3	<i>NTN3</i>	Netrin 3	-3.975	0.043
4	<i>COL1A1</i>	Collagen Type I Alpha 1 Chain	-2.834	0.002
5	<i>SLC16A4</i>	Solute Carrier Family 16 Member 4	-2.705	0.034
6	<i>TERF1</i>	Telomeric Repeat Binding Factor 1	-2.619	0.014
7	<i>NOC2L</i>	NOC2 Like Nucleolar Associated Transcriptional Repressor	-2.406	0.024
8	<i>COL3A1</i>	Collagen Type III Alpha 1 Chain	-2.279	0.000
9	<i>COL6A3</i>	Collagen Type VI Alpha 3 Chain	-2.219	0.042
10	<i>SPARCL1</i>	SPARC Like 1	-2.130	0.009
11	<i>SPARC</i>	Secreted Protein Acidic and Cysteine Rich	-2.128	0.001
12	<i>SMG6</i>	SMG6 Nonsense Mediated MRNA Decay Factor	-2.027	0.001

### 3. 1.1.5. Highly up-regulated genes in the bovine endometrium (NP vs. C)

The highly up-regulated genes in the non-pregnant endometrium (vs. cyclic) are *NBEA*, *TNC*, *LRRC8C*, *COL1A1*, *DNAH11*, *MAPK7*, *ST3GAL6*, *COL3A1*, *SLC46A1*, and *TRADD* (Table 9). Among many genes, *NBEA* was highly up-regulated with log2foldchange 3.871, followed by genes *TNC*, *LRRC8C*, *COL1A1*, and *DNAH11* with log2foldchange 3.792, 3.373, 2.271, and 1.392, respectively.

**Table 9:** Up-regulated genes in the bovine endometrium (NP vs. C).

S.N.	Gene name	Gene description	Fold Change (log2)	padj
1	<i>NBEA</i>	Neurobeachin	3.871	0.006
2	<i>TNC</i>	Tenascin C	3.792	0.006
3	<i>LRRC8C</i>	Leucine-Rich Repeat Containing 8 VRAC Subunit C	3.373	0.004
4	<i>COL1A1</i>	Collagen Type I Alpha 1 Chain	2.271	0.024
5	<i>DNAH11</i>	Dynein Axonemal Heavy Chain 11	1.392	0.044
6	<i>MAPK7</i>	Mitogen-Activated Protein Kinase 7	1.272	0.001
7	<i>ST3GAL6</i>	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 6	1.265	0.013
8	<i>COL3A1</i>	Collagen Type III Alpha 1 Chain	1.248	0.035
9	<i>SLC46A1</i>	Solute Carrier Family 46 Member 1	1.147	0.050
10	<i>TRADD</i>	TNFRSF1A Associated Via Death Domain	1.126	0.022

### 3.1.1.6. Highly down-regulated genes in the bovine endometrium (NP vs. C)

The highly down-regulated genes in the non-pregnant endometrium (vs. cyclic) are *BPIFB1*, *PENK*, *AMMECR1L*, *ARL2BP*, *DAAM2*, *COLQ*, *MPST*, *P2RX6*, *TMEM151B*, and *PDE3B* (Table 10). Among many genes, *BPIFB1* was highly down-regulated with log2foldchange -6.550, followed by genes *PENK*, *AMMECR1L*, *ARL2B*, and *DAAM2* with log2foldchange -4.218, -3.726, -3.035, and -2.711, respectively.

**Table 10:** Down-regulated genes in the bovine endometrium (NP vs. C).

S.N.	Gene name	Gene Description	Fold Change (log2)	padj
1	<i>BPIFB1</i>	BPI Fold Containing Family B Member 1	-6.550	0.000
2	<i>PENK</i>	Proenkephalin	-4.218	0.003
3	<i>AMMECR1L</i>	AMMECR1 Like	-3.726	0.001
4	<i>ARL2BP</i>	ADP Ribosylation Factor Like GTPase 2 Binding Protein	-3.035	0.001
5	<i>DAAM2</i>	Dishevelled Associated Activator of Morphogenesis 2	-2.711	0.000
6	<i>COLQ</i>	Collagen Like Tail Subunit of Asymmetric Acetylcholinesterase	-2.665	0.044
7	<i>MPST</i>	Mercaptopyruvate Sulfur transferase	-2.552	0.000
8	<i>P2RX6</i>	Purinergic Receptor P2X 6	-2.521	0.006
9	<i>TMEM151B</i>	Transmembrane Protein 151B	-2.514	0.022
10	<i>PDE3B</i>	Phosphodiesterase 3B	-2.367	0.001
11	<i>GNL2</i>	G Protein Nucleolar 2	-2.314	0.013
12	<i>PPP1R12C</i>	Protein Phosphatase 1 Regulatory Subunit 12C	-2.209	0.039
13	<i>TPCNI</i>	Two Pore Segment Channel 1	-2.059	0.027
14	<i>PCIF1</i>	PDX1 C-Terminal Inhibiting Factor 1	-2.011	0.004

### 3.1.2. Gene Ontology analysis

The Gene ontology analysis demonstrates Type-1 interferon signaling, Immune response, and extracellular matrix organization were important functional pathways observed in the biological process. Similarly, Ion transporters such as *SLC34A2*, *SLC2A1*, and *SLC16A11* were

important in the molecular functions. The cellular component functions on the Endoplasmic reticulum lumen were governed by genes such as *WNT5B*, *IL23A1*, and *PENK* (Table 11).

The Gene Ontology analysis of non-pregnant endometrium on which genes such as *MAPK7*, *PCIF1*, *PKMYT1*, and *PPP1R12C* are expressed as negative regulator of hydrolase. *TRADD* is expressed as a death-inducing signaling complex (Table 12).

**Table 11:** Gene Ontology analysis in the bovine pregnant endometrium.

<b>Gene ontology</b>	<b>Pregnant endometrium</b>
Biological process	Type-1 interferon signaling ( <i>MX1</i> , <i>MX2</i> , <i>IF16</i> , <i>IRF1</i> , and <i>ISG15</i> ) Immune response ( <i>IL23A</i> , and <i>RSAD2</i> ) Extracellular matrix organization ( <i>COL1A1</i> , <i>COL1A2</i> , <i>COL3A1</i> , and <i>TIMP2</i> )
Molecular functions	Ion transporters ( <i>SLC34A2</i> , <i>SLC2A1</i> , <i>SLC16A11</i> , <i>SLC16A4</i> and <i>ATP1B1</i> ) Platelets derived factors, telomerase activity ( <i>HMBOX1</i> , and <i>TERF1</i> ) and ATPase activities ( <i>P2RX6</i> and <i>DNAJB1</i> )
Cellular component	Endoplasmic reticulum lumen ( <i>WNT5B</i> , <i>IL23A1</i> , <i>PENK</i> , <i>TNC</i> , <i>SPARCL1</i> , and <i>B2M</i> )

**Table 12:** Gene Ontology analysis in the bovine non-pregnant endometrium.

Nonpregnant endometrium	Negative regulator of hydrolase ( <i>MAPK7</i> ; <i>PCIF1</i> ; <i>PKMYT1</i> ; <i>PPP1R12C</i> ) Negative regulator of dephosphorylation and phosphatase, ( <i>PCIF1</i> ; <i>PKMYT1</i> ; <i>PPP1R12C</i> ) Death-inducing signaling complex ( <i>TRADD</i> ) Platelet-derived growth factor binding ( <i>COL1A1</i> ; <i>COL3A1</i> )
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### 3.1.3 KEGG Pathway

In the KEGG pathway, both groups (P vs. C) and (P vs. NP) had some common pathways, i.e., the mineral absorption pathway. On the other hand, Th17 cell differentiation, Endocrine, and other factor-regulated calcium reabsorption and Progesterone-mediated oocyte maturation pathways were highly enriched in (P vs. C) (Table 13) and ECM-receptor interaction, C-type lectin receptor signaling pathway and IL-17 signaling pathway in (P vs. NP) (Table 14). Collagen genes are found abundantly in bovine pregnant endometrium.

**Table 13:** KEGG Pathway (P vs. C).

<b>Term</b>	<b>Odds Ratio</b>	<b>Genes</b>
Progesterone-mediated oocyte maturation	6.23	<i>PDE3B; PKMYT1; ADCY6</i>
Th17 cell differentiation	5.50	<i>IL23A; FOS; JAK3</i>
Parathyroid hormone synthesis, secretion, and action	5.24	<i>SLC34A2; FOS; ADCY6</i>
<b>Mineral absorption</b>	8.49	<i>SLC34A2; ATP1B1</i>
cAMP signaling pathway	3.54	<i>PDE3B; FOS; ATP1B1; ADCY6</i>
Endocrine and other factor-regulated calcium reabsorption	6.79	<i>ATP1B1; ADCY6</i>
Regulation of lipolysis in adipocytes	6.67	<i>PDE3B; ADCY6</i>

**Table 14:** KEGG Pathway (P vs. NP).

<b>Term</b>	<b>Odds Ratio</b>	<b>Genes</b>
Protein digestion and absorption	9.07	<i>COL1A1; COL3A1; COL1A2; COL6A3</i>
ECM-receptor interaction	7.37	<i>COL1A1; COL1A2; COL6A3</i>
C-type lectin receptor signaling pathway	5.46	<i>IL23A; IRF1; IRF9</i>
<b>Mineral absorption</b>	9.27	<i>SLC46A1; TRPV6</i>
Platelet activation	4.89	<i>COL1A1; COL3A1; COL1A2</i>
IL-17 signaling pathway	4.48	<i>MAPK7; TRADD</i>
Focal adhesion	3.07	<i>COL1A1; COL1A2; COL6A3</i>
Arginine biosynthesis	10.71	<i>GPT2</i>

### 3.1.4 Ingenuity Pathway Analysis

#### 3.1.4.1 Canonical Pathway

In the Ingenuity Canonical pathways, two pathways were common in both groups. They were interferon signaling, and IL-12 signaling and production in macrophages. Other uncommon pathways to the group were the Th17 activation pathway, MIF Regulation of Innate Immunity, and IL-23 Signaling Pathway (P vs. C) (Table 15). In contrast, Oxidative Phosphorylation, GP6 signaling pathway, and Inhibition of Matrix Metalloproteases were some pathways that were expressed in the group (P vs. NP) (Table 16)

**Table 15:** Ingenuity Canonical Pathway (P vs. C).

<b>Ingenuity Canonical Pathways</b>	<b>-log (p-value)</b>	<b>Molecules</b>
Th17 Activation Pathway	4.40	<i>HIF1A, HSP90AA1, HSP90AB1, IL10, IL23A, JAK3, NFKB1, PTGER2, STAT4</i>
<b>Interferon Signaling</b>	4.38	<i>IFI6, IFIT1, IRF1, IRF9, ISG15, MX1</i>
Epithelial-Mesenchymal Transition Pathway	4.30	<i>EGR1, FGF14, FGF9, HIF1A, HNF1A, JAK3, MET, MMP-2, NFKB1, NOTCH4, PIK3R1, TWIST1, WNT5B</i>
PI3K/AKT Signaling	3.47	<i>CDKN1B, HSP90AA1, HSP90AB1, INPP5J, ITGA3, JAK3, NFKB1, PIK3R1, PPP2R3A, PTGS2, TP53</i>
<b>IL-12 Signaling and Production in Macrophages</b>	3.17	<i>APOB, FOS, IL10, IL23A, IRF1, MST1R, NFKB1, PIK3R1, STAT4</i>
MIF Regulation of Innate Immunity	3.03	<i>CD74, FOS, NFKB1, PTGS2, TP53</i>
CD40 Signaling	2.95	<i>FCER2, FOS, JAK3, NFKB1, PIK3R1, PTGS2</i>
IL-23 Signaling Pathway	2.94	<i>HIF1A, IL23A, NFKB1, PIK3R1, STAT4</i>
PPAR Signaling	2.55	<i>AIP, FOS, HSP90AA1, HSP90AB1, NFKB1, PTGS2</i>

**Table 16:** Ingenuity Canonical Pathway (P vs. NP).

<b>Ingenuity Canonical Pathways</b>	<b>-log (p-value)</b>	<b>Molecules</b>
<b>Interferon Signaling</b>	7	<i>IFI6, IFIT1, IRF1, IRF9, ISG15, MX1, STAT1, TAP1</i>
Oxidative Phosphorylation	4.09	<i>ATP5F1C, COX11, COX4I2, MT-CO1, MT-CYB, MT-ND1, MT-ND2, MT-ND3, MT-ND5</i>
GP6 Signaling Pathway	3.80	<i>CERT1, COL12A1, COL1A1, COL1A2, COL3A1, COL4A2, COL5A1, COL6A1, COL6A3</i>
Mitochondrial Dysfunction	3.25	<i>ATP5F1C, COX11, COX4I2, MT-CO1, MT-CYB, MT-ND1, MT-ND2, MT-ND3, MT-ND5, PDHA1</i>
Inhibition of Matrix Metalloproteases	2.42	<i>MMP-14, MMP-2, SDC1, TIMP2</i>
<b>IL-12 and Macrophage production Signaling</b>	2.2	<i>APOB, CLU, IL23A, IRF1, STAT1, STAT4, TGFB3</i>

### 3.1.4.2 IPA Networks

In the IPA Network, cell cycle (P vs. C) molecules such as *ABHD16B*, *ATIC*, *BPIFB1*, *CD96*, *CER1*, *Cma2/Mcpt9*, *CRYBG2*, *CTNNB1*, *CYB5A*, *CYP2W1*, *ESM1*, *EVX1*, *EZH2*, *F2*, *FALEC*, *GATA2*, *GPATCH11*, *HESX1*, *IFNG*, *LECT2*, *LUCAT1*, *MAN2B1*, *MINK1*, *MPZL2*, *MTHFD1L*, *P2RX6*, *PLEKHA6*, *PMCH*, *Ppbb*, *PROX2*, *TMEM50B*, *TSEN15*, *TSEN54*, and *TSPAN32* are included (Figure 6).

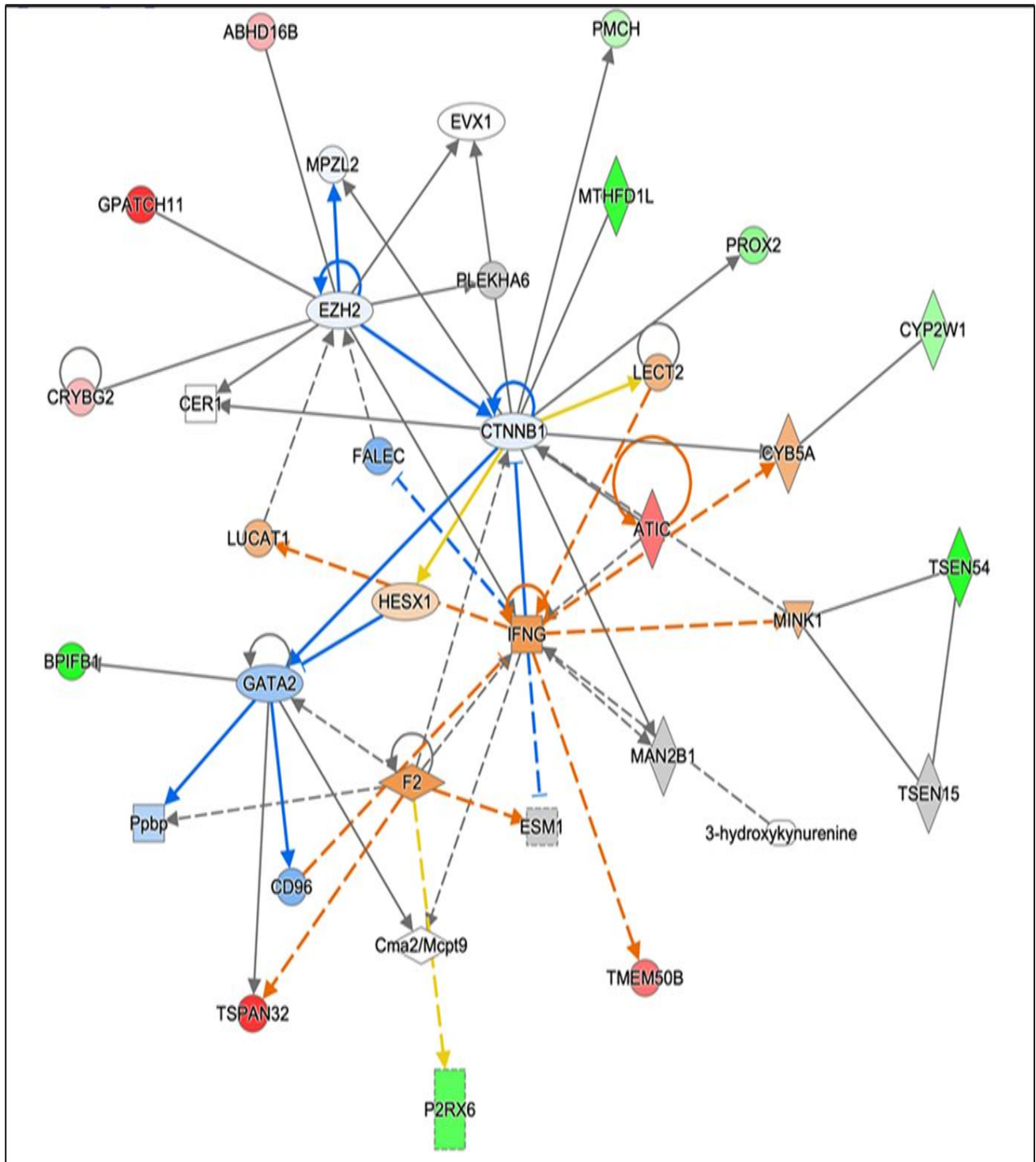
In IPA network, cell morphology (P vs. C) molecules such as *AGTR1*, *CLCCI1*, *CYP3A7*, *CYP4F3*, *CYREN*, *ERI2*, *FETUB*, *FZD3*, *GHRHR*, *GPR160*, *GTPBP4*, *H2AB3*, *HNFA4*, *INPP5J*, *JKAMP*, *KCNQ5*, *LYPDI*, *MCOLN3*, *NAALADLI*, *PPP1R3F*, *PRKDC*, *SLC12A9*, *SLC16A11*, *SLC9A1*, *SYBU*, *TAPT1*, *TMEM151B*, *TMEM39B*, *TPCN1*, *tretinoin*, *TSPAN14*, *TSPOAP1*, *ZNF133*, and *ZNF692* are included (Figure 7).

In IPA network, lipid metabolism (P vs. C) molecules such as *Alp*, *CDH1*, *COLQ*, *EIF3M*, *EIF4A1*, *EIF4A3*, *ELOA*, *EPAS1*, *FARP1*, *Fgf*, *HELZ*, *HISTONE*, *Histoneh3*, *HNFA1A*, *Insulin*, *KMT2E*, *mediator*, *MMP2*, *NFIA*, *NOC2L*, *PTEFb*, *POLR2B*, *Proinsulin*, *RBBP4*, *RNAPolymeraseII*, *Rnr*, *RPH3AL*, *RPSA*, *SKIDA1*, *SLC16A4*, *TCF/LEF*, *TMEM132A*, *TMPRSS2*, *ZFC3H1*, and *ZNHIT2* (Figure 8).

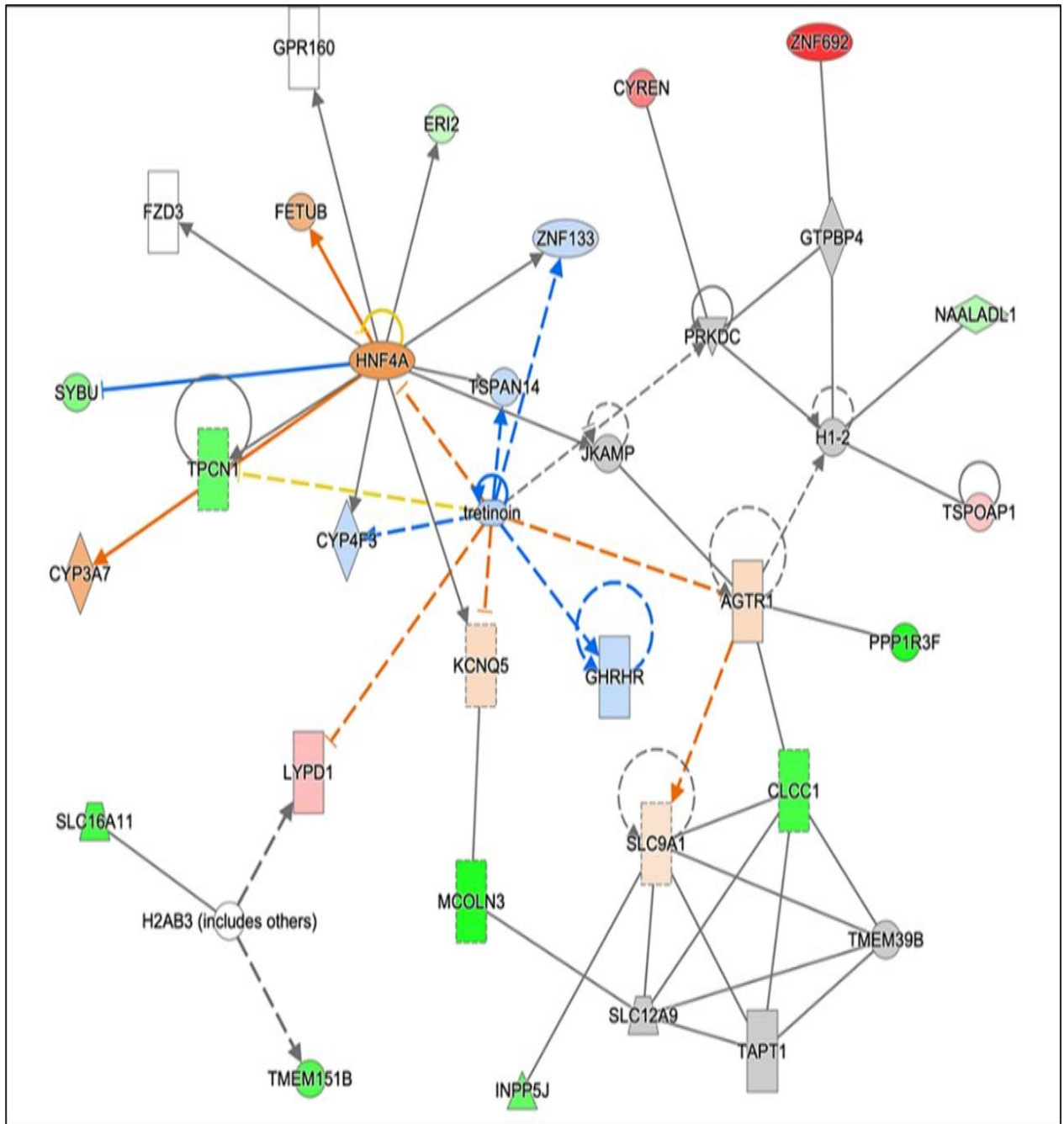
In IPA network, cell morphology (P vs. NP) molecules such as *ATPase*, *BAG3*, *CFTR*, *Ck2*, *COASY*, *CTSZ*, *CYREN*, *cytokine*, *DDX39B*, *DNAJB1*, *DNAJB8*, *EEF1A1*, *FBLN1*, *FKBP4*, *GRN*, *histone deacetylase*, *Histone h3*, *HSP*, *Hsp70*, *HSPA6*, *HSPH1*, *IL12 (complex)*, *KDM1A*, *KHNYN*, *Ku*, *LRATD1*, *MHC Class II (complex)*, *NBEA*, *PACSINI*, *PTCH2*, *TERF1*, *TKT*, *Ubiquitin*, *ZNF687*, and *ZNFX1* are included (Figure 9).

In IPA network, cell cycle (P vs. NP) molecules such as *ARSL*, *CCT2*, *CLBA1*, *CNDP2*, *CUL1*, *DYM*, *EHBPI*, *ENOSF1*, *EVA1B*, *FANCD2*, *FLVCR2*, *GCAT*, *GPT2*, *H12*, *HNRNPL*, *ISG20L2*, *KIAA1109*, *LONP1*, *MRS2*, *MTERF2*, *MYO1A*, *NAALADLI*, *PDXK*, *PDZD8*, *PNPLA1*, *RASGEF1C*, *RECQL4*, *SCNN1D*, *SGTA*, *SMG6*, *SPIDR*, *SRD5A3*, *TNK2*, *TLL3*, and *ZNF133* are included (Figure 10).

In IPA network, lipid metabolism (P vs. NP) molecules such as *ACAT2*, *Alpha 1 antitrypsin*, *ALT*, *Ap2*, *APOB*, *BIVM*, *C1q*, *C1S*, *CLU*, *EMB*, *ENTPD1*, *Fibrinogen*, *GPLD1*, *GPT2*, *Growth hormone*, *HDL*, *HDL-cholesterol*, *hemoglobin*, *IDII*, *IgG*, *IL23*, *LDL*, *LDL cholesterol*, *LIPA*, *NADPH oxidase*, *NETO2*, *PAIP2B*, *Pkc(s)*, *SAA*, *SDC1*, *STAB1*, *STARD4*, *STAT5a/b*, *TMPRSS6*, and *VLDL-cholesterol* are included (Figure 11).

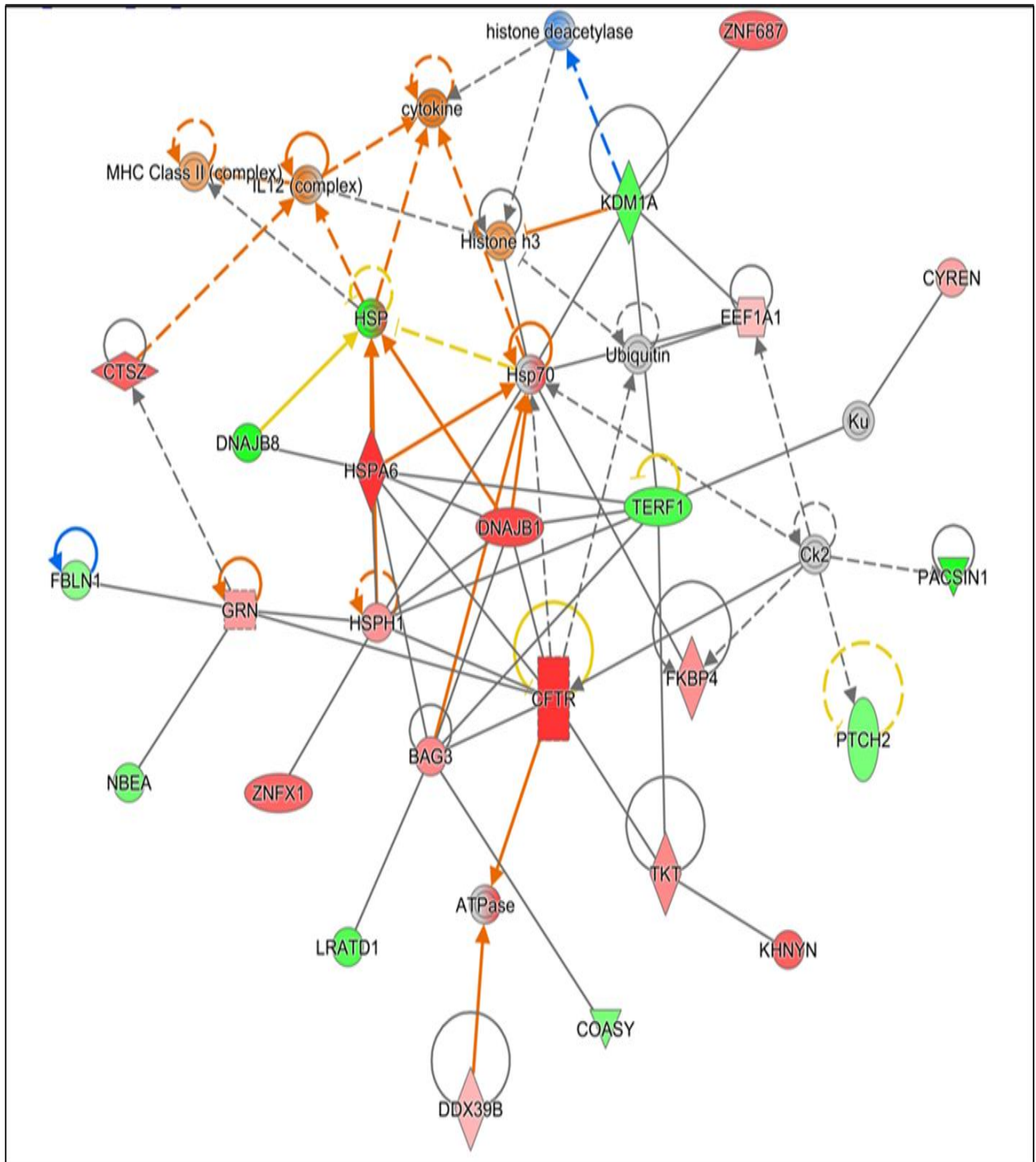


**Figure 6:** Network 1, Cell Cycle (P vs. C). The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes; with red representing up-regulation and green, down-regulation in Pregnant versus Cyclic endometrium.

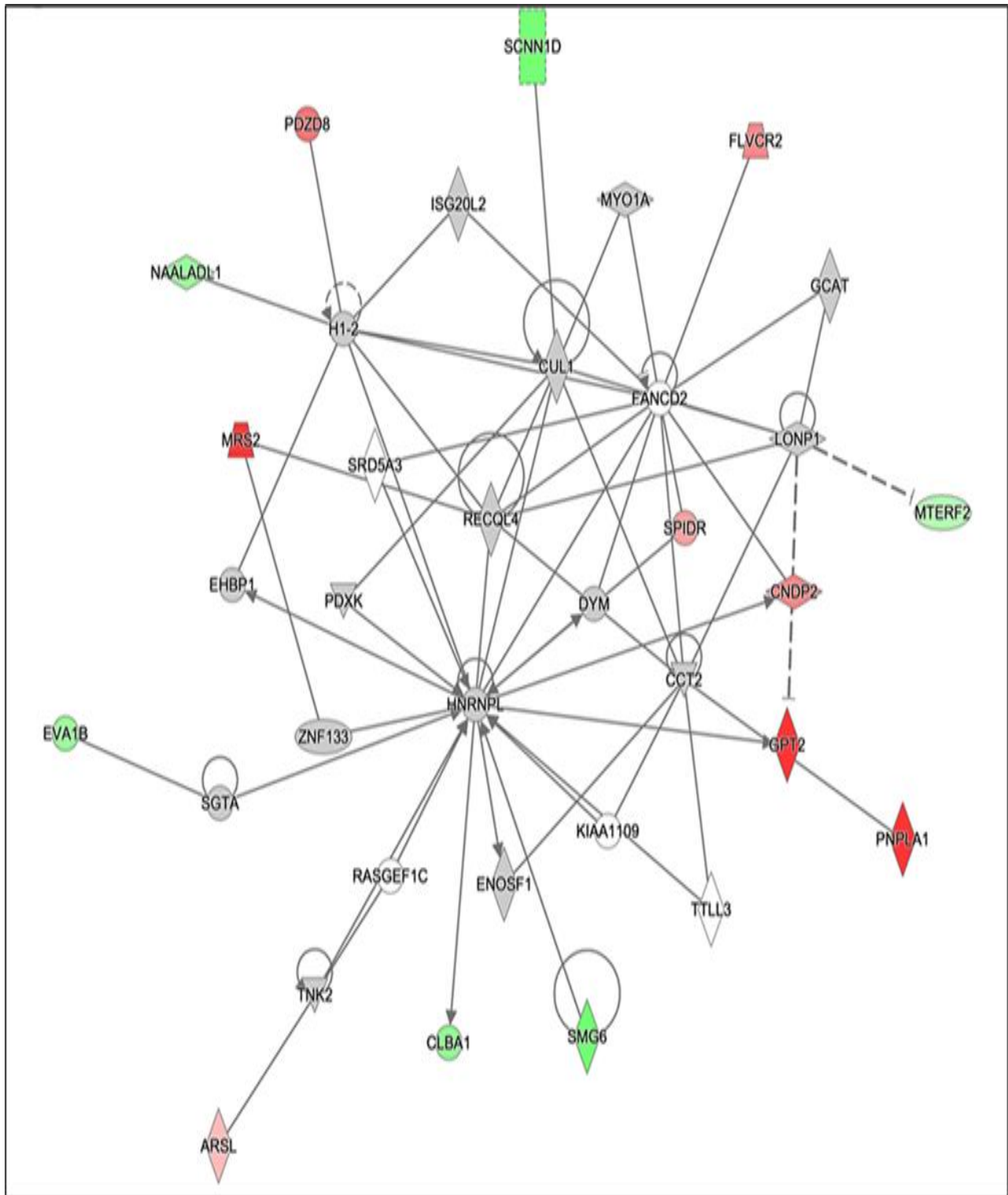


**Figure 7:** Network 2, Cell Morphology (P vs. C). The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes; with red representing up-regulation and green, down-regulation in Pregnant versus Cyclic endometrium.

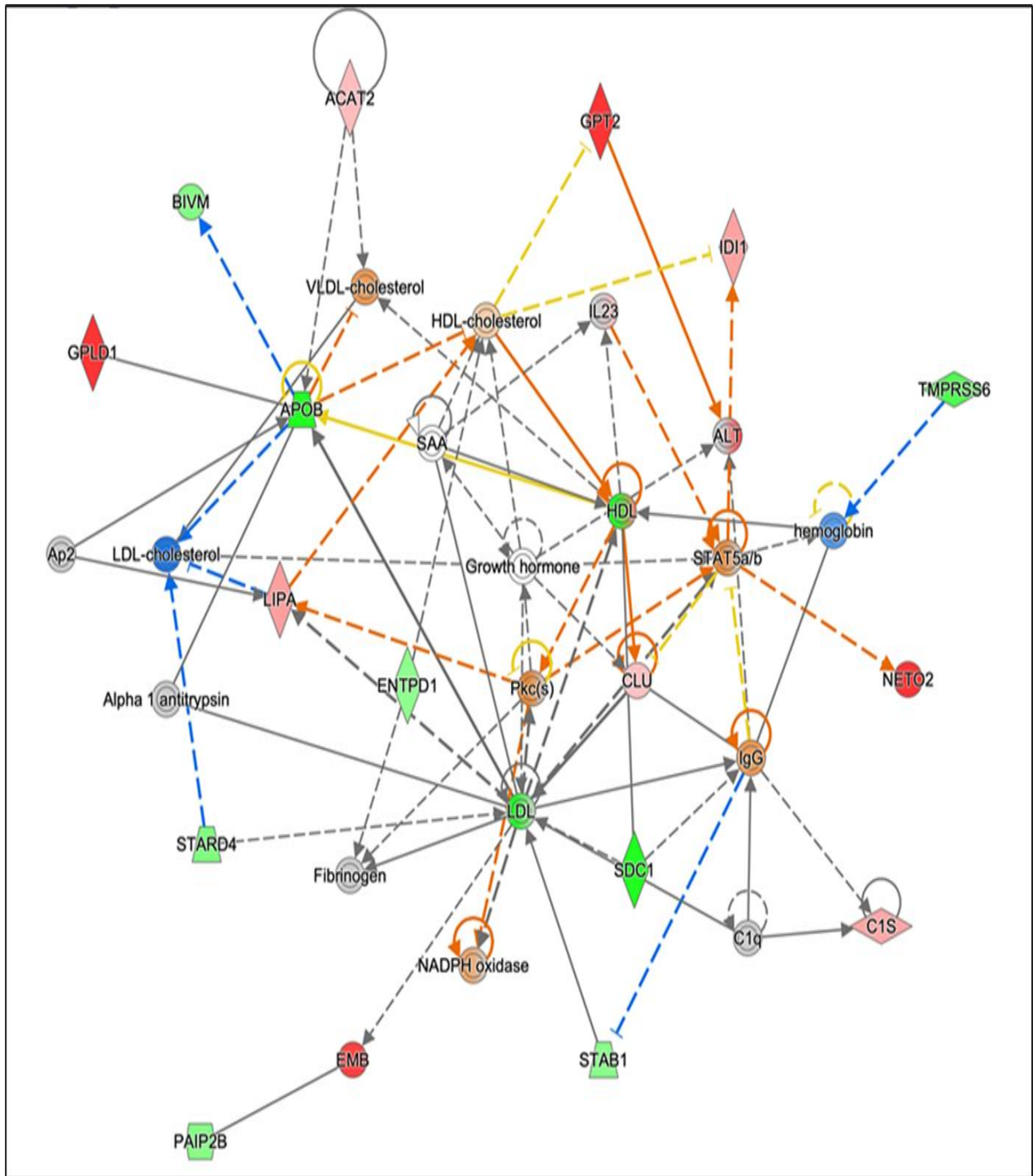




**Figure 9:** Network 4, Cell Morphology (P vs. NP). The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes; with red representing up-regulation and green, down-regulation in pregnant versus cyclic endometrium.



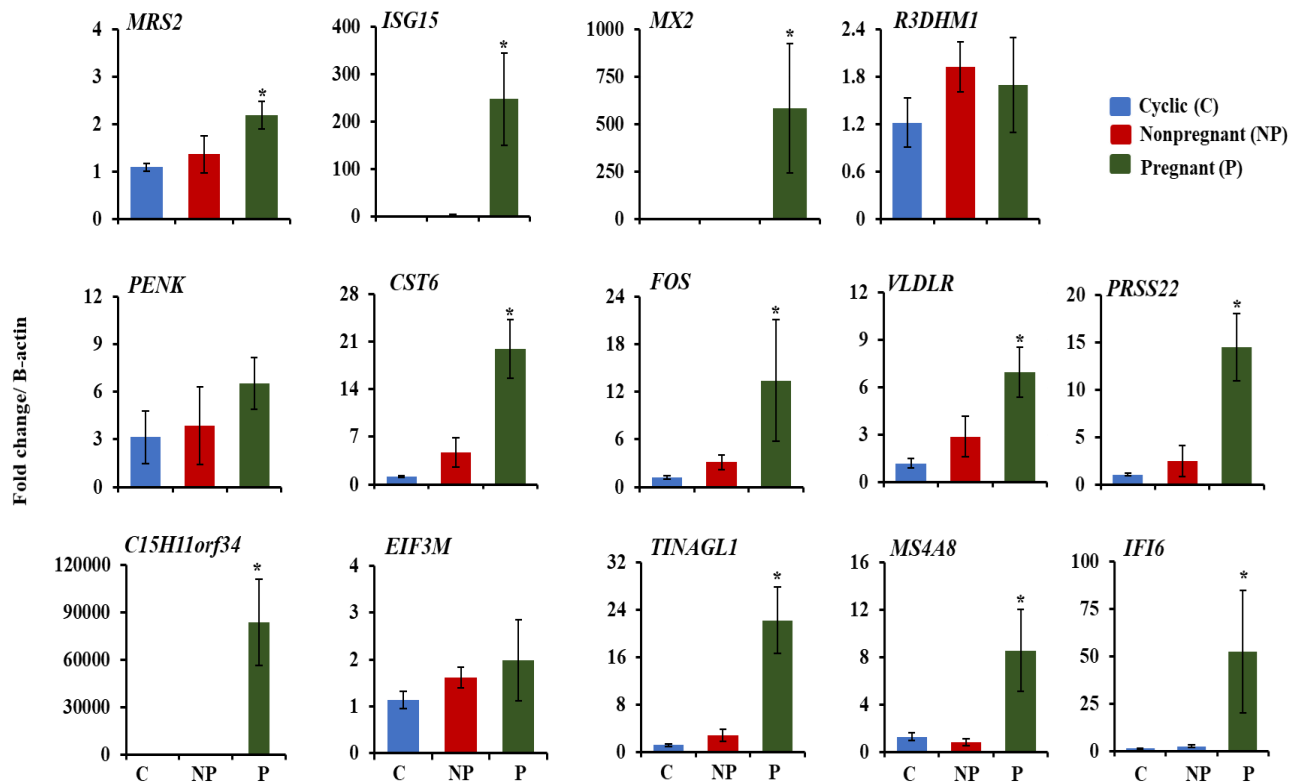
**Figure 10:** Network 5, Cell Cycle (P vs. NP). The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes; with red representing up-regulation and green, down-regulation in pregnant versus cyclic endometrium.



**Figure 11:** Network 6, Lipid Metabolism (P vs. NP). The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes; with red representing up-regulation and green, down-regulation in pregnant versus cyclic endometrium.

### 3.1.5 Gene expression profiles using qPCR in the pregnant bovine endometrium

The RNA-Seq data identified the differentially expressed genes in the pregnant bovine endometrium. Among the most highly up-regulated genes, fourteen candidate genes (*MRS2*, *CST6*, *FOS*, *VLDLR*, *ISG15*, *IFI6*, *MX2*, *C15H11ORF34*, *EIF3M*, *PENK*, *PRSS22*, *MS4A8*, *TINAGL1*, and *R3HDM1*) were selected for validation using qPCR. The results of relative fold change for candidate genes obtained from qPCR are shown in figure 12. *MRS2*, *MS4A8*, *PRSS22*, *CST6*, *VLDLR*, *IFI6*, *C15H11ORF34*, *ISG15*, *TINAGL1*, and *MX2* were significantly higher ( $p < 0.05$ ) in the pregnant endometrium compared to NP and C, whereas *R3DHM1*, *EIF3M*, *FOS*, and *PENK* remained unchanged.



**Figure 12:** Validation of the gene expression in the endometrium. The fold changes were normalized with the  $\beta$ -actin. Data represented as the mean  $\pm$  standard error. The x-axis represents the different physiological status of the cows (C; cyclic, NP; nonpregnant, and P; pregnant cattle). Y-axis represents relative fold change for gene expression. \* denote significance at  $P$ -value  $< 0.05$ ).

## CHAPTER 4: DISCUSSION

Reproductive efficiency is crucial to the production of animals and the overall profitability of the farm. Early pregnancy failure is one of the causes that affect the long-term management conditions and economic output of the farm. Early embryonic loss is one of the critical fertility issues in the beef industry (Davoodi, 2015).

In ruminant, the successful establishment of pregnancy requires the intricate dialogue between the uterus and growing conceptus. During the maternal recognition of pregnancy, a conceptus-derived signal (INF tau) leads to the persistence of the corpus luteum and induces the endometrial transcript to establish gestation (Mansouri-Atiia et al., 2009). Endometrium undergoes dynamic changes during the estrous cycle, peri-implantation period, and different stages of gestation to support conceptus growth and development (Mishra et al., 2010; Mishra et al., 2012). Previous studies have suggested that ovarian hormones (estradiol and progesterone) regulate the endometrial remodeling and induces the transcriptomes during the estrous cycle (Bauersachs et al., 2005) and peri-implantation periods (Klein et al., 2006; Mansouri-Attia et al., 2009; Walker Caroline et al., 2010). Bovine embryos of different developmental stages induce endometrial gene expression (Bauersachs et al., 2009). Recent studies have suggested that bovine embryos around the peri-implantation period induce endometrial gene expression in the intercaruncular region for the maternal recognition and establishment of gestation (Davoodi, 2015; Dunne et al., 2000; Mansouri-Attia et al., 2009). Despite these studies, the changes in the caruncular endometrial transcriptome during the maternal recognition of pregnancy (Day16 of gestation) are not completely understood.

The caruncular endometrium is the site for embryo implantation in cattle and undergoes morphological and biochemical changes in the presence of an embryo. This study investigated the

bovine caruncular endometrium during the maternal recognition of pregnancy (Day 15-17) using RNA-Sequencing and further validated using qPCR. The endometrial gene expression during the maternal recognition of pregnancy was compared with non-pregnant and cyclic cows. A total of 107 genes (pregnant vs. cyclic) and 98 genes (pregnant vs. Nonpregnant) were differentially expressed in the pregnant endometrium. Differentially expressed genes in the pregnant endometrium (P vs. C) and (P vs. NP) were 50 and 47, respectively. The most highly up-regulated genes in the pregnant endometrium (vs. cyclic) were *MRS2*, *CST6*, *FOS*, *VLDLR*, *ISG15*, *IFI6*, *MX2*, *C15H11ORF34*, and *EIF3M*. The most highly up-regulated genes in the pregnant endometrium (vs. nonpregnant) were *ISG15*, *IFI6*, *PENK*, *PRSS22*, *MS4A8*, *CLDN4*, *C15H11ORF34*, *MRS2*, *TINAGL1*, and *R3HDM1*. In our study, *MRS2* has a highly expressed gene with log<sub>2</sub> fold change 7.72 in the pregnant endometrium (vs. cyclic), whereas *LRFN4* was highly down-regulated with log<sub>2</sub> fold change -3.276. Similarly, *ISG15* was highly expressed with log<sub>2</sub>foldchange 6.520 in the pregnant endometrium (vs. nonpregnant). On the other hand, *SNX20* was highly down-regulated in the pregnant endometrium (vs. nonpregnant) with log<sub>2</sub>foldchange -5.182 in (P vs. NP). In the nonpregnant endometrium (vs. cyclic), *NBEA* was highly up-regulated with log<sub>2</sub> fold change 3.871, whereas *BPIFB1* was highly down-regulated with log<sub>2</sub>foldchange -6.550. Most interestingly, this study identified some novel genes (*MRS2*, *C15H11ORF34* and *PRSS22*) along with pre-identified genes in the caruncular endometrium around the maternal recognition of pregnancy.

## 4.1 Pathways of candidate genes in pregnant endometrium

### 4.1.1 Interferon Signaling genes

The Interferon Signaling pathway is important in the pregnant endometrium around the peri-implantation period (Forde et al., 2013; Mansouri-Atiia et al., 2009). In this study, interferon signaling was highly enriched in the pregnant endometrium. Under interferon signaling, we identified several genes such as *MX2*, *IFI6*, *IFIT1*, *IRF1*, *IRF9*, *ISG15*, *MX1*, *STAT1*, and *TAP1*. Further, we validated *ISG15*, *MX2*, and *IFI6* using qPCR.

### 4.1.2 Collagen genes

Collagen, the most abundant extracellular protein in mammals, is the main structural protein in the extracellular matrix in the various connective tissues (Di Lullo et al., 2002). In this study, GO analysis detected several collagen genes (*COL1A1*, *COL1A2*, and *COL3A1*) involved in the extracellular matrix organization in the pregnant bovine endometrium. We also found that endometrial collagen genes have a predicted role in platelet-derived growth factor (s). The KEGG pathway revealed many collagen genes in the pregnant endometrium having different functions. For example, in protein digestion and absorption, genes such as *COL1A1*, *COL3A1*, *COL1A2*, and *COL6A3* were detected in the pregnant endometrium. Similarly, *COL1A1*, *COL1A2*, and *COL6A3* were involved in ECM-receptor interaction. Moreover, *COL1A1*, *COL3A1*, and *COL1A2* are involved in platelet activation. Collagen genes have an essential role in cell adhesion. Some of the important collagen genes that help in adhesion are *COL1A1*, *COL1A2*, and *COL6A3*. According to our findings from Ingenuity Canonical Pathways analysis, *COL12A1*, *COL1A1*, *COL1A2*, *COL3A1*, *COL4A2*, *COL5A1*, *COL6A1*, and *COL6A3* are involved in Glycoprotein 6 (GP 6) Signaling Pathway.

### **4.1.3 Solute carrier genes**

Solute carrier (SLC) genes, consisting of 52 families, are mostly located in the cell membrane and code for membrane transport proteins (Cedernaes et al., 2011). The function of the SLC gene includes the transport of glucose, electrolytes, and amino acids. Since numerous nutrients and electrolytes need to be transported from the blood to the uterine environment, SLC genes play a critical role in the bovine endometrium. *SLC2A1* was among the top 20 most up-regulated SLC genes in the pregnant endometrium. Conversely, *SLC16A11* (Monocarboxylate transporter), *SLC16A4* (Monocarboxylate transporter), and *SLC7A4* (Cationic amino acid transporter/glycoprotein-associated amino acid) were found to be down-regulated in the pregnant endometrium. *SLC46A1* was up-regulated in the non-pregnant endometrium. Gene ontology analysis showed *SLC34A2* (Type-II Na<sup>+</sup>/HPO<sub>4</sub><sup>2-</sup> co-transporter), *SLC2A1* (glucose transporter), *SLC16A11* (Monocarboxylate transporter), and *SLC16A4* (Monocarboxylate transporter) (He et al., 2009) involved in ion transportation. *SLC34A2* was included in parathyroid hormone synthesis, secretion, and action. This gene was also found in the mineral absorption pathway. The *SLC46A1* (Folic acid transporter) was also in the pregnant bovine endometrium. These results suggest that transporter molecules transport nutrients from blood circulation to the endometrial cells for their growth and development and then transported to the uterine lumen to nourish the embryo.

### **4.1.4 Extracellular matrix remodeling and matrix metalloproteinases family**

Matrix metalloproteinases (MMP) are known to degrade the ECM for cellular proliferation, differentiation, migration, and apoptosis (Mishra et al., 2010). A high amount of extracellular matrix remodeling (ECM) and cellular remodeling occur in the endometrium during the estrous cycle, peri-implantation period, and different gestation stages (Mishra et al., 2012). MMP activities are induced by extracellular matrix metalloproteinase inducers (EMMPRIN) and inhibited by

tissue inhibitors (*TIMPs*) (Mishra et al., 2012). In our study, Ingenuity canonical pathway showed inhibition of Matrix Metalloproteases that included *MMPs* such as *MMP-14* and *MMP-2*. It is well-known that *MMP-14* regulates the *MMP-2* through binding *TIMP-2*. This *MMPs* cascade regulated the endometrial cell functions required for embryo implantation (Mishra et al., 2010; Mishra et al., 2012). A study on mice shows scarcity in *MMP-14* leads to premature aging, short lifespan, and cell senescence, suggesting an essential role of *MMP-14* in extracellular matrix remodeling during aging. *MMP-2* is found to be involved in tumor invasion (Löffek et al., 2011).

## **4.2 Most highly up-regulated genes in pregnant endometrium**

RNA-Seq data revealed several genes differentially up-regulated in the bovine pregnant endometrium during the maternal recognition of pregnancy compared to non-pregnant and cyclic endometrium. We further selected the top 14 highly up-regulated genes and were validated using qPCR. Among them, 5 were common between the group (P vs. C) and (P vs. NP).

### **4.2.1 Magnesium Transporter *MRS2* (*MRS2*)**

Magnesium Transporter *MRS2* (*MRS2*) is one of the highly up-regulated genes in the pregnant endometrium. *MRS2* is a protein-coding gene and located in mitochondria (Li et al., 2020). Our data revealed that *MRS2* is a highly up-regulated gene in pregnant endometrium than cyclic and non-pregnant. The pathway analysis from our result showed that *MRS2* plays a significant role in the cell cycle, cellular assembly, and organization while having a significant role in DNA replication, recombination, and repair. According to GO analysis, *MRS2* is associated with magnesium ion transportation. Magnesium transporter mediates the influx of magnesium into the mitochondrial matrix required to express the mitochondrial respiratory complex I subunits (Stelzer et al., 2016). *MRS2* gene expression has been reported in the endometrium of pregnant

goats at the morula stage (Li et al., 2020). For the first time, this study reported *MRS2* in the bovine endometrium during the maternal recognition of pregnancy. However, the spatiotemporal expression of *MRS2* is completely unknown in the bovine endometrium.

#### **4.2.2 Cystatin 6 or Cystatin E/M (*CST6*)**

Cystatin 6 or Cystatin E/M (*CST6*) is among the highly up-regulated genes in the pregnant endometrium. This gene is located on chromosome 29 in cattle, and the homolog in humans is located in chromosome 11 (Keppler, 2011). Cystatin superfamily consists of three important families: Type 1 cystatins (stefins), Type 2 cystatins, and the kininogens. Mutations in this gene resulted in connective tissue disorders, developmental disorders, and neurological diseases. GO annotations related to this gene include cysteine-type endopeptidase inhibitor activity.

*CST6* is an inhibitor of cathepsins (CTSs), including CTSB, CTSL, and legumain (LGMN) (Patrick et al., 2009). *CST6* are lysosomal cysteine proteases that act on the degradation of extracellular matrix molecules and activation of intracellular pre-proteins (Claus et al., 1998). CSTs were expressed in the endometrium of rodents, humans, ruminants, and pigs (Salamonsen, 1999; Song et al., 2010; Spencer et al., 2007). The function of the *CST6* includes uterine endometrial and placental tissue remodeling and facilitates transplacental transport of nutrients. *CST6* expression was detected in the endometrium during the estrous cycle and in pregnancy. The expression of *CST6* in chorionic epithelia of the placental membrane was at increasing levels during late pregnancy (Salamonsen, 1999), suggesting that cell type-specific expression and function of *CST6* is critical for appropriate maternal-fetal interactions.

#### **4.2.3 FOS Proto-Oncogene, AP-1 Transcription Factor Subunit (*FOS*)**

*FOS* is among the highly up-regulated gene in pregnant bovine endometrium. Ingenuity canonical pathway analysis from this study revealed that *FOS* impacts IL-12 signaling and production in Macrophages and has an important immune function (e.g., anti-cancer factors), and regulates innate immunity. It acts on the cAMP signaling pathway. Furthermore, the KEGG pathway shows that *FOS* takes part in endocrine activities such as Parathyroid hormone synthesis, secretion, and action, and it has a significant role in Th17 cell differentiation. GO annotations related to this gene include DNA-binding transcription factor activity and transcription factor binding. The altered expression and distribution of *FOS* protein prompted endometriosis in the baboon (Hastings et al., 2006).

#### **4.2.4 Very Low-Density Lipoprotein Receptor (*VLDLR*)**

Very Low-Density Lipoprotein Receptor (*VLDLR*) is highly up-regulated in the pregnant endometrium. *VLDLR* belongs to the low-density-lipoprotein (LDL) transmembrane receptor family that localizes to the plasma membrane and is located at chromosome 9 (Go & Mani, 2012). *VLDLR* consists of cell surface proteins involved in receptor-mediated endocytosis of specific ligands. This gene encodes a lipoprotein receptor that is a member of the LDLR family and plays a vital role in VLDL-triglyceride metabolism. It shows an essential function in the synaptogenesis signaling pathway. *VLDLR* is considered as a potential mediator of P4-dependent signaling through membrane progesterone receptors (mPR) to drive oocyte maturation and meiosis progression. It was found that the knocking down of *VLDLR* inhibited the oocyte maturation and meiosis progression. In contrast, overexpression of the *VLDLR* showed the exact opposite action of what it did when it was knocked down, confirming its importance on P4-dependent oocyte maturation (Nader et al., 2018). *VLDLR* is known to permit cholesterol to reach tissues from the

bloodstream, and it may be used as energy sources. During pregnancy, endometrial cells secrete large amounts of cytokines, growth factors, and other molecules for embryonic growth and development. Therefore, *VLDLR* might play an important role in endometrial cells' function for the establishment of pregnancy.

#### **4.2.5 ISG15 Ubiquitin Like Modifier (*ISG15*)**

*ISG15* is among the highly up-regulated gene in the pregnant endometrium. Gene Ontology and Ingenuity pathway analysis from our study revealed that *ISG15* functions in the Interferon Signaling Activation pathway in the pregnant endometrium, which is very important for the maternal recognition of the pregnancy. Conversely, this gene was not found in both the control groups (non-pregnant and cyclic cattle), stating *ISG15* is only found during the peri-implantation period. It was found in organelles and compartments of endometrial epithelial cells and stromal cells: nucleus, perinuclear space, cytosol, mitochondria, rough endoplasmic reticulum, and cell membrane in bovine endometrium (Austin et al., 2004). *ISG15* was expressed in the ruminant uteri in response to interferon (IFN)- $\tau$ , and its expression was detected in the stromal cells (SC) and glandular epithelial cells (GE) (Forde et al., 2011, Bazer et al., 2012). It takes part in the interferon signaling pathway. GO annotations related to this gene include protein tag. *ISG15* exhibits antiviral activity towards DNA and RNA viruses, including influenza A, HIV-1, and Ebola. The secreted form of *ISG15* can induce natural killer cell proliferation, acting as a chemotactic factor for neutrophils. IFNT from the elongating conceptus up-regulates *ISG15* (Mansouri-Atiia et al., 2009; Forde et al., 2011). A study showed that *ISG15* is found in the uterotubal junction in the uterine horn ipsilateral to CL (Sponchiado et al., 2017).

In bovine, after endometrial *ISG15* is detected on day 16 of pregnancy, their levels get altered. For example, *ISG15* level peaks between days 18-23, while their level declined by day 45 (Austin et

al., 2004). *ISG15* is generated from a precursor by a cleavage which is common among ubiquitin-like proteins (Potter et al., 1999). Previous studies have reported its secretion from human monocytes and lymphocytes, emphasizing its properties as an interferon-induced cytokine (D’Cunha et al., 1996). Bovine *ISG15* conjugates to various uterine cytosolic proteins during early pregnancy (Johnson et al., 1998). *ISG15* can alter the function of different proteins involved in many activities such as transcription, DNA repair, signal transduction, apoptosis, and cell-cycle by conjugating itself to those target proteins (Kerscher et al., 2006). *ISG15* can regulate the innate immunity of embryonic cells.

#### **4.2.6 Interferon Alpha Inducible Protein 6 (*IFI6*)**

*IFI6* was highly up-regulated in the pregnant endometrium and is part of the interferon signaling pathway. *IFI6* is not mapped yet in cattle, but the human *IFI6* gene is located on HSAP1p35, which is homologous to a chromosomal region between cattle and humans (Stelzer et al., 2016). This gene was first identified as one of the many genes induced by interferon (Stelzer et al., 2016). They are mostly expressed in the placenta, salivary glands, and lungs. One of the functions of *IFI6* is to bind double-stranded DNA; it is also involved in the innate immune response by recognizing viral dsDNA in the cytosol and probably in the nucleus. Additionally, it has anti-inflammatory activity and inhibits the replication of herpesviruses. The timing of the up-regulation of ISGs, such as *IFI6* in pregnant heifers, was observed in previous studies (Forde et al., 2011). It was localized in the myometrial side in the uterine horn ipsilateral to the CL (Sponchiado et al., 2017). Our study, as well as previously reported, suggests that *IFI6* signaling is required to maintain the endometrium's immune status required for embryonic survival and maternal recognition of pregnancy.

#### **4.2.7 MX Dynamin Like GTPase 2 (*MX2*)**

In the present study, *MX2* was among the highly up-regulated genes. *MX2*, which is recognized as intracellular antiviral proteins, belongs to a large GTPase family. They are mostly associated with the functions that defend against viruses and increase immune response (Haller & Kochs, 2002; Horisberger et al., 1983; Racicot et al., 2008). The protein encoded by this gene has a nuclear and a cytoplasmic form and is a member of both the dynamin family and large GTPases (Racicot et al., 2008). It takes part in the interferon signaling pathway and innate immune system. In our study, GO annotations related to this gene include GTP binding and GTPase activity. *MX2* is up-regulated in response to IFNT from the elongating conceptus (Mansouri-Attia et al., 2009). *MX2* expression in response to elongating conceptus is consistent across many different mammal species, including cattle, sheep, and humans. For example, the *MX2* expression was increased in the pregnant endometrium of ewes (Ott et al., 1998) and cows (Hicks et al., 2003) in response to IFNT. It is reported that *MX2* mRNA is detectable in pregnant animals' peripheral blood lymphocytes and was also more expressed by day 16 in pregnancy than non-pregnant cows (Ott et al., 1998). Our study and the previous report suggested that *MX2* is an important antiviral gene in the uterus during the preimplantation period.

#### **4.2.8 Placenta Expressed Transcript 1 (*C15H11ORF34*)**

*C15H11ORF34*, also known as Placenta Expressed Transcript 1 (PLET1), is highly up-regulated in the pregnant endometrium. RNA-Seq data identified unannotated genes such as *C15H11ORF34* up-regulated in embryos derived from T-cells cells (Srirattana & St John, 2017). This gene was among the top 10 up-regulated genes in our study, and the qPCR validation result showed that it was the most highly expressed gene in the pregnant bovine endometrium. The spatiotemporal expression and function of this gene are entirely unknown.

#### **4.2.9 Eukaryotic Translation Initiation Factor 3 Subunit M (*EIF3M*)**

Eukaryotic Translation Initiation Factor 3 Subunit M (*EIF3M*) is among the up-regulated genes in the pregnant endometrium. *EIF3M* is found in the cytosol (Stelzer et al., 2016). *EIF3M* functions in lipid metabolism, molecular transport, protein synthesis. Furthermore, it is also involved in cell cycling, cell morphology, and apoptosis. IPA analysis from the study shows it is associated with the regulation of eIF4 (Eukaryotic Initiation Factor-4) signaling.

#### **4.2.10 Tubulointerstitial Nephritis Antigen Like 1 (*TINAGLI*)**

*TINAGLI* is among the highly up-regulated gene in the pregnant endometrium. *TINAGLI* is important in antimicrobial response, cell signaling, and inflammatory response. The previous study has shown an increased expression of *TINAGLI* on days 13 in pregnant heifers (Forde et al., 2013), and quantitative real-time PCR analysis revealed the expression of *TINAGLI* in the endometrium ( $P < 0.05$ ) as the early pregnancy progressed (Forde et al., 2013).

#### **4.2.11 Proenkephalin (*PENK*)**

Proenkephalin (*PENK*) is a highly up-regulated gene in the pregnant endometrium. *PENK* is a member of the opioid polypeptide hormone found in various mammals, rodents, and avian species. It is known to play a role in many physiologic functions, including pain perception and stress responses. The previous study showed an increased *PENK* expression on day 13 in heifers' endometrium (Forde et al., 2013). Besides its dominance in the CNS, it is also expressed in the oviducts in chicken (Jeong et al., 2012) and is associated with eggshell calcification (Jeong et al., 2012; Brionne et al., 2014). However, its mechanistic role in the bovine uterus has not been established.

*PENK* was detected in the myometrial region of the pregnant mouse's uterus until day 18 of pregnancy and helped in maternal adaptation to pregnancy and in supporting the embryo growth. *PENK* detected in the uterus were suggested to have multiple material adaptation roles to pregnancy and support embryo growth (Zhu & Pintar, 1998).

#### **4.2.12 PRSS22-Serine Protease 22 (*PRSS22*)**

*PRSS22* is a highly up-regulated gene in the pregnant endometrium. *PRSS22* is located on chromosome 16, and has a predicted function related to serine-type endopeptidase activity. *PRSS22* has been reported in the endometrial region of the mouse and human (Pelch et al., 2010) but has not been reported in the cattle.

#### **4.2.13 Membrane Spanning 4-Domains A8 (*MS4A8*)**

*MS4A8* is highly up-regulated in the pregnant bovine endometrium. It is assumed that *MS4A8* might have an important role in signal transduction and immune response activity. It has membranous and cytoplasmic gene expression, especially in fallopian tubes and respiratory epithelium in humans (Stelzer et al., 2016).

#### **4.2.14 R3H Domain Containing 1 (*R3HDM1*)**

*R3HDM1* is among the up-regulated gene in the pregnant endometrium. According to Entrez Gene, *R3HDM1* maps on chromosome 2, at 2q21.3. GO annotations related to this gene include nucleic acid-binding.

### **4.3 Pathway analysis**

Gene ontology analysis revealed that the biological process related to Type-1 interferon signaling (*MX1*, *MX2*, *IF16*, *IRF1*, and *ISG15*), immune response (*IL23A*, and *RSAD2*), and

extracellular matrix organization (*COL1A1*, *COL1A2*, *COL3A1*, and *TIMP2*) were significantly enriched in the pregnant endometrium. Ion transporters (*SLC34A2*, *SLC2A1*, *SLC16A11*, *SLC16A4* and *ATP1B1*), platelets derived factors, telomerase activity (*HMBOX1*, and *TERF1*), and ATPase activities (*P2RX6*, and *DNAJB1*) were significantly enriched as molecular functions, and endoplasmic reticulum lumen (*WNT5B*, *IL23A1*, *PENK*, *TNC*, *SPARCL1*, and *B2M*) was the most significantly enriched cellular component. In the nonpregnant endometrium, negative regulators of hydrolase, dephosphorylation, phosphatase, death-inducing signaling complex, and apoptosis were significantly enriched. Ingenuity canonical pathways analysis revealed that the interferon signaling, hepatic stellate cell activation, oxidative phosphorylation, GP6 signaling, coenzyme A Biosynthesis, and sirtuin signaling made up the highly enriched pathways. The biological pathway analysis shows that an embryo's presence induced the endometrial transcripts related to endometrial remodeling, immune response, nutrients, ion transporters, and interferon signaling pathways. Further, in the absence of the embryo, these transcripts are down-regulated in the endometrium.

Although cyclic and non-pregnant cows lacked conceptus, interestingly, in our study, we found some molecules such as *COL1A1*, *TRADD*, and *COL3A1* were up-regulated in both non-pregnant and pregnant endometrium. One reason could be those collagen genes (*COL1A1* and *COL3A1*) are abundantly present in platelet-derived growth factor binding. In contrast, *TRADD* is essential for a death-inducing signaling pathway important for pregnant and non-pregnant cattle.

Our study showed a significant difference in gene expression between pregnant versus cyclic and non-pregnant cows. The RNA-Seq results of the top up-regulated genes were further validated by the qPCR analysis and confirmed a variation in the gene expression among the cows' different reproductive stages. Hence, we found some distinct contrast among pregnant vs. non-pregnant vs.

cyclic cows while identifying the differentially expressed genes and biological pathways. Further studies are warranted to prove this hypothesis.

#### **4.4 Conclusions and future direction**

Both the RNA-Seq and qPCR analysis confirmed the differential expression of several pre-discovered and novel genes and their biological pathways in the pregnant endometrium compared to cyclic and non-pregnant endometrium. Interferon signaling, immune response, nutrient transporter, synthesis, and secretion of proteins are crucial pathways during the maternal recognition of pregnancy. This study found some important molecules such as *MX1*, *MX2*, *IF16*, *IRF1*, and *ISG15* related to Type-1 interferon signaling, ion transportation (*SLC34A2*, *SLC2A1*, *SLC16A11*, and *SLC16A4*), and ECM organization (*COL1A1*, *COL1A2*, and *COL3A1*). Overall, this study identified the differentially expressed genes and pathways in the pregnant caruncular endometrium compared to cyclic and non-pregnant. In this study, we found some novel genes (*MRS2*, *C15H11ORF34*). The study demonstrated that conceptus at day 15-17 of gestation could actively affect the endometrial gene expression during the preimplantation phase. In summary, this study provides a comprehensive dataset of transcripts associated with maternal recognition of pregnancy.

This research could be used for future perspectives as a reference for different studies on individual gene or groups of genes, serum hormonal profile, and metabolomics. Further studies are required to analyze the spatiotemporal expression and potential functions of novel genes such as *MRS2*, *C15H11ORF34*, and *PRSS22* in the bovine endometrium during early pregnancy.

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