CECÍLIA CONSTANTINO ROCHA

Studies of early pregnancy-induced transcripts in peripheral blood immune cells in cattle

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Dissertation submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Sciences of the University of São Paulo to obtain the Master's degree in Sciences.

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Advisor:

Prof. Guilherme Pugliesi, Ph.D.

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CERTIFICADO

Certificamos que a proposta intitulada "Estudo do perfil transcriptômico em células imunes polimorfonucleares do sangue periférico no início da gestação em novilhas de corte", protocolada sob o CEUA nº 6810070817 (ID 004305), sob a responsabilidade de **Guilherme Pugliesi** *e equipe; Cecília Constantino Rocha* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 06/12/2017.

We certify that the proposal "Study of the transcriptomic profile in peripheral blood polymorphonuclear immune cells at the beginning of gestation in cutting heifers", utilizing 30 Bovines (30 females), protocol number CEUA 6810070817 (ID 004305), under the responsibility of **Guilherme Pugliesi** *and team; Cecília Constantino Rocha* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 12/06/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 09/2017 a 12/2017 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	24 a 30 meses	N:	30
Linhagem:	Nelore			Peso:	250 a 350 kg		

Local do experimento: O experimento será realizado na Universidade de São Paulo, campus de Pirassununga no Departamento de Reprodução Animal

São Paulo, 08 de novembro de 2019

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
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EVALUATION FORM

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Date: _____/_____/_____

Committee Members

Prof._____

Institution:_____ Decision:_____

Prof._____

Institution:_____ Decision:_____

Prof._____

Institution :_____ Decision:_____

DEDICATION

I dedicate this dissertation to my mom, Denilce Constantino. The one who taught the value of knowledge and character. The one who never let me alone and during the hard times always hold me with the best hug of the world.

All my love to you.

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RESUMO

ROCHA, C.R. **Estudo de transcritos induzidos pela gestação inicial em células imunes do sangue periférico de fêmeas bovinas.** [Studies of early pregnancy-induced transcripts in peripheral blood immune cells in cattle]. 2019. 118 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

Em bovinos grande parte das perdas embrionárias ocorrem até as três primeiras semanas de gestação. Estuda-las e detecta-las o mais precocemente, são pontos importantes para melhorar a eficiência reprodutiva. O fato do concepto possuir 50% do material genético "estranho" ao sistema imune materno, evidencia a importância de elucidar os acontecimentos imunes nesse período inicial. Além disso, nesse momento, a principal glicoproteína sinalizadora do concepto (IFN-τ) é liberada na circulação e estimula a expressão de genes (ISGs). O estudo da expressão gênica dos mais comuns ISGs (*ISG15*, *OAS1*, *MX1* e *MX2*) em células imunes do sangue periférico já foi utilizado como forma de diagnóstico precoce de gestação, entretanto a acurácia foi baixa. Considerando-se a deficiência de pesquisas sobre a imunologia de gestação, testou-se as hipóteses de que (1) no dia 18 de gestação novos genes além dos tradicionais ISGs seriam diferencialmente expressos (DE) em células imunes de animais gestantes e não gestantes, que permitiriam a seleção de potenciais marcadores da gestação antes do dia 20. E (2) que os genes DE descobertos, estariam relacionados com vias da resposta imunológica as quais seriam diferencialmente expressas entre categoria animal de paridade. Esta dissertação consistiu em 2 estudos. O primeiro estudo foi realizado para detecção de novos genes através da técnica de sequenciamento de RNA (RNAseq). Para isso foi utilizado células mononucleares do sangue periférico (PBMC) de novilhas Nelore, no dia 18 após a inseminação artificial (IA) em tempo fixo. O perfil de transcritos selecionados, foram estudados em PBMC e células polimorfonucleares do sangue periférico (PMN) entre os dias 10 e 20 após a IA por qPCR. Conclui-se que dos 20 genes avaliados, 9 demonstraram-se potenciais marcadores da gestação em PBMC e 5 em PMN. No segundo estudo os resultados de RNAseq foram submetidos a analises de enriquecimento (GO, KEGG e escore de regulação de vias) para a elucidação das principais vias no início da gestação. Os genes pertencentes a via selecionada foram validados no sangue total de vacas e novilhas Holandesas nos dias 16, 19 e 23 após inseminação. Outro experimento *in vitro* foi realizado, para testar se a via era estimulada por IFN-τ. Para isso cortes endometriais foram cultivados com meio de cultura, PBS e IFN-τ por 2 e 24 horas. Como resultados a via de receptores de reconhecimento de padrões (PRRs) foi a mais significativa e consequentemente avaliada. De 18 genes avaliados sete apresentaram interação entre status gestacional, categoria animal e tempo, os quais estão envolvidos com receptores Toll like e Nod

like. A interação aconteceu por aumento de expressão no dia 23 em novilhas gestantes. Entretanto os resultados do experimento in *vitro* apresentaram efeitos de cultivo para todos os grupos, impossibilitando definir se os genes estudados são estimulados por IFN-τ. Conclui-se que a gestação inicial exerceu influência no sistema imune de novilhas de corte e de leite, diferentemente de vacas. E que a diferença entre categorias acontece no período após a implantação (D23). Sendo assim, aceita-se a hipótese de que novos transcritos além dos tradicionais ISGs podem ser utilizados como marcadores de gestação e que a principal via em células imunes durante a gestação está relacionada a resposta imune, a qual é regulada em ambas subespécies e diferencialmente expressa entre categorias de paridade.

Palavras chave: concepto, interferon-tau, sistema imune, receptores toll like, diagnóstico de gestação.

ABSTRACT

ROCHA, C.R. **Studies of early pregnancy-induced transcripts in peripheral blood immune cells in cattle**. [Estudo de transcritos induzidos pela gestação inicial em células imunes do sangue periférico de fêmeas bovinas]. 2019. 118 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

In cattle, the largest part of embryonic loss occur during the first three weeks of pregnancy. Thus, to study and detect those loses as early as possible some points are key to improve reproductive efficiency. Half of the genetic material of the conceptus is allogenic to the maternal immune system. In this regard, the elucidation of the immune events is important during early pregnancy. Moreover, that moment is coincident with the release by the conceptus of the main glycoprotein signal, interferon-τ (IFN-τ). Interferon-τ is released in the circulation and stimulates some genes (ISGs). The study of the expression of the most common ISGs (*ISG15*, *OAS1*, *MX1* e *MX2*) in immune cells has already been used before as an early pregnancy diagnosis, however, the accuracy was low. Therefore, considering the deficiency of studies on the immunology of pregnancy we tested the follow hypotheses: (1) at day 18 of pregnancy, novel genes beyond the traditional ISGs would be differentially expressed (DE) in immune cells of pregnant and non-pregnant heifers, which would allow the selection of potential pregnancy markers before day 20. Also, (2) the DE genes discovered would be linked with pathways of the immune response, which would be differentially expressed between animal parity categories and regulated in subspecies (*taurus* and *indicus*). This dissertation consists of two studies. The first study was designed to detect novel genes by RNA sequence (RNAseq). Peripheral blood mononuclear cells (PBMC) RNA from day 18 after timed artificially inseminated (TAI) Nelore heifers were subject to RNAseq. The transcripts profile was evaluated in PBMC and peripheral blood polimorphonuclear cells (PMN) among days 10 to 20 by qPCR. In summary, 9 and 5 out of the 20 genes evaluated were considered potential pregnancy markers in PBMC and PMN respectively. In the second study, the RNAseq results were submitted to enrichment analysis (GO, KEGG, and pathway score regulation) to elucidate the main pathway regulated in early pregnancy. The genes found were evaluated in whole blood from Holstein cows and heifers on D16, 19 and 23 post AI. Another *in vitro* experiment was performed to test if the genes linked to the pathway were stimulated by IFN-τ. For this purpose, uterine explants were cultured with mammalian medium with no supplementation, with PBS, and with recombinant IFN-τ, for 2 and 24 hours. As a result, the most significant pathway was linked with the pattern recognition receptors (PRRs). Eighteen genes were evaluated and seven

of them showed an interaction between pregnancy status, animal category and time. Where for that seven genes on D23 the P-HF had a greater expression than NP-HF. All seven genes belong to the Toll like or Nod like receptors pathway. However, the *in vitro* results showed an effect of culture for all groups and we could not concluded if the regulation was mediated only by IFN-τ. In conclusion, early pregnancy induces differential changes on immune system of beef and dairy heifers, compared with cows. The difference became obvious specially during the post implantation period (D23). In summary, we accepted the hypothesis that novel transcripts beyond the traditional ISGs could potentially be used as pregnancy markers and that the main pathway during early pregnancy in immune cells is linked with the immune response, which is regulated between subspecies and differentially expressed between parity category.

Keywords: conceptus, interferon tau, immune system, toll like receptors, pregnancy diagnosis.

LIST OF ABBREVIATION

µL	Microliter
mL	Milliliter
mg	milligram
g	gram
18S	18 S Ribosomal RNA
A2M	Alpha-2-Macroglobulin
ACTB	Actin Beta
AI	Artificial insemination
AKR1B1	Aldo-keto reductase family 1 member B
ANG	Angiogenin
BPI	Bactericidal permeability increasing protein
C1R	Complement C1r
CARD9	Caspase recruitment domain family member 9
CASP1	Caspase 1
CD14	Monocyte differentiation antigen
cDNA	Complementary DNA
CL	Corpus Luteum
CLEC3B	C-type lectin domain family 3 member B
COX-2	Cyclooxygenase-2
Cq	Quantification cycle
DDX58	DExD/H-box helicase 58
DEGs	Differentially expressed genes
DMKN	Dermokine
DRAM1	DNA damage regulated autophagy modulator 1
ESR	Estradiol receptor
ET	Embryo transfer
FDR	False discovery rate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEO	Gene expression omnibus
GO	Gene ontologies
IFI44	Interferon alpha protein 4
IFI6	Interferon alpha protein 6
IFITM2	Interferon induced transmembrane protein 2

IFN	Interferon
IFNAR	Interferon Receptor
IFN-β	IFN-beta
IFN-γ	IFN-gamma
IFN-τ	Interferon-tau
IL1β	Interleukin 1 beta
IRAK	Phosphor-interleukin-1 receptor associated kinase
IRFs	Interferon regulatory factors
ISG15	Ubiquitin like modifier 15
ISGs	Interferon stimulated genes
JAK	Janus kinase
KEGG	Kyoto encyclopedia of genes and genomes
LGALS3BP	Galectin 3 Binding Protein
LIG1	DNA Ligase 1
LPS	Like bacterial lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MHC-I	Major histocompatibility complex class I
MIC1	Major histocompatibility class 1 related protein
MX1	MX dynamin like GTPase 1
MX2	MX dynamin like GTPase 2
MyD88	Myeloid Differentiation factor 88
NC1	Non-classical monomorphic major histocompatibility complex class I
NCBI	National center of biotechnology information
NF-κβ	Nuclear factor-κβ
NK	Natural Killer
NLRs	NOD-like receptors
NLRs	Nucleotide oligomerization domain-like receptors
NP	Non-pregnant
NP-C	Non-pregnant cows
NP-HF	Non-pregnant heifers
OAS1	2’-5’-Oligoadenylate synthetase 1
OAS2	2’-5’-Oligoadenylate sinthetase 2
OXTR	Oxytocin receptor

P	Pregnant
P4	Progesterone
PAGs	Pregnancy associated proteins
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
P-C	Pregnant cows
PGE_2	Prostaglandin E2
PGF2α	Prostaglandin F2alfa
PGFM	Prostaglandin F metabolite
PGs	Prostaglandins
P-HF	Pregnant heifers
PI3K-AKT1	Phosphatidylinositol 3-kinase thymoma viral proto-oncogene 1
PL	Placental lactogens
PLSCR2	Phospholipid Scramblase 2
PMN	Peripheral blood polymorphonuclear cells
PPIA	Ciclofilin
PRRs	Pattern recognition receptors
PRS	Pathway regulation score
PTGES	Prostaglandin E synthase
PTGFR	Prostaglandin F receptor
qPCR	Quantitative real-time polimerase chain reaction
RHOT1	Ras Homolog family member T1
RIG-1	Retinoic acid inducible gene 1
RLRs	Retinoic acid-inducible gene I-like receptors
RNAseq	RNA sequence
ROC	Receiver operator characteristic
RPL15	Ribosomal Protein L15
RPL19	Ribosomal Protein L19
RPL30	Ribosomal Protein L30
RSAD2	Radical S-Adenosyl methionine domain containing 2
SIGLEC1	Sialic acid binding Ig-like lectin 1
SORD	Sorbitol Dehidrogenase
STAT	Signal transducers and activators of transcription
TAI	Fixed time artificial insemination

Th	Lymphocytes T helper
TIR	Toll/interleukin 1 receptor
TLR4	Toll like receptor 4
TLRs	Toll-like receptors
TMM	Trimmed mean of M-values
TNFSF	Tumor necrosis factor superfamily
TNFSF13B	Tumor necrosis factor superfamily 13b
TNFα	Tumor necrosis factor alfa
tRNA	Total RNA

SUMMARY

1 INTRODUCTION

Brazil has the largest commercial herd in the world, reaching 214.7 million cattle in 2018(ABIEC, 2019). Consequently, the improvement of bovine production is important for the national economy as in 2018 this segment produced benefits for R$ 597.22 billion (ABIEC, 2019). In addition, the international economy is also influenced by the Brazilian beef herds, as Brazil is the leader in beef exportation in the world (ABIEC, 2019). Although the Brazilian beef industry is on the rise, the reproductive efficiency is still low and deserves attention. For example, the calving interval in Brazilian beef cattle operations is about 16.3 months, far from the optimal inter-calving period of 12-13 months (BARUSELLI *et al*., 2016).

In this regard, one of the main reasons for low reproductive efficiency is the long postpartum anestrous and the interval from calving to conception. Consequently, the TAI in beef herds has increased as this biotechinique has a great beneficial impact to reduce the anestrus period and to increase pregnancy rates (ABIEC, 2019). However, in TAI programs only 50% of the animals become pregnant after a single AI (SA FILHO *et al.*, 2014). Thus, to reach a greater proportion of animals pregnant with semen from proven bulls, a second TAI can be performed after detection of non-pregnant animals from the first TAI. Nowadays, the most common methodology used to detect the presence of a conceptus is by B-mode ultrasonography on day 28 post-TAI. Nevertheless, this technology does not allow the identification of non-pregnant animals at the time of the first estrus at about 21 days after first TAI (FORDE *et al.*, 2011). Therefore, the development of new technologies to detect non-pregnant animals earlier than day 28 is highly desired.

In addition, the largest rates of early embryonic loss (60%) occur before day 17 post-mating (DISKIN; SREENAN, 1980). Therefore, in TAI programs the best alternative would be to detect the embryo loss or the conception failure as soon as it happens. During this critical period the conceptus starts releasing great amounts of IFN-τ by trophoblast cells as a signal of recognition of pregnancy (SPENCER; HANSEN, 2015). IFN- τ increases on day 15, peaks on day 20 and decreases to basal concentrations near day 25 (BAZER *et al.*, 2009). The release of this protein is important to the maintenance of the CL. Interferon-τ acts in the endometrial cells down regulating ESR and OXTR (SPENCER *et al.*, 2007). As a consequence the pulsatile release of PGF2α, induced through oxytocin bind OXTR, does not happen and the CL remains active and continues producing P4 for the maintenance of pregnancy.

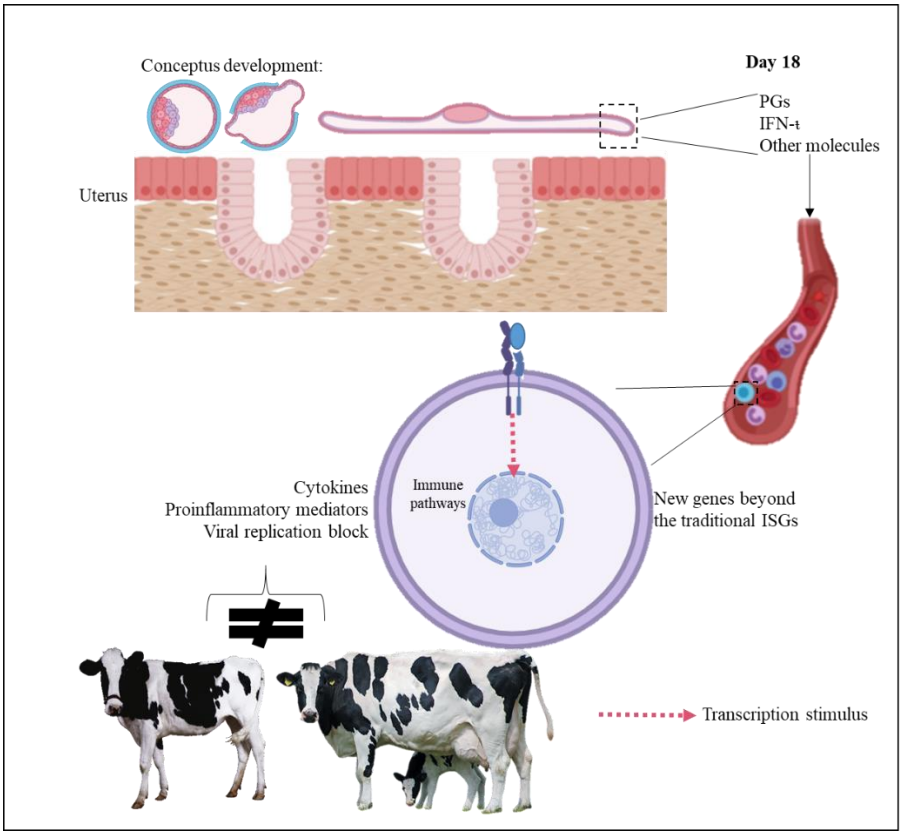
In the last years, advances in early pregnancy researches allowed the use of ISGs in peripheral blood immune cells to indirectly detect the presence of the conceptus on days 18 to

20 of pregnancy (GREEN *et al.*, 2010; SHIRASUNA *et al.*, 2012; KIZAKI *et al.*, 2013; PUGLIESI *et al.*, 2014). The increase of ISGs abundance in PBMC (lymphocyte and monocyte) has been reported in ewes (YANKEY *et al.*, 2001), dairy cows (GREEN *et al.*, 2010; SHIRASUNA *et al.*, 2012) and beef cattle (PUGLIESI *et al.*, 2014; MELO *et al.*, 2019). The expression of ISGs follows the same pattern of IFN-τ release, which could be indicative of pregnancy, earlier than other conventional methods. Also, the expression of classic ISGs has been reported on PMN (neutrophil, eosinophil and basophil) in cattle (KIZAKI *et al.*, 2013; TOJI *et al.*, 2017; MELO *et al.*, 2019), and with some evidences of higher sensitivity to IFN-τ stimulation than PBMC (KIZAKI *et al.*, 2013). However, when the ISGs expression in PBMC and PMN was used to prospectively diagnose pregnancy on day 20 post-TAI the accuracy was low (maximum 87%) (PUGLIESI *et al.*, 2014; MELO *et al.*, 2019).

This low accuracy is a result of the high false positive rate, which can be due to other type 1 IFN involved in the immune response to non-detected viral diseases. Secondly, the embryo losses where less amount of IFN-τ is released, enough to stimulate the ISGs, but not to establish pregnancy. The most traditional ISGs used to detect pregnancy are *ISG15*, *OAS1*, *MX1* and *MX2* (KIZAKI *et al.*, 2013; PUGLIESI *et al.*, 2014). Therefore, a possibility to improve the methodology would be studying the physiology of the immune cells during early pregnancy. This approach would allow to understand the pathways regulated by the presence of the conceptus and discover novel genes beyond the traditional ISGs, which may be used as a pregnancy marker with higher accuracy. Therefore, the aims of this study were (1) to discover novel early pregnancy-induced transcripts in PBMC of beef heifers after TAI, (2) to evaluate the mRNA profile of novel transcripts in PBMC and PMN during the first weeks of pregnancy in beef heifers and (3) to study the most significant pathway overrepresented in immune cells during early pregnancy.

Consequently**, the hypotheses of this work were (1) that new transcripts other than the classic ISGs would be differently expressed between pregnant and non-pregnant beef heifers and its profile would allow the selection of new potential pregnancy markers in bovine immune cells before day 20 post-TAI. (2) On day 18 of pregnancy the genes stimulated through conceptus presence would overrepresent pathways linked with the immune system, which would be differentially expressed between animal parity (heifers and cows) (Figure 1)**.

Figure 1 - Graphical Hypothetic Model



Source: (ROCHA, C. C., 2019)
Description: The image illustrates that on day 18 of pregnancy the conceptus secrets IFN-τ, PGs and other molecules that exit the uterus, come into the circulation, bind specific receptors and then stimulate the transcription of genes beyond the classic ISGs and linked to the immune system pathways in immune cells.

The use of molecular markers to detect pregnancy is already a reality in dairy herds in United states, where the circulating concentrations of PAGs on days 25-38 post-IA are in use. In addition, the color Doppler ultrasonography to evaluate the CL functionality has been recently used to detect non-pregnant cattle on day 20 post-TAI (PUGLIESI *et al.*, 2014). Despite the Doppler ultrasonography being a good and easy alternative to improve reproductive efficiency, the number of false positives among dairy cows and beef heifers can reach up to 25% (SIQUEIRA *et al.*, 2013; PUGLIESI *et al.*, 2018). Thus, the implementation of molecular alternatives may help to reduce this high false positive rate.

This dissertation was divided in three chapters. Chapter 1 reviews important topics raised during the experiments. Chapter 2 describes the study of novel early pregnancy-induced markers in *Bos taurus indicus* heifers and it was submitted to Biology of Reproduction. Finally, Chapter 3 comprises the investigation of the overrepresented pathway during early pregnancy

in *Bos taurus taurus* cows and heifers and will submit to Journal of Animal Science and Biotechnology. At the end of this dissertation, there are final considerations that summarize the research results and discuss the perspectives.

It is expected that the study of novel pregnancy markers and pathways regulated during this period allows to understand the immune regulation and embryonic loss during early pregnancy and further improve the reproductive efficiency in beef and dairy herds.

2 CHAPTER 1: LITERATURE REVIEW

2.1 PREGNANCY SIGNALING IN RUMINANTS

In ruminants the establishment of pregnancy depends on the conceptus signals, which starts during the first weeks of it (HANSEN *et al.*, 2017) followed by implantation and placentation. Around 4-5 days after mating the morula-stage embryo enters the uterus and develops to become a blastocyst by day 7 (SPENCER *et al.*, 2007). The zona pellucida from the blastocyst hatches on days 9-10 and the conceptus develops to a tubular structure which begins to elongate on day 15 to a filamentous form occupying the whole ipsilateral uterine horn on day 19 (WANG *et al.*, 2009). The trophoblastic cells belong to the conceptus. They develop within the embryo and at about day 16 begin the apposition and transient attachment to the uterine epithelium that result after day 19 in the adhesion to the luminal epithelium, when placentation begins (GUILLOMOT *et al.*, 1981).

The conceptus releases molecules that prevent the luteolysis to allow the establishment of pregnancy, being IFN-τ secretion by the trophoblast cells the major one (ROBERTS *et al.*, 2008). There are evidences showing that IFN-τ release in starts already at the blastocyst stage on day 7, more specifically where it makes contact with the endometrium (SPONCHIADO *et al.*, 2017). Circulating immune cells are also stimulated on day 7 in recipient cows bearing 10 embryos (RASHID *et al.*, 2018). Besides this early secretion, the significant increase on IFN-τ begins at day 15 approximately, when the conceptus begins the elongation phase and reaches a peak on IFN-τ secretion on day 20 (SPENCER *et al.*, 2016). The IFN-τ secretion prevents luteolysis that would normally occurs between day 15 and 18, and consequently the CL remains active producing progesterone and maintaining pregnancy (SPENCER; HANSEN, 2015). In this regard, luteolysis is inhibited in non-pregnant cattle submitted to intrauterine infusion of recombinant ovine IFN-τ (SPENCER *et al.*, 1995). Also, the insufficient production of IFN-τ by the conceptus allows for embryonic loss due to the inability to inhibit luteolysis (MANN; LAMMING, 2001). Furthermore, luteolysis is prevented by inhibiting estrogen-induced increase in *ESR* and *OXTR* expression (SPENCER *et al.*, 1995). These events are needed to stimulate the PGF2α pulses by the endometrium, that are responsible for the luteolysis in ruminants.

Oxytocin induces the release of PGF2α through OXTR activation, which increases the cytosolic calcium concentration and activates protein kinase C. These events in the uterus result on the activation of COX-2, the main enzyme involved in PGF2α production (ASSELIN *et al.*,

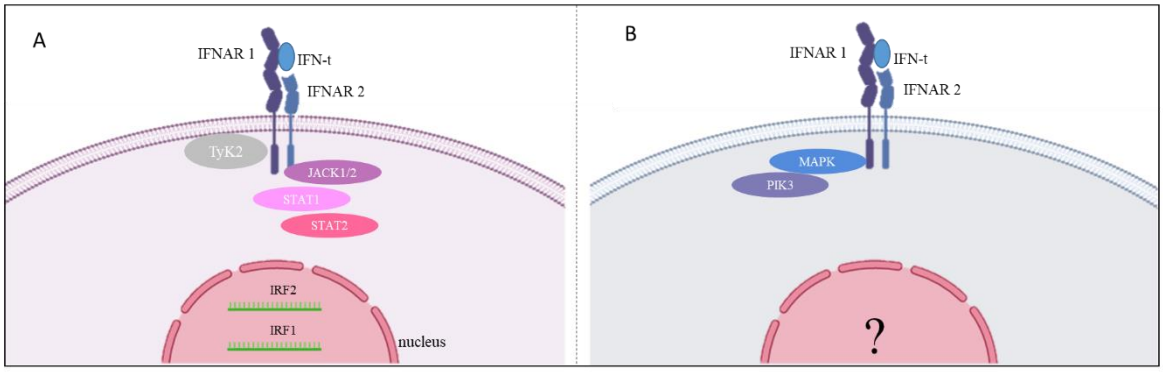
1997; THATCHER *et al.*, 2001), which allows the pulsatile release of PGF2α. Another interesting mechanism where IFN-τ works to prevent luteolysis is changing the dynamic production of PGs. Thus, it stimulates the COX-2 in the epithelial and stromal uterine cells to change the primary synthesis from PGF2α to PGE_2. PGE_2, in contrast, has a luteoprotective effect (ASSELIN *et al.*, 1997). Although IFN-τ prevents luteolysis by inhibiting the pulsatile release of PGF2α, it does not inhibit the basal production, which is higher in pregnant than non-pregnant ruminants (DORNIAK *et al.*, 2011). Beyond IFN-τ, there is evidence that conceptuses produce other molecules, such as PAGs, PL, PGE_2 and PFG2α (BAZER *et al.*, 2009). However, the role of PAGs and PGs secreted by the conceptus during early pregnancy is not clear (OTT *et al.*, 2014).

2.2 AUTOCRINE, ENDOCRINE AND PARACRINE ACTIONS OF INTERFERON-τ

Like other Type 1 IFNs, IFN-τ signals for specific α and β receptors IFNAR and regulate the transcription by stimulation of the STAT and IRFs pathways (SPENCER *et al.*, 1998). The IFNAR are surface receptors ligand-induced with two sub units IFNAR1 and IFNAR2 (STARK *et al.*, 1998). Once activated the IFNAR signaling can stimulate the canonical (most traditional) or non-canonical pathways. The canonical signaling pathway involves JAK-STAT-IRF that results in ISGs expression. In contrast, the non-canonical pathway is not well described and is uncommon (HANSEN *et al.*, 2017). It is activated by MAPK and PI3K-AKT1 (STARK *et al.*, 1998) (Figure 2). The most common IFN-τ activated IRFs in the uterus are IRF1 and IRF2, which have important roles to prevent the luteolysis events (SPENCER *et al.*, 1998). Therefore, the IRF2 is important to inhibit OXTR expression independent of ESR expression in bovine endometrium, while in sheep the mechanism is only related to ESR down-regulation, without effects on OXTR expression (TELGMANN[1] revised by (HANSEN *et al.*, 2017).

[1] TELGMANN R, BATHGATE RA, JAEGER S, TILLMANN G & IVELL R 2003 Transcriptional regulation of the bovine oxytocin receptor gene. **Biology of Reproduction** 68 1015–1026. doi:10.1095/ biolreprod.102.008961

Figure 2 - IFN- τ Signals by Canonical and Non-canonical IFNAR Pathway

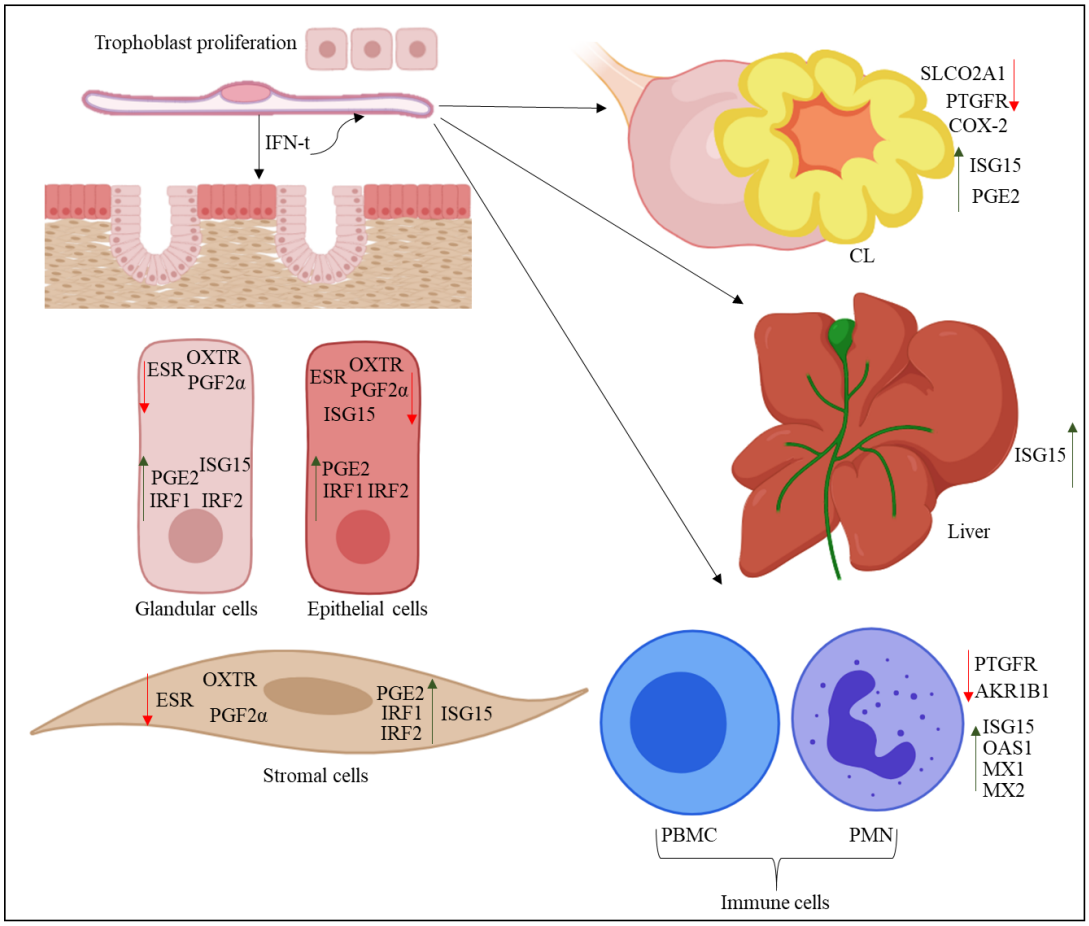


Source: (ROCHA, C. C., 2019; Adapted from (HANSEN *et al.*, 2017)[2]
Description: Image represents (A) canonical and (B) non-canonical pathways stimulated by the IFN-τ bound in IFNAR of bovine cells. The pathway begins with IFNAR stimulation by IFN-τ, however, the following actions are only triggered if TyK2 is associated with IFNAR1.

Beyond preventing luteolysis, IFN-τ stimulates expression of classic and non-classic ISGs in the endometrium (HANSEN *et al.*, 2017), which are important for uterine receptivity (OKUMU *et al.*, 2011) and to prevent the immune rejection of conceptus (CHOI *et al.*, 2003). In addition, IFNAR has already been found in the elongating conceptus of ewes, indicating an embryotrophic autocrine role of IFN-τ (WANG *et al.*, 2013). This autocrine role is thought to be involved in trophoblast cell proliferation as a dose dependent *in vitro* effect of IFN-τ is observed in trophoblast cells (WANG *et al.*, 2013). All endocrine and paracrine actions described in this section are represented on Figure 3.

[2] HANSEN, T. R., SINEDINO, L. D. P., & SPENCER, T. E. (2017). Paracrine and endocrine actions of interferon tau (IFNT). **Reproduction**, *154*(5), F45-F59. doi:10.1530/rep-17-0315

Figure 3 – Autocrine, Paracrine and Endocrine Actions of IFN-τ



Source: (ROCHA, C. C., 2019)
Description: Image illustrates that the IFN-t released by the trophoblast cells stimulates the conceptus development (autocrine) and the transcription in the epithelial, glandular and stromal endometrial cells (paracrine), liver, CL and immune cells (endocrine). The effects written inside the cells represent endocrine actions, whereas outside them represent paracrine actions. Red arrows represent down regulation and green arrows represent up regulation of genes.

The endometrial modulation by IFN-τ is not limited to preventing CL regression, but also stimulates the development of the conceptus. In this regard, retarded and malformed sheep conceptuses are found in knockout ewes for IFNAR in the endometrium (BROOKS; SPENCER, 2015). Therefore, the embryotrophic actions by IFN-τ are both autocrine and paracrine. Furthermore, *in situ* hybridization reveled the expression of IFNAR in the luminal, glandular and stromal cells from ovine endometrium at day 14 post mating (ROSENFELD *et al.*, 2002). However, the most common ISGs are interestingly not express on luminal epithelium, they are in contrast expressed in the glandular and stromal cells (JOHNSON *et al.*, 2001). This inhibition of the luminal epithelium is modulated by IRF2. The role of IRF2 is to regulate the immune system to prevent conceptus rejection (CHOI *et al.*, 2001).

The endocrine effects of IFN-τ have already been demonstrated by the expression of ISGs in the liver (MEYERHOLZ *et al.*, 2015), luteal cells (BRIDI *et al.*, 2018) and immune blood cells (KIZAKI *et al.*, 2013; PUGLIESI *et al.*, 2014). Despite the evidences of immune stimulation, no antiviral activity was detected in the main vessels of the circulatory system. However, the IFN-τ antiviral activity has been detected in blood of the uterine vein (SCHALUE-FRANCIS *et al.*, 1991) from day 15 in pregnant ewes, but not in the uterine artery or the ovarian vein. Further evidence that IFN-τ exits the uterus is the presence of antiviral activity in the extracellular vesicles released by the conceptus (NAKAMURA *et al.*, 2016). These last findings can suggest two important hypotheses that would explain why other studies did not detect IFN-τ in the main circulation (KAZEMI *et al.*, 1988; LEE *et al.*, 2012). The first based on rapid clearance from the systemic circulation (HANSEN *et al.*, 2017), as only 58 antiviral units per ml of IFN-τ were detected in the uterine vein (SCHALUE-FRANCIS *et al.*, 1991). A second hypothesis was that IFN-τ may stimulate ISGs expression through extracellular vesicles, explaining the impossibility to detect it free in the circulation (HANSEN *et al.*, 2017). In this regard, the affinity of IFN-τ for IFNAR is remarkably high, thus, low circulating concentrations are enough for IFNAR stimulation (LI; ROBERTS,1994).

The identification of high ISGs expression in the luteal tissue has been explained by the endocrine actions of IFN-τ over the CL (OLIVEIRA *et al.*, 2008). Also, the infusion of IFN-τ into the uterine vein on day 10 of the estrous cycle in the absence of a conceptus extends the life span of the CL in ewes (BOTT *et al.*, 2010). The most common ISG is *ISG15* (HANSEN *et al.*, 2017). It has been detected in luteal cells of ewes on day 15 of pregnancy (YANG *et al.*, 2010). The endocrine actions of IFN-τ in the CL prevent luteolysis in a different way than the endometrial regulation. One study (ANTONIAZZI *et al.*, 2013) proved that the infusion of IFN-τ into the uterine vein for 24 hours followed by and injection of PGF2α i.m. down regulates the main membrane transporter of PGs (BANU *et al.*, 2008), *SLCO2A1*, and the main PGF2α receptor *PTGFR* in the CL tissue. These evidences suggest an endocrine luteoprotective effect of IFN-τ in the CL. In addition, the up regulation of *PTGES* and down regulation of *COX-2* in the CL are also IFN-τ regulated, which modulates, like in the endometrium, the production of the luteoprotective PGE_2 (Arosh *et al.*, 2004). A single reduced dose of PGF2α (4mg / 58kg) administered in pregnant and non-pregnant ewes caused similar serum concentration of PGFM. However, luteolysis is only induced in non-pregnant animals (SILVIA; NISWENDER, 1984). Furthermore, intrauterine IFN-τ infusion did not affect the peak of PGFM in response to the administration of oxytocin on day 15 of the estrous cycle in ewes (OTT *et al.*, 1997). All these

actions in the CL are regulated by IFNAR, mainly in the large luteal cells (OLIVEIRA *et al.*, 2008).

In this context, pregnant and non-pregnant ewes received bovine serum albumin (BSA) or 200 µg of IFN-τ uterine vein infusion. As a result, a similar up regulation of *ISG15* was detected in endometrium, CL and liver in the pregnant and non-pregnant IFN-τ infused groups when compared to the non-pregnant BSA-infused group (BOTT *et al.*, 2010). Therefore, *ISG15* expression in liver and CL during pregnancy is an effect of IFN-τ stimulation. Expression of ISGs has been detected in hepatocytes in culture (RUHMANN *et al.*, 2017) following IFN-τ supplementation and in liver biopsies of pregnant cows (MEYERHOLZ *et al.*, 2015), however, no function has been determined yet for the ISGs regulation in that tissue. Therefore, a direct effect of IFN-τ or an indirect effect through the IFN-τ-stimulated immune cells could be involved (RUHMANN *et al.*, 2017) and need further investigation.

The expression of classic ISGs in immune cells has been well studied in ruminants. Independently of the cellular type, PBMC or PMN, the expression profile is similar to the IFN-τ secretion by the trophoblast cells (SHIRASUNA *et al.*, 2012; KIZAKI *et al.*, 2013; PUGLIESI *et al.*, 2014). In cattle, after ISGs expression reaches a peak on day 20 of pregnancy, a decrease to basal levels is observed near day 25 (SHIRASUNA *et al.*, 2012; PUGLIESI *et al.*, 2014). The amount of IFN-τ released by the uterine horn correlates with the *ISG15* expression levels in the immune cells (MATSUYAMA *et al.*, 2012). This is in agreement with the positive correlation found between ISGs expression on immune cells and concentration of exogenous IFN-τ administered (MATSUYAMA *et al.*, 2012). A study evaluated the ISG expression in whole blood from the uterine vein, uterine artery and jugular vein. However, no differences were found among the vessels in the expression of *ISG15* and *OAS1* (OLIVEIRA *et al.*, 2008). Thus, the immune cells may be stimulated when they come in the uterine vein. In this regard, in this cellular type the IFNAR is the main receptor (TOJI *et al.*, 2017) and, as stated above, the affinity between both is high. Therefore, another possible hypothesis is that IFN-τ stimulates ISG expression in peripheral vessels, even when it is not detected.

The mesenteric lymph nodes also express *ISG15* (OLIVEIRA *et al.*, 2008), for this reason the immune cells present in the endometrium may leave the uterus through the lymphatic drainage which stimulates the lymphatic system. The most common ISGs studied in the immune cells are the *ISG15*, *OAS1*, *MX1* and *MX2* (KIZAKI *et al.*, 2013; PUGLIESI *et al.*, 2014; HANSEN *et al.*, 2017). However, there is no specific function determined for each one of them. The only evidence proved is that ISGs expression is important for regulating the immune system, preventing the rejection of the conceptus (WEGMANN, 1988). This action is important

to prevent spontaneous abortions. Furthermore, in cows the intrauterine administration of PBMC in the uterine horn ipsilateral to the CL improves endometrial receptivity (IDETA *et al.*, 2010). During the estrous cycle, enzymes related to luteolysis (*PTGF*R and *AKR1B1*) are up regulated in the uterus, the CL and immune cells (YANG *et al.*, 2016). Also, *PTGFR* and *AKR1B1* genes were down regulated when compared to non-pregnant animals on day 18 of pregnancy in cattle (YANG *et al.*, 2016). These findings suggest a luteoprotective effect of IFN-τ also in the immune cells. Even though the response to IFN-t appear earlier in PMN (KIZAKI *et al.*, 2013; TOJI *et al.*, 2017), more evidence are needed as other studies showed similar profiles of the traditional ISGs for PMN and PBMC during early pregnancy (MELO *et al.*, 2019).

2.3 IMMUNE SYSTEM AND EARLY PREGNANCY

There are two events during early pregnancy when the immune system has an important role to avoid the rejection of the paternal antigen. Initially, during mating when the maternal tissue gets in contact with the semen. Secondly, when the trophoblast cells begin the contact with the maternal tissue (FAIR, 2015). However, in contrast with human and mouse, researches on the immune system carried in cattle during early pregnancy are very limited (FAIR, 2015).

The first studies in this field proposed three mechanisms whereby the allogenic conceptus evades the maternal immune system: immunosuppression of mother, conceptus being inert and not expressing histocompatibility antigens, and the placenta being a key barrier between conceptus and maternal immune cells (OTT *et al.*, 2014). However, recent studies in cattle have shown that the MHC-I genes are expressed during embryo development (DOYLE *et al.*, 2009) and in trophoblast cells (DAVIES *et al.*, 2000). This is in disagreement with the theory of the inert conceptus also as there is a positive association between MHC-I mRNA abundance, embryo development and embryo quality in cows (FAIR *et al.*, 2004). In trophoblast cells, the NC1 is activated by IFN-τ, IFN-γ and high concentrations of P4 (O'GORMAN *et al.*, 2010). The main role of NC1 is to protect the conceptus from the NK cells attack (OTT *et al.*, 2014).

As cited above, the immunosuppression of the mother is associated with the success of pregnancy. In ruminants, the small amount of studies in the immune regulation are focused in IFN-τ because it appears to be the key regulator of the maternal immune response (OTT *et al.*, 1997). There are evidences that IFN-τ may suppress the immune function in the uterus by stimulating immunosuppressive molecules (HANSEN, 2007). Furthermore, the infusion of

IFN-τ plays a role in the blood distribution of leukocytes, inducing lymphopenia and neutropenia in lambs (TUO *et al.*, 1998). When IFN-τ binds IFNAR in the endometrial tissue, beyond the classic ISGs, the *MCP1* and 2 *MCP2* are up regulated (MANSOURI-ATTIA *et al.*, 2012). Both proteins are potent chemotactic factors for monocytes, which is one of the most important cell types during the immune maternal response to the embryo in cattle (VELÁZQUEZ *et al.*, 2019). The maternal innate immune response is also IFN-τ regulated with a down regulation of *IL1β* and NF-κβ system (MUÑOZ *et al.*, 2012). In fact, the maternal immune response during pregnancy is not immunosuppressed, but it has a different modulation of immune functions in the uterus (VELÁZQUEZ *et al.*, 2019).

Some findings indicated that the lymphocytes through the Th cells play a central role in modulating immune pregnancy responses (MAEDA *et al.*, 2013). They modulate the balance between the Th1 and Th2 responses. That response (Th1 and Th2) are balanced according to the cytokines secreted (MOSMANN *et al.*, 1986). The Th1 cells generate cytokines such as IFN-γ, IL1β and TNFα. The Th2 cells produce IL4, IL5, IL6, IL10 and IL13 and down regulate the Th1 responses (MOSMANN *et al.*, 1986). In this sense, the Th1 cells in *in vitro* culture are able to inhibit the growth of the trophoblast human cells (BERKOWITZ *et al.*, 1988). In contrast, the cytokines released by the Th2 immune response induced conceptus tolerance in mice (LI *et al.*, 1998). Therefore, the Th2 cytokines were helpful for the success of pregnancy, while Th1 cytokines in the endometrium were unfavorable to the maintenance of pregnancy (ZHANG *et al.*, 2015). Thus, the immune control of pregnancy occur mainly through the Th1 and Th2 response, where the Th2 is predominant. Further studies are still needed to clarify the mechanisms by which the conceptus avoids rejection during early pregnancy in cattle.

2.4 USE OF ISGs IN IMMUNE CELLS FOR EARLY PREGNANCY DIAGNOSIS

The ISG expression profile in immune cells follows the same of IFN-τ secretion (HANSEN *et al.*, 2017), thus, several research groups have tried to develop an early pregnancy diagnostic test different than the traditional methodologies such as mode B ultrasonography. Most of the studies identified day 18 or 20 of pregnancy as the best time for comparison of *ISG15*, *MX1*, *MX2* and *OAS1* expression between pregnant and non-pregnant cattle (GREEN *et al.*, 2010; SHIRASUNA *et al.*, 2012; PUGLIESI *et al.*, 2014). This profile is conserved among the studies and independent of the status and breed. Also, similar levels of ISGs expression were observed in PBMC of pregnant animals from AI or ET (MATSUYAMA *et al.*,

2012). In addition, the use of ISGs expression in PBMC before day 17 did not result in an accurate pregnancy test based on ROC curves (GREEN *et al.*, 2010).

The ISGs expression in immune cells was tested as a prospective diagnostic method to detect pregnancy on day 20 in beef cows (PUGLIESI *et al.*, 2014). The use of *MX2* and *OAS1* expression in PBMC on day 20 proved an accuracy of 62 to 80% to detect the presence of a conceptus (PUGLIESI *et al.*, 2014). These results are lesser than when Doppler ultrasonography was used to diagnose non-pregnant females based on luteolysis detection on day 20 after insemination (PUGLIESI *et al.*, 2014). Recently, the expression of *OAS1* and *ISG15* genes in PMN were also evaluated in beef cows and heifers, resulting in an accuracy of 71 to 80% (MELO *et al.*, 2019). Therefore, to improve the accuracy of this methodology for pregnancy prediction an alternative approach is to measure the expression of ISGs at two time points, one at the time of AI and on day 18 post-AI (GREEN *et al.*, 2010). Using this approach, the ratio expression is calculated and used to predict pregnancy, resulting in a higher accuracy when compared to the single expression evaluation on day 18, due to the reduced overlap between pregnancy status (GREEN *et al.*, 2010).

Therefore, the low accuracy was a consequence of the ability of IFNAR to stimulate the expression of ISGs. IFNAR is a non-selective receptor and is stimulated by any type 1 IFN, which means that other IFNs could bind it and stimulate ISGs expression like in viral infections (HANSEN *et al.*, 2017). In this same way, most embryo losses happen before day 18 of pregnancy (DISKIN; SREENAN, 1980), but an earlier secretion of IFN-τ could have occurred in animals that underwent embryo loss, which increases the false positive rate (PUGLIESI *et al.*, 2014). In this regard when pregnant, non-pregnant and embryo death (by PGF2α on day 18 of pregnancy) were evaluated in ewes, no difference was found for embryo death and pregnant ewes in the *ISG15* and *MX1* expression on day 21 and 23 post-mating respectively (KOSE *et al.*, 2016). Both genes are reliable to detect the embryonic death only on day 25 post mating in ewes (KOSE *et al.*, 2016).

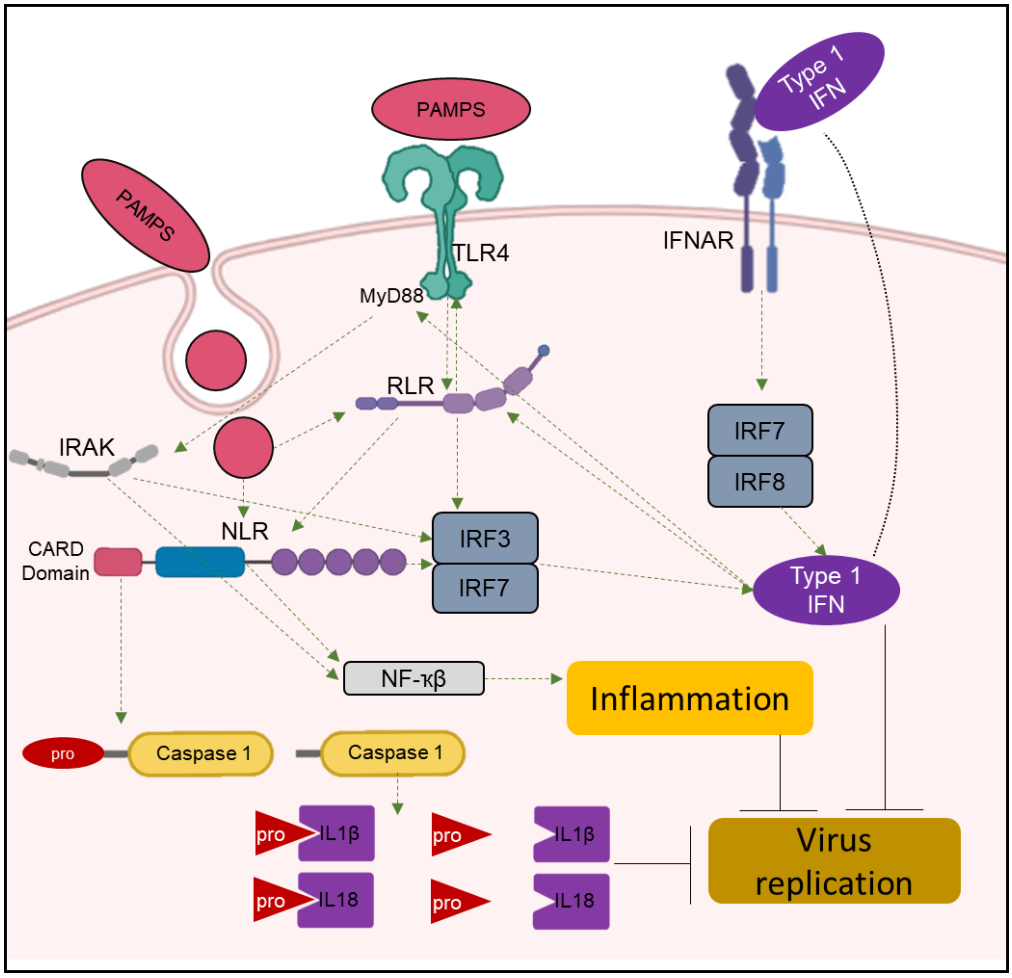
2.5 PATTERN RECOGNITION RECEPTORS AND TYPE 1 INTERFERON RESPONSE

The cells of the innate immune system identify pathogens through the PRRs by engaging PAMPs (THOMPSON *et al.*, 2011). The most studied PRRs are the TLRs, which are a type 1 transmembrane proteins responsible of the detection of PAMPs, like bacterial LPS and viral

nucleic acids in the extracellular environment (THOMPSON *et al.*, 2011). In addition, nucleic acids from viruses have emerged as major components of the TLRs response, and more particularly, TLR4 is an essential member of the TLRs family (DAI *et al.*, 2016). The TLR4 receptor functionality relays in an activated subdomain, the MyD88 (BALLOY *et al.*, 2008) pathway. The Myd88 domain is an important protein adapter in the cellular cytoplasm and has at the same time two domains, the death domain and TIR domain, which is stimulated by type 1 IFN (DAI *et al.*, 2016).

In the cytoplasm, there are other PRRs components involved in the immune response, like RLRs and NLRs (THOMPSON *et al.*, 2011). When the PRRs pathway is activated the main result is the transcription of essential factors, such as IRFs (*IRF3*, 7 and 8), cytokines and maturation of IL1β and IL18 (Figure 4). As the PRRs play a role in the viral response, they are also regulated by type 1 IFNs. Therefore, the mRNA of several TLRs (*TLR2*, *TLR4*, *TLR5*, *TLR6*, *TLR7*, *TLR8*, and *TLR10*) has been detected in the ovine trophoblast on day 13 post mating (KAYA *et al.*, 2017), but not in the immune cells. Furthermore, IRFs are the key mediator of TLRs immune response and thus, *IRF7* and 6 were found in the exosomes released by trophoblast cells in the uterine lumen of ewes from days 13 to 16, with the highest expression on day 14 post mating (RUIZ-GONZALEZ *et al.*, 2015), suggesting a role of TLRs in conceptus development.

Figure 4 - Synergic Collaboration Among the PRRs



Source: (ROCHA, C. C., 2019)
Description: Image illustrates the representative synergistic collaboration among the PRRs by PAMPs and type 1 IFN stimulation, where TLR4 represents the most traditional Toll like receptors. The green arrows indicate stimulation, while black strokes indicate attenuation.

There is a synergistical relationship among PRRs (OVIEDO-BOYSO *et al.*, 2014). For example, NLRs are activated intracellular by TLRs and RLRs, which play a role in type 1 IFN response in dendritic human cells (KAWAI, AKIRA, 2011) (Figure 4). In this regard, RLRs act as a cytoplasmic sensor of viral RNA and type I IFN production. The activated RLRs interact with the mitochondrial antiviral signaling adaptor (FEKETE *et al.*, 2018). This recruitment interaction downstream the signaling factor activate NF-κβ and IRFs, both crucial to the induction of type I IFN (LOO; GALE, 2011) (Figure 4). In addition, dendritic cells may activate their TLRs by the RLRs activation and type 1 IFN induction (LOO; GALE, 2011). The TLRs can also stimulate the IRAK, which contributes to interleukin production in the immune response (WANG *et al.*, 2017). In this context, there is a member of the RLR family, termed RIG-1, which is an enzyme encoded by the *DDX58* gene (WU *et al.*, 2019). This last gene has

already been found as an ISGs up regulated in the endometrium on day 16 in pregnant cattle (FORDE *et al.*, 2012). In this way, there is a human model where TLRs mediate the early phase of type 1 IFN production while the RLR participate of the late phase of IFN response (AGOD *et al.*, 2017) and NLRs are also stimulated by type 1 IFN (FEKETE *et al.*, 2018) in human dendritic cells.

The NLRs constitute a large family of intracellular receptors in the PRRs group, they can be classified in three subgroups: inflammasome (NLRP1, NLRP3, NLRP6, NLRC4 and NLRC5), reproductive NLRs (not classified yet) and regulatory NLRs (NOD1, NOD2, NLRP12, NLRX1 and NLRC3) (COUTERMARSH-OTT *et al.*, 2016). They are characterized by a tripartite domain structure with a N-terminal domain that is either typically Pyrin or Card domains, a central nucleotide and a C-terminal (SCHRODER; TSCHOPP, 2010). The NLRs have been found modulating important signaling pathways such as NF-κβ and type 1 IFN responses (COUTERMARSH-OTT *et al.*, 2016). In addition, two NLRs (*NLRC5* and *NLRX1*) have been reported to be linked to the RIG-1 signaling pathway and consequently mediating the viral immune response (COUTERMARSH-OTT *et al.*, 2016). Interestingly, as the NLRs, TLRs and RLRs are interlinked, when the PRRs are activated the final outcome will be the activation or formation of multiprotein complexes that have the capacity to modulate inflammation or the immune response (COUTERMARSH-OTT *et al.*, 2016). Therefore, some consequences of the pathway activation are ILs and CASP1 maturation, IRFs up regulation, and ISGs expression including *ISG15* and *MX1* (Wei *et al.*, 2016). The modulation of NLRs in reproduction was first described in human oocytes and ovaries in a meta-analysis (HRUZ *et al.*, 2008). In this regard, NLRP7, 5 and 9 proteins are expressed during human follicle development (SENA *et al.*, 2009). However, no role for NLRs in bovine reproduction was explained yet.

In front of the all statements shown here. Even with long time of studies, the embryo maternal communication during early pregnancy stills shallow explored. In this regard, the endocrine stimulation by IFN-τ has to be more studied in other tissues than the already published. Even of that, the immune cells specifically, opened a window for more exploration. For that, the main field, which should be deliberate, is the association of *in vitro* and *in vivo* studies to reveal the responses levels in PMN and PBMC in front of IFN-τ stimulation. Followed the same way, we should know the function of the main ISGs during early pregnancy. Those finds could facilitate the physiology knowledge, improving the reproductive efficiency.

This review reiterates that the immune system response during early pregnancy has to be more explored. With the evidences demonstrated here, we could prove the importance of the immune regulation to the maternal receptivity. In this regard, the development of approaches

to understand the systemic maternal response during early pregnancy has a potential power to improve the fertility results. Our group, specific, have some unpublished data about that, and intend to explore more. The understanding of the immune physiology during early pregnancy can be one of the main explanation for the big losses that occur during the early pregnancy period, which is focus for a large number of research groups.

3 CHAPTER 2: EARLY PREGNANCY-INDUCED TRANSCRIPTS IN PERIPHERAL BLOOD IMMUNE CELLS IN BEEF HEIFERS[3]

3.1 INTRODUCTION

In cattle, pregnancy success depends on the maintenance of a functional CL beyond the time of luteolysis, which normally occurs between days 15 to 18 of the estrous cycle [1, 2]. The CL maintenance in ruminants occurs in response to IFN-τ secreted by the conceptus [3]. The INF-τ glycoprotein binds to its receptors and consequently inhibits the pulsatile secretion of PGF2α from the endometrium, preventing luteolysis [4]. Consequently, the CL remains active and secretes P4 in levels sufficient for the establishment of pregnancy [1]. Success in this sequence of events determines the outcome of pregnancy. However as much as 50% of pregnancies fail until day 17 after AI [5].

The mechanisms that mediate embryo survival and death are incompletely understood, but the immune maternal system plays an important role in embryo development during the pre-attachment period [6]. A functional connection between the maternal immune system and the developing embryo is IFN-τ. The day-4 bovine embryo is already capable of synthesizing IFN-τ, which regulates the local immune environment in the oviduct [7]. In addition, the *in vitro* development of bovine embryos from morula (day-5) to hatched blastocyst stage (day-9), starts signaling of the uterine epithelial and immune cells in co-culture to modulate the anti-inflammatory response mediated by IFN-τ [8]. These changes contribute to conceptus growth and maternal immune modulation to prevent conceptus rejection [9]. Therefore, an immunological crosstalk between embryo and immune cells exist locally in the uterus, however, how those signals influence the immune cells is currently unclear.

Studies in ruminants during the last decades have reported that the IFN-τ up regulates the expression of ISGs during early pregnancy in various tissues, such as the endometrium [10], luteal cells [11], liver [12] PBMC and PMN [13]. The transcriptional profile of classic ISGs, such as *ISG15*, *OAS1*, *MX1* and *MX2* is closely associated with the IFN-τ secretion by the conceptus. Indeed, transcription usually increases from day 15 post-AI, reaches a peak on day 20 and reduces from day 22 on [14-16]. These findings suggested that IFN-τ may modulate the immune system during early pregnancy. Such information was used in recent studies that

[3] This study was submitted to the journal Biology of Reproduction as an original research article (BIOLRE-2019-0531). The proof submission of the manuscript is in the Attachment.

established that ISGs are diagnostic markers of pregnancy between days 18 and 20 days post-AI [14, 15].

In this context, early detection of pregnancy loss after AI is critical for dairy and beef cattle operations. The method to diagnose pregnancy that is used mostly in the beef industry is based on the ultrasonographic visualization of a viable embryo between 28 and 35 days after breeding [17]. Thus, in timed-AI (TAI) programs where the second TAI depends on the efficient detection of non-pregnant females from the first TAI, the interval between two TAI is usually 32-40 days. For this reason, development of earlier diagnostic methods (≤ 20 days post-TAI) are desirable, because they would make possible to shorten the interval between subsequent TAIs, leading to improved reproductive efficiency [18, 19]. Recently, earlier resynchronization strategies have been developed based on the detection of structural luteolysis using color Doppler ultrasonography to evaluate the luteal blood perfusion [14, 20, 21]. Although this method can be used as early as day 20 post-TAI and the occurrence of false-negative results are minimal, the presence of the conceptus is not detected and there is a 15-25% of false-positive diagnostics in dairy cows and beef heifers [22, 23].

A greater understanding of the role of immune cells during early pregnancy may result in improvements in the practical use of ISG expression as a tool to diagnose early pregnancies in cattle. For example, one study that compared ISG expression between PBMC and PMN reported that PMN may have an earlier response to IFN-τ secretion [13], but that requires confirmation. Furthermore, the accuracy of using the expression of the classic ISG transcripts on PBMC for pregnancy detection on day 20 post-TAI is only about 60 to 83% in beef cows [14, 24]. Hence, one potential strategy to improve this accuracy and to better understand the immune physiology during early pregnancy is the discovery of novel transcripts stimulated by the presence of the conceptus and use them as pregnancy markers in PBMC or PMN.

Therefore, we aimed with the present study: 1) to discover novel potential pregnancy markers in through transcriptomic analysis using PBMC on day 18 post-AI in beef heifers; and 2) to evaluate the pregnancy-induced mRNA profile of those novel markers in PBMC and PMN during the first weeks post-AI in beef heifers. We hypothesized that newly-identified transcripts, other than the classic ISGs reported in immune cells, are differently expressed between pregnant and non-pregnant beef heifers. We anticipate that such differences will serve as the basis for the selection of novel pregnancy markers in bovine immune cells before day 20 post-TAI. Furthermore, clues on novel mechanisms through which the conceptus signals its presence to the maternal system and modulates immune cell function will be gleaned from the current results.

3.2 MATERIAL AND METHODS

3.2.1 Animals

Animal welfare guidelines and handling procedures recommended by the São Paulo State (Brazil) law number 11.977 were strictly followed through Animals Ethics Committee of the School of Veterinary Medicine and Animal Science (CEUA-FMVZ number: 6810070817). Nelore (*Bos taurus indicus*) nulliparous beef heifers (n = 29) weighing 340 kg ± 32, with a body condition score between 3 and 4 (scale 1-5), with no gross reproductive anomalies were maintained in grazing conditions and received water and mineral supplementation *ad libitum*. Pre-breeding examinations were performed approximately 45 days before the experiment to ensure that all heifers were pubertal (presence of CL and a developed reproductive tract), as indicated by Holm [25].

Heifers were subjected to an estrous synchronization protocol for timed TAI based on P4 and estradiol treatments. Briefly, on D−10 (D0 = TAI) an intravaginal P4 device (1.0 g of P4; Sincrogest; Ourofino Saúde Animal, Cravinhos, SP, Brazil) was inserted, and 1mL i.m. of estradiol benzoate (2mg; Sincrodiol; Ourofino Saúde Animal) and 2mL i.m. of PGF2α (500 µg; of sodium cloprostenol; Sincrocio; Ouro Fino Saúde Animal) were injected. Eight days later, the P4 device was withdrawn and the animals were treated i.m. with a second PGF2α dose (Sincrocio; Ouro Fino Saúde Animal), 1mL of estradiol cypionate (1mg; Sincro CP; Ourofino Saúde Animal) and 1.5mL of equine chorionic gonadotropin (300 U.I; Sincro eCG; Ouro Fino Saúde Animal). Two days after (D0), all heifers were treated i.m. with a gonadotropin-releasing hormone analog (10 µg; of buserelin acetate; Sincroforte; Ouro Fino Saúde Animal) and were artificially inseminated by a single operator using thawed semen from a single Nelore sire.

On D10, D14, D16, D18 and D20, blood samples (40mL) were collected from the jugular vein into evacuated tubes (BD Vacutainer®; São Paulo, Brazil) containing sodium heparin for isolation of immune cells and evaluation of plasma P4 concentration. On the same days, B-mode and color Doppler ultrasonography exams were performed to measure the area and blood perfusion of CL, respectively. Pregnancy diagnostics were performed on D28 and heifers were assigned to the P or NP groups, based on the presence or absence of an embryo with heartbeat.

3.2.2 Isolation of immune cells

Blood samples collected for PBMC and PMN isolation (≈30mL) were submitted to a Ficoll® gradient protocol. To isolate each group of cells, whole blood was mixed with an equal volume of PBS, and the solution was layered onto 15mL of Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden), placed in a 50mL conical tube, and then centrifuged at 1100Xg for 30 min 20°C to obtain the buffy coat, as described previously [14]. After the gradient formation, the buffy coat was utilized for PBMC isolation and the last layer containing the granulocytes and red blood cells was utilized for PMN isolation, as reported previously [26]. The PBMC and PMN were subject to successive lyses steps with hypertonic solutions and then PBS restored the isotonicity. The resulting pellet was stored in a 1.5mL conical tube at −80°C until RNA extraction. To check the purity of the PBMC and PMN, freshly isolated samples of each cell type were placed on a slice and stained with fast panotic method for morphological identification of cells by light microscopy under 400x magnification. The purity > 95% for all samples.

3.2.3 Plasma progesterone concentrations

For P4 analysis, blood samples were stored in a box with chopped ice immediately after collection, plasma was harvested by centrifugation (2700g/15min/4°C) and stored at −20 °C. Concentrations of P4 were assayed with a solid-phase Radioimmunoassay kit (ImmuChem coated tube, MP Biomedicals, Costa Mesa, USA). The intra-assay CV for high and low reference controls and sensitivity for P4 were respectively, 2.32%, 2.03% and 0.01 ng/mL.

3.2.4 Ultrasound evaluations

Ultrasound scanning was performed at D0 to detect a dominant follicle and 48 hours later to confirm ovulation. On D10, 14, 16, 18 and 20, ultrasonography evaluations were executed to measure the area and blood perfusion of CL using the color Doppler mode. A duplex B-mode (gray-scale) and pulse-wave color Doppler ultrasound instrument (MyLab Delta Vet Gold; Esaote Healthcare; Italy) equipped with a multifrequency linear transducer (3.5-7.5 MHz) in B mode (RES-A, gain 50%, P 74 mm, X/M, PRS 1) and Doppler mode (gain 61%, PRF 730 Hz, frequency 6.3 MHz, WF 4, PRS 3, PRC M/2) was used. Area of the CL was determined using a B-mode still image and the tracing function. For CLs with anechoic fluid-filled cavities, the area of the cavity was subtracted from the total area [27]. The percentage of luteal area with color Doppler signals of blood flow at each examination was determined as described

previously [14, 28].

3.2.5 Study 1: discovery of novel pregnancy markers

To discover novel pregnancy markers, PBMC cells collected on D18 from six P and six NP heifers were submitted to RNAseq. Females were selected based on the CL blood perfusion and area on D18 (heifers bearing the largest CLs with at least >40% of blood perfusion for the P group, and heifers bearing the smallest CLs with <10% of blood perfusion for the NP group). These criteria was expected to generate contrasting groups of animals as the CL blood perfusion and area are strong correlate with P4 concentrations [27], which is required during pregnancy.

3.2.5.1 RNA extraction, library preparation, and RNA sequencing

The PBMC samples selected for RNAseq (n = 6 P and n = 6 NP), were submitted to RNA extraction. Cell pellets were thawed and immediately mixed with the lysis buffer from the RNeasy mini column kit (Quiagen, Hilden, Germany), according to the manufacturer's instructions. The RNA yield was quantified using the Qubit RNA Broad Range Assay Kit (Eurogene, USA) on a Qubit Fluorometer, and integrity was assessed on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) using an Agilent RNA 6000 Nano chip (Bioanalyzer, Agilent, Santa Clara, USA). All Samples used for RNAseq obtained RIN values ranging between 7.1 and 9.4. Libraries were prepared with 1µg of RNA in the TruSeq Stranded mRNA Library Prep kit (Illumina, San Diego, USA) following manufacturer's instructions. The Library size distribution was estimated through the Agilent DNA 1000 chip (Agilent Technologies) and the library concentration was measured through qPCR with a KAPA Library Quantification kit (KAPA Biosystems, Basileia, Switzerland). Samples were diluted, pooled in equimolar amounts and then sequenced at the "Centro Genômico Funcional Aplicado a Agropecuária e Agroenergia" at the University of São Paulo (São Paulo-SP, Brazil) using a HiSeq 2500 Sequencer (Illumina).

3.2.5.2 Statistical analysis

Statistical analyses of P4 concentrations and CL characteristics were performed using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). Heifer was considered the experimental

unit. Data were evaluated for the detection of outliers using Dixon test and for the normal distribution according to the Shapiro-Wilk test. When the raw data did not follow a normal distribution, they were transformed into natural logarithms or ranks. The plasma P4 concentrations, area, and blood perfusion of CL on D18 were analyzed using ANOVA by the MIXED procedure (SAS), considering the random effect of heifer.

3.2.5.3 Bioinformatics analyses

The sequences generated were filtered to remove low-quality sequences and contaminated reads using the SeqyClean 1.9.9 (https://github.com/ibest/seqyclean), and only high-quality paired-end sequences (with average PhredScore over 24) were used for further analysis. The samples were mapped against the *Bos taurus taurus* genome (ARS-UCD1.2) using the function –quantMode from the package STAR [29]. The significance of differential gene expression was assessed with the edgeR program [30]. These analyses were run in R/Bioconductor [31], for each comparison analyses were conducted separately. Count data were first normalized by TMM [32] for differences in sequencing effort and proportionality across libraries using the calcNormFactors function, while common dispersions were calculated using the estimate CommonDisp function [33]. We considered only DEGs with the FDR<0.05.

3.2.5.4 Enrichment analyses

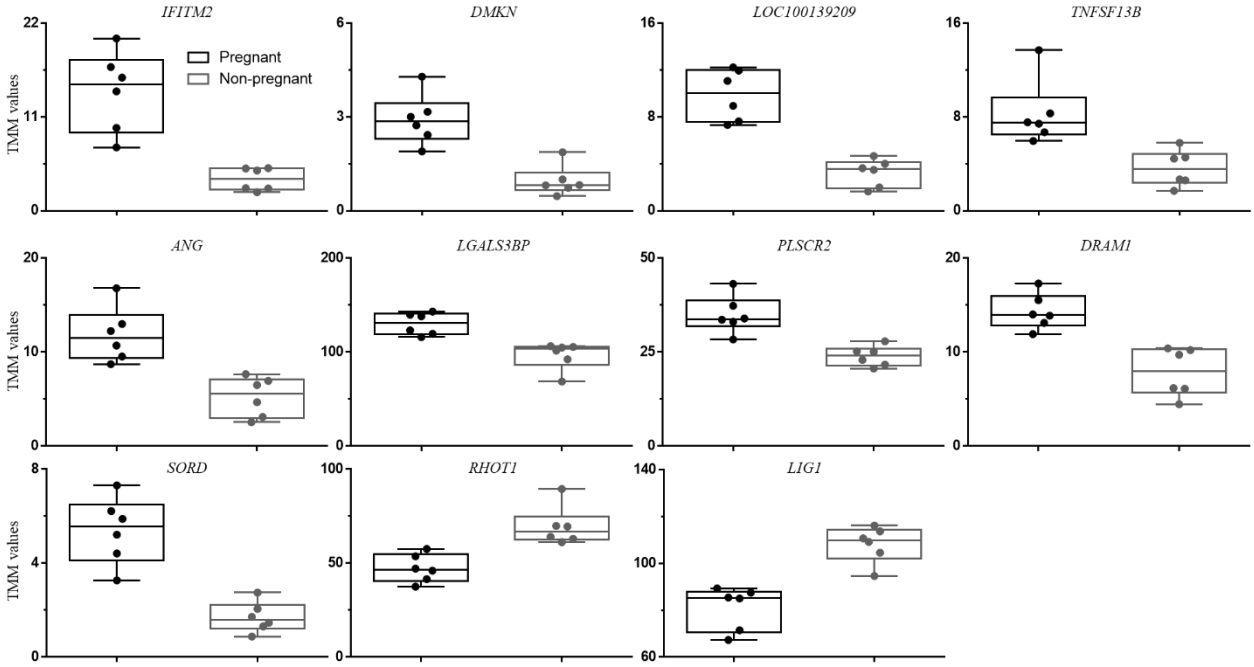
The list of DEGs, was analyzed using DAVID Bioinformatics Resources [34] for gene enrichment analysis relying on *Bos taurus* annotation from the GO consortium (geneontology.org). KEEG pathways were also assessed through the DAVID Bioinformatics Resources [34]. Both enrichment analyses adopt the Hypergeometric Test along with the Benjamini & Hochberg p-value adjustment method ($P < 0.05$).

3.2.6 Study 2: Expression profile of the novel markers in immune cells

The expression of 20 novel transcripts, that were identified as differentially expressed between P and NP groups in Study 1 was evaluated in PBMC and PMN samples collected of the same heifers (n = 29) from Study 1, on D10, 14, 16, 18, and 20. Twelve heifers were selected randomly from the P (n = 6) and NP (n = 6) groups. The criteria used to select the genes

discovered in Study 1 were: 1) DEGs that did not present overlap in the RNAseq TMM values between NP and P groups (Figure 5); and 2) the DEGs with the highest log fold change values.

Figure 5 - Box plot showing the maximum and minimum TMM for the eleven genes with absence of overlap evaluated on qPCR.



Source: (ROCHA, C. C., 2019)
Description: Each circle represents the specific TMM of each animal (n = 6 P and n = 6 NP).

3.2.6.1 RNA extraction, cDNA synthesis, and qPCR

Total RNA extraction of PBMC was performed using TRIzol® reagent (TRIzol reagent; Life Technologies; Frederick, USA) in accordance with manufacturer's guidelines. The PMN samples were submitted to RNA extraction through TRIzol® and chloroform gradient, after the RNA obtaining the samples were passed through a column of the kit mini prep Direct-zol (Zymo Research, USA) for purification in accordance to manufacturer's guidelines. During the PMN extraction, the RNA was treated with DNase I included in the kit (mini prep Direct-zol) for 15 minutes at room temperature. Total RNA extracted from both cells was separately eluted with 20 µL of RNAse free water. Concentrations and purity of RNA in extracts were evaluated using spectrophotometry (NanoVueTM Plus Spectrophotometer, Ge Healthcare. UK) and absorbance ratios values (260/280) ranged between 1.7 and 2.

Before the reverse transcription, the isolated RNA from PBMC was treated with DNase I (DNaseI, Amplification Grade; Life Technologies) for genomic DNA contamination

as per manufactures instructions. We synthesized cDNA from 500ng (PMN) and 1000ng (PBMC) of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). A master mix (10 µL) containing random primers, reverse transcriptase enzymes and deoxynucleotides were added to 11 µL of samples. Samples were incubated at 25°C for 10 minutes and then at 37°C for 2 hours, subjected to reverse transcriptase inactivation at 85°C for 5 minutes, and stored at -20°C until qPCR analysis. The final RT reaction was diluted 1:80 and this cDNA was used as a template for each qPCR reaction.

Analyses of relative abundance of transcripts were performed using SYBR Green PCR Master Mix (Life Technologies) for the amplification reactions in a Step One Plus thermocycler (Applied Biosystems Real-Time PCR System; Life Technologies). The optimized primer pairs were designed using primer design platform from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) based on the mRNA sequence of target genes obtained from the RefSeq database, on Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and the specificity of the primer were checked by BLAST (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The qPCR products were submitted to SANGER-DNA sequencing, and identities of target genes were confirmed. Details of primers were provided in Table 1. In order to select reference genes, the Normfinder Microsof Excel applet was used [35]. The *GAPDH* and *ACTB* were the most stable genes in PMN and *GAPDH* and *PPIA* were the most stable genes on PBMC, therefore, were selected as reference genes among the others evaluated *RPL30*, *RPL15* and *18S*).

Determination of qPCR efficiency and Cq values per sample were performed with LinRegPCR software. Quantification was obtained after normalization of the target genes expression values (Cq values) by the geometric mean of the endogenous control expression values [36]. Twenty target genes were evaluated on PBMC cells. For PMN, genes with an increased expression in P or NP groups in PBMC were selected and only those validated in this immune cell type were evaluated (n = 9).

3.2.6.2 Statistical analysis

The data were evaluated for detection of outliers using Dixon test and the significant ($P < 0.05$) outliers detected were excluded from the analyses. The data that were not normally distributed according to the Shapiro-Wilk test were transformed with natural logarithm, rank and square root. The plasma P4 concentrations, area and blood perfusion of CL and the abundance of each transcript were analyzed by split-plot ANOVA using the MIXED procedure of SAS software (Version 9.2; SAS Institute) with a REPEATED statement to account for the

autocorrelation between sequential measurements. The main effects of group (P or NP) and time (D10, 14, 16, 18 or 20) and their interaction (group-by-time) were evaluated for each variable, considering the significant ($P \leq 0.05$) and approached significant effects ($P \leq 0.1$). Heifers-within-group was used as a random effect. For the ratio and difference between D18 to D10 we considered each heifer as an experimental unit, using ANOVA by the MIXED procedure (SAS), heifer as a random effect, considering the significant ($P \leq 0.05$) and approached significant effects ($P \leq 0.1$).

Table 1 - Bovine specific oligonucleotide forward and reverse primer sequences (5'-3'), primer efficiency in the standard curve and amplicon length of the genes evaluated on qPCR.

Target name	**Gene Number**	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**	**Efficiency (%)**	**Length (bp)**
A2M	NM_001109795.1	TGCAACACAGTCTGGTCTCC	AGCACCATGTATTGCGGTTTT	2.07	143
ANG	NM_001078144.1	GGCCGAGGAGCCTTTGTTG	CATTCCGGCCCTTTGGTTTG	1.95	164
BPI	NM_173895.2	GCTGCCAGTGACAACCAAAC	TACACCATGCGATCGTGGTC	1.95	189
CLEC3B	NM_001046212.1	TGCCAAGAAAGATGCTGTGAG	TGGAAGGTCTTCGCTTGGAC	1.94	177
CR1	NM_001034407.1	GGCCTTGAGAAATGTGGCTCT	AGGGTAAGGCTTGGGGAACA	2.09	128
DMKN	NM_001082463.1	CAGGATGGCAAGACGCAGTA	CGGAAGTGCTTGCCTACCAA	1.91	192
DRAM1	NM_001031767.2	TGTACACGCTCCTGCAATCC	GAAGCACAGGCAATCATGGG	1.97	133
IFI44	XM_002686295.6	TCTGCCCATTGCTGAAGGAC	CCACATGGACCACATCAGACT	1.97	141
IFI6	NM_001075588.1	TGCTCTCCTCCAAGATACGGT	CAGAAGCTCGAGTCGCTGTT	2.06	161
IFITM2	NM_001078054.2	GCACATCGATCTCCCTACACA	GGTGTTGAACAGGGACCACA	2.04	157
LGALS3BP	NM_001046316.2	GGACTCGAGGCGTGAAAGAC	ATGTTCTCACACACCGTCCC	1.93	112
LIG1	NM_001102548.1	AACGGAAAGTCCCTGGTACG	TCACCGACTGCTCCAAGAAC	1.92	151
LOC100139209	XM_024989137.1	CTCCATCCACTACATGGGGC	CACTGGAGGGTCACATTCCC	1.98	183
OAS2	NM_001024557.1	GATCCCACTGACCCAACCAA	GTGATGCAGGCAGAACATTCC	1.95	139
PLSCR2	NM_001034436.1	GTGTACCAAAGAAGGACACACT	ACACTTGAGGGATCTGAAAACT	1.97	152
RHOT1	NM_001046082.1	CGTAGCTGCAAAGTCAGACC	CTTGTGTCACGTGCGGGTA	1.96	182
RSAD2	NM_001045941.1	TGGTTCCAGAAGTACGGTGAA	ACCACGGCCAATAAGGACAT	1.97	90
SIGLEC1	XM_025001078.1	GAGACAGCGGCACTTCGAG	GGTTCTGTTGCCTTGTTCTTTG	1.93	187
SORD	NM_001037320.1	CGGCATCTACGCCACTCATT	GGGTGACCAAGGGCTTTACA	1.95	194
TNFSF13B	NM_001114506.1	CTGCCCTGAAACAGCGGAT	CGTATAGTGGGCGTGTCACT	1.99	154

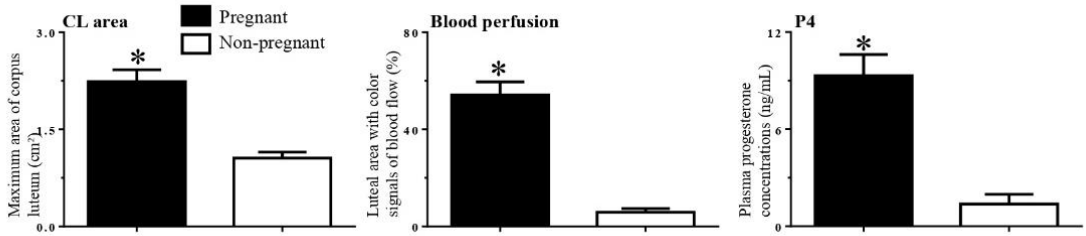
3.3 RESULTS

The probabilities (significance or approached significance) for a group effect, a time effect and the group-by-time interaction were shown in the figures, and the probabilities for differences in discrete endpoints were given in the text.

3.3.1 Study 1

The pregnancy rate on D28 post-TAI in the heifers that ovulated (n = 21) to the protocol was 43% (9/21). For the heifers selected for the transcriptomic analyses (n = 6 P and n = 6 NP), as expected, a greater CL area (P = 0.0002) blood perfusion (P < 0.0001) and plasma P4 concentrations (P = 0.003) on D18 was observed in the P group than in the NP group (Figure 6).

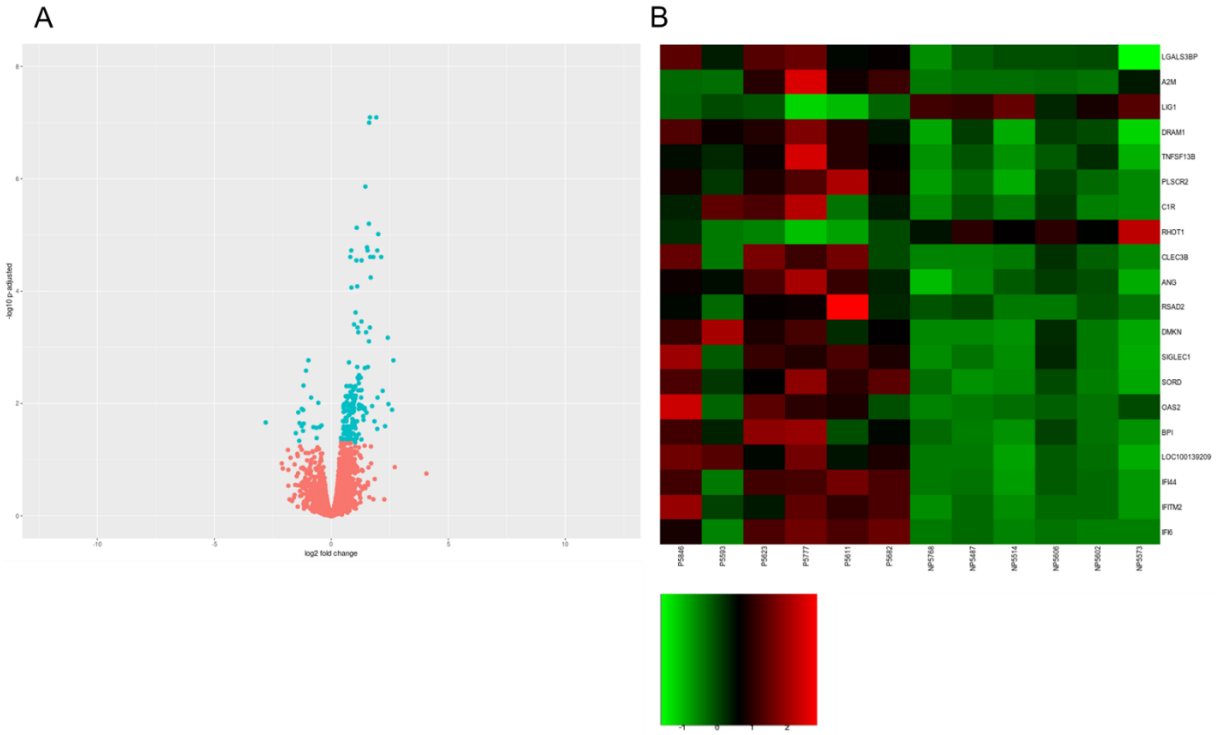
Figure 6 - Mean ± SEM for CL area, CL blood perfusion and plasma P4 concentrations on D18 post-TAI of the pregnant and non-pregnant heifers select for RNAseq (6/group).



Source: (ROCHA, C. C., 2019)
Description: * Means indicate differences (P < 0.05) between groups.

The RNAseq produced a total of ~240 million reads with an average of 20 million reads for each sample. Six biological replicates were analyzed for each group with the reads ranging from 17–21 million per sample after filtering. After use STAR, approximately ~90% of the total reads uniquely mapped to the reference genome, excluding also reads that aligned ambiguously. After applying the variance and minimal value of base Mean filtering, a total of 13,434 genes were included in the differential expression analysis. A total of 220 out of the 13,434 analyzed genes showed differential expression, of which, 200 were up-regulated (FDR: P < 0.05) on P group and 20 down-regulated on P group (FDR: P < 0.05) (Figure 7A). All read sequences (raw files and processed files) and an overview of this data has been deposited in NCBI's GEO and is accessible through GEO Series accession number GSE136102.

Figure 7 – Volcano Plot and Heat map



Source: (ROCHA, C. C. 2019)
Description: Panel A: Volcano Plot showing pregnant (P; n = 6) and Non-pregnant (NP; n = 6) gene expression, in terms of the DEGs (FDR < 0.05). While Panel B: Heat map constructed showing the 20 genes selected using the criteria of overlap absence and highest log fold change, for the comparison between Pregnant (P; n = 6) and Non-pregnant (NP; n = 6) heifers. The colors in the map display the relative standing of the reads count data; Green indicates a count value that is lower than the mean value of the row while red indicates higher than the mean. The shades of the color indicate distance from each data point to the mean value of the row. Columns represent individual samples of P and NP group.

After excluding seven out of 220 DEGs that were classic ISGs in immune cells (*ISG15*, *MX1*, *MX2*, *OAS1Y*, *OAS1X*, *OAS1Z and MIC1*), we selected 20 DEGs for further evaluation, based on the two criteria: 1) DEGs that did not present overlap in the RNAseq TMM values between NP and P groups and 2) the DEGs with the highest log fold change (logFC) values (Figure 7B). Eleven DEGs were selected based on the first criterion: *ANG*, *DMKN*, *DRAM1*, *IFITM2*, *LIG1*, *LGALS3BP*, LOC100139209, *PLSCR2*, *RHOT1*, *SORD* and *TNFSF13B*. Using the second criterion, the other nine DEGs analyzed were: *A2M*, *BPI*, *C1R*, *CLEC3B*, *IFI6*, *IFI44*, *OAS2*, *RSAD2* and *SIGLEC1* (Table 2).

Table 2 - List of the genes selected using the criteria of: highest log fold change (FC) (first column) and absence of overlap criteria (third column) with their respectively FC e

Without overlap	Log FC value	FDR	Greatest Log FC	Log FC value	FDR
IFITM2	1.9224	0.0000	*C1R*	2.4414	*0.010*
*LOC112448753**	1.6828	0.0001	*SLCO2B1**	2.4124	*0.0007*
SORD	1.6632	0.0000	*A2M*	2.2973	*0.02*
DMKN	1.6092	0.0008	*CLEC3B*	2.1951	*0.006*
LOC100139209	1.6062	0.0000	*OAS2*	2.1323	*0.0000*
*LOC100852090**	1.4337	0.03	*IFI6*	1.6506	*0.0000*
*LOC782062**	1.4317	0.002	*SIGLEC1*	1.6480	*0.02*
*FRMD4A**	1.2734	0.008	*RSAD2*	1.5611	*0.002*
TNFSF13B	1.1912	0.02	*BPI*	1.5484	*0.0000*
ANG	1.1761	0.004	*IFI44*	1.4556	*0.005*
*LOC112443203**	0.9729	0.005			
DRAM1	0.8653	0.01			
PLSCR2	0.5459	0.01			
*CASP1**	0.5245	0.03			
*SLC1A5**	0.5199	0.02			
LGALS3BP	0.4285	0.05			
LIG1	-0.4165	0.02			
RHOT1	-0.5568	0.01			

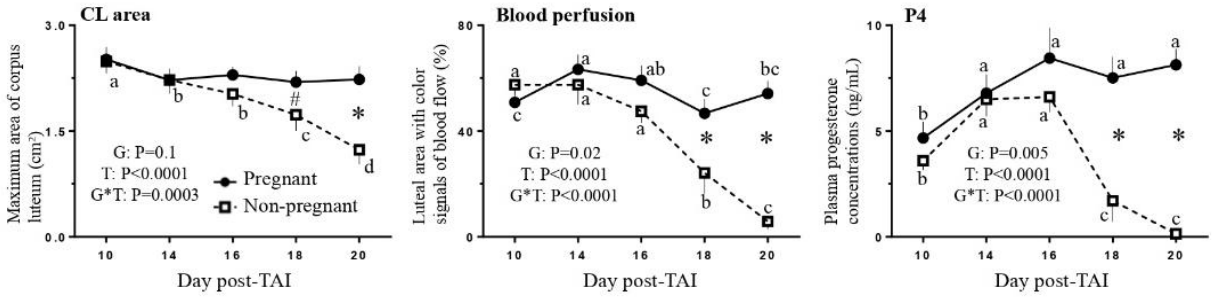
Description: The negative FC value means genes down-regulated on pregnant group, while positive FC value means genes up-regulated on pregnant group. * means genes that were not validated by qPCR .

Although the main purposes of the herein study is to identify novel transcripts in immune cells for using as a maker for pregnancy detection, to understand the functional implication of DEGs between P and NP groups, we performed a GO enrichment analysis. The GO analysis with the up-regulated DEGs on P group resulted in 42 chart records overrepresented for biological process category, 17 for cellular component category and 19 for molecular function category (Appendix A). The same DEGs were submitted to KEGG pathway enrichment analysis and 19 overrepresented KEGG domains were identified. There were no overrepresented significant charts for the DEGs downregulated on P group in the GO enrichment and KEGG pathway analysis (Appendix B).

3.3.2 Study 2

The main effects of time (days) and group and the interaction of group-by-time were significant for CL area, CL blood perfusion and plasma P4 concentrations (Figure 8). The interaction of group-by-time represented a greater ($P < 0.05$) or approached greater ($P \leq 0.1$) CL area and blood perfusion and plasma P4 concentrations on D18 and D20 in the P group than in the NP group.

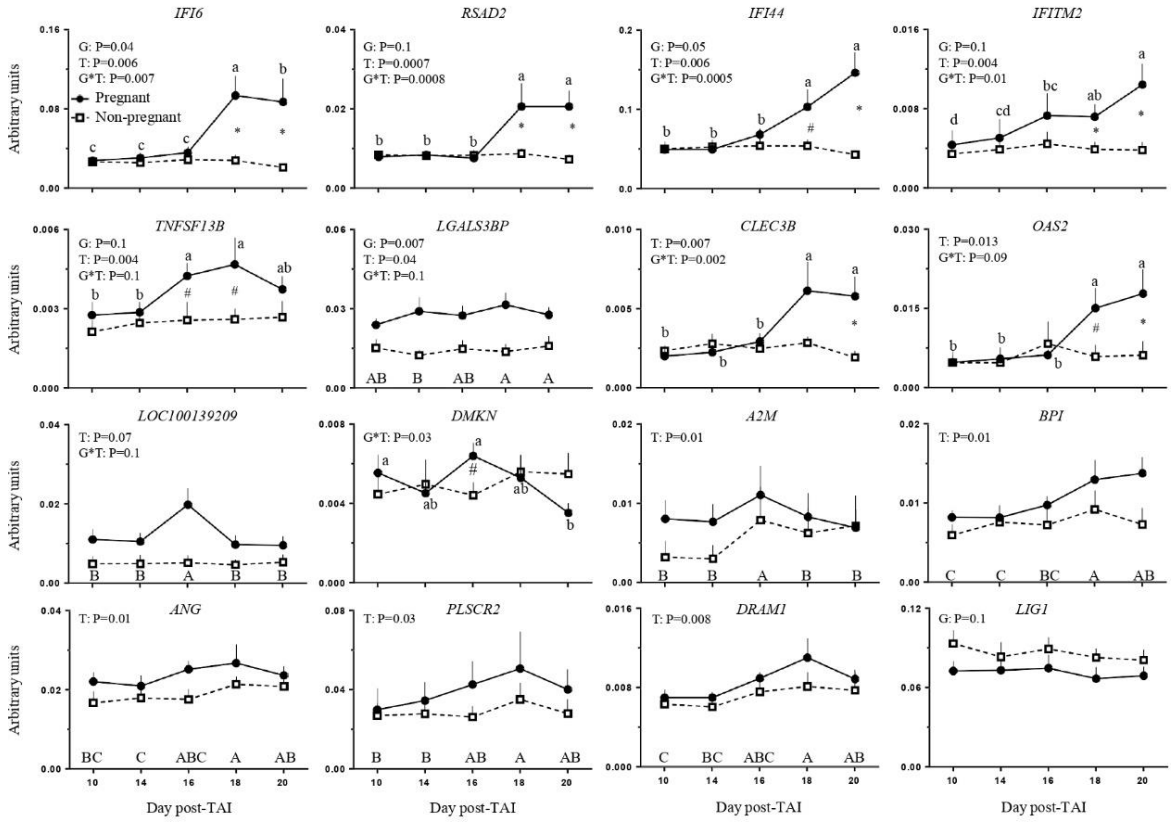
Figure 8 - Mean ± SEM for CL area, CL blood perfusion and plasma P4 concentrations from days 10 to 20 post-TAI of pregnant (n = 6) and non-pregnant (n = 6) heifers.



Source: (ROCHA, C. C., 2019)
Description: Probabilities are shown for significant main effects of group (G) and time (T) and the interaction for group-by-time (G*T). *# Means indicate differences ($P \leq 0.05$ and $P \leq 0.1$ respectively) between groups in specifics days. abcd Means without a common letter within a group indicate differences ($P < 0.05$) between days.

Transcription of the 20 genes selected was analyzed in PBMC by qPCR from D10, D14, D16, D18 and D20 after TAI. For *IFI6*, *RSAD2*, *IFI44*, *IFITM2*, *TNFSF13B* and *LGALS3BP*, a significance ($P < 0.05$) or approached significance ($P \leq 0.1$) effects of time and group and the interaction of group-by-time were observed. The interaction for *IFI6*, *RSAD2*, *IFI44* and *IFITM2* (Figure 9) reflected that initially the transcript levels start similar between P and NP group, becoming significant ($P < 0.05$) different in the P group between D16 and D18, resulting in about 2-fold change greater expression on D18 and D20 in the P group than the NP group. For *TNFSF13B* (Figure 9), however, the significant ($P < 0.05$) increases on P group was between D14 and D16, resulting in approaching ($P < 0.1$) greater expression (1.5-fold change) on D16 and D18 in the P group than in the NP group. Exception is *LGALS3BP*, where no differences between P and NP groups within a day were observed. Therefore, a significance group effect indicated that the relative expression of *LGALS3BP* was always 50% greater from D10 to D20 in the P group than in the NP group, while a significant time effect reflected a greater expression on D18 and D20, regardless the group (Figure 9).

Figure 9 - Mean ± SEM for relative expression by qPCR on PBMC of the genes with significant effects during statistical analysis, from days 10 to 20 post-TAI of pregnant and non-pregnant heifers (6/group).



Source: (ROCHA, C. C., 2019)
Description: Probabilities are shown for significant main effects of group (G) and time (T) and the interaction for group-by-time (G*T). *# Means indicate differences ($P \leq 0.05$ and $P \leq 0.1$ respectively) between groups in specifics days. abcd Means without a common letter within a group indicate differences ($P < 0.05$) between days. ABCD Means difference between days in all groups ($P < 0.05$).

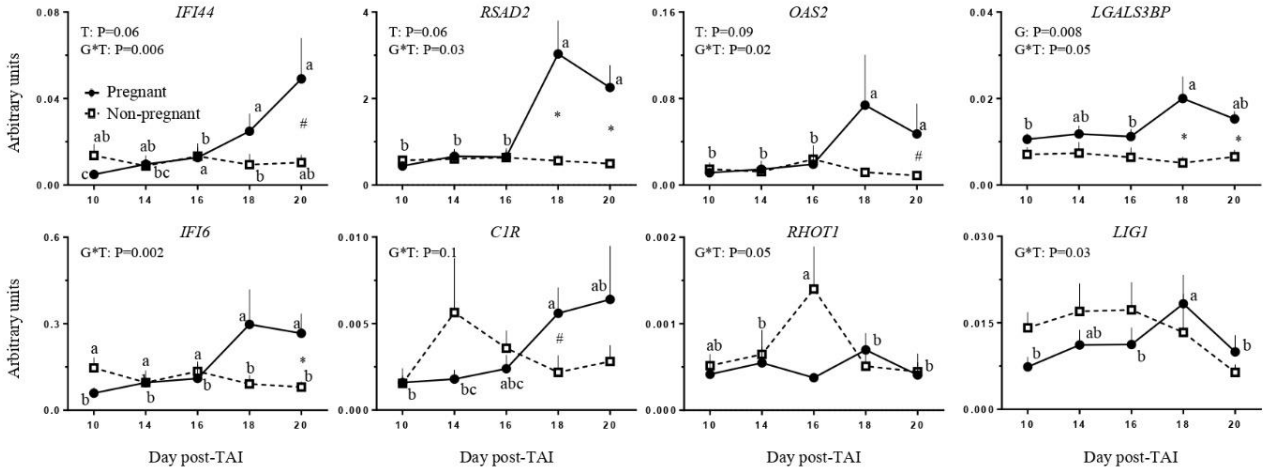
For *CLEC3B*, *OAS2* and LOC100139209 (Figure 9), the main effect of time and the interaction of group-by-time were significance ($P < 0.05$) or approached significance ($P \leq 0.1$). The interaction for *CLEC3B* and *OAS2* reflected that initially the transcript levels start similar between P and NP, becoming significant ($P < 0.05$) different in the P group between D16 and D18, and resulted in about 1.5 to 2.5-fold change greater in the P group than the NP group on D18 for *OAS2* and on D20 for both genes. While no effects were found when the interaction for LOC1001139209 was explored, the time effect reflected a significant increased ($P < 0.05$) expression on D16 followed by a decrease on D18, regardless the group ($P < 0.05$). For *DMKN* (Figure 9), only an interaction of group-by-time was detected, which reflected 0.5-fold change of decrease ($P < 0.05$) between

D10 and D14 followed by a similar increase (1.5) ($P < 0.05$) on D16 and a second decrease ($P < 0.05$) on D18 in the P group.

Although a significance main effect of group or group-by-time interaction was not detected for *A2M*, *BPI*, *ANG*, *PLSCR2* and *DRAM1* (Figure 9), a significance ($P < 0.05$) time effect was detected. For *A2M*, the highest ($P < 0.05$) relative expression value was on D16, regardless of the group (P or NP); while for *BPI*, *ANG*, *PLSCR2*, and *DRAM1*, was on D18. For *LIG1* (Figure 9), only an approached significance ($P \leq 0.1$) effect of group was observed, as indicated by about 1.5-fold change greater expression in the NP group than in the P group. For *SIGLEC1*, *C1R*, *SORD* and *RHOT1* (Appendix C), no significance effects of group, time or interaction of group-by-time were detected. Finally, nine out of the 20 evaluated genes in PBMC were validated with the RNAseq, resulting in 45% of agreement.

As the PMN are difficult the get good RNA quality, we selected only the main nine genes for the qPCR. For them, a significance ($P < 0.05$) or approached significance ($P < 0.1$) effects of time or group and interaction of group-by-time were observed for *IFI44*, *RSAD2*, *OAS2* and *LGALS3BP* (Figure 10). The interaction reflected that initially the transcript levels start similar between the P and NP groups, becoming significant different in the P group between D16 and D18, and resulted in about 2 to 2.5-fold change greater expression on D18 for *RSAD2* and *LGALS3BP* and on D20 for these four genes in the P group than in the NP group. For *IFI6*, *C1R*, *RHOT1* and *LIG1* (Figure 10), only a significance ($P \leq 0.05$) or approached significance ($P \leq 0.1$) interaction of group-by-time was detected. For *IFI6* and *C1R* the interaction mainly reflected an increased expression in the P group with 2-fold change greater expression on D20 for *IFI6* and on D18 for *C1R* in the P group than in the NP group. For *RHOT1* and *LIG1* only a 1.5 to 2.5 -fold change greater expression was observed in the P group on D16 for *RHOT1* and on D18 for *LIG1* compared to D10. For *IFITM2* (Appendix D), no significance effects of group, time or interaction of group-by-time were detected.

Figure 10 - Mean ± SEM for relative expression by qPCR of all genes with significate effects during statistical analysis on PMN from days 10 to 20 post-TAI of Pregnant (n = 6) and Non-pregnant (n = 6) heifers.



Source: (ROCHA, C. C., 2019)
Description: Probabilities are shown for significant main effects of group (G) and time (T) and the interaction for group-by-time (G*T). *# Means indicate differences (P ≤ 0.05 and P ≤ 0.1 respectively) between groups in specifics days. abcd Means without a common letter within a group indicate differences (P < 0.05) between days.

When the expression of the genes affected by pregnancy status or with an interaction of group-by-time was analyzed according to the relative abundance between D18 and D10 post-TAI (Table 3), a greater (P < 0.05) or approaching greater (P ≤ 0.1) relative transcript abundance (difference between D18 and D10 and D18/D10) in *IFI6*, *IFI44*, *RASD2*, *OAS2*, *LGALS3BP*, *IFITM2* and *CLEC3B* was detected in P versus NP heifers for PBMC samples. For PMN, a greater (P < 0.05) or approaching greater (P ≤ 0.1) relative transcript abundance between D18 and D10 was detected in *IFI6*, *IFI44*, *RASD2* and *LGALS3BP* heifers, but not for *OAS2* in P versus NP. In addition, when the relative expression between D18 to D10 in the P heifers was compared between PBMC and PMN, a greater (P < 0.05) ratio in *RASD2* and an approaching greater (P ≤ 0.1) ratio in *IFI44* were observed on PMN than PBMC.

3.3.3 Putative pregnancy markers

To selected the best putative pregnancy markers, we made a thin evaluation about the novel pregnancy markers. Thus, through the qPCR results we created one selection criteria. For that, the novel potential pregnancy marker should present significant interaction group by time, with significant increase in one of those days. In this regard, we found nine genes in PBMCs,

which presented significant interaction and increases during the days, that are: *IFI6*, *RSAD2*, *IFI44*, *OAS2*, *LGALS3BP*, *IFITM2*, *TNFSF13B*, *CLEC3B* and *DMKN*. While in PMN, we found five genes using the same criteria (*IFI6*, *RSAD2*, *IFI44*, *OAS2* and *LGALS3BP)*.

Table 3 – Table 3. Mean ± SEM difference and ratio of the relative expression of genes between Days 10 and 18 between P and NP on *Study 2*.

Endpoint	PBMC			PMN		
	Pregnant	Non-pregnant	P value	Pregnant	Non-pregnant	P value
IFI6						
Difference between D18 to D10[1]	0.06 ± 0.02	0.001 ± 0.006	0.006	0.2 ± 0.1	-0.05 ± 0.02	0.03
D18/D10[2]	3.4 ± 0.6	1.1 ± 0.2	0.007	5.6 ± 1.3	0.6 ± 0.2	0.003
RSAD2						
Difference between D18 to D10	0.01 ± 0.005	0.0003 ± 0.001	0.02	2.6 ± 0.8	-0.007 ± 0.1	0.007
D18/D10	2.4 ± 0.4[B]	1.1 ± 0.1	0.01	10.4 ± 3.1[A]	1.1 ± 0.2	0.01
IFI44						
Difference between D18 to D10	0.06 ± 0.02	0.004 ± 0.006	0.01	0.02 ± 0.009	-0.004 ± 0.001	0.02
D18/D10	2.5 ± 0.4[Y]	1.1 ± 0.2	0.01	9.6 ± 3.1[X]	0.8 ± 0.4	0.02
OAS2						
Difference between D18 to D10	0.01 ± 0.004	0.001 ± 0.002	0.08	0.06± 0.04	-0.003 ± 0.002	0.1
D18/D10	4.4 ± 1.1	1.8 ± 0.5	0.05	4.9 ± 1.8	1.4 ± 0.8	0.1
LGALS3BP						
Difference between D18 to D10	0.008 ± 0.002	-0.001 ± 0.003	0.04	0.009± 0.006	-0.002 ± 0.001	0.07
D18/D10	1.3 ± 0.09	0.9 ± 0.1	0.04	2.1 ± 0.5	0.7 ± 0.2	0.03
IFITM2						
Difference between D18 to D10	0.004 ± 0.001	0.0005 ± 0.0006	0.03	-	-	
D18/D10	2.6 ± 0.5	1.3 ± 0.3	0.07	-	-	
TNFSF13B						
Difference between D18 to D10	0.002 ± 0.0005	0.0005 ± 0.0003	0.04	-	-	
D18/D10	1.6 ± 0.1	1.4 ± 0.2	0.4	-	-	
CLEC3B						
Difference between D18 to D10	0.004 ± 0.001	0.0005 ± 0.0004	0.04	-	-	
D18/D10	3.1 ± 0.7	1.3 ± 0.3	0.03	-	-	
DMKN						
Difference between D18 to D10	-0.0002 ± 0.001	0.0011 ± 0.001	0.4	-	-	
D18/D10	1.09 ± 0.2	1.4 ± 0.2	0.3	-	-	

Description: The P value means significant differences between P and NP in the same cellular type. Superscript letters in the same line indicate significant ([AB]) or approached ([XY]) differences between the immune cell types of pregnant heifers. [1]The difference was calculated for each individual relative expression on day 18 and subtracted of the same value on day 10. [2]The individual relative expression of each animal on day 18 was contrasted with the same animal on day 10.

3.4 DISCUSSION

In cattle, the early pregnancy period is associated with paracrine conceptus signaling by IFN-τ to the endometrium, which modulates the immune responses. In addition, endocrine actions of IFN-τ have been proposed to affect several cells and tissues such as the immune cells. In the herein study we described novel DEGs in the PBMCs and PMNs between P and NP heifers during the critical period of conceptus signaling and survival. Determination of the DEGs expression profile on immune cells during the first weeks of pregnancy provides the opportunity to further understand the immune modulation of the conceptus-originated stimuli on peripheral immune cells during the preimplantation period. Also, this effort prospected the identification of novel pregnancy markers that can be used to develop an earlier pregnancy detection method in cows based on indirect recognition of conceptus signaling. The development of a novel pregnancy diagnosis method before day 20 of pregnancy has a great impact compared to the traditional method on day 28-30 and a potential improvement on novel approaches to shorten the time from AI to resynchronization and rebreeding.

Our hypothesis that novel transcripts beyond the classic ISGs reported in immune cells would be differently expressed between P and NP beef heifers was supported. Only seven out of the 200 DEGs up-regulated in P heifers were classic ISGs already reported in immune cells. Thus, we were able to select DEGs that are ISGs reported for the first in immune cells (*IFI44*, *IFI6* and *OAS2*), genes not previous related to pregnancy establishment (*RHOT1*, *LIG1*, *DMKN* and *DRAM1*) and even non-named genes like LOC100139209. In addition, the classic ISGs, *ISG15* and *OAS1X* genes were in the top 10 transcripts, up-regulated in P heifers of our results, but with overlapping in TMM values between P and NP samples. This reinforces that the use of these known ISGs as markers for pregnancy diagnosis may result in a high rate of false negative, as recently reported in PBMC and PMN on day 20 of pregnancy in beef cattle [14, 24]. Despite the high number of DEGs found on RNAseq, when we used the criterion of absence of overlap on TMM results to find novel markers, only few candidates were detected. Therefore, the presence of a greater number of DEGs without overlapping could have occurred if we have compared samples obtained from pregnant animals and from heifers non-inseminated. However, our main goal was to find pregnancy markers for use as a method to detect pregnancy in inseminated animals, and the occurrence of early pregnancy loss before days 18 after TAI can reach 40-50% in cattle [5]. In this

regard, we found a difference in CL function between P and NP heifers on D18, which are important characteristics for pregnancy maintenance. Therefore, we believe that the present approach allowed the discovery of the genes stimulated by a viable conceptus and that its expression is not or less stimulated in heifers that failed to conceive or after an early pregnancy loss.

The hypothesis that the profile of the DEGs would allow the selection of novel pregnancy markers in bovine immune cells before day 20 post-TAI was also supported. Five genes (*IFI6*, *IFI44*, *RASD2*, *OAS2* and *LGALS3BP*) evaluated in both types of immune cells, have a similar increased expression profile. Other three genes were up regulated before D20, but the different profiles between the immune cell types may represent a more responsiveness to the conceptus presence for *C1R* and *RHOT1* on PMN and for *IFTM2* on PBMC. In this regard, during early pregnancy the main immune cell type attracted by endometrium is the monocyte [37]. But, previous studies [13, 38] suggested that PMN may have an earlier response to INF-τ secretion. Also, Toji et al [39] indicated that PMNs are more sensitive to IFN-τ and that the shorten lifespan (few hours) of neutrophils, may impact on the PMN sensitivity to IFN-τ compared to other immune cell types. In this regard, the IFN-τ acts on endometrial cells by JAK/STAT pathway [40], but the intracellular mechanisms of IFN-τ on immune cells is not well described.

The profile of several genes on both immune cell types followed the expected IFN-τ secretion during trophectoderm expansion. That is, the profile expression of *IFI6*, *IFI44*, *RSAD2*, *OAS2*, *IFITM2* and *CLECL3B* on PBMC and of *IFI6*, *IFI44*, *RSAD2* and *OAS2* on PMN, which increases from day 15-16 post-AI and reaches a peak on about day 20 [16, 41]. In addition, the expression profile observed for these genes corroborates with the expression of the classic and most studied ISGs in immune cells (*ISG15*, *MX1*, *MX2*) [14, 15]. Therefore, the reported antiviral response mediated by type I IFN on the expression of *IFI6*, *IFI44*, *RSAD2*, *OAS2* and *IFITM2* [42-44] indicate that these genes are also stimulated by the conceptus through the IFN-τ secretion. However, to our knowledge, only *IFITM1* and *IFITM3* [45] and not *IFITM2* have been reported to respond to the IFN-τ stimuli during early pregnancy. The *CLEC3B* also presented an expression profile similar to the classic ISGs. In this regard, transcriptomics studies in cattle reported that *CLEC3B* [46] and also the other two genes, members of the same domain but with different families, *CLEC4F* [47] and *CLEC2B* [44], are stimulated by conceptus presence in the

endometrium tissue. Considering these reports and the present results is suggested that *CLEC3B* is also a gene stimulated by IFN-τ on peripheral immune cells.

The *TNFSF* was also previous related to viral response mediated by type I IFN [48, 49]. In the present study, we analyzed on PBMC the expression profile of *TNFSF13B*, which was previously reported as a DEG in the endometrium on day 16 of pregnancy in heifers [44] and in the granulocytes with IFN-τ culture cells [39]. Interestingly, we observed its expression on immune cells, with an earlier increased expression on PBMC than most ISGs previously reported [14, 15]. A greater expression of *TNFSF13B* was observed on D16 and D18 in the P heifers. This suggests that this gene has an earlier response than the classic ISGs and can be stimulated by INF-τ or another molecule secreted from the conceptus, but probably using a different pathway as it is a pro-apoptotic gene [50]. The *C1R* is important for the modulation of the maternal immune system, preventing the embryo rejection on bovine endometrial cells during the pre-attachment period [51]. The *C1R* was expressed in the endometrium of *Bos taurus taurus* heifers analyzed by transcriptomic analyses on day 16 [44] and 18 [52] post-estrus and by microarray analysis on day 21 of pregnancy in PMN [39]. Considering this later report and our present results on immune cells, it is suggested that the CR1 is involved in the early immune response to conceptus presence during the pre-attachment period of pregnancy, but its use for detection of pregnancy in immune cell is limited as we only detected an up regulation of this gene on D18 in PMN.

Another interesting DEG evaluated that resulted in a potential pregnancy marker was the *LGALS3BP*. Previous studies [51-53] described the involvement of this gene during the early pregnancy, but more related to the cellular adhesion process in the bovine endometrium during the pre-attachment period. In humans, the *LGALS3BP* is stimulated by type I IFN on peripheral immune blood cells [54]. The different expression profile between the two immune cell types is curious as this indicates that the PBMC response to conceptus was earlier than in PMN, which is the opposite outcome to the results previously reported by Kizaki et al [13]. Also, a previous study [55] reported the involvement of *LGALS2BP* with macrophage activation and mediation of immune response regulated by type I IFN and neopterin, without granulocytes involvement. The *LGALS2BP* gene may acts with high intensity on cells of the monocyte-macrophage lineage [55], which could explain the different *LGALS3BP* expression profiles between PMN and PBMC in the present study.

The *DMNK* increased abundance in PBMC on D16, also indicated that the conceptus presence stimulated its up-regulation in peripheral immune cells. This gene is abundant in stratified epithelia and in differentiating primary human keratinocytes, mainly related to inflammatory skin disorders [56]. During inflammatory disorders, the *DMKN* expression is regulated by cytokines such TNFα and IFN-β [56]. The IFN-β like IFN-τ is one of type I IFNs, which suggests that the IFN-τ can stimulate the *DMKN* expression in immune cells. Additionally, other studies have shown the expression of one specific isoform *DMKNα* in the mouse and humans placenta [57, 58], but without a function determined yet. However, its expression was down-regulated on D18 in the present study, which indicates that this gene is a poor pregnancy marker in cattle during this period.

Although the *A2M*, *BPI*, *ANG*, *PLSCR2* and *DRAM1* were not affected by the pregnancy status, a temporal change on expression of these genes was observed. One possible explanation for this result is that the normal changes on steroid hormones during estrous cycle could have a major effect on expression of these genes rather than the pregnancy status. Therefore, the increase on plasma P4 concentrations from D10 to D16 regardless the pregnancy status group could have affected the expression of these genes. One alternative explanation regarding the up regulation on *BPI*, *ANG*, *PLSCR2* and *DRAM*1 is that all heifers were inseminated, and NP heifers may have experienced the presence of an embryo during the first two weeks and then underwent pregnancy loss around the period of pregnancy recognition (days 15 to 18), resulting in up regulation on the expression of these genes on D18. In addition, the *ANG* family gene has already been associated with the growth or demise of luteal tissue through alterations in vascularity [59], that is necessary independent of the pregnancy status and can be regulated during the luteolysis around day 18 of the estrous cycle, the same moment of higher expression of *ANG* in the present study. Increased expression of *BPI* and *PLSCR2* has also been associated with the immune response of cows to bacterial and virus infection, respectively [60, 61]. In this regard, the *PLSCR2* in the viral response is stimulated by activation of STAT pathway induced by type I IFN [60], as the classic ISGs. However, a difference in the expression profile between P and NP heifers as observed in ISGs was not observed in the herein study.

In summary, the presence of a viable conceptus stimulates a large number of DEGs in peripheral leucocytes on D18. The comprehension of the temporal changes of 20 novel DEGs between P and NP beef heifers during early pregnancy allowed the discovery of nine potential pregnancy markers on PBMC (*IFI6*, *RSAD2*, *IFI44*, *OAS2*, *LGALS3BP*, *IFITM2*, *TNFSF13B*,

CLEC3B and *DMKN*) and five on PMN (*IFI6*, *RSAD2*, *IFI44*, *OAS2* and *LGALS3BP*). The discovery of these novel early-pregnancy markers on immune cells retrieved from peripheral blood can be used for the development of new methods to predict positive pregnancies earlier than the traditional diagnostic methods.

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4 CHAPTER 3: EARLY PREGNANCY-INDUCED TRANSCRIPTIONAL CHANGES IN CIRCULATION: DOES THE IMMUNE RESPONSE DIFFER BETWEEN PREGNANT COWS AND HEIFERS?[4]

4.1 INTRODUCTION

In cattle, the average calving rate is ~ 55% (Diskin and Sreenan, 1980) with a fertilization rate of ~ 90%. In addition, the majority of embryonic loss (70% to 80%) occurs post-fertilization and the peri-implantation period (Diskin and Morris, 2008). During this time period, the embryo undergoes a number of significant developmental stages. On day 4 post mating, the morula-stage embryo enters the uterus and undergoes further cell divisions to become a blastocyst. Once the embryo hatches from the zona pellucida it forms an ovoid (Day 13) and then tubular conceptus (Day 14) (Forde and Lonergan, 2017). The outer layer of trophectoderm cells undergoes rapid elongation and secreted increasing quantities of IFN-τ, the pregnancy recognition signal in ruminants (Spencer et al., 1995).

The IFN-τ is a glycoprotein, and a member of type I IFN family (Roberts et al., 1992). Release of IFN-τ by the mononuclear trophoblast cells (Wang et al., 2009) facilitates the paracrine and endocrine actions to induce maternal recognition of pregnancy. The most important paracrine action of IFN-τ, is the inhibition of the luteolytic mechanism in the cells of the endometrium. Which is made by the down regulation of ESR and OXTR, that when up regulate allow the pulsatile releases of PGF2α (Asselin et al., 1997). Consequently the CL and P4 production are maintained, allowing early pregnancy to be established (Thatcher et al., 1989). A threshold concentration of IFN-τ is required by Day 16 post-estrus and its output from the conceptus peaks by day 20 and then declines to undetectable levels on day 25 (Roberts et al., 1999). This process if fundamental for early pregnancy success. The IFN-τ signals by IFNAR (alpha and beta), that is a cell membrane receptor with two subunits IFNAR1 and IFNAR2 (Stark et al., 1998). The IFNAR is a ligand-induced receptor stimulates by immune response, viral infection (Welsch et al., 2019) and pregnancy (Shirozu et al., 2017). In the endometrium IFN-τ modifies the endometrial response inducing a type 1 IFN transcriptional response (Bauersachs et al., 2006, Mansouri-Attia et al., 2009, Forde et al., 2011) typical of members of the ISGs family. However, it has also been shown that

[4] This study after the corrections of the evaluators will be submitted to the Journal of Animal Science and Biotechnology.

IFN-τ can stimulate non-classic ISGs in the endometrium (Hansen et al., 2017). The non-classic ISGs also allow the conceptus development. In this regard, the non-classic has been related, modulating the immune system and preventing the pregnancy loss (Fair, 2015).

In addition to pregnancy recognition, a key event that is required to facilitate successful pregnancy is immune tolarization of the conceptus. Like all type I IFN, the IFN-τ up regulated traditional genes, with important role in viral infection and pregnancy. For example, the endometrium of both, pregnant and with Borna-virus infection cows express *ISG15* (Henkes et al., 2015, Takino et al., 2016). However, even all type I IFN bind to IFNAR, during the pregnancy, interestingly, the IFN-τ should control that immune stimulation to avoid the conceptus rejection by the female (Hansen et al., 2017).

Recent studies have investigated the changes in the bovine endometrium immune system during the pregnancy, demonstrating immune modulation in early pregnancy (Ott et al., 2014, Fair, 2015, Velázquez et al., 2019). One evidence is the role of cytokines during pregnancy. Thus, the most important inflammatory cytokine, interleukin 1β (IL1β), is down regulated in the endometrium by IFN-τ during early pregnancy (Muñoz et al., 2012). In addition, the endometrial cells, such as glandular, epithelial and stromal cells are differentially regulated by IFN-τ (Fair, 2015). The most classic stimulated IFN-τ gene (*ISG15*) is only expressed in the deep glandular and stromal cells of the endometrium, but, not in the luminal (Hansen et al., 2017). In addition, in cattle endometrium the IFN-τ up regulates the MCP1 and MCP2 (Mansouri-Attia et al., 2012), which play role in the monocytes attraction. Therefore, during early pregnancy the IFN-τ, appears to work within the immune modulation by leucocytes helps. All these finds proving that both immune response, innate and adaptive are important to the pregnancy success. Where, the innate immune system works specially by the dendritic, natural killer and leukocytes cells (Fair, 2015). While, the adaptive works by lymphocyte B and T. In this regard, the TH1 and TH2 play a balance in the endometrium immune adaptive response to prevent the conceptus rejection (Li et al., 1998).

In addition, IFN-τ exerts effects in extra-uterine tissues (Oliveira et al., 2008) and can regulate the transcriptional response in other cells, like hepatocytes (Ruhmann et al., 2017), large luteal cells (Bridi et al., 2018), PBMC (Pugliesi et al., 2014) and PMN (Kizaki et al., 2013). In this regard, a recent study in PMN revealed that heifers had 1.5-fold change greater of ISG15 expression when compared with cows. Which can suggest differences between the response patterns in the parity category. While we know of some of the changes that occur in immune cells and their

responses in early pregnancy in cattle, we do not know specifically about the extra uterine tissues effects and if these are all regulated by the IFN-τ.

Therefore, we aimed with the present study to (1) discover what were the key pathways modified in specific immune cells in circulation in response to early pregnancy, and (2) determine how members of these pathways may be regulated. We hypothesize that, in early pregnancy the most overrepresented pathway is linked with the immune response and that one is differentially expressed between parity categories (nulliparous and multiparous).

4.2 MATERIAL AND METHODS

4.2.1 Animal ethical approvals

In Experiment 1, the animal welfare guidelines and handling procedures recommended by the São Paulo State (Brazil) law number 11.977 were strictly followed (CEUA-FMVZ number: 6810070817). Nelore (*Bos taurus indicus*) heifers with no gross reproductive anomalies were maintained in grazing conditions and received water *ad libitum*. In Experiment 2 the animal welfare guidelines and handling procedures recommended by the University of Liverpool (England) approved by local ethics board (PB5D4D273). *Bos taurus taurus* female with no gross reproductive anomalies were maintained in grazing conditions and received water *ad libitum*.

4.2.2 Experiment 1: Identification of pathways modified of specific immune cells in circulation in response to successful early pregnancy

4.2.2.1 Animal model

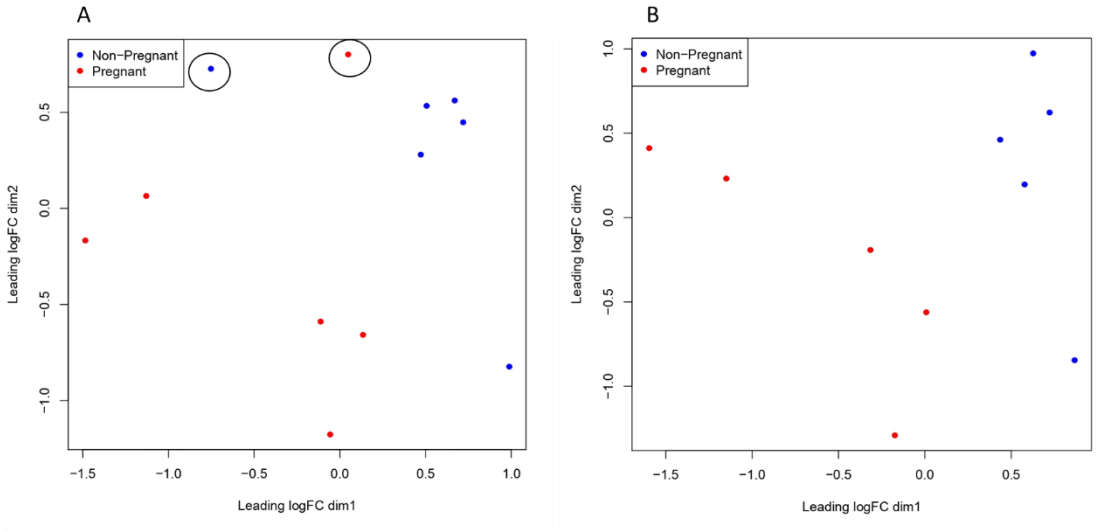
The animal model has been previously described (Chapter 2). Briefly, Nelore heifers (n=29), from Pirassununga Campus of the University of São Paulo in Brazil (Latitude - 21.953833; Longitude – 47.453143), with a body condition score between 3 and 4 (scale 1-5, were 1 mean extremely thin and 5 extremely fat), were submitted to pre-breeding examinations approximately 45 days before the experiment to ensure that all heifers were pubertal (presence of CL and a developed reproductive tract), as indicated by (Holm et al., 2009).

The animals were subjected to an eight day estrous synchronization protocol for TAI based on estradiol (1mg) and P4 (1g) (Chapter 2). All heifers were artificially inseminated (AI) on Day 0 [D0] by a single operator using thawed semen from a single sire. On D18 (initiation of implantation), 30mL was collected from the jugular vein for isolation of PBMC (Chapter 2). Briefly, PBMCs were isolated using a Ficoll gradient protocol. Whole blood was mixed with an equal volume of PBS, and the solution was layered onto 15mL of Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden), placed in a 50mL conical tube, and then centrifuged at 1100 g for 30 min 20°C to obtain the buffy coat. Animals were retrospectively assigned to either P and NP groups based on the presence or absence of a embryo heartbeat at D28. Six PBMC samples from each of the confirmed P and NP heifers were subjected to RNA sequencing analysis, with the Illumina protocol using a HiSeq 2500 Sequencer (Illumina) (Chapter 2).

4.2.2.2 Pathways overrepresented in PBMCs from confirmed pregnant compared to non-pregnant animals

Sequencing data generated from PBMC RNA were subjected to quality control and alignment against the *Bos taurus taurus* reference genome were performed according to our recent publication (Chapter 2). Two animals (NP 5606 and P 5846) considered outlier by MDS plot (Figure 11A) were excluded of the analysis, resulting in five animals per group. Therefore, pregnant (n = 5) and non-pregnant (n = 5) heifers were subjected to differential expression analysis using edgeR version 3.8 (Robinson et al., 2010), using the "Classic Exact Test" method with an FDR threshold of 0.05. Enhanced Volcano (https://bioconductor.org/packages/release/bioc/html/EnhancedVolcano.html) and heatmap.2 (https://www.rdocumentation.org/packages/gplots/versions/3.0.1.1/topics/heatmap.2) were used for summarizing the overall DE result on both volcano plot and heatmap charts. All analyses were run under the R environment version 3.5.2. All read sequences (raw files and processed files) and an overview of this data has been deposited in NCBI's GEO and is accessible through GEO Series accession number GSE136102.

Figure 11 – Multi-Dimensional Scaling (MDS) plot



Source: (ROCHA, C. C., 2019)

Description: The figures represents a multidimensional reduction analyses using a MDS plot summarizing 6 pregnant (red) and 6 non-pregnant (blue) animals (panel A) or 5 pregnant (red) and 5 non-pregnant animals (blue) (panel B). The black circles are in the two outlier animals that were excluded to create the MDS in panel B.

The list of DEGs, were subjected to down-stream analysis to determine overrepresented pathways and GO associated with PBMCs from confirmed P and NP animals on D18. Overrepresented ontologies were determined using BinGO (Maere et al., 2005) in Cytoscape version 3.7.1 (Shannon et al., 2003, Smoot et al., 2011) using *Bos taurus taurus* annotation from the GO consortium (geneontology.org). The pathways were assessed through the KEEG pathways in the DAVID Bioinformatics Resources (Huang et al., 2008). Both enrichment analyses adopt the Hypergeometric Test along with the Benjamini & Hochberg P-value adjustment method. The "bubble" chart depicting all enriched KEGG pathways was generated using matplot lib in Python 3.6, where red circles indicate pathways that passed the adjusted p-value threshold (< 0.05). Circle sizes correspond to the proportion of DEGs within the pathway. The significant impact of the DEGs on signaling pathway was accessed by analysis of PRS as described before previously (Tarca et al., 2008). This topology-based pathways analysis was conducted using ToPAseq package (Ihnatova and Budinska, 2015).

4.2.3 Experiment 2: Assessment of putative candidate pathway in an independent set of samples

4.2.3.1 Animal model

Twenty four Holstein females (8 heifers aging 15 months and 16 cows between 3-6 months postpartum), based at the University of Liverpool Veterinary School, had presumptive estrus determined following activity monitoring (pedometer), and were AI (D0) 12 hours later by a single operator using frozen-thawed semen of six different Angus sires. Three mL of blood was collected of the tail vein via Tempus^{TM} blood RNA tubes (Applied Biosystems, Woolston, UK) on D16 (day of pregnancy recognition), D19 (initiation of implantation) and D23 (post estrous cycle). The blood tubes were immediately inverted 10 times and placed on ice until storage at −80°C prior to RNA extraction. On D30, the animals were retrospectively assigned to either confirmed as P or NP by ultrasound examination and then, classified in each group by the parity category following, P-HF, P-C, NP-HF and NP-C.

4.2.3.2 RNA extraction and RT-qPCR analysis

Samples were thawed at room temperature and tRNA extracted using Tempus^{TM} spin RNA isolation kit (Applied Biosystems, Woolston, UK) in accordance to manufacturer's guidelines. Initially, the total blood was transferred to 50 mL Falcon tubes, diluted with 3mL of PBS, passed through the column and submitted to consecutives centrfugations to wash the tRNA. The tRNA was eluted with 100 µL of DNase RNase free water. Before the reverse transcription, the isolated RNA was treated with DNA-free kit (Invitrogen, Graiciuno, Lithuania) for genomic DNA contamination as per manufactures instructions. Then, the concentration of RNA was evaluated using spectrophotometry (NanoDrop, Microvolume Spectrophotometer and Fluorometer, Thermo Fisher Scientific, UK) and absorbance ratios values (260/280) ranged between 1.9 and 2.1. cDNA was synthesized from 35 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Graiciuno, Lithuania). A master mix (10µL) containing primers, enzymes and deoxynucleotides were added to 10 µL of samples with 35 ng total. Samples were incubated at 25°C for 10 minutes and then at 37°C for 2 hours, subjected

to reverse transcriptase inactivation at 85°C for 5 minutes, and stored at −20°C until PCR analysis. The final reaction was diluted to 2.5ng and this cDNA was used as template for each PCR reaction.

Analyses of relative abundance of transcripts were performed (n = 4 P-HF, 4 NP-HF, 6 P-C and 10 NP-C) using SYBR Green I Master (Roche, Indianapolis, United States) for the amplification reactions in a LightCycler 480 instrument II 384 well (LightCycler 480 System, Roche). Samples were submitted to pre-incubation at 95°C for 5 minutes, amplification 45 cycles at 95°C for 10 seconds, 53°C for 10 seconds, 72°C for 20 seconds, and then melting curve with 95°C during 5 seconds, 65°C 1 minute and continuous increase to 97°C. The optimized primer pairs were designed based on the mRNA sequence of target genes obtained from the RefSeq database, on Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and the specificity of the primer were checked by BLAST (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Details of primers were provided on Appendix E. In order to select reference genes, the Normfinder Microsoft Excel applet was used (Andersen et al., 2004). The *GAPDH* and *ACTB* were the most stable genes, therefore, were selected as reference genes among the others evaluated (*RPL19* and *PPIA*). Determination of quantification was obtained after normalization of the target genes expression values (Cq values) by the geometric mean of the endogenous control expression values (Pfaffl, 2001). A list with all target genes evaluated and which part of the pathway they being was available on Table 4.

Table 4 - Target genes evaluated in Experiment 2. All genes from the PRRs pathways and were up regulated on P group from RNAseq results of Experiment 1.

Membrane Receptors	Phagosome	Cytosolic Receptors	Final Product
TLR4	*NOD2*	*IRAK3*	*IL1β*
TLR2	*NLRP1*	*DDX58*	*CASP1*
CD14	*NLRP3*		*IRF3*
MyD88	*NLRP5*		*IRF7*
	NLRP12		
	NLRC4		
	NLRX1		
	CARD9		

4.2.3.3 Statistical analysis

The data were evaluated for detection of outliers using Dixon test and the significant ($P<0.05$) outliers detected were excluded from the analyses. The transcripts abundance was

analyzed using split-plot ANOVA MIXED of SAS software (Version 9.2; SAS Institute) procedure with a REPEATED statement to account for the autocorrelation between sequential measurements, and considering the fixed effects of pregnancy status group (P or NP), parity category (HF or C), day (D16, D19 and D23) and their interaction. Animal was used as a random effect. The data that were not normally distributed according to the Shapiro-Wilk test were transformed to natural logarithms, rank and square root. However, the data in the graphs is presented as untransformed values.

4.2.4 Experiment 3: To determine if selected transcripts are regulated by IFN-τ by *in vitro* model

To determine if selected transcripts that were differentially expressed in Experiment 2 were regulated by IFN-τ, we used an *in vitro* system. Endometrial explants were isolated from the reproductive tracts of female cows (n=3) sourced from a local abattoir. The reproductive tracts were collected at slaughter and transported to the laboratory on a cooler box within 1 hour. Endometrial intercaruncular sections length about 5cm were cut out from the middle part of the uterine horn ipsilateral to CL from animals at the early luteal stage of the estrous cycle, as determined by Ireland et al. (1980). Intact endometrial explants were washed with PBS and placed in culture vials containing 2 mL of mammalian cell medium RPMI 1640 (Gibco, #11835-063) supplemented with 10% charcoal-stripped FBS (PAA, Cambridge, UK #A151092424) and 1% ABAM (Sigma-Aldrich, Hilden, Germany #A5955) and preincubated *in vitro* in a humidified atmosphere of air with 5% CO2 at 37.5°C for 1 hour. Before the incubation, the explants were washed with PBS and then six explants of each animal were incubated *in vitro*. Explants were exposed to the following conditions: 1) 20µL of control medium; 2) 20µL of PBS (Vehicle group [where the IFN-τ was eluted]); or 3) 1µg/ml of recombinant ovine IFN-τ, for either 2 or 24 hours (n=3 explants/treatment/time). The times were selected based on the early and late IFN-τ response (Forde et al., 2012). Recombinant ovine IFN-τ was obtained from *Saccromyces* and re-suspended in PBS (Van Heeke et al., 1996). Two and 24 hours after the treatment the explants were washed on PBS and immediately frozen in a 2 mL tube on −80°C until the RNA extraction. The IFN-τ concentration was previously determined by our group (Forde et al., 2011).

4.2.4.1 RNA extraction and RT-qPCR analysis

The total RNA extraction was performed using miRNeasy Mini kit (Quiagen, Hilden, Germany) in accordance to manufacturer's guidelines included the DNase treatment. The explants were homogenized in a homogenizer for 1 minute with QIAzol lysis reagent, followed for a chloroform gradient, where the upper aqueous phase was transfer to the column and submitted to successive washing with wash solutions. The apparatus to homogenize was cleaned with 70% ethanol and RNaseZAP (Sigma-Aldrich, Hilden, Germany) among each sample. The tRNA was eluted with 50 µL of DNase RNase free water. Concentrations and purity of RNA in extracts were evaluated using spectrophotometry (NanoDrop Microvolume Spectrophotometer and Fluorometer, Thermo Fisher Scientific, UK) and absorbance ratios values (260/280) ranged between 1.97 and 2.06. cDNA was synthesized from 500ng of tRNA follow the same principles as Experiment 2. The final RT reaction was diluted to 5ng and this cDNA was used as template for each PCR.

Three target genes involved on the cytoplasmic PRRs (*NLRC4*, *NLRP3* and *NOD2)* on Experiment 2 were selected to evaluate the expression on endometrium explants. Two reference genes (*RPL19* and *PPIA*) were used with the same criteria as Experiment 2.

4.2.4.2 Statistical analysis

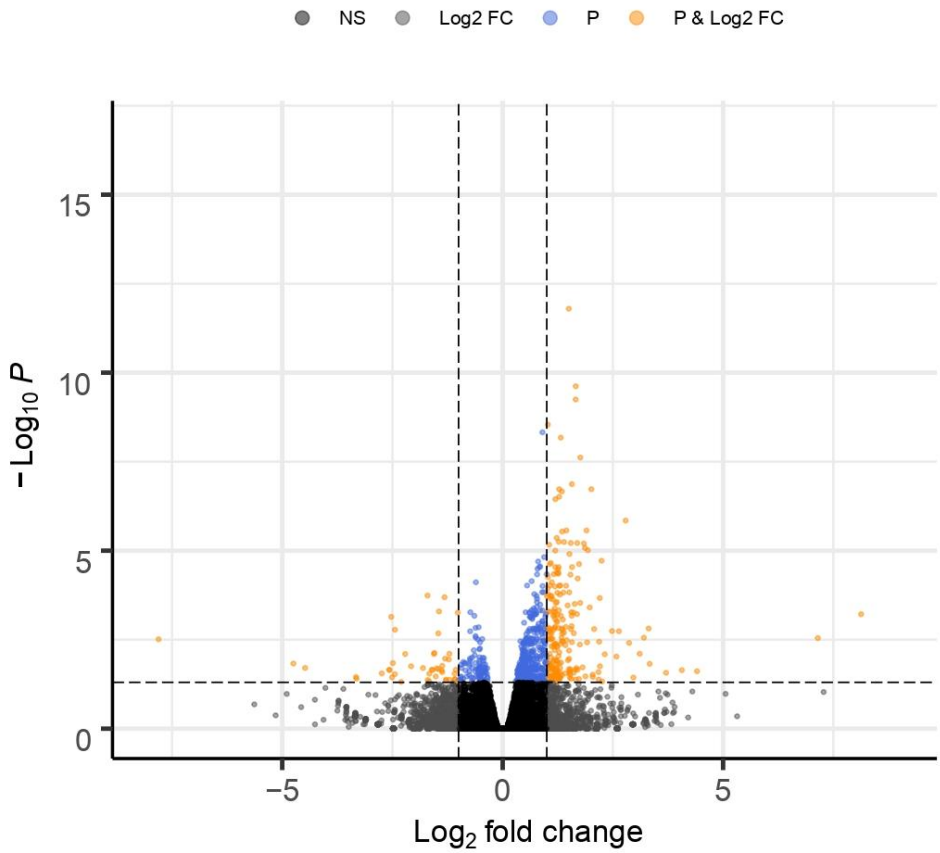
The data were evaluated for detection of outliers using Dixon test and the significant ($P<0.05$) outliers detected were excluded from the analyses. The transcripts abundance were analyzed using split-plot ANOVA MIXED of SAS software (Version 9.2; SAS Institute) procedure and considering the fixed effects of treatment group (Control, Vehicle or IFN-t), hour (Hour 2 or Hour 24) and their interaction. Explant of each animal within hour was used as a random effect. The data that were not normally distributed according to the Shapiro-Wilk test were transformed to natural logarithms, rank and square root.

4.3 RESULTS

4.3.1 Experiment 1

The Figure 11B shows a multi-dimensional scaling (MDS) plot, how homogenous groups overall contribution to transcriptomic signature. A total of 724 DEGs were found between P and NP heifers, of which 585 were up regulated on P group and 139 down regulated on P group (Figure 12 and Appendix F). The 50 most significance DEGs found between P and NP contrast can be accessed on Figure 13.

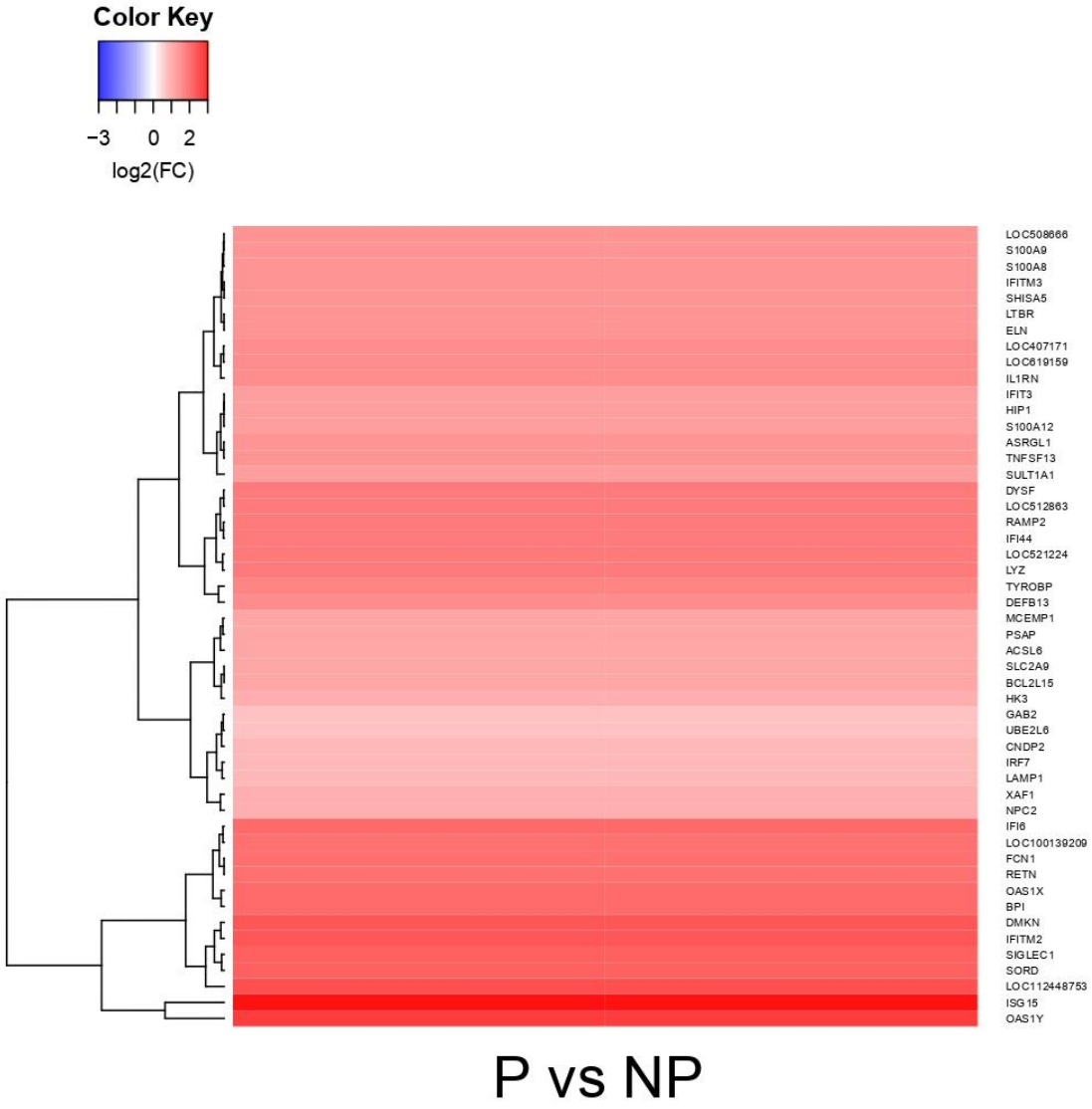
Figure 12 - Volcano Plot



Source: (ROCHA, C.C., 2019)

Description: The image shows pregnant (n=5) and Non-pregnant (n=5) gene expression, in terms of the differentially expressed genes. FDR < 0.05.

Figure 13 - Heat map constructed showing the 50 genes with lowest FDR for the comparison between Pregnant (P; n = 5) and Non-pregnant (NP; n = 5) heifers.



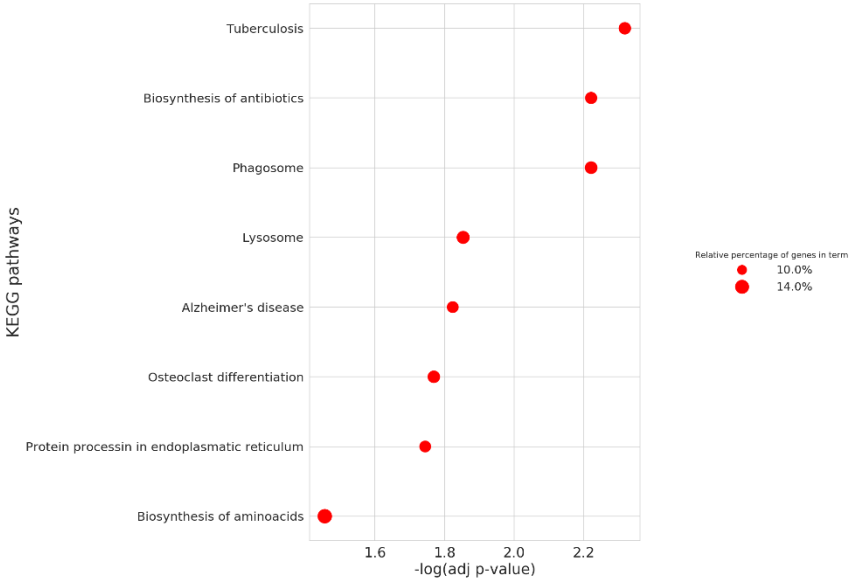
Source: (ROCHA, C.C., 2019)

Description: The colors in the map display the relative standing of the reads count data; blue indicates a count value that is lower than the mean value of the row while red indicates higher than the mean. The shades of the color indicate distance from each data point to the mean value of the row.

Given the limited number of DEGs down regulated in pregnant heifers, no overrepresented GO or overrepresented pathways were identified. In contrast, 215 GO terms were overrepresented

in the list of the up regulated DEGs identified between P compared to NP group, where 159 belong to the biological process, 30 to cellular components and 26 to molecular function. Detailed GO terms associated with up-regulated genes in the P group were provided in Appendix G. In the KEGG pathways analysis, eight pathways were overrepresented in the list of DEGs increased in expression in the immune cells of pregnant heifers on Day 18 including Tuberculosis (20/181), Biosynthesis of antibiotics (22/206), Phagosome (18/158), Lysosome (15/126), Alzheimer's disease (18/178), Osteoclast differentiation (15/134), Protein processing in endoplasmatic reticulum (17/169) and Biosynthesis of amino acids (10/71) (Figure 14 and Appendix H).

Figure 14 - KEGG pathways-based gene enrichment analysis on the list of 585 up-regulated genes in D18 pregnant heifers.



Source: (ROCHA, C.C., 2019)

Description: The "bubble" chart depicts all significant KEGG pathways (adjusted p-value < 0.05, or –log adj. p-value > 1.3), where red circles' size indicates the proportion of DEGs within the pathway in comparison to the total number of genes in the same pathway.

In an attempt to gain insight into the underlying biology of DEGs between P and NP heifers, a topology-based pathway analysis approach was used. The PRS revealed which pathways were more significant based of the main receptors of that and not about the number of genes like KEGG analysis (Tarca et al., 2008). In this evaluation, the top ten highest pathway regulation were involved with the immune response their names and respectively scores (nPRS) were Legionellosis

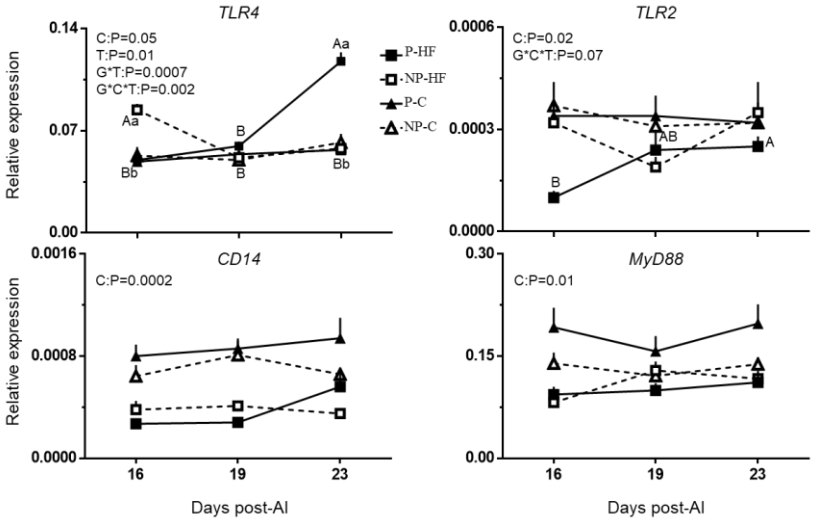
(46.5), Osteoclast differentiation (35.4), Pertussis (34.7) Systemic lupus erythematosus (32.3), Tuberculosis (27.8), Staphylococcus aureus infection (27.6) NOD-like receptors signaling pathway (27.3), Amoebiasis (23.7), Salmonella infection (21.9) and Influenza A (21.8) all significant PRS pathways (n = 37) their respectively nPRS and p value can be accessed on Appendix I, while the most significant pathway can be visualized on Appendix J.

4.3.2 Experiment 2

In order, all results presented in that experiment is about DEGs selected through Experiment 1. The probabilities for a group effect, a time effect, parity category effect, and their interactions resulted from the factorial analyses were shown in the figures, and the probabilities for differences in discrete endpoints are given in the text. When the parity category effects were not significant, the graph was designed with heifers and cows in the same P group. A total of 37.5 % (6/16) cows and 50% (4/8) heifers were detected pregnant on day 30 by ultrasonography.

As the PRRs was the most representative in the top ten nPRS and significant KEGG pathways in Experiment 1. Thus that pathway was the main investigation focus on Experiment 2. For that, we chose all representative DEGs of that pathways, that were up regulated in the RNAseq results and investigated in more depth and at different time-points. Four genes involved with the PRRs surface receptors were evaluated. For the *TLR4*, the main effects of category, time, and the interactions of group-by-time and group-by-category-by-time were significant ($P \leq 0.05$). The triple interaction was primarily for a decreased ($P<0.05$) relative expression in the NP-HF between D16 and D19 and an increased ($P<0.05$) relative expression between D19 and D23 in the P-HF, which reflected greater ($P<0.05$) relative expression on D16 for NP-HF than P-HF, and on D23 for P-HF than NP-HF (Figure 15). For the *TLR2* the main effects of category and the interaction of group-by-category-by-time were significant ($P<0.05$) or approached ($P<0.1$) significant, respectively (Figure 15). The triple interaction was primarily for an increase ($P<0.05$) on relative expression in the P-HF group between D16 and D19, while no difference ($P>0.1$) was detected among the days for the NP-HF group. In addition, when the the NP-HF and P-HF groups were compared in each day, no significant ($P>0.1$) effects were detected. For *CD14* and *MyD88*, only an increased ($P<0.05$) relative expression was detected in heifers compared to cows, regardless the pregnancy status (Figure 15).

Figure 15 - Mean ± SEM for relative expression of whole blood by qPCR of the surface receptors belong to the PRRs pathway



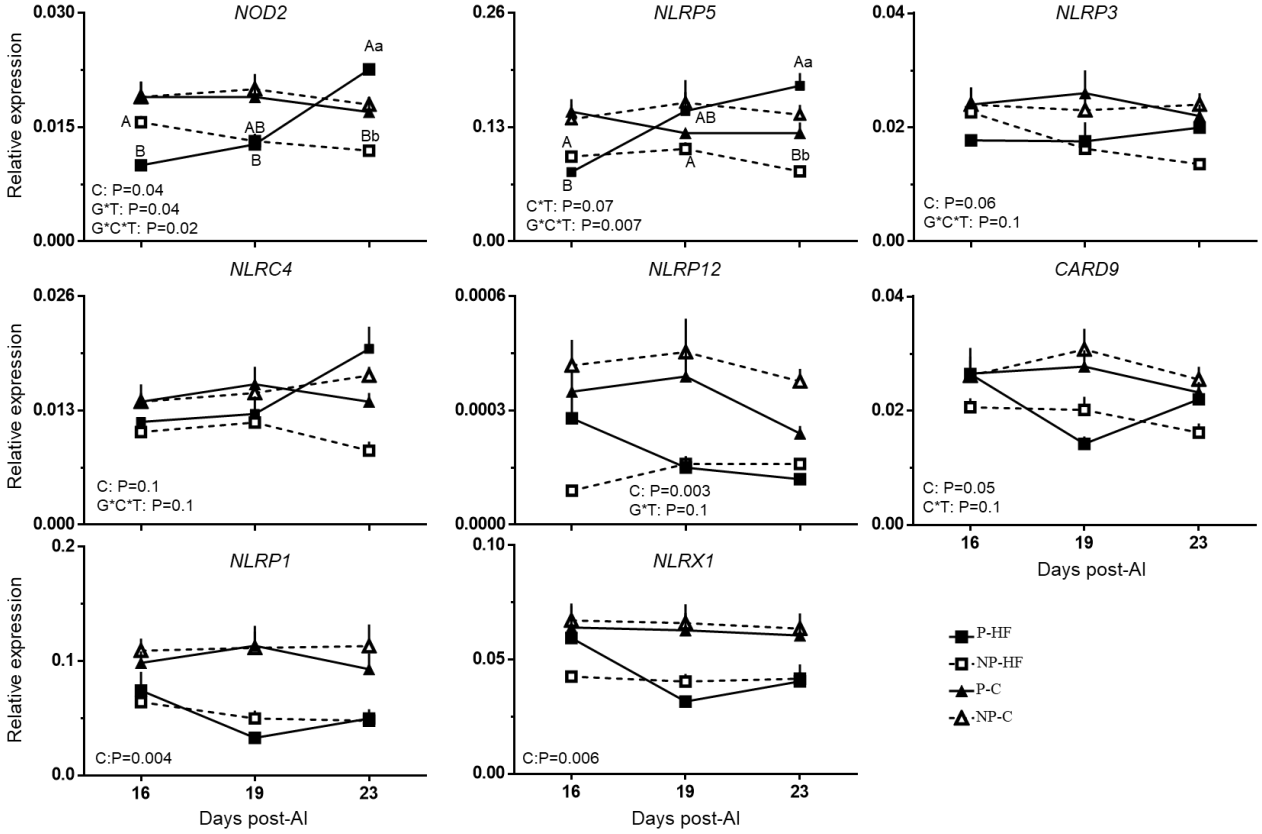
Source: (ROCHA, C. C., 2019)

Description: The figure represents the relative expression of pregnant and non-pregnant heifers (P-HF [n = 4] [closed square] and NP-HF [n = 4] [opened square]) and cows (P-C [n = 6] [closed triangle] and NP-C [n = 10] [opened triangle]). Probabilities are shown for significant main effects of group (G), time (T), category (C), interaction for group-by-time (G*T), for group-by-category (G*C), for category-by-time (G*T) and for group-by-category-by-time (G*C*T). There were no other interaction. [ABCD] Means without a common letter within a group in the same category indicate differences (P<0.05) between days. [ab] Means difference between the groups in the same parity category on specifics days.

Eight genes linked with NOD/NLR receptors pathway were explored (Figure 16). For *NOD2*, the main effects of category, the interaction of group-by-time and group-by-category-by-time were significant (P<0.05). The triple interaction was primarily for an increase on relative expression between D19 and D23 in the P-HF group, which reflected in greater (P<0.05) relative expression in P-HF than NP-HF on D23. For the *NLRP5* the interaction category-by-time and group-by-category-by-time were significant. The triple interaction was primarily for an increase (P<0.05) on relative expression between D19 and D23 in P-HF and a decreased (P<0.05) between D19 and D23 in NP-HF, that reflected in greater (P<0.05) relative expression in the P-HF than the NP-HF group on D23. For the *NLRP3* and *NLRC4* the main effects of category and the triple interaction of group-by-category-by-time were significant (P<0.05) or approached (P≤0.1) significant. Although no difference (P>0.1) between days was observed in each group when the

categories were evaluated separately, the relative expression of *NLRP3* and *NLRC4* was increased ($P<0.05$) in heifers than cows, regardless the pregnancy status. For the *NLRP12* and *CARD9*, the main effects of category and the interaction group-by-time were significant ($P<0.05$) or approached of significant ($P\leq0.1$), respectively, but no significant ($P>0.1$) effects were observed when groups were compared in each time-point. For the *NLRP1* and *NLRX1* only an increased ($P<0.05$) expression in heifers compared to cows was observed, regardless the pregnancy status.

Figure 16 - Mean ± SEM for relative expression of whole blood by qPCR of the cytosolic NOD-like receptors belong to the PRRs pathway.



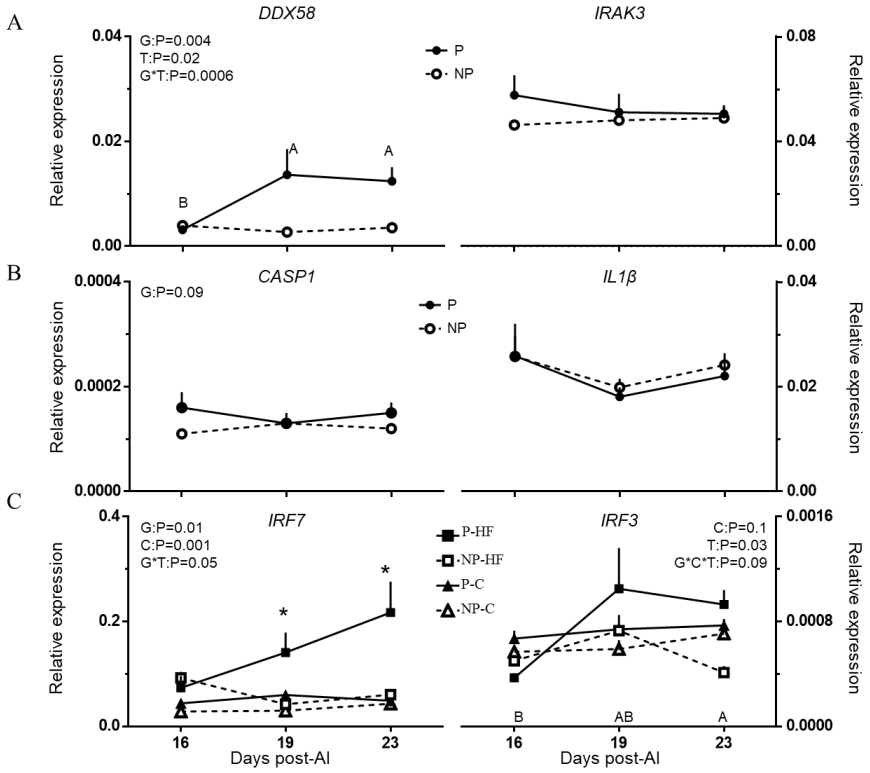
Source: (ROCHA, C. C., 2019)

Description: the figure represents the relative expression of pregnant and non-pregnant heifers (P-HF [n = 4] [closed square] and NP-HF [n = 4] [opened square]) and cows (P-C [n = 6] [closed triangle] and NP-C [n = 10] [opened triangle]). Probabilities were shown for significant main effects of group (G), time (T), category (C), interaction for group-by-time (G*T), for group-by-category (G*C), for category-by-time (G*T) and for group-by-category-by-time (G*C*T). There were no other interaction. [ABCD] Means without a common letter within a group in the same category indicate differences (P<0.05) between days. [ab] Means difference between the groups in the same days.

Other cytosolic receptors belonging to the PRRs family were analyzed (Figure 17A). For the *DDX58* the main effects of group, time, and interaction group-by-time were significant (P<0.05). The interaction was primarily for an increase (P<0.05) on the relative expression in the P group between D16 and D19. However, for *IRAK3* no significant (P>0.1) effects were detected. In addition, two important regulators of the inflammatory process stimulated by PRRs were also evaluated (Figure 17B). For the *CASP1*, only an increased (P<0.05) relative expression was

detected in pregnant females compared to non-pregnant. For *IL1β,* no significant (P>0.1) effects were detected.

Figure 17 - Mean ± SEM for relative expression of whole blood by qPCR



Source: (ROCHA, C. C., 2019)

Description: The panel A represents other cytosolic receptors out the NOD-like, while the panels B and C was about the main transcripts of the PRRs pathway results. The results of Panel A and B represents pregnant (P [n = 10] [●]) and non-pregnant (NP [n = 14] [○]) cattle, regardless the category, where ^{ABCD} Means indicate differences (P<0.05) between days. While in Panel B the results were for pregnant and non-pregnant heifers (P-HF [n = 4] [■] and NP-HF [n = 4] [□]) and cows (P-C [n = 6] [▲] and NP-C [n = 10] [△]). Where, * means differences (P < 0.05) between groups regardless category during specific days. AB means above the X axis, represent differences between days regardless group and category. For all graphs the probabilities are shown for significant main effects of group (G), time (T), category (C), interaction for group-by-time (G*T), for group-by-category (G*C), for category-by-time (G*T) and for group-by-category-by-time (G*C*T). There were no other interaction.

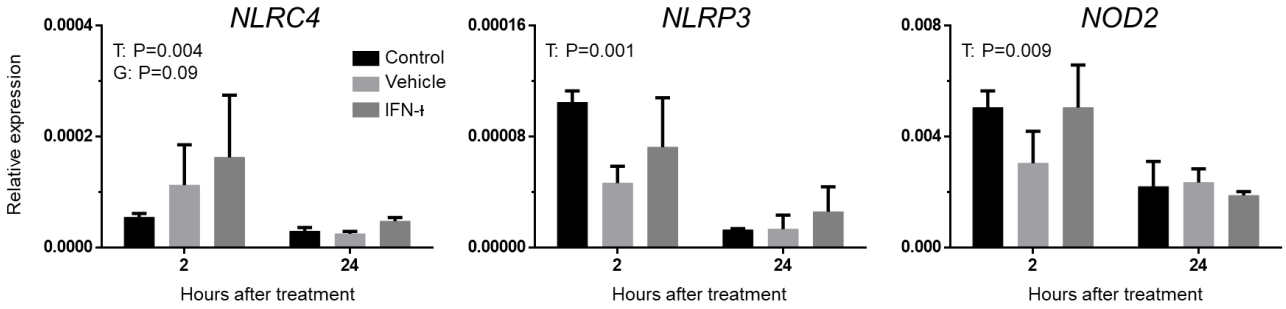
The main IRF stimulated by the PRRs were explored too (Figure 17C). For the *IRF7* the main effects of group, category and interaction group-by-time were significant (P<0.05). The interaction was primarily for an increased (P<0.05) relative expression on D19 and 23 in the P-HF

and P-C groups. For *IRF3* the main effects of category, time and triple interaction group-by-category-by-time were significant (P<0.05) or approached significant (P≤0.1). The triple interaction was explored, but no significant (P<0.05) results were detected between groups within a day or between days within a group or category. The time effect reflected an increase (P<0.05) in the relative expression between D19 and D23, regardless the group and category.

4.3.3 Experiment 3

Based on the results of Experiment 2, we chose to examine expression of three cytosolic receptors that were stimulated for the main membrane receptor with significant results (*TLR4*) in immune cells. Therefore, we chose to examine if they were regulated by the IFN-τ, the predominant molecule involved in the conceptus maternal interaction (Figure 18). For the *NLRC4* the main effects of group and time were approached significant (P<0.1) or significant (P<0.05), respectively. The time effect reflected a decrease on relative expression at 24 hours, regardless the treatment group. However, a significant difference was not detected between treatment groups, when they were compared regardless of the time. For the *NLRP3* and *NOD2*, only a decreased (P<0.05) relative expression of these genes at 24 hours of culture was detected, regardless the treatment group.

Figure 18 - Mean ± SEM for relative expression by qPCR of NOD-like receptors in endometrial explants



Source: (ROCHA, C. C., 2019)

Description: The figure represents endometrial explants (n = 3 explants/group/time) treated with culture medium (Control), PBS (Vehicle) and IFN-τ during 2 and 24 hours. Probabilities were show for significant main effects of group (G), time (T) and interaction of group-by-time (G*T). There were no other interaction. [ab] Means differences between the treatment in specifics times.

4.4 DISCUSSION

This study provided the chance to understand the biology of the conceptus stimuli on immune cells during the early pregnancy period. In addition, studies about the immune modulation during early pregnancy are still scarce and the large part of them are related to the major histocompatibility complex (Davies et al., 2000, Doyle et al., 2009). However, we revealed a novel pathway linked with the innate and adaptive immune response which were determinant during early pregnancy (Berkowitz et al., 1988). Our results, confirmed our hypothesis, that in early pregnancy the most overrepresented pathway is linked with the immune response and differentially expressed between nulliparous and parous cows. This was mainly indicated by the overexpressed PRRs pathways on PBMCs at day 18 of pregnancy and the parity influence on expression of several circulating transcripts related to immune response during early pregnancy (days 16 to 23).

In the Experiment 1, the removal of two clear outliers from the sample set identified a larger number of DEGs (increased 200%) compared with the previous study (Chapter 2). The different profile of these animals compared to the group they belong (P or NP) could be caused by an early pregnancy loss at the maternal recognition period in the outlier NP animal as all heifers were inseminated. Consequently, that exclusion allowed us to detect the PRRs pathway. We focused on PRRs as at least one member of that were present in some of the ten most overrepresented PRS identified in PBMC. Moreover, recent data have demonstrated that increased expression of PRRs and components of the pathway were important in mediating type 1 IFN responses in immune cells (Coutermarsh-Ott et al., 2016, Fekete et al., 2018).

During the pregnancy period evaluated in our study (days 16 to 23), important events happened in embryo development. After the embryo came in the uterus on day 4-5 post mating, and follows the development to an elongate structure, the bovine conceptus started the attachment process on day 19 (Forde and Lonergan, 2017). The embryo is an allogenic structure and have to prevent his rejection (Fair, 2015). Initially, the main molecule involved with that regulation is the IFN-τ (Hansen, 2007), that modulates the cells transcription. However, after de attachment period, the amounts of IFN-τ decreases and reaches basal concentrations until day 25 (Bazer et al., 1991). On day 25 the placentation begins and protects the conceptus of the immune system attacks by a physical barrier (Imakawa et al., 2017). In addition, during this transitory period (attachment and placentation) the binucleate cells play a fundamental role for conceptus immune protection, making

a barrier between deep endometrium and conceptus (Imakawa et al., 2017). The binucleate cells are the combination of the trophoblast and endometrial epithelial cells.

Our main changes in the circulating blood influenced by pregnancy occurred only on day 23 of pregnancy. Where, the *TLR4*, *TLR2*, *NOD2* and *NLRP5* expression increased in pregnant animals. However, this enhanced expression of the TLRs and NLRs occurred when a reduction on IFN-τ secretion is expected in ruminants (Hansen et al., 2017). Therefore, we suggested that the IFN-τ during early pregnancy plays a distinctive effect compared with the other type 1 IFN, down regulating that pathway among days 16 to 19 of pregnancy. In this regard, on day 23 the attachment has already started (Spencer et al., 2007) and other protective mechanisms beyond the IFN-τ, as the presence of the binucleate cells start to play an important role in pregnancy establishment (Imakawa et al., 2017). However, changes on relative expression of TLRs and NLRs was only increased in nulliparous pregnant animals. In this regard, the PRRs belong to the innate and adaptive immune system (Tran et al., 2020). Thus, our suggestion was that cows that had already experienced others pregnancies, which soften the immune response by the adaptive immune system. Supporting that, we detected a parity effect on relative expression in 14 out of 18 genes evaluated. In addition, the results of experiment 3 showed that besides the culture involvement, the IFN-τ does not stimulate that pathway in the cytosolic receptors of endometrium cells. Thus, we could not prove the involvement of IFN-τ in that pathway, which does not mean this not happen, as the cellular expression response variates among the cellular types.

The *TLR4* is a founding member of the TLRs family (Thompson et al., 2011). When the bacterial LPS, binds, makes a complex TLR4-LPS-CD14 (Kawai and Akira, 2011). In contrast, during viral infections the *TLR4*, transmit signals through *MyD88* (Dai et al., 2016). The *MyD88* has the TIR domain (Muzio et al., 2000) which is regulated by type 1 IFN during viral infections (Wang et al., 2017). Therefore, we hypothesized that *TLR4* and *MyD88* would be modified similar, but the *TLR4* profile is only similar to changes in *NOD2* and *NLRP5*. Those finds indicated that the synergic collaboration among the PRRs between a cytosolic (NLRs) and membrane receptors (TLRs) already reported in viral response (Coutermarsh-Ott et al., 2016), also occurs during early pregnancy. The NLRs work associated to domains to destroy the pathogen in the phagosome structure (Ip et al., 2005). In our study, only the *CARD9* domain was detected as a DEG in PBMCs at day 18 of pregnancy, but the changes in relative expression of *CARD9* was different compared

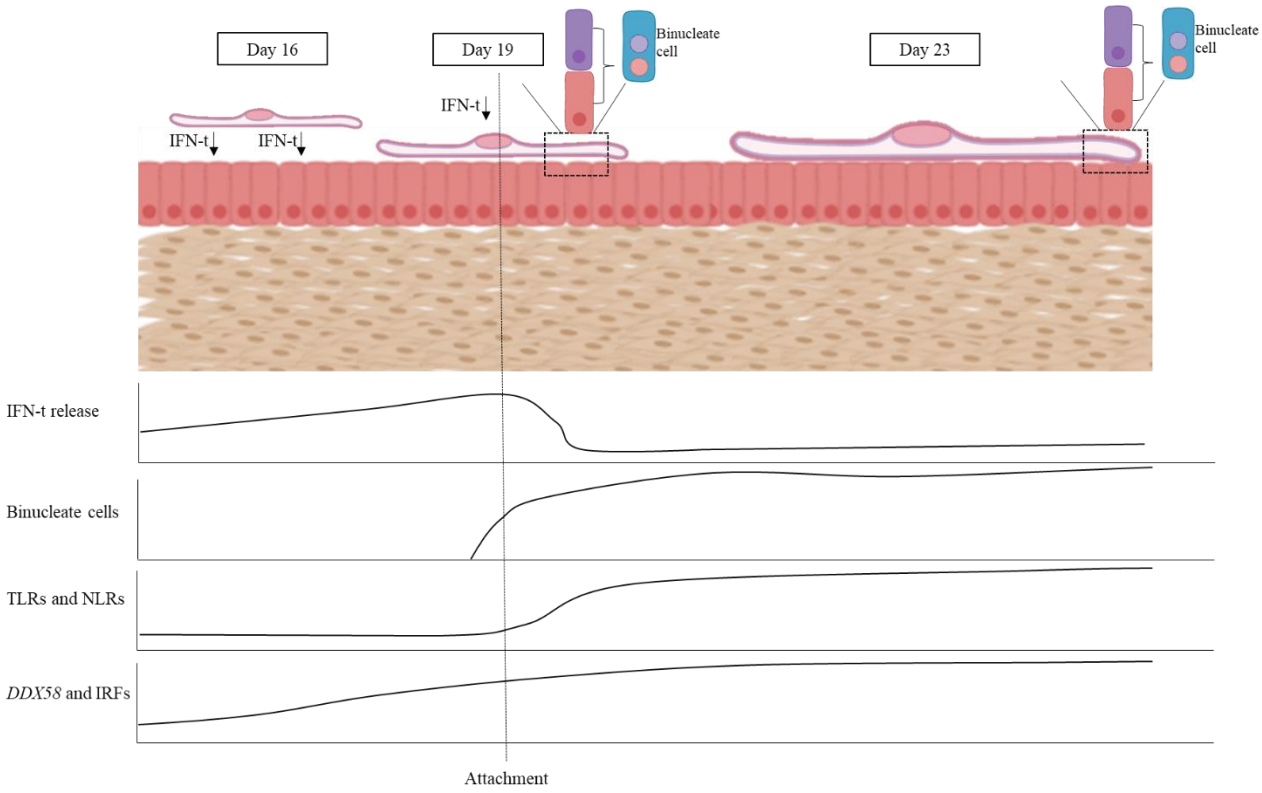
to the NLRs. The possible explanation for this disruption between *CARD9* and NLRs expression is that besides allogenic, the conceptus does not produce molecules like PAMPs, that are needed for induction of phagocytose. Thus, the pathway is stimulated but the phagosome formation is not required. To support that, the main function of CARD domain is to induce maturation of CASP1 followed by IL1β maturation (Maharana, 2018). In our study, the *CASP1* expression increased over days 16 to 23 in pregnant animals, but *IL1β* expression was not affected by pregnancy status.

We also evaluated other cytosolic receptors, the *IRAK3* and *DDX58*. The significant effects found for *DDX58* was earlier than other PRRs. In this regard, the *DDX58* has been considered one ISG in the endometrium (Forde et al., 2011). Thus, we can suggest, that the *DDX58* stimulation was by IFNAR as the other ISGs and not by PRRs. Therefore, because the different profiles for both cytosolic receptors (*DDX58* and *IRAK3*) compared to TLRs and NLRs, we proposed that the immune control until day 23 of early pregnancy in immune cells is only through TLRs and NLRs. The *IRF7*, agreed with the *DDX58* profile in our study. To support that, the *IRF7* and *IRF3* were considered ISGs up regulated on day 16 in the cow pregnant endometrium (Forde et al., 2011), but in our study the *IRF3* increased during the days regardless the pregnancy status and parity. However, the *IRF3* is not the most stimulated IRF during early pregnancy. Other IRFs such as *IRF9*, *IRF1* and *IRF2* have been reported as the main IRF transcripts stimulated by IFN-τ in ruminants (Shirozu et al., 2016, Hansen et al., 2017).

For *TLR4*, a greater expression was also found in non-pregnant nulliparous on D16 compared to pregnant nulliparous. That difference, reiterates the influence of the pregnancy/IFN-τ in that pathway. Thus, our running hypothesis is that non-pregnant nulliparous were not previously exposed to the allogenic conceptus and the PRRs pathway has not been inhibited by IFN-τ, but alterations in the estrous cycle, such as hormone variation, may stimulate the immune system. In addition, in our first study (Chapter 2) none of all these PRRs genes were considered DEGs. Thus, we believed that the non-pregnant animal excluded has experienced one failed pregnancy. Consequently, is possible that the PRRs pathway was up regulated on D18 in that heifer by the conceptus presence, but not in the others. To support our hypothesis, the main cytokine product of the TLRs and NLRs pathways is the *IL1B*, which has to be down regulated during the elongation period, because is detrimental to the trophoblast cells (Mosmann et al., 1986).

In summary, our study was the first to evaluate the profile expression of the PRRs pathways during early pregnancy in immune cells in cattle. The transcriptional changes in the blood of pregnant and non-pregnant cattle, suggested that pregnancy stimulates the PRRs pathways in the immune cells by TLRs and NLRs, regardless the subspecies. Also, heifers have a different immune response compared to cows. With these finds, we created a hypothetical model about the immune regulation in heifers. Thus, in *Bos taurus taurus* heifers, during the expected decrease of IFN-τ (day 23) the expression of PRRs is unblocked. The conceptus stills allogenic at the IFN-τ decrease stimulated the PRRs genes (NLRs and TLRs) as a physiologic immune response. However, the rejection does not happen, because the binucleate cells already developed could protect the conceptus (Figure 19). Once, as cows have already experienced pregnancy, the adaptive immune system softs the signals on day 23 of pregnancy similar to non-pregnant animals. The finds of herein study elucidates the different immune responses between nulliparous and parous females, which add valuable information to mitigate the mechanisms involved in the immune tolerance of the conceptus during early pregnancy. However, more studies are needed, specially, to understand the direct action of IFN-τ or the conceptus presence on the immune system in cattle.

Figure 19 – Schematic representation of the hypothetical model how the immune system works in *Bos taurus taurus* heifers during early pregnancy



Source: (ROCHA, C. C., 2019)

Description: The image illustrates a hypothetical model about the relationship among the immune system response, embryo development and biology events of early pregnancy. During the expected decrease of IFN-τ (day 23) the expression of PRRs is unblocked. The conceptus stills allogenic at the IFN-τ decrease stimulates the PRRs genes (NLRs and TLRs) as a physiologic immune response. However, the rejection does not happen, because the binucleate cells already developed can protect the conceptus. In contrast, as *DDX58* and IRFs are considered ISGs, they are up regulated by other mechanism which allows the increase before TLRs and NLRs.

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5 FINAL CONSIDERATIONS

The modulation of the immune system during early pregnancy has been elected as the main factor for pregnancy establishment and an important cause of embryonic loss. In addition, detection of pregnancy as early as possible may contribute to improve the reproductive efficiency in cattle operations worldwide. Here we studied the immune cell response during early pregnancy in *Bos taurus indicus* and *Bos taurus tarus* females, using different type of samples, such as PBMC, PMN and whole blood.

In the first study described in Chapter 2, nine novel early pregnancy markers were detected and are indicated for use in further studies aiming to evaluate them as pregnancy predictors as early as day 20 of pregnancy. Therefore, new studies are needed to test the accuracy of each gene for pregnancy diagnosis in dairy and beef cattle, as well their use as pregnancy loss predictors. This can impact on improvement of the molecular based test using classic ISGs to detect pregnancy in cattle. The definition of an accurate early pregnancy diagnosis on about day 20 of gestation open new possibilities for improvement of reproductive efficiency in cattle operations, mainly, in countries where fixed-time programs are used. Also, the temporal transcriptional changes for the 20 genes evaluated in immune cells bring new opportunities for understanding the physiologic mechanisms and the involvement of immune cells during early pregnancy in ruminants.

In addition, the study of the transcriptional changes in the main pathways modified by the pregnancy establishment (Chapter 3) contribute for understand of the immune system response to the allogenic conceptus. The immune pathways studied here, has been studied well during viral infections, specially in humans. Therefore, this study is the first to show the involvement of one viral pathway response during early pregnancy. In addition, we could prove a different pathway of the innate immune system involved with early pregnancy. The differences in immune related transcripts between cows and heifers indicate that after the first parous the immune response is differently modulated in a subsequent exposure to the allogenic conceptus.

Still, the exact mechanisms involved in the differential expression of the large part of these genes presented here are not well known. First, new studies are need to elucidate what are the molecules secreted or induced by the bovine conceptus that cause these immunologic changes. In this regard, the development of *in vitro* studies for studying of the in immune cell response to conceptus secreted molecules, as IFN-τ are indicated. For example, PBMC and PMN culture systems can be used to evaluate the response on transcript and protein abundance to the IFN-τ

treatment. These latter results can also drive for a better selection of circulating transcripts for use as potential pregnancy markers in studies using a larger number of animals in field conditions.

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ATTACHEMENT - Proof of submission

Biology of Reproduction

Early pregnancy-induced transcripts in peripheral blood immune cells in beef heifers

--Manuscript Draft--

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Abstract:	Immune cells play a central role in early pregnancy establishment in cattle. We aimed to: 1) to discover novel early-pregnancy-induced genes in peripheral blood mononuclear cells (PBMC); and 2) to characterize the temporal pattern of early-pregnancy-induced transcription of select genes in PBMC and peripheral blood polymorphonuclear cells (PMN). Beef heifers were artificially inseminated (AI) on D0 and pregnancies were diagnosed on D28. On D10, 14, 16, 18, and 20, blood was collected for isolation of PBMC and PMN from heifers that were retrospectively classified as pregnant (P) or non-pregnant (NP). PBMC samples from D18 were submitted to RNA sequencing and 220 genes were differentially expressed between P and NP heifers. The temporal abundance profile of 20 select transcripts was compared between P and NP animals, both in PBMC and PMN. In PBMC, pregnancy stimulated transcription of IFI6 , RSAD2 , IFI44 , IFITM2 , CLEC3B , OAS2, TNFSF13B, DMKN and LGALS3BP as early as D18. Expression of IFI44 , RSAD2 , OAS2, LGALS3BP, IFI6 and C1R in PMN was stimulated in the P group from D18. The novel early-pregnancy induced genes discovered in beef heifers will allow both the further understanding of the role of immune cells during the pre-attachment period and the development of technologies to detect early pregnancies in beef cattle.

APPENDIX A- Spreadsheet with results of GO enrichment

The Appendix A is an excel file with the GO enrichment results. It can be accessed in the CD-room attached in that dissertation

Description: GO enrichment showing the overrepresented terms on biological process, molecular functions and cellular components with their respectively genes, of up-regulated DEGs on P group.

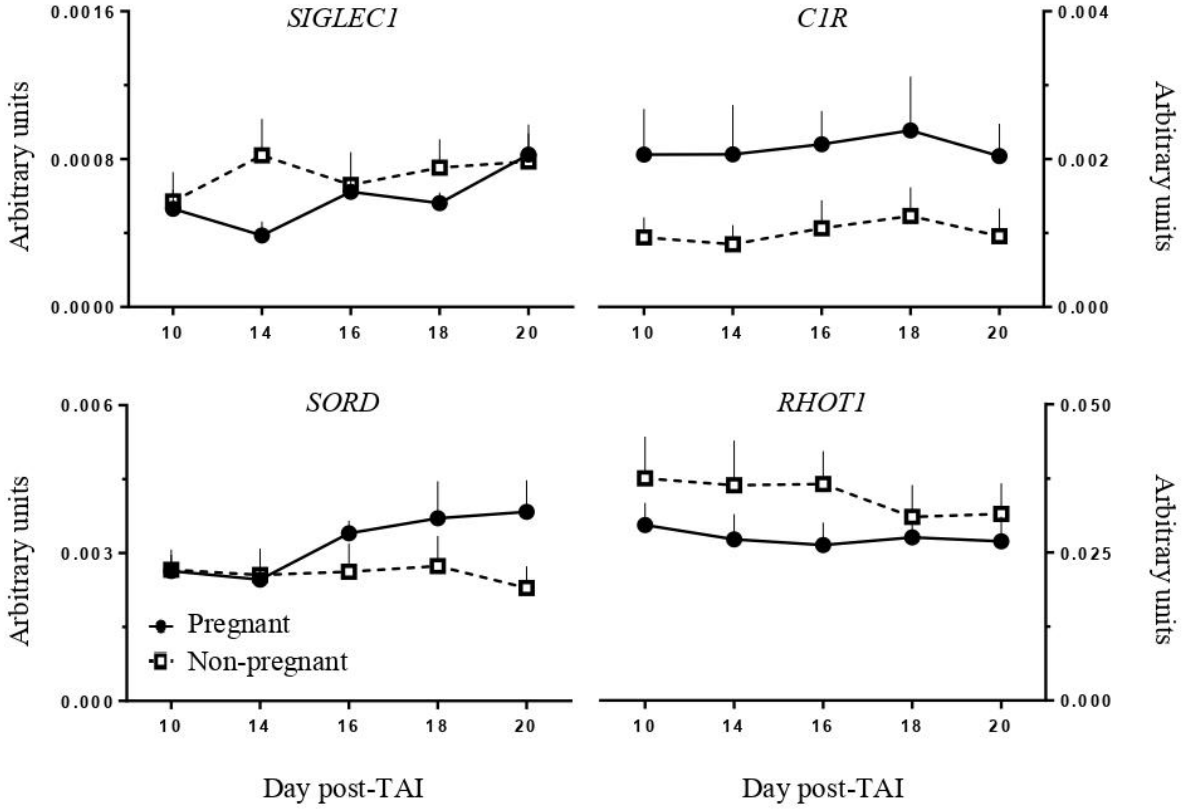
APPENDIX B - Spreadsheet with results of KEGG pathways analyses

The appendix B is an excel file with the KEGG pathways results. It can be accessed in the CD-room attached in that dissertation

Description: KEGG pathways showing all overrepresented pathways and their respectively genes of up-regulated DEGs on P group.

APPENDIX C – Figure of the genes without significance difference in PBMC

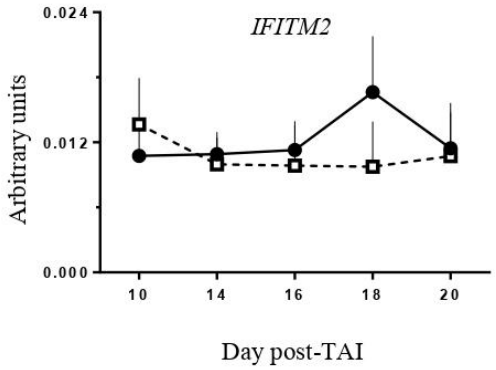
A figure with mean ± SEM for relative expression by qPCR on PBMC of the genes without a significant effects (P > 0.1) during statistical analysis: *SIGLEC1*, *C1R*, *SORD*, *RHOT1*, from days 10 to 20 post-TAI of pregnant (n = 6) and non-pregnant heifers (n = 6).



Source: (ROCHA, C. C. 2019)

APPENDIX D - Figure of the genes without significance difference in PMN

A figure with Mean ± SEM for relative expression by qPCR of *IFITM2* gene, that did not results in significate effects, from days 10 to 20 post-TAI of Pregnant (n = 6) and Non-pregnant heifers (n = 6).



Source: (ROCHA, C. C. 2019)

APPENDIX E – Primer sequences

Bovine specific oligonucleotide forward and reverse primer sequence (5'-3') and PCR product length.

Gene	Identification	Foward and Reverse sequences	Eficiency	Product Lenght
ACTB	NC_037352.1	5'CGCCATGGATGATGATATTGC	98	144
ACTB	NC_037352.1	3'AAGCCGGCCTTGCACAT	98	144
CARD9	NC_037338.1	5'CCAGGAGGATCTGGAGGATACC	88	150
CARD9	NC_037338.1	3'GTGCTGAAGGTCAAGACAGGAG	88	150
CASP1	NC_037342.1	5'AGACGGAGCTGATGTTGATACC	106	168
CASP1	NC_037342.1	3'AGGTCCGATGCTCTGAGTGAG	106	168
CD14	NC_037334.1	5'TGAACATTGCCCAAGCACACT	110	167
CD14	NC_037334.1	3'GAGACTGGGATTGTCAGACAGGT	110	167
DDX58	NC_037335.1	5'TCCGAACCAACAGAGACAGC	101	146
DDX58	NC_037335.1	3'TCTGCCTCTGGTCTGGATCA	101	146
GAPDH	NC_037332.1	5'GCCATCAATGACCCCTTCAT	100	141
GAPDH	NC_037332.1	3'TGCCGTGGGTGGAATCA	100	141
IL1B	NC_037338.1	5'CTCCGACGAGTTTCTGTGTGAC	112	152
IL1B	NC_037338.1	3'CAGAACACCACTTCTCGGTTCA	112	152
IRAK3	NC_037332.1	5'TCCGTCATCCGAACATACTGGA	98.9	149
IRAK3	NC_037332.1	3'GGCTGTGTCACCTACACACTG	98.9	149
IRF3	NC_037345.1	5'AAAGCTCAACTGACGGGAAGTG	102	149
IRF3	NC_037345.1	3'ATGGTCTGGCCTAAGTGTTGGG	102	149
IRF7	NC_037356.1	5'GTGACACGCCCATCTTTGACTT	89.7	151
IRF7	NC_037356.1	3'CAAGTAGATGGTGTAGTGCGGG	89.7	151
MyD88	NC_037349.1	5'GTGGTCTCTGACGAATACCTGC	102	151
MyD88	NC_037349.1	3'AGGATGCTGGGGAACTCTTTCT	102	151
NLRC4	NC_037338.1	5'CTTGGACCACTGTGGAGACCTA	100	151
NLRC4	NC_037338.1	3'GCAGGACATCCTCGTTCACATT	100	151
NLRP1	NC_037346.1	5'TGATGAAGGAGGTACAGCACGA	108	163
NLRP1	NC_037346.1	3'GGAGAATGAGGAATACAGGGCTGA	108	163
NLRP12	NC_037345.1	5'CAGACTCCAGAAACTGCTCGAC	85	150
NLRP12	NC_037345.1	3'CAGGGCGTTGTTGGTCAGATAA	85	150
NLRP3	NC_037334.1	5'AAGACTTTCTGGACTCTGACCG	99	152
NLRP3	NC_037334.1	3'AGCCCTTCTGACTGGGATAGTC	99	152
NLRP5	NC_037345.1	5'TGTCTTCCTGCCTCAAGTCTCA	79	162
NLRP5	NC_037345.1	3'TAGCTTCGTCCCTTACACCACA	79	162
NLRX1	NC_037342.1	5'ACAGACTAGAAGGCAGTGTGCT	85	100
NLRX1	NC_037342.1	3'GGATACGTTCATCTGCTGGCTG	85	100
NOD2	NC_037345.1	5'CACCATTCTGGAAGTCTGGCTC	99	150
NOD2	NC_037345.1	3'AACACACTGAGCTGAACACTGG	99	150
PPIA	NC_037331.1	5'GCCATGGAGCGCTTTGG	98	145
PPIA	NC_037331.1	3'CCACAGTCAGCAATGGTGATCT	98	145
RPL19	NC_037346.1	5'GGGTATAGGTAAGCGAAAGGG	95	148
RPL19	NC_037346.1	3'TCACGGTATCGTCTAAGCAGC	95	148
TLR2	NC_037344.1	5'CTACGGACGGACAGTCAGCG	102	146
TLR2	NC_037344.1	3'TTGCCTGAAGAGCAGATGAGG	102	146
TLR4	NC_037335.1	5'CACAGAGCCACTTCTGGTCAC	85	150
TLR4	NC_037335.1	3'GTTAGGAACAACCTGTACGCAAG	85	150

APPENDIX F – Spreadsheet with the DEGs found on Chapter 3

The Appendix F is an excel file with the DEGs up e downs regulated on Pregnant group. It can be accessed in the CD-room attached in that dissertation

Description: DEGs resultant of the pregnant with non-pregnant contrast on Experiment 1 of the Chapter 3.

APPENDIX G- Spreadsheet with results of GO enrichment

The Appendix G is an excel file with the GO enrichment results. It can be accessed in the CD-room attached in that dissertation

Description: GO enrichment showing the overrepresented terms on biological process, molecular functions and cellular components with their respectively genes, of up-regulated DEGs on P group on Experiment 1 of Chapter 3.

APPENDIX H - Spreadsheet with results of KEGG pathways analyses

The appendix H is an excel file with the KEGG pathways results of Chapter 3. It can be accessed in the CD-room attached in that dissertation

Description: KEGG pathways showing all overrepresented pathways and their respectively genes of up-regulated DEGs on P group on Experiment 1 of Chapter 3.

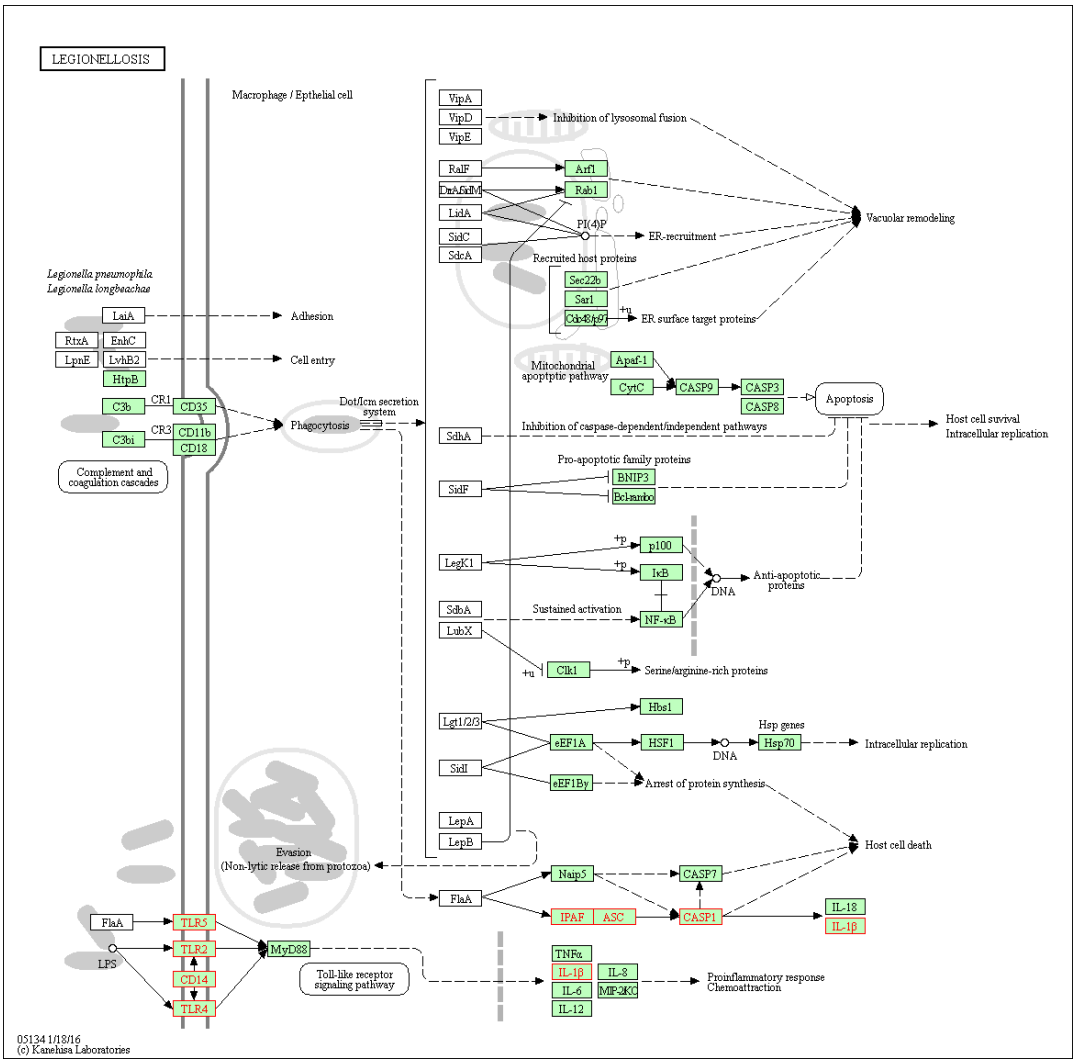
APPENDIX I - Spreadsheet with results of PRS analyses

Pathway regulation score (PRS) results showing the scores (nPRS) of all significant pathways, of the up regulated DEGs on P group on Experiment 1 of Chapter 3.

KEGGpathway	nPRS	p.value	q.value(FDR)
Legionellosis	46.4769	0.000999	0.011268731
Osteoclast differentiation	35.40941	0.000999	0.011268731
Pertussis	34.75831	0.000999	0.011268731
Systemic lupus erythematosus	32.34062	0.000999	0.011268731
Tuberculosis	27.75987	0.000999	0.011268731
Staphylococcus aureus infection	27.58552	0.000999	0.011268731
NOD-like receptor signaling pathway	27.29233	0.000999	0.011268731
Amoebiasis	23.66241	0.000999	0.011268731
Salmonella infection	21.84421	0.000999	0.011268731
Influenza A	21.78567	0.000999	0.011268731
Glycolysis / Gluconeogenesis	20.87016	0.000999	0.011268731
C-type lectin receptor signaling pathway	19.77105	0.000999	0.011268731
Asthma	19.14352	0.000999	0.011268731
Herpes simplex infection	16.41069	0.000999	0.011268731
Vitamin B6 metabolism	16.35104	0.000999	0.011268731
Complement and coagulation cascades	15.91586	0.000999	0.011268731
Cytokine-cytokine receptor interaction	14.59374	0.000999	0.011268731
Arginine and proline metabolismo	14.59072	0.000999	0.011268731
Intestinal immune network for IgA production	13.32336	0.000999	0.011268731
Leishmaniasis	13.28128	0.000999	0.011268731
NF-kappa B signaling pathway	13.03634	0.000999	0.011268731
Cytosolic DNA-sensing pathway	12.48944	0.000999	0.011268731
Pyruvate metabolismo	10.93858	0.000999	0.011268731
Necroptosis	9.231194	0.000999	0.011268731
Measles	8.461195	0.000999	0.011268731
Sulfur metabolismo	12.21775	0.001998	0.020122734
Toll-like receptor signaling pathway	7.545934	0.001998	0.020122734
Fluid shear stress and atherosclerosis	6.247899	0.001998	0.020122734
Drug metabolism - cytochrome P450	9.106846	0.002997	0.028171828
Fructose and mannose metabolismo	8.557106	0.002997	0.028171828
Tryptophan metabolismo	7.240445	0.003996	0.033143327
Protein processing in endoplasmic reticulum	7.095241	0.003996	0.033143327
Epstein-Barr virus infection	6.948274	0.003996	0.033143327
Huntington disease	6.155511	0.003996	0.033143327
Natural killer cell mediated cytotoxicity	5.19377	0.004995	0.040245469
Chemokine signaling pathway	6.09977	0.005994	0.045684046
Allograft rejection	5.924793	0.005994	0.045684046

Description: False discovery rate (FDR)

APPENDIX J – The graphical representation of the most significant PRS pathway



Source: (ROCHA, C. C., 2019)

Description: The graphical representation with all genes belong to the Leginellosis pathway. The genes with a square red means genes up regulated in the results of the Experiment 1 (Chapter 3) that regulate this pathway.