GABRIELA DALMASO DE MELO

Early pregnancy diagnosis in beef cattle through the expression of Interferon-tau stimulated genes in peripheral blood polymorphonuclear cells

Pirassununga

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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 Sciences.

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Prof. Guilherme Pugliesi, Ph.D.

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CERTIFICADO

Certificamos que a proposta intitulada "Diagnóstico precoce da gestação em bovinos através da expressão de genes estimulados por interferon-tau em neutrófilos", protocolada sob o CEUA nº 3554190717 (ID 006222), sob a responsabilidade de **Guilherme Pugliesi** *e equipe; Gabriela Dalmaso de Melo; Igor Garcia Motta* - 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 07/03/2019.

We certify that the proposal "Early pregnancy diagnosis in cattle through the expression of interferon-tau stimulated genes in neutrophils", utilizing 279 Bovines (279 females), protocol number CEUA 3554190717 (ID 006222), under the responsibility of **Guilherme Pugliesi** *and team; Gabriela Dalmaso de Melo; Igor Garcia Motta* - 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 03/07/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2017 a 07/2019 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	18 a 24 meses	N:	30
Linhagem:	Nelore			Peso:	290 a 400 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	18 a 24 meses	N:	103
Linhagem:	Nelore			Peso:	300 a 500 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 9 anos	N:	146
Linhagem:	Nelore			Peso:	400 a 700 kg		

Local do experimento: Universidade de São Paulo - Campus de Pirassununga

São Paulo, 07 de março 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

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Date: ___/___/___

Committee members

Prof. _____ Institution: _____

Decision: _____

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Prof. _____ Institution: _____

Decision: _____

I dedicate this dissertation to my mother, Vivian Dalmaso, whose affection, love, encouragement, prays and unfaltering support made me able to get here.

I love you with all my heart.

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RESUMO

MELO, G. D. **Diagnóstico precoce da gestação em fêmeas de corte por meio da expressão de genes estimulados por interferon-tau em células polimorfonucleares do sangue periférico.** [Early pregnancy diagnosis in beef cattle through the expression of interferon-tau stimulated genes in peripheral blood polymorphonuclear cells]. 2019. 94 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2019.

Considerando que o interferon-tau (IFNT) é capaz de estimular diversos transcritos (ISGs) em células mono (PBMCs) e polimorfonucleares (PMNs) do sangue periférico materno, nós testamos a hipótese de que a expressão de ISGs em PMNs ocorreria de forma mais precoce e intensa, o que permitiria um diagnóstico de gestação precoce e mais acurado. A presente dissertação consiste em dois estudos. No primeiro estudo, nós comparamos abundância de transcritos de ISGs (*ISG15, OAS1, MX1* e *MX2*) e receptores de IFNT do tipo 1 (*IFNAR I* e *II*) em PMNs e PBMCs de fêmeas Nelore inseminadas gestantes (n=8) e não gestantes (n=9), do D0 (IATF) até o D20. Nesse estudo, concluímos que as PMNs não respondem mais precocemente ao estímulo do concepto, e que a abundância de *ISG15* e *OAS1* nas PMNs e nas PBMCs pode ser utilizada como um marcador adequado para o diagnóstico de gestação no D18 e D20 pós IATF. Ainda, o status gestacional e o tipo celular não afetam a expressão do *IFNAR I* e *II*. No segundo estudo, nós comparamos a abundância do *ISG15* e *OAS1* em PMNs no D20 pós IATF de novilhas (n=103) e vacas (n=144) Nelore como preditores da gestação com o diagnóstico por meio da ultrassonografia Doppler (Doppler-US) e mensuração das concentrações de progesterona (P4) no D20, e mensuração das concentrações de glicoproteínas associadas à gestação (PAGs) no D25 pós IATF. As fêmeas foram artificialmente inseminadas no D0. A gestação foi diagnosticada por meio da ultrassonografia em modo B no D30 e D70, e após o diagnóstico final, as fêmeas foram divididas em quatro grupos: Gestantes; Não gestantes; CL funcional mas não gestantes (CL-NP) e Perdas gestacionais (PL). Após a seleção de valores de corte por meio de curva ROC, a Sensibilidade, Especificidade, Valores Preditivos Positivos, Valores Preditivos Negativos e Acurácia foram determinados para cada método. Todos os métodos avaliados provaram ser preditores significativos (P<0.0001) da prenhez, mas melhores acurácias foram obtidas por meio da mensuração das concentrações de PAGs e P4 e pelo Doppler-US, devido ao menor número de resultados falso-positivos e falso-negativos. Nós

concluímos que, apesar de serem marcadores significativos da gestação, a execução prática de um diagnóstico de gestação por meio da abundância de ISGs em PMNs ainda não é viável devido aos consideráveis números de resultados falso-negativos e positivos. A determinação das concentrações de PAGs e P4, e da luteólise por meio da Doppler-US oferecem melhores acurácias. Os diagnósticos por meio da Doppler-US e concentrações de P4 podem ser feitos mais precocemente, mas um diagnóstico em tempo real somente é possível por meio da Doppler-US.

Palavras-chave: PMNs, PBMCs, ISGs, diagnóstico precoce de gestação

ABSTRACT

MELO, G. D. **Early pregnancy diagnosis in beef cattle through the expression of interferon-tau stimulated genes in peripheral blood polymorphonuclear cells.** [Diagnóstico precoce da gestação em fêmeas de corte por meio da expressão de genes estimulados por interferon-tau em células polimorfonucleares do sangue periférico]. 2019. 94 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2019.

Considering that interferon-tau (IFNT) can stimulate several transcripts (ISGs) in mono (PBMCs) and polymorphonuclear cells (PMNs) at the maternal peripheral circulation, here we tested the central hypothesis that ISGs expression in PMNs would occur earlier and more intensely, which would allow a more accurate pregnancy diagnosis. This dissertation consists of two studies. In the first study, we compared the transcript abundance of ISGs *(ISG15, OAS1, MX1* and *MX2*) and IFNT type 1 receptors (*IFNAR I* and *II*) in PMNs and PBMCs of pregnant (n=8) and non-pregnant (n=9) inseminated Nelore heifers, from D0 (FTAI) to day D20. In this study, we concluded that PMNs do not respond earlier to the conceptus stimuli, and *ISG15* and *OAS1* expression both in PMNs and PBMCs can be used as a suitable marker for pregnancy diagnosis on D18 and D20 post-FTAI. Also, gestational status and cell type do not affect *IFNAR I* and *II* expression. In the second study, we compared the abundance of *ISG15* and *OAS1* in PMNs on D20 post-FTAI in Nelore heifers (n=103) and cows (n=144) as pregnancy predictors to the diagnosis performed by Doppler ultrasonography (Doppler-US) and measurement of peripheral plasma progesterone (P4) on D20, and measurement of pregnancy-associated glycoproteins (PAGs) on D25 post-FTAI. Females were artificially inseminated on D0. Pregnancy was diagnosed by B-mode ultrasonography on D30 and D70, and after the final diagnosis, females were divided in four groups: Pregnant; Non-pregnant; Functional CL but non-pregnant (CL-NP) and Pregnancy loss (PL). After determining cutoff values through a ROC curve, the Sensitivity (SE), Specificity (SP), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (ACC) were determined for each method. All the methods evaluated were significant (P<0.0001) predictors of pregnancy, but better accuracies were obtained through PAGs and P4 concentrations, and Doppler-US, due to the lower number of false positive and false negative results. We concluded that, despite being significant pregnancy markers, the practical execution of a pregnancy diagnosis through ISGs abundance in PMNs is still not viable due to the considerable number of false-positive and false-negative results. The determination of PAGs and P4 concentrations and

luteal blood perfusion by Doppler-US offer better accuracies. The Doppler-US and P4 methods can be performed earlier, but a real-time diagnosis is only possible by Doppler-US.

Keywords: PMNs, PBMCs, ISGs, early pregnancy diagnosis

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SUMMARY

1. INTRODUCTION

With approximately 215 million heads (ABIEC, 2019), Brazil holds the largest commercial herd in the world. Agribusiness is a significant contributor to the country's economy, accounting for about 20% of the gross domestic product (CEPEA, 2019), and beef cattle represent 8.7% of this income (ABIEC, 2019). Due to its strong performance in the sector, Brazil also stood out as one of the largest beef exporters, exporting about 30% of its domestic production to countries in Asia, Europe and North America (ABIEC, 2019). The insertion of national products in a competitive and demanding market highlights that Brazil has been increasingly improving in terms of productivity and product quality. However, even holding 15% of the world's herd, the country's total beef production is still below to the ones reported in other countries due to poor reproductive efficiency. Currently, the herds have an average birth rate of 74%, and a weaning rate of 68% (ANUALPEC, 2018). This represents about 21 million cows that do not produce one calf per year, which also generates millions of hectares with low productivity. Thus, in order to maximize productivity and meet world food demands, it is necessary to invest in reproductive technologies that are commercially efficient and applicable, bringing positive economic returns to the producers.

A prolonged calving interval is one of the factors responsible for the reduced reproductive efficiency, and it is directly affected by the long calving to conception interval. Thus, to shorten the period in which females remain open, a possible approach would be the early detection of pregnancy and embryonic losses, which would allow them new chances of conception. For that matter, Lucy et al. (2004) suggested a reproductive program aiming to detect pregnancy within 18 days after fixed-time artificial insemination (FTAI) and re-insemination 21 days after the first FTAI. For this program to be successful, an earlier method of pregnancy diagnosis is required. The conventional method is predominantly executed by B-mode ultrasonography from day 28 of gestation, when it is possible to visualize an embryo with heartbeats (PIETERSE et al., 1990). However, despite being an accurate method, the diagnosis is still performed after estrus return, later than the ideal model proposed.

The development of an early pregnancy diagnosis method relies on the understanding of the mechanisms involved in conceptus's development, and the

maternal recognition process plays an essential role in this regard. After the development to a tubular shape, the embryo initiates its elongations process, from day 12 after fertilization until day 21, approximately (SPENCER et al., 2007). During this process, the embryo trophectoderm cells secrete interferon-tau (IFNT), the main signaling component of gestation in ruminants (BAZER et al., 1986; GUILLOMOT et al., 1990; GRAY et al., 2002). In the absence of the embryo, around 12 days after estrous, estrogen derived from follicles binds to its receptors in the endometrium and induces the expression of more oxytocin receptors (SPENCER et al., 2007). The oxytocin, secreted by posterior pituitary and by the corpus luteum (CL) (SCHALLENBERGER et al., 1984; CROWLEY; ARMSTRONG, 1992), binds to its receptors in a greater intensity, leading to prostaglandin F2α (PGF2α) release and triggering the luteolytic cascade (MCCRACKEN; CUSTER; LAMSA, 1999). When the conceptus is present, the IFNT released during its elongation will bind to type 1 interferon-receptors (IFNARs) in the endometrium, acting through the JAK-STAT pathway (SPENCER et al., 2007). IFNT blocks the transcription of estrogen receptors by activation of a transcription inhibitor factor (IRF2), which avoids the transcription of oxytocin receptors and PGF2α release (WATHES; LAMMING, 1995), thus maintaining CL function and progesterone (P4) release. Also, from day 14-16, trophectoderm mononuclear cells start to differentiate into giant binucleate cells (SPENCER et al., 2007). These cells appear in the uterine luminal epithelium by days 19-21 of gestation and secrete their contents, which are mainly proteins and hormones (POHLER et al., 2015).

In non-pregnant females, the CL usually regresses between days 15 and 18 of the estrous cycle (GINTHER; SHRESTHA; BEG, 2010), and during luteolysis, decreased P4 secretion is associated with decreased CL blood perfusion in response to PGF2α (NISWENDER et al., 2000). Thus, the evaluation of CL blood perfusion during its regression by Doppler ultrasonography (Doppler-US) was suggested by many authors as a tool to identify luteolysis (HERZOG et al., 2010; PUGLIESI et al., 2014; SCULLY et al., 2015). The execution of this technique 20 days after FTAI showed high accuracy and sensitivity in beef (PUGLIESI et al., 2014) and dairy cattle (SIQUEIRA et al., 2013). Ever since, Doppler-US has been indicated as an accurate method for pregnancy diagnosis, with great efficiency in detecting non-pregnant females due to false-negative rates close to 0% (PUGLIESI et al., 2018). Furthermore,

it is a method with satisfactory field results when used in early and super-early resynchronization protocols of inseminated females, allowing re-insemination 22-24 days after the first FTAI (BARUSELLI, 2017; PUGLIESI et al., 2019). However, this technique still has a considerable rate of false-positive results due to the presence of a functional CL (blood perfusion > 25%) at the time of pregnancy diagnosis, but absence of a viable embryo on the confirmatory diagnosis. False-positive results can have several causes, such as late ovulation to synchronization protocols, long estrous cycle and early pregnancy losses (PUGLIESI et al., 2018). Nevertheless, although they decrease the accuracy of the diagnosis, these results are still more desirable than false negatives. Pregnant females misdiagnosed as non-pregnant could receive a PGF2α injection at the resynchronization protocol, which would lead to gestational loss.

The IFNT has also been explored as a pregnancy marker in bovine females. Still, its applicability on the early pregnancy diagnosis is controversial due to difficulties encountered in the assay performance and variation of the reported results. In addition to its role in the process of maternal recognition of pregnancy in ruminants, IFNT is also able to induce the expression of several genes (ISGs) in the endometrium (MIRANDO et al., 1991). Furthermore, studies indicated that IFNT possesses an endocrine function, since it can reach the peripheral maternal circulation through the uterine vein (OLIVEIRA et al., 2008; BOTT et al., 2010), thus leading to the expression of ISGs in extrauterine sites, such as the CL (OLIVEIRA et al., 2008), liver (RUHMANN et al., 2017), vaginal and cervical mucosa (KUNII et al., 2018) and peripheral blood leukocytes (YANKEY et al., 2001; HAN et al., 2006; KIZAKI et al., 2013).

Despite being an excellent indicator of the presence of the conceptus, so far no assay has been sensitive enough to differentiate IFNT concentrations in the peripheral circulation of pregnant and non-pregnant females. Thus, the expression of ISGs in peripheral blood leukocytes has been suggested as a potential tool for pregnancy diagnosis (GREEN et al., 2010a; PUGLIESI et al., 2014). The IFNT release window, from day 14 to 21 days of gestation (EALY; YANG, 2009), allows an early detection of pregnant females. In addition, the expression pattern of ISGs could reflect the embryo viability, as the amounts of IFNT released by the conceptus correlate to the expression of ISGs (MATSUYAMA et al., 2012). Several ISGs are induced by IFNT, but some of them, the so-called classic ISGs, are often used in studies aiming to diagnose

pregnancy, such as *ISG15*, *OAS1*, *MX1* and *MX2*. The function of these genes has not yet been fully elucidated. *ISG15* is known to play important roles in uterine receptivity (KLEIN; SCOGGIN; TROEDSSON, 2011) and development and survival of the embryo (HENKES et al., 2015; ZHAO et al., 2017). The functions of *OAS1*, *MX1* and *MX2* are related to the innate immune response (STEVENSON et al., 2007; ALEX et al., 2018).

The use of ISGs abundance for pregnancy diagnosis was already reported in whole leukocytes (HAN et al., 2006; STEVENSON et al., 2007; GREEN et al., 2010a), peripheral blood mononuclear cells (PBMCs; GIFFORD et al., 2007; PUGLIESI et al., 2014) and peripheral blood polymorphonuclear cells (PMNs; MATSUYAMA et al., 2012; SHIRASUNA et al., 2012; KIZAKI et al., 2013; YOSHINO et al., 2018). In PMNs, ISGs expression was suggested to occur earlier and more prominently when compared to expression in PBMCs or total leukocytes. Kizaki et al. (2013), for instance, described a significant difference in the expression of these genes in PMNs between pregnant and non-pregnant females 14 days after FTAI, while in PBMCs, this difference was reported only 18 days post-FTAI (GIFFORD et al., 2007; KIZAKI et al., 2013; PUGLIESI et al., 2014). The authors attributed the difference between the cell groups to the characteristics of the cellular immune response, as neutrophils participate in the first line of defense on the immune response. Few studies, however, have reported the accuracy of this method of diagnosis when applied to the field. Pugliesi et al. (2014) reported an accuracy close or below to 80% in the diagnosis through ISGs abundance in PBMCs 20 days post-FTAI in beef females. Yoshino et al. (2018) also described an accuracy close to 80% when this method was used in PMNs of dairy cows on days 20-22 post-FTAI. Still, no study evaluated the accuracy of ISGs expression in PMNs in beef females.

Other pregnancy diagnosis techniques consider the direct stimulus of the conceptus during its initial development and were, therefore, pointed as potential markers of pregnancy and embryonic viability. Pregnancy-associated glycoproteins (PAGs) constitute a large family, exhibiting different temporal and spatial expression patterns throughout pregnancy (WALLACE et al., 2015). They are produced in the trophoblast, mainly by binucleate cells (GREEN et al., 2000), and reach maternal bloodstream at the time of these cells' migration (GREEN et al., 2005). Thus, PAGs were discovered as reliable markers of pregnancy, and its concentrations were initially

measured by radioimmunoassay around the fourth week of gestation (SASSER; RUDER, 1987; ZOLI et al., 1992; MIALON et al., 1993, 1994). Subsequently, an ELISA assay was developed using only PAGs secreted in the early gestational period as an attempt to reduce false-positive results (GREEN et al., 2005), since some of these glycoproteins are released in greater amounts immediately before parturition, and also have a long half-life in the post-partum period (ZOLI et al., 1992; DE SOUSA et al., 2003). Ever since, PAGs have been measured by commercial assays on milk samples around 30 days of gestation (RICCI et al., 2015) and blood samples (serum or plasma) around 28 days of gestation (POHLER et al., 2016a), with satisfactory field results. This test accuracy has been reported at 92-99%, and false-positive rates ranged from 1-5% (SILVA et al., 2007; PIECHOTTA et al., 2011; POHLER et al., 2013; KAREN et al., 2015; FOSGATE et al., 2017). Recently, the 24th day after FTAI was also suggested as a suitable moment for pregnancy diagnosis trough PAGs concentrations, obtaining good performances in dairy (REESE et al., 2019) and beef cattle (FILHO et al., 2020). In addition, studies pointed that PAGs concentrations could also reflect the embryonic viability and placenta function (POHLER et al., 2013, 2016a; FILHO et al., 2020), since females that experienced embryonic losses showed lower concentrations of this protein at the time of sample collection than females that maintained pregnancy. However, despite the significant advantages in terms of accuracy and prediction of gestational losses, this is still a late method for pregnancy diagnosis when compared to Doppler-US.

In the present study, our main goal was to verify the feasibility and applicability of pregnancy diagnosis through the ISGs transcripts levels in PMNs of Nelore females, since so far, no study has explored the implementation of this technique in beef females. Our central hypothesis was that the abundance of these genes would occur earlier and more intensely in PMNs, which would allow a more accurate pregnancy diagnosis.

The specific objectives of the present study were: 1) to characterize the abundance of ISGs in PMNs of pregnant and non-pregnant beef heifers, and compare with the expression in PBMCs from D0 (FTAI) to D20; 2) to characterize the expression of interferon receptor transcripts in PMNs and PBMCs; 3) to evaluate the accuracy of ISGs expression in PMNs on D20 post-FTAI as a method of pregnancy diagnosis; 4)

to compare the accuracy of this method between heifers and cows; 5) to compare the efficiency of ISGs for diagnosing pregnancy with other earlier methods, and 6) To evaluate the feasibility of using ISGs expression in PMNs as predictors of pregnancy losses.

This dissertation was divided into two chapters. The Chapter 1 compares the abundance of classic ISGs between PMNs and PBMCs of Nelore heifers from D0 (FTAI) to D20 post-FTAI, as well as characterizes the abundance of IFNT receptor transcripts in both cell groups. We also selected the genes with the best performance in PMNs, as well as the day with the least overlap of gene expression between pregnant and non-pregnant females. This study served as the basis for the development of the second experiment, which was described in the Chapter 2. In Chapter 2, we tested the accuracy of *ISG15* and *OAS1* expression in PMNs as a pregnancy diagnosis method, and compared with other techniques, such as evaluation of CL function by Doppler-US, and measurement of PAGs and P4 concentrations. The Chapter 1 was submitted for publication in Reproductive, Fertility and Development (Attachment A), and Chapter 2 was organized in a journal manuscript format for publication following the considerations of the members of the committee. The final considerations at the end of this dissertation summarize the most important findings of each study and discuss future perspectives.

2. CHAPTER 1: PROFILE OF RECEPTORS FOR INTERFERON-TAU AND ITS STIMULATED GENES IN MONO AND POLYMORPHONUCLEAR CELLS AT EARLY PREGNANCY IN BEEF HEIFERS[1]

2.1. INTRODUCTION

In order to reduce the calving to conception interval is fundamental to detect conception and embryonic losses early after the first insemination in fixed-time artificial insemination (FTAI) programs (GREEN et al., 2010a). However, pregnancy is mainly identified in cattle submitted to FTAI programs through the detection of a viable embryo by using B-mode ultrasonography after the fourth week of gestation (PIETERSE et al., 1990). The development of earlier methods for detection of pregnancy has been performed by measurement of pregnancy-associated glycoproteins (PAGs) produced by the placenta (WOODING; ROBERTS; GREEN, 2005) and by evaluation of corpus luteum (CL) function using color Doppler ultrasonography (PUGLIESI et al., 2014). Nonetheless, a significant increase in circulating PAGs concentrations to distinguish pregnant and non-pregnant females only occur after day 24 of gestation (RICCI et al., 2015; POHLER et al., 2016b). Moreover, Doppler ultrasonography only allows the detection of non-pregnant females that underwent luteolysis between days 20 and 22 after FTAI (SIQUEIRA et al., 2013; PUGLIESI et al., 2014), and the conceptus or a marker of a viable conceptus is not detected.

The interferon-tau (IFNT), a glycoprotein secreted by cells from the trophoblast and the main component of the maternal recognition of pregnancy mechanism, could be explored as a pregnancy marker. It starts to be released at the late morula and early blastocyst stage (FARIN; IMAKAWA; ROBERTS, 1989; LONERGAN, 2003), increasing significantly from D14 and peaking around D21 of gestation (EALY; YANG, 2009). The IFNT acts on the luminal epithelium and superficial endometrial glands by binding to its receptors (*IFNAR I* and *II*) (SPENCER; BAZER, 2004), preventing the release of prostaglandin F2α (PGF2α) luteolytic pulses (WATHES; LAMMING, 1995) and inducing the expression of IFNT-stimulated genes (ISGs). However, due to reduced IFNT concentrations in blood (SHEIKH et al., 2018), no IFNT assay has been

[1]This study was submitted to the journal Reproduction, Fertility and Development. The proof of submission of the manuscript is in Attachment A.

developed to distinguish pregnant from non-pregnant females. Alternatively, ISGs transcripts can be retrieved in peripheral blood leukocytes (HAN et al., 2006; GIFFORD et al., 2007; STEVENSON et al., 2007), as IFNT reaches different tissues and cells through blood circulation (OLIVEIRA et al., 2008; BOTT et al., 2010; ANTONIAZZI et al., 2013).

The increase in abundance of several ISGs in peripheral mononuclear leukocytes (PBMCs; lymphocytes and monocytes) between 18 and 20 days after breeding has been reported in sheep (YANKEY et al., 2001) and cattle (GIFFORD et al., 2007; GREEN et al., 2010a; JIEMTAWEEBOON et al., 2011; PUGLIESI et al., 2014). However, later studies (JIEMTAWEEBOON et al., 2011; KIZAKI et al., 2013; MANJARI et al., 2018) have indicated that polymorphonuclear cells (PMNs; neutrophils, basophils and eosinophils) may respond earlier to IFNT stimuli. Kizaki *et al.* (2013) reported a greater abundance of ISGs transcripts in PMNs than in PBMCs on day 14 of pregnancy, but a direct comparison of the ISGs expression profile in PMNs and PBMCs throughout early pregnancy is still not known. Also, although Toji *et al.* (2017) indicated that there is no difference on *IFNAR I* and *II* relative levels between PMNs and PBMCs in pregnant cows on day 21 of pregnancy, a different pattern of these genes expression before the IFNT peak may interfere on the immune cell type response to IFNT during the first three weeks of pregnancy.

Therefore, in the present study we aimed: 1) to characterize the abundance of ISGs in PMNs and compare with the expression in PBMCs from D0 (FTAI) to D20 of pregnancy; 2) to evaluate the feasibility of using the expression of ISGs in PMNs as pregnancy markers; and 3) to characterize the relative levels of transcripts for interferon receptors type I and II in PMNs and PBMCs. We hypothesized that the expression of ISGs in PMNs would occur earlier and more intensely than in PBMCs, and that *IFNAR I* and *II* transcripts would be up-regulated in pregnant heifers, and that this response would be more prominent in PMNs than in PBMCs.

2.2. MATERIAL AND METHODS

2.2.1. Animals

The experiment was conducted at the Animal Reproduction Department of the University of São Paulo, in Pirassununga, SP, Brazil. A total of 26 pubertal Nelore (*Bos indicus*) heifers between 18-20 months of age, weighing 314.2 kg ± 31.3kg (average ± SEM) and with adequate uterine and ovarian conditions were selected. Animals were maintained on *Brachiaria brizantha* pastures with free access to mineralized salt and water. Heifers were handled in accordance with the Institutional Committee for Ethics in Research of the University of São Paulo (CEUA-FMVZ number: 3554190717).

2.2.2. Experimental design and animal handling

Heifers were submitted to an estradiol (E2) and progesterone (P4) based protocol for synchronization of ovulation, as follows. On D-10, heifers received a second-time used intravaginal P4-releasing device (1 g, Sincrogest, Ourofino Saúde Animal, Cravinhos, SP, Brazil) along with an administration of estradiol benzoate (2 mg, i.m., Sincrodiol, Ourofino Saúde Animal, SP, Brazil) and a PGF2α analog (0.526 mg of sodium cloprostenol; i.m., Sincrocio, Ourofino Saúde Animal, SP, Brazil). On D-2 the P4 device was removed and the animals received another treatment with a PGF2α analog (0.526 mg of sodium cloprostenol; i.m., Sincrocio, Ourofino Saúde Animal, SP, Brazil), estradiol cypionate (1 mg, i.m., SincroCP, Ourofino Saúde Animal, SP, Brazil) and equine chorionic gonadotropin (200IU, i.m., SincroeCG. Ourofino Saúde Animal, SP, Brazil). On D0, heifers were treated with a gonadotropin-releasing hormone analogue (10 µg, i.m., buserelin acetate; Sincroforte; Ourofino Saúde Animal, SP, Brazil) and were artificially inseminated by a single operator using thawed semen from a single sire.

2.2.3. Ultrasound scanning

Ultrasound scanning of ovaries was performed on D-10, D-2, D0 (FTAI), D1, D10, D14, D16, D18 and D20 by a single operator using a duplex ultrasound equipment (MyLab™30 Vet Gold, Esaote, São Paulo, Brazil) with a linear multi-frequency (3.5-7.5 MHz) B-mode and Doppler transducer. On D-10, D-2, D0 and D1, ultrasound

examinations were performed in B mode to measure the diameter of the largest follicles observed and identify the dominant follicle, as well as to detect their disappearance (ovulation). Heifers that did not ovulate within 24 hours after FTAI (n=4) or with double ovulation (n=1) were excluded.

On D10, D14, D16, D18 and D20 post-FTAI, ultrasonographic evaluations by color Doppler were performed to measure the blood perfusion of corpus luteum (CL). The maximum area of the CL was determined using the tracing function from B-mode ultrasonography, as previous reported (PUGLIESI et al., 2014). When the CL presented cavity, its size was subtracted from the total luteal tissue. Color Doppler was used in order to access luteal blood perfusion through the estimation of the luteal area with colorful signs in relation to its total area. Ultrasound evaluations for pregnancy diagnosis were conducted on D20, D25 and D28 post-TAI by transrectal ultrasonography. A female was considered pregnant when a functional CL was present on D20 (luteal blood perfusion >25%) and the presence of an embryonic vesicle and heartbeats were detected on D25 and D28.

2.2.4. Blood collection

For the remaining heifers (n=21), blood samples (35ml) were collected from the jugular vein in 10ml sodium heparinized tubes (BD Life Sciences, New Jersey, USA) on D0, D10, D14, D16, D18 and D20 post-FTAI to measure the P4 circulating concentrations and for isolation of PMNs and PBMCs. After collection and manual homogenization, samples were placed in chopped ice until its processing. Blood samples used in P4 assay (10ml) were centrifuged at 2500 x g for 15 minutes at 4ºC. The resulting plasma was decanted and stored at -20ºC in 1.5ml sterile microtubes until the assay was performed. The blood samples destined to PMNs and PBMCs isolation (25ml) were submitted to the protocol right after animal handling.

2.2.5. Isolation of PMNs and PBMCs

Isolation of PMNs and PBMCs was performed by a density gradient using a Ficoll-Paque solution (Ficoll-Paque Plus, GE Healthcare, Chicago, IL, USA), and all the procedures were executed at room temperature. Whole blood (25ml) was mixed with

an equal volume of PBS in a 50ml conical tube, and the solution was layered onto 15ml of Ficoll-Paque solution and centrifuged at 1100 x g for 30 minutes, 21°C, according to the protocol previously described (JIEMTAWEEBOON et al., 2011). After centrifuging, three phases were formed: plasma, buffy-coat and red blood cells together with PMNs. Plasma was discarded and mononuclear (PBMCs, buffy coat) and PMNs were disposed in different tubes for further processing.

The protocol used for PBMCs isolation was previously described by Pugliesi *et al.* (2014). Briefly, the buffy-coat fraction was centrifuged with PBS at 900 x g for 15 minutes, 10°C, and the contaminating red blood cells were lysed in a hypertonic solution for 10 minutes. Isotonicity was restored by suspending the cells in PBS and centrifuging at 900 x g for 15 minutes, 10°C. The resultant pellet was suspended in PBS and the final solution was transferred to a 1,5ml RNAse free microtube. The isolated cells were centrifuged at 3300 x g for 8 minutes, 4°C, and after removal of the supernatant, the remaining pellet was stored at -80^{0}C until RNA extraction. The purity of PBMCs was checked immediately after the procedure by staining fresh isolates samples with the quick panoptic protocol. Samples were considered pure when 95% of the 200 counted cells were mononuclear cells.

For PMNs isolation, procedures described by Jiemtaweeboon *et al.* (2011) were followed with some modifications. The fraction with PMNs and red blood cells was washed in hypotonic distilled water for 10 seconds, and isotonicity was restored after the addition of twice-concentrated PBS. Samples were centrifuged at 900 x g for 10 minutes at 10°C. After discarding the supernatant, the lysis process with distilled water was repeated, and after centrifugation the remaining pellet was lysed with 10ml of a hypertonic solution. After 10 minutes, 30ml of PBS were added and samples were centrifuged at 900 x g for 15 minutes, 10°C. The pellet was suspended in PBS and transferred to a 1.5ml RNAse free microtube, and the following steps were the same as for PBMCs. The purity of PMNs was also checked by staining fresh isolates samples with the quick panoptic protocol. Samples were considered pure when 95% of the 200 counted cells were polymorphonuclear cells.

2.2.6. Assay of circulating progesterone concentrations

Plasma samples collected on D10, D14, D16, D18 and D20 post-FTAI were assayed for P4 using a solid phase ^{125}P4 RIA kit following the manufacturer's instructions (MP Biomedicals, Lot PK1831, Irvine, CA, USA). The sensitivity of the assay was 0.01 ng/ml. The intra-assay coefficient of variation (CV) for quality control samples was 2.3% (low standard) and 2.03% (high standard).

2.2.7. RNA extraction and cDNA synthesis

Samples from 9 pregnant and 9 non-pregnant heifers were randomly selected for RNA extraction. Samples of isolated PBMCs were thawed on ice and RNA extraction was performed by using Trizol (Thermo Fisher Scientific, Waltham, USA) protocol, following the manufacturer's instructions. The RNA from PMNs was extracted by using Trizol reagent associated with the DirectZol-RNA kit (Zymo Research, Irvine, CA, USA). For that, after thawing PMNs samples on ice, 600µl of Trizol was added to each sample. The pellets were dissolved by vortexing the tubes for 2-5 min, and after incubation for 5 minutes at room temperature, 200µl of chloroform was added to the samples, followed by vortexing and 2 min incubation at room temperature. After that, the samples were centrifuged at 12000 x g for 15 min at 4°C. The supernatant was transferred to another tube, and 600µl of 100% ethanol was added to each sample. The remaining solution was moved to the columns provided by the kit, and after this step the manufacturer's instructions were followed.

Total RNA samples from PBMCs and PMNs were treated with DNAse I (Life Technologies, Carlsbad, USA) for 15 min at room temperature in a 10µl reaction volume. The concentration and purity (A260/A280) of total RNA extracts was measured using a spectrophotometer (NanoVue, GE Healthcare, Chicago, USA), and one sample from a pregnant heifer was not used due to low RNA concentration. Therefore, the isolated RNA (0.3µg) from 8 pregnant and 9 non-pregnant heifers was subjected to reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Life Technologies) according to the manufacturer's instructions. The cDNA of each sample was stored at -20°C until qPCR analysis.

2.2.8. Quantitative Polymerase Chain Reaction

The real-time qPCR analyses were performed using SYBR Green (Life Technologies, Carlsbad, CA, USA), the reactions were run in triplicates, using the same qPCR settings as described previously (SPONCHIADO et al., 2017), and were carried out using a Step One Plus apparatus (Life Technologies). The selected target genes were *ISG15*, *OAS1*, *MX1* and *MX2*, which are classic ISGs, and the primers have been previously described (OLIVEIRA et al., 2008; GREEN et al., 2010a; SHIRASUNA et al., 2012; PUGLIESI et al., 2014; ARAÚJO et al., 2016) for cattle and are provided on Table 1. *MX1* and *MX2* expression in PMNs were analyzed with fewer replicates (6 and 4 pregnant and 9 and 7 non-pregnant for *MX1* and *MX2*, respectively) due to amplification failures. The genes *GAPDH*, *ACTB*, *PPIA*, *RPL30* and *18S* were tested in all PMN and PBMC samples as housekeeping genes, and those with less variation, selected by the NormFinder software, were used. For PMNs, *GAPDH* and *ACTB* were selected for normalization; whereas for PBMCs, the most stable genes for normalization were *GAPDH* and *PPIA*. The reactions were run in triplicates on a 96-well plate. The raw fluorescence data were extracted from the Step One Plus apparatus and analyzed using the LinReg PCR software for baseline correction, determination of qPCR efficiency and cycle quantification values per sample, as described by Ruijter *et al.* (2009). The log-linear portion of the amplification curve used for the analysis performed with the LinReg PCR software contained four points with the highest R^2 value. The expression of the target genes evaluated by qPCR was normalized with the two reference genes by the comparative Ct method (PFAFFL, 2001). The results are expressed as relative arbitrary units.

Table 1 – Forward (F) and reverse (R) primers sequences of target and reference genes analyzed using qPCR

Gene	GeneBank	Primer sequence	Reference
OAS1	NM_001040606.1	F: TAGCCTGGAACATCAGGTC R: TTTGGTCTGGCTGGATTACC	Shirasuna et al., 2012
MX1	NM_173940.2	F: GTACGAGCCGAGTTCTCCAA R: ATGTCCACAGCAGGCTCTTC	Green et al., 2010
MX2	NM_173941	F: CTTCAGAGACGCCTCAGTCG R: TGAAGCAGCCAGGAATAGT	Green et al., 2010
ISG15	NM_174366	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT	Oliveira et al., 2008
GAPDH	NM_001034034.2	F: GCCATCAATGACCCCTTCAT R: TGCCGTGGGTGGAATCA	Araujo et al., 2016
PPIA	BF230516.1	F: GCCATGGAGCGCTTTGG R: CCACAGTCAGCAATGGTGATCT	Pugliesi et al., 2014
ACTB	NM_173979.3	F: GGATGAGGCTCAGAGCAAGAGA R: TCGTCCCAGTTGGTGACGAT	Araujo et al., 2016

Source: (MELO, G.D., 2019)

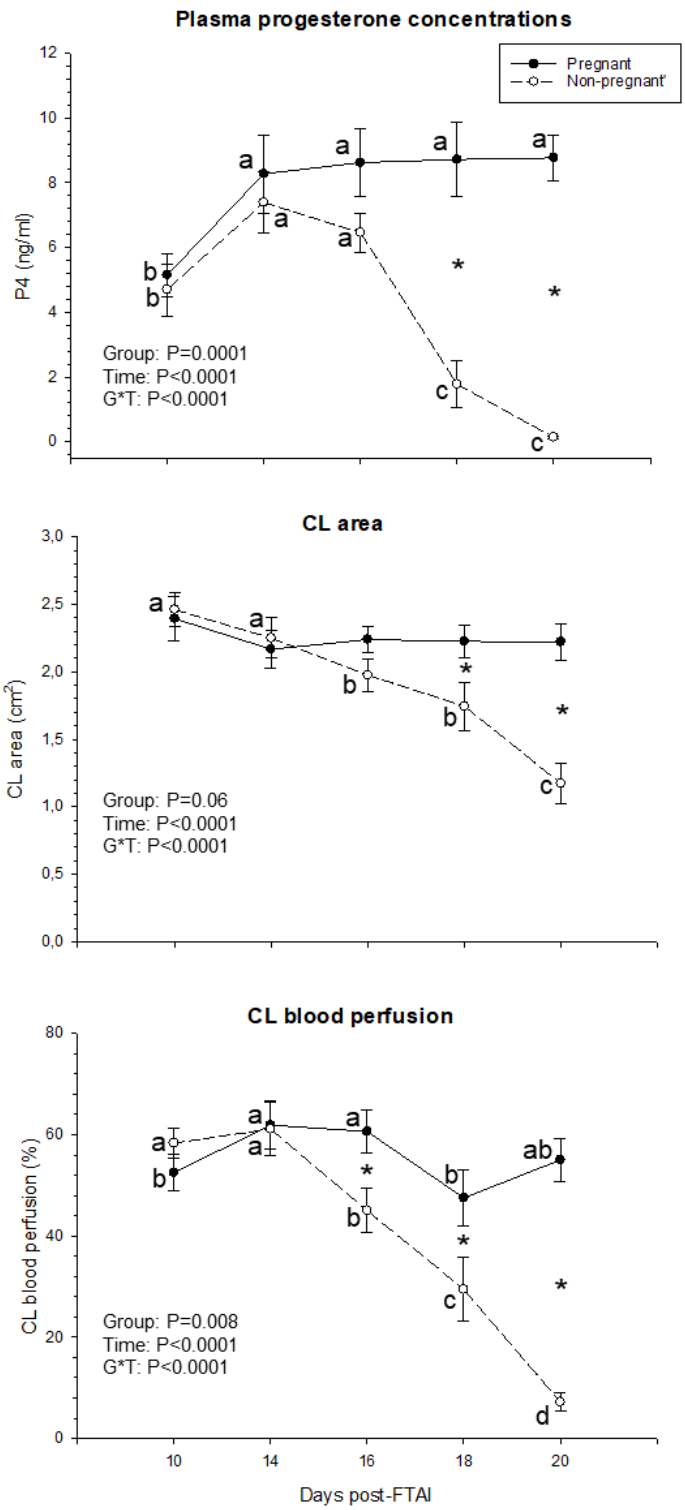
2.2.9. Statistical analyses

Data that were not normally distributed according to Shapiro-Wilk test were transformed to natural logarithms or ranks. Plasma P4 concentrations, CL area and blood perfusion and gene expression were analyzed using Analysis of Variance (ANOVA) with repeated measures of time using the PROC mixed of SAS (SAS Institute, version 9.2, Cary, USA) and considering the random effects of the heifers and fixed effects of group (pregnant and non-pregnant), time and their interaction. For comparisons between cell type groups (PBMCs vs. PMNs), the arbitrary expression values were normalized by calculating the relative expression in each day for each animal to D0. The least significant difference test was used when comparisons between days were made within a group. Pearson's correlation between ISGs and IFNARs expression in pregnant and non-pregnant heifers was analyzed by MedCalc software (Mariakerke, Belgium). Results were presented as MEANS ± S.E.M. Probabilities of $P \leq 0.05$ indicate significant difference while probabilities of $0.05 > P \leq 0.10$ indicate that significance was approached.

2.3. RESULTS

Pregnancy was diagnosed on 43% (9/21) of the ovulated heifers by the detection of a viable embryo on days 25 and 28 after FTAI. When evaluating plasma P4 concentrations, effects of group, time and their interaction were detected (Figure 1). The plasma P4 concentrations were higher ($P<0.05$) in the pregnant than in the non-pregnant group (8 ± 0.5 vs. 4.1 ± 0.5 ng/ml), and the interaction reflected a significant decrease on P4 concentrations in non-pregnant heifers from D18, resulting in a significant ($P<0.05$) difference between the groups on D18 (8.7 ± 1.1 vs. 2 ± 0.7 ng/ml) and D20 (9.0 ± 0.7 vs. 0.14 ± 0.03 ng/ml) post-FTAI (Figure. 1). The effects of time and interaction between group and time were also detected for CL area and blood perfusion, but a group effect was only detected for CL blood perfusion (Figure 1). For CL size, the interaction reflected a progressive decrease ($P<0.05$) on CL area in non-pregnant heifers from D16, resulting in larger ($P<0.05$) CLs in the pregnant heifers than the non-pregnant heifers on D18 (2.2 ± 0.1 vs. 1.74 ± 0.2 cm^2) and D20 (2.22 ± 0.1 vs. 1.2 ± 0.1 cm^2) post-FTAI (Figure 1). For CL blood perfusion, a progressive decrease ($P<0.05$) was also observed from D16 in the non-pregnant females, and the difference between the groups was detected earlier. The CL from pregnant heifers had a greater ($P<0.05$) proportion of blood perfusion than the CL from non-pregnant heifers on D16 (60 ± 4 vs. 50 ± 4 %), D18 (50 ± 6 vs. 30 ± 5%) and D20 (55 ± 3 vs. 7 ± 3%) post-FTAI (Figure 1).

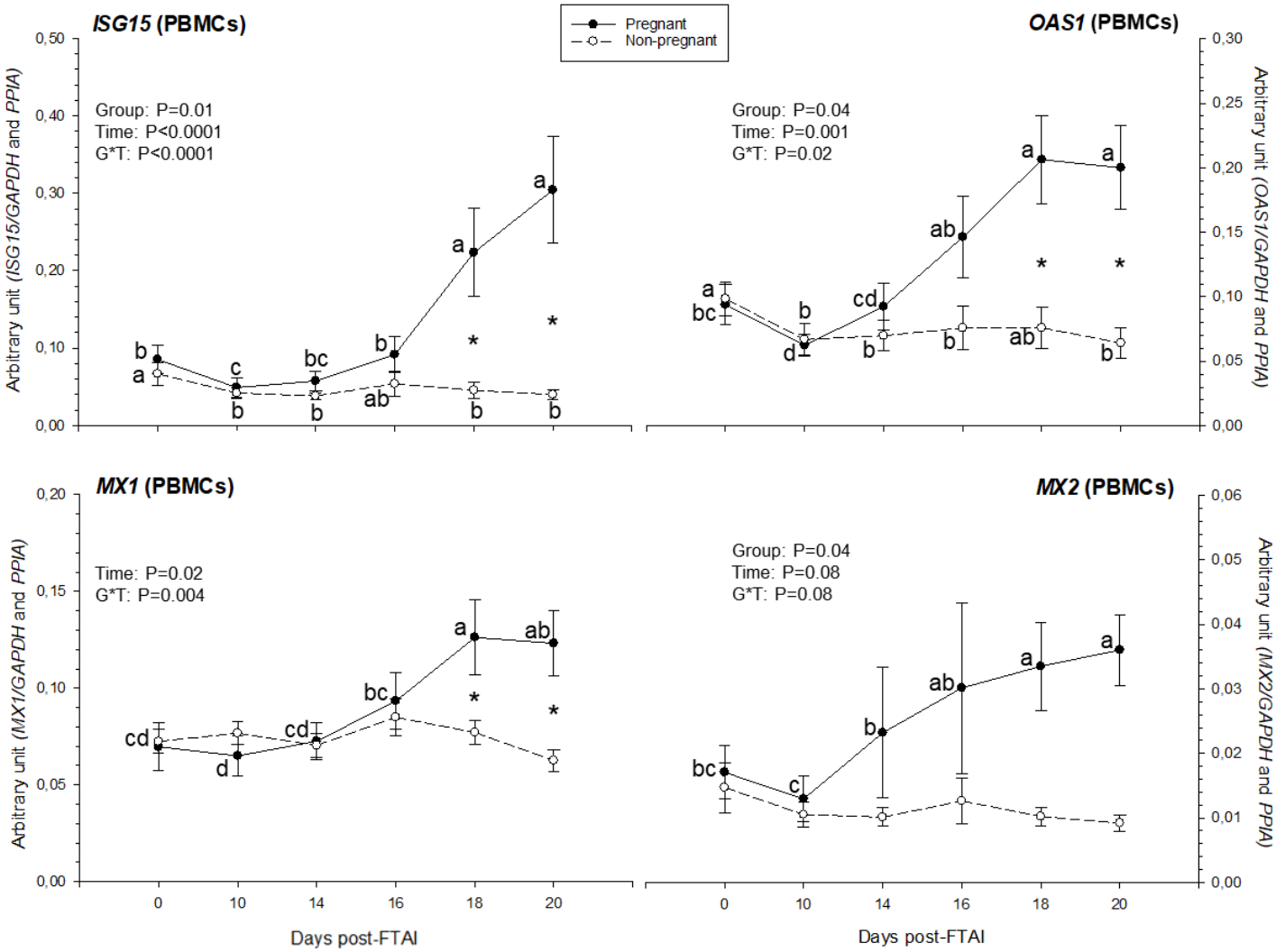
Figure 1 - Mean ± SEM of plasma P4 concentrations, CL area and CL blood perfusion during the first weeks post-AI in pregnant (n=8) and non-pregnant (n=9) beef heifers.



Source: (MELO, G.D., 2019)
Legend: The mains effects of group (G), time (T) and their interaction (G*T) are indicated. The asterisks indicate differences (P<0.05) between groups within a day; abcd, the means without a common letter are different within the groups (P<0.05).

The expression of the ISGs evaluated in PBMCs is shown in Figure 2. Significant (P<0.05) effects of group, time and their interaction were verified on *OAS1* and *ISG15* expression. The group effect indicated that expression of *OAS1* and *ISG15* was, respectively, 1.8 and 2.6-fold greater (P<0.05) over D0 to D20 in the pregnant than in the non-pregnant heifers. The interaction reflected a decrease (P<0.05) on the expression of these genes between D0 and D10, followed by a progressive increase (P<0.05) from D16 in pregnant heifers. For non-pregnant heifers, only a decrease (P<0.05) between D0 and D10 was observed. Also, a greater (P<0.05) abundance in the pregnant group than in non-pregnant was observed on D18 and D20 for *OAS1* and *ISG15* transcripts. For *MX1*, significant (P<0.05) effects of time and interaction between group and time were detected. The interaction reflected an increase (P<0.05) of its expression from D18 in the pregnant heifers; whereas, no difference (P>0.1) was detected along the days evaluated in the non-pregnant. Additionally, a greater (P<0.05) abundance of *MX1* transcript was observed in pregnant heifers on D18 and D20. For *MX2*, a significant group effect (P<0.05) and an approached (P=0.08) significant effect of time and interaction of group-by-time were observed. The expression of *MX2* was 2-fold greater in the pregnant heifers and the interaction reflected a progressive increase (P<0.05) of its expression from D16 to D20 in the pregnant group; whereas no difference (P>0.1) was observed in the non-pregnant heifers.

Figure 2 - Mean ± SEM of abundance of *ISG15*, *OAS1*, *MX1* and *MX2* transcripts in PBMCs of pregnant (n=8) and non-pregnant (n=9) beef heifers.

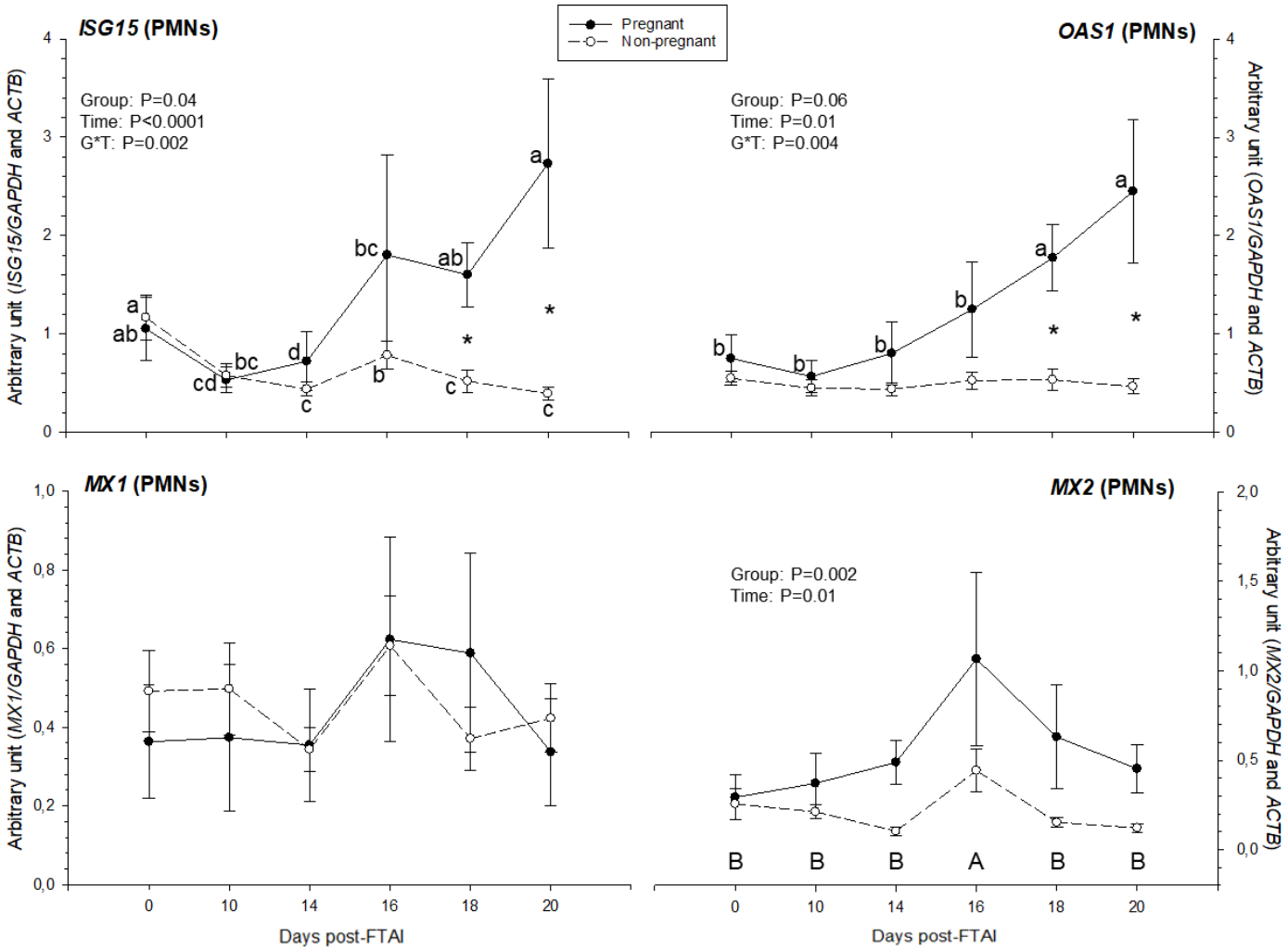


Source: (MELO, G.D., 2019)
Legend: The main effects of group (G), time (T) and their interaction (G*T) are indicated. The asterisks indicate differences (P<0.05) between groups within a day; abcd, the means without a common letter are different (P<0.05) within the groups.

The expression of the four ISGs in PMNs is shown in Figure 3. For *ISG15*, significant (P<0.05) effects of group, time, and their interaction were detected. The group effect was represented by a 2.3-fold greater expression of this gene in the pregnant heifers. The group by time interaction reflected a decrease (P<0.05) of *ISG15* expression between D0 and D10, followed by a progressive increase (P<0.05) from D16 in pregnant heifers. For non-pregnant heifers, a decrease (P<0.05) on this gene expression between D0 and D10 was also observed, followed by an increase (P<0.05) on D16 and a second decrease (P<0.05) on D18. Also, a greater (P<0.05) *ISG15*

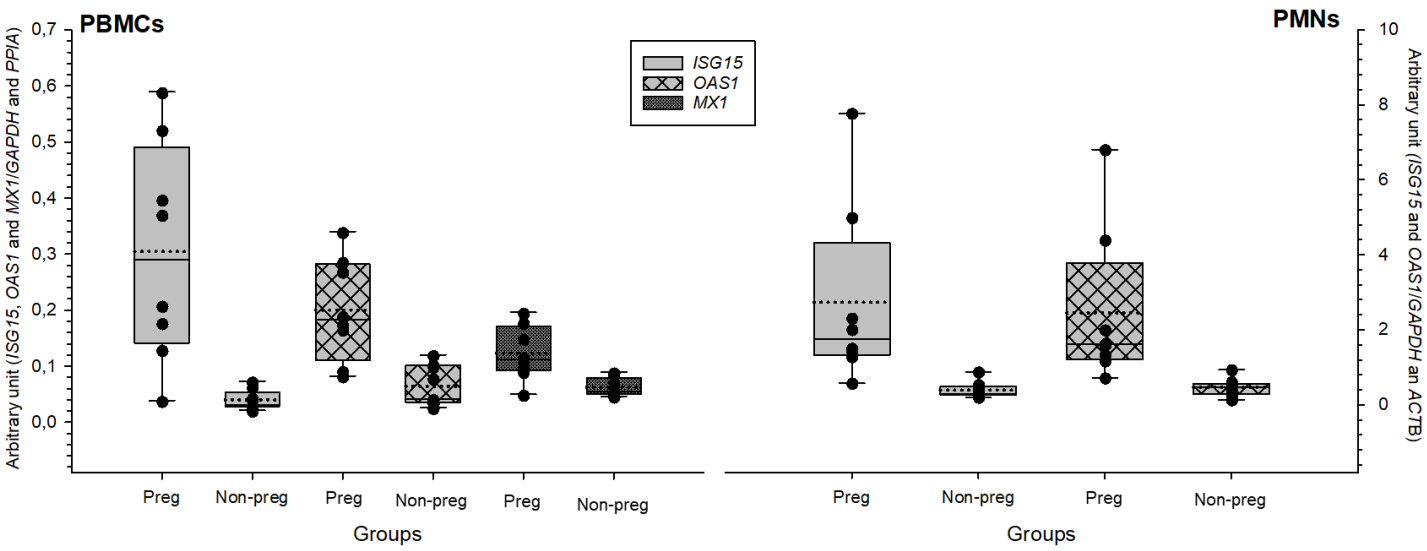
expression on D18 and D20 was observed in the pregnant heifers. For *OAS1*, a significant (P<0.05) effect of time and an interaction of group by time were detected. The *OAS1* expression progressively increased (P<0.05) from D16 in the pregnant heifers; whereas no difference (P>0.1) was detected along the days in the non-pregnant heifers. Also, a greater *OAS1* expression on D18 and D20 was observed in the pregnant heifers. In addition, pregnant heifers tended (P=0.06) do have a 2.6-fold greater expression of this gene. For *MX2*, a 2.6-fold greater expression was detected in the pregnant group, while a time effect (P<0.05) represented a greater expression of *MX2* on D16, regardless of the pregnancy status of the females. For *MX1*, no significant (P>0.1) effect of group, time or interaction of group by time was detected on its expression. The individual expression of the ISGs with a significant difference between the groups on D20 in PBMCs (*ISG15*, *OAS1* and *MX1*) and PMNs (*ISG15* and *OAS1*) is represented in Figure 4.

Figure 3 - Mean ± SEM of abundance of *ISG15*, *OAS1*, *MX1* and *MX2* transcripts in PMNs of pregnant (n=8) and non-pregnant (n=9) beef heifers.



Source: (MELO, G.D., 2019)
Legend: The main effects of group (G), time (T) and their interaction (G*T) are indicated. The asterisks indicate differences (P<0.05) between groups within a day; abcd, the means without a common letter are different (P<0.05) within the groups; AB, the means without a common letter represent the time effect (P<0.05) for both groups.

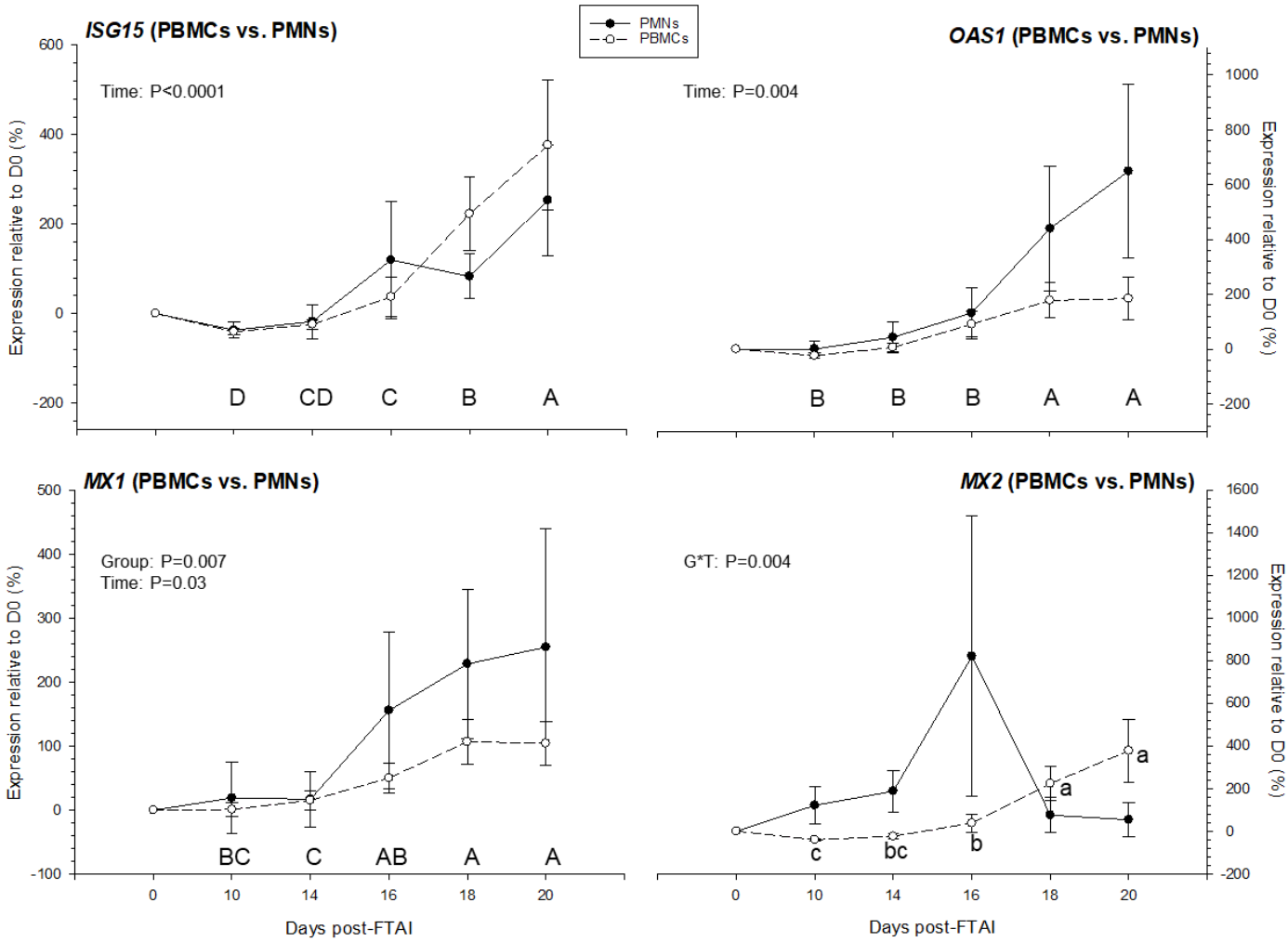
Figure 4 - Box plot representing the individual values for the abundance of *ISG15*, *OAS1* and *MX1* transcripts in PBMCs and *ISG15* and *OAS1* transcripts in PMNs on D20 post-FTAI in pregnant (Preg; n=8) and non-pregnant (Non-preg; n=9) beef heifers.



Source: (MELO, G.D., 2019)
Legend: Mean – dotted line; median – continuous line

When comparing the relative expression of each gene to the D0 in PMNs and PBMCs of pregnant heifers (Figure 5), only a time effect was detected on the expression of *ISG15, MX1* and *OAS1*, indicating an increased (P<0.05) expression from D16 or D18. For *MX1*, a group effect (P<0.05) was also detected, represented by a 2.4-fold greater expression in PMNs than in PBMCs. For *MX2*, a significant (P<0.05) interaction of group by time was detected and reflected a progressive increase (P<0.05) on MX2 expression in PBMCs from 16; whereas, no difference (P>0.1) was observed in PMNs.

Figure 5 - Comparison of *ISG15*, *OAS1*, *MX1* and *MX2* relative expression to D0 between PMNs and PBMCs of pregnant (n=8) beef heifers.

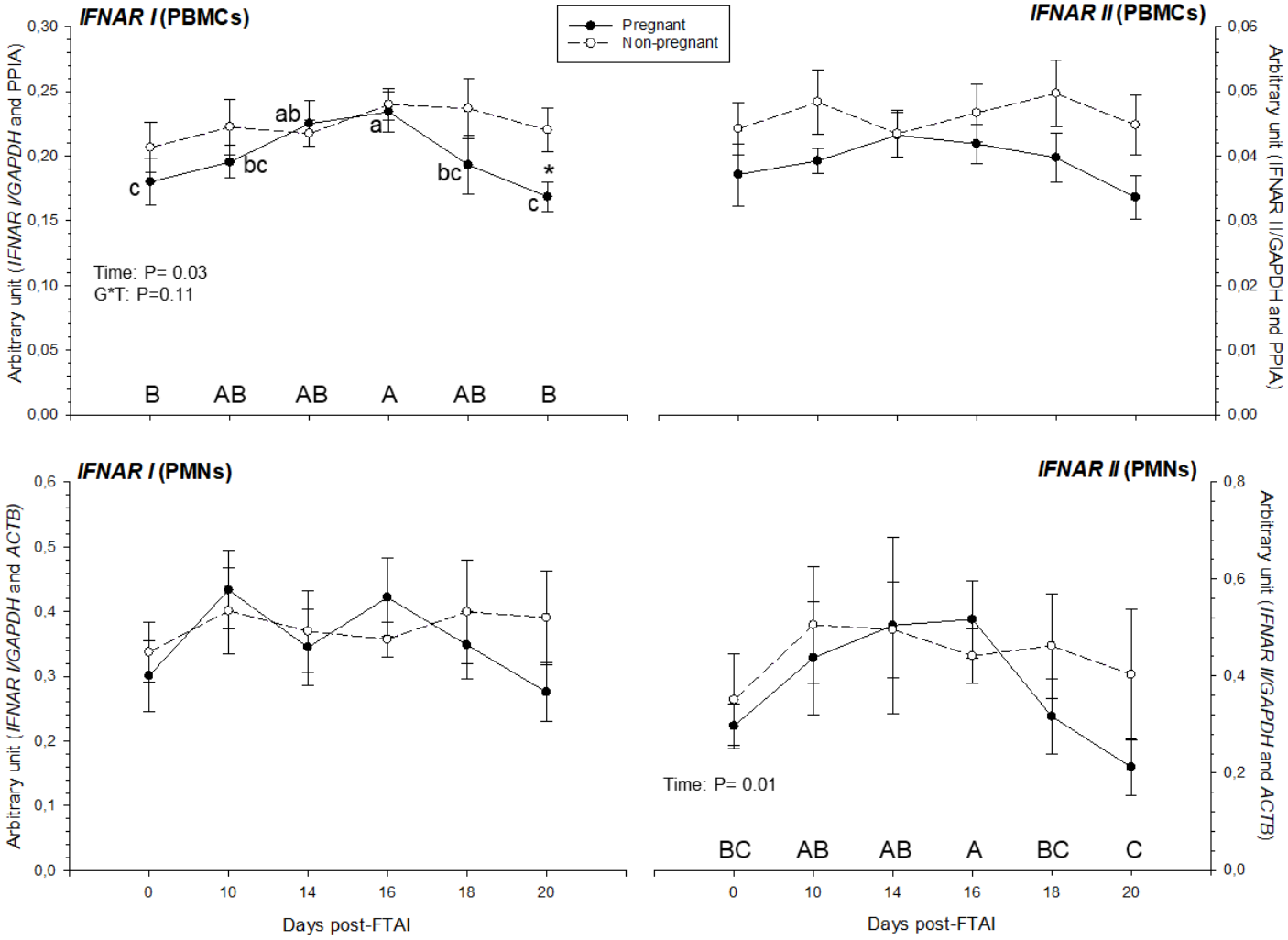


Source: (MELO, G.D., 2019)
Legend: The main effects of group (G), time (T) and their interaction (G*T) are indicated. abc, the means without a common letter are different (P<0.05) within the cellular types; ABCD the means without a common letter represent the time effect (P<0.05) for both cellular types.

The expressions of *IFNAR I* and *II* in PMNs and PBMCs are shown in Figure 6. For PBMCs, only a time effect (P<0.05) was observed for *IFNAR I* expression, characterized by an increase (P<0.05) on the *IFNAR I* transcript abundance between D10 and D16, followed by a progressive decrease (P<0.05) on D18 and D20. Although interaction of group by time was not significant (P=0.11), further analysis indicated that *IFNAR I* abundance in PBMCs of non-pregnant heifers was greater (P<0.05) compared to pregnant heifers on D20. No significant (P>0.1) effects were detected on *IFNAR II* expression in PBMCs. For PMNs, no significant (P>0.1) effects were detected on *IFNAR I* expression, while for *IFNAR II*, a time effect (P<0.05) was observed, indicating an increase (P<0.05) on the *IFNAR II* transcript abundance from D0 to D16, and a

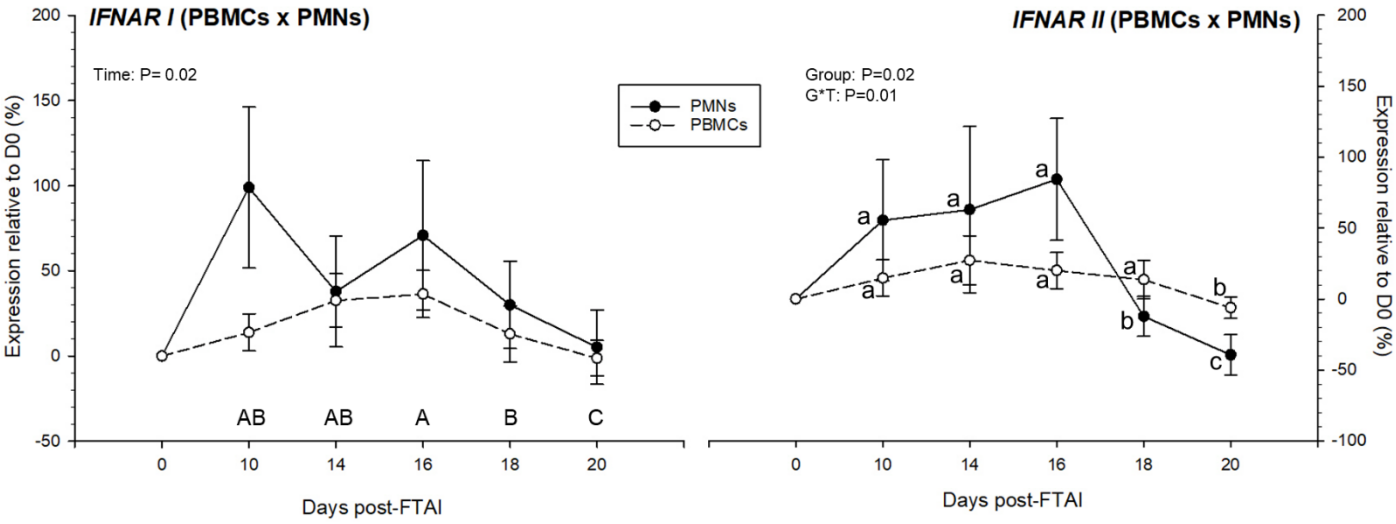
decrease (P<0.05) on D20, regardless the pregnancy status group. The comparison between the relative expression of both *IFNAR*s to D0 in PMNs and PBMCs is demonstrated in Figure 7. For *IFNAR I*, only a time effect (P<0.05) was detected, represented by a decreased (P<0.05) expression of this gene on D18 and D20, regardless of the immune cell type. For *IFNAR II*, significant (P<0.05) effects of group and a group by time interaction were detected. The interaction reflected a decrease (P<0.05) on *IFNAR II* expression only on D20 in PBMCs and a progressive decrease (P<0.05) in PMNs from D18 to D20.

Figure 6 - Mean ± SEM of abundance of *IFNAR I* and *IFNAR II* transcripts in PMNs and PBMCs of pregnant (n=8) and non-pregnant (n=9) beef heifers.



Source: (MELO, G.D., 2019)
Legend: The main effects of time (T) and group by time interaction (G*T) are indicated. abc, the means without a common letter are different (P<0.05) within the groups; ABC the means without a common letter represent the time effect (P<0.05) for both groups.

Figure 7 - Comparison of *IFNAR I* and *IFNAR II* relative expression to D0 between PMNs and PBMCs of pregnant (n=8) beef heifers.



Source: (MELO, G.D., 2019)
Legend: The main effects of group (G), time (T) and their interaction (G*T) are indicated. abc, the means without a common letter are different (P<0.05) within the groups; ABC the means without a common letter represent the time effect (P<0.05) for both groups.

When the abundance of ISGs was analyzed according to the relative expression between D18 and D10 (D18/D10) or between D20 and D10 (D20/D10; Table 2), a greater (P<0.05) relative transcript abundance (D18/D10 and D20/D10) in PBMCs was detected for all ISGs in the pregnant than in non-pregnant heifers. For PMNs, a greater (P<0.05) relative transcript abundance (D18/D10 and D20/D10) in pregnant heifers was only detected for *ISG15* and *OAS1*. Also, the relative ISGs expression in the pregnant heifers did not differ (P>0.1) between PMNs and PBMCs (Table 2).

Table 2 - Mean ± SEM for relative abundance between D16 and D10, D18 and D10 and D20 and D10 of *ISG15*, *OAS1*, *MX1* and *MX2* transcripts in PMNs and PBMCs of pregnant (n=8) and non-pregnant (n=9) beef heifers.

Endpoint	PBMCs			PMNs		
	Pregnant	Non-pregnant	P value	Pregnant	Non-pregnant	P value
ISG15						
D16/D10	2.13 ± 0.5	1.2 ± 0.1	NS	2.64 ± 0.7	1.75 ± 0.4	NS
D18/D10	5.16 ± 1.00	1.10 ± 0.1	0.01	4.01 ± 0.8	1.10 ± 0.2	0.01
D20/D10	7.78 ± 2.00	0.99 ± 0.01	0.01	6.6 ± 1.5	0.86 ± 0.2	0.01
OAS1						
D16/D10	2.36 ± 0.4	1.13 ± 0.2	0.01	2.03 ± 0.3	1.4 ± 0.3	NS
D18/D10	3.43 ± 0.5	1.3 ± 0.3	0.01	5.72 ± 1.6	1.5 ± 0.4	0.03
D20/D10	3.53 ± 0.6	1.01 ± 0.1	0.004	7.4 ± 2.00	1.3 ± 0.3	0.02
MX1						
D16/D10	1.5 ± 0.2	1.14 ± 0.1	NS	2.5 ± 0.5	1.7 ± 0.3	NS
D18/D10	2.05 ± 0.3	1.04 ± 0.1	0.01	6.69 ± 2.5	1.01 ± 0.2	0.06
D20/D10	2.08 ± 0.4	0.83 ± 0.1	0.01	7.5 ± 3.4	1.5 ± 0.6	NS
MX2						
D16/D10	2.25 ± 0.6	1.4 ± 0.4	NS	4.57 ± 2.00	2.88 ± 0.8	NS
D18/D10	3.43 ± 0.7	1.17 ± 0.2	0.02	3.32 ± 5.6	0.97 ± 0.2	NS
D20/D10	3.93 ± 0.8	1.01 ± 0.1	0.01	4.59 ± 2.7	1.10 ± 0.7	NS

Source: (MELO, G.D., 2019)
Legend: NS – Non-significant

Pearson's correlation results between ISGs and IFNARs expression in all groups and cells on D16, D18, D20, and D0-D20 are indicated in Table 3. We verified a significant (P<0.05), but a weak positive correlation between *MX2* and *IFNAR II* in PMNs (r=0.27), and *MX1* and *IFNAR II* in PBMCs on D0-D20 (r=0.20). Also, a significant (P<0.05) and low positive correlation was found between *MX1* and *IFNAR I* in PMNs on D1-D20 (r=0.36); and a significant (P<0.05) and moderate positive correlation was reported between *MX2* and *IFNAR II* on D16 (r=0.66).

Table 3: Pearson's coefficient correlation and P values between ISGs and IFNARs expression in PMNs and PBMCs on D16, D18, D20 and D0 to D20 of pregnant and non-pregnant heifers.

	IFNAR I				*IFNAR II*			
	D16	D18	D20	D0 to D20	D16	D18	D20	D0 to D20
PMNs								
ISG15	0.40 (NS)	0.27 (NS)	0.02 (NS)	-0.05 (NS)	0.31 (NS)	-0.13 (NS)	-0.32 (NS)	-0.04 (NS)
OAS1	0.43 (NS)	0.11 (NS)	0.04 (NS)	-0.004 (NS)	-0.14 (NS)	-0.23 (NS)	-0.26 (NS)	-0.10 (NS)
MX1	0.30 (NS)	0.37 (NS)	0.23 (NS)	0.36 (0.0005)	0.37 (NS)	0.20 (NS)	0.21 (NS)	-0.14 (NS)
MX2	0.47 (NS)	-0.11 (NS)	0.26 (NS)	-0.14 (NS)	0.66 (0.01)	-0.16 (NS)	-0.16 (NS)	0.27 (0.02)
PBMCs								
ISG15	0.08 (NS)	0.06 (NS)	-0.34 (NS)	-0.05 (NS)	0.01 (NS)	-0.04 (NS)	-0.44 (NS)	-0.11 (NS)
OAS1	0.02 (NS)	0.15 (NS)	-0.34 (NS)	-0.05 (NS)	0.06 (NS)	0.006 (NS)	-0.66 (NS)	-0.33 (NS)
MX1	0.09 (NS)	0.17 (NS)	0 (NS)	0.07 (NS)	0.06 (NS)	-0.0001 (NS)	-0.16 (NS)	0.20 (0.03)
MX2	-0.25 (NS)	-0.10 (NS)	-0.35 (NS)	-0.005 (NS)	-0.15 (NS)	-0.12 (NS)	--0.36 (NS)	-0.07 (NS)

Source: (MELO, G.D., 2019)

2.4. DISCUSSION

Understanding the stimuli of a viable conceptus on the maternal immune system may contribute to the development of new technologies to promote a more efficient and early pregnancy diagnosis, benefiting livestock productions. The present research provides evidence that the expression of ISGs in both PMNs and PBMCs could be used as a potential tool on the detection of pregnant bovine females, as it can detect the presence of the conceptus on early stages of development in heifers with a functional CL on days 18 and 20 after FTAI.

The present results related to CL function supported that the luteal characteristics observed by real-time Doppler ultrasonography are powerful indicators of pregnancy status, since it was possible to observe in non-pregnant heifers reduced plasma P4 concentrations and CL area from D18, and reduced CL blood perfusion from D16. These results corroborate with (PUGLIESI et al., 2014), which highlighted the difference in CL function between pregnant and non-pregnant Nelore cows during the luteolytic period. The effectiveness of Doppler Ultrasonography as an early method of pregnancy diagnosis for detecting non-pregnant females between days 20 and 22 has proved to be highly efficient in beef cows, but the accuracy is reduced in beef heifers due to a greater proportion of false-positive results (PUGLIESI et al., 2014, 2018). Thus, the detection of a functional CL by Doppler ultrasonography does not confirm

the presence of a viable conceptus, as an active CL on days 20-22 after FTAI can result from several reasons, such as non-synchronized ovulation, a longer estrous cycle or early pregnancy losses (PUGLIESI et al., 2018). Therefore, the development of an early diagnosis method that can identify pregnancy by detecting the conceptus or its markers could reduce the false positive results and improve the accuracy in females with a functional CL on about 20 days after FTAI.

The expression of ISGs in peripheral blood leukocytes (HAN et al., 2006; GIFFORD et al., 2007; STEVENSON et al., 2007) can indirectly represent the IFNT secretion, but the use of specific immune cell groups for evaluation of ISGs expression could be more advantageous as inconsistent results were reported when the total leukocyte fraction was used (HAN et al., 2006; GREEN et al., 2010a; KIZAKI et al., 2013). In our study, we characterized the expression of the four classic ISGs in PBMCs and PMNs before, during and after the period of pregnancy recognition in cattle (D15 to D18). For both immune cell types, it was possible to observe a greater abundance of ISGs in the pregnant group right after the pregnancy recognition period. In PBMCs, *ISG15*, *OAS1* and *MX1* expression increased from D16 in pregnant females, with greater abundance compared to the non-pregnant heifers on D18 and D20. These results are consistent with those reported in previous studies in beef cows (PUGLIESI et al., 2014) and dairy heifers and cows (HAN et al., 2006; GIFFORD et al., 2007; GREEN et al., 2010a). In all the prior studies using PBMCs or the total fraction of peripheral blood immune cells, as well as in the present one, days 18 and 20 of pregnancy were classified as the most appropriate for diagnosing pregnancy due to lesser data overlapping between pregnant and non-pregnant animals. The minor data overlap at this time point results from greater IFNT secretion around day 20 of gestation, since it increases proportionally to the growth of the trophectoderm (THATCHER; MEYER; DANET-DESNOYERS, 1995; ROBERTS et al., 2008). Regarding *MX2* expression in PBMCs, a significant difference between pregnant and non-pregnant heifers within a day was not detected, which corroborates with Stevenson *et al.* (2007), but contradicts other findings (YANKEY et al., 2001; PUGLIESI et al., 2014). We first hypothesized that the lack of response on *MX2* expression could be related to the use of heifers (nulliparous) in the present study, since most studies were conducted in cows. However, Green *et al.* (2010) reported a greater expression of this gene in heifers and primiparous cows when compared to

multiparous. We also hypothesized an abnormal response of this gene expression in response to viral infections, due to its role on the regulation of the innate immune response (STEVENSON et al., 2007). The other ISGs, though, also play a role in the immune response against viral infections (SCHOGGINS, 2014, 2019), and in the present study, only *MX2* expression was impaired in PBMCs.

Previous studies (SHIRASUNA et al., 2012; KIZAKI et al., 2013) suggested an earlier response of PMNs to IFNT stimuli between the first and second weeks of pregnancy in dairy cows. Also, *in vitro* studies suggested a greater sensitivity of PMNs to the stimuli of the conceptus as these cells respond rapidly to low IFNT concentrations (TOJI et al., 2017; MANJARI et al., 2018). Therefore, we hypothesized that ISGs would be detected earlier and more intensely in PMNs than in PBMCs. Few studies reported the expression of ISGs in PMNs during early pregnancy in dairy cattle (SHIRASUNA et al., 2012; KIZAKI et al., 2013; MANJARI et al., 2018; SHEIKH et al., 2018; YOSHINO et al., 2018), but to our knowledge, this is the first time that a direct comparison of ISGs expression between PMNs and PBMCs was made simultaneously at several specific time points of the first three weeks of pregnancy in cattle. In the present study, however, the hypothesis of an early response in PMNs was not supported, as significant increases in the expression of these genes in pregnant heifers were only evident from D16 or D18. Furthermore, significant differences between pregnant and non-pregnant heifers were only found for *ISG15* and *OAS1* expression in PMNs on D18 and D20. Evidence of a more intense response to the conceptus stimuli in PMNs was also not supported, since a cell type effect was only detected on *MX1* expression in PBMCs versus PMNs comparison. However, interesting profiles were observed for *MX1 and MX2* expression in PMNs. The *MX2* expression was greater over the days evaluated in the pregnant group, while *MX1* expression did not differ between pregnant and non-pregnant heifers. Nevertheless, the increased expression of *MX2* on D16 followed by a decrease on D18 and D20, regardless the pregnancy group, indicates that the conceptus may have stimulated this gene for a shorter period in both groups, but with greater intensity in heifers that become pregnant than in those probably undergoing a pregnancy loss in the non-pregnant group. These results indicate a different response of these genes to the conceptus presence between PMNs and PBMCs.

It is necessary to highlight that the analyses of *MX1* and *MX2* expression in PMNs by qPCR had several amplification problems, which compromised the number of samples analyzed. We believe that even though the primers were carefully selected according to previous studies and validated for PMNs and PBMCs used herein, the shortened lifespan of PMNs could have resulted in more degradable mRNA for these genes (Figure 8). PMNs are known to have a shorter lifespan (MCCRACKEN; ALLEN, 2014), and in the present study, cell isolation protocol was performed within five hours. White blood cells harvested from peripheral blood circulation are often vulnerable to metabolic stress during collection and further processing (HÄRTEL et al., 2001), which could alter the mRNA profile from these cells (BAECHLER et al., 2004). Also, some studies reported difficulties in obtaining good quality mRNA from human neutrophils (LAKSCHEVITZ et al., 2015) and PMNs (KUHNS et al.,) Therefore, we believe that the mRNA integrity of our PMNs samples could have been compromised by the long-lasting processing, from blood collection until cell isolation.

We first hypothesized that *IFNAR I* and *II* transcripts would be up-regulated in pregnant heifers, which could represent a positive feedback mechanism to the increased IFNT concentrations, contributing to the maintenance of pregnancy. This hypothesis, however, was not supported, since no difference was detected between pregnant and non-pregnant females on IFNARs abundance. However, an interesting response was identified on *IFNAR I* expression in PBMCs and *IFNAR II* expression in PMNs, characterized by an increase of these genes' abundance from D10 to D16, followed by a progressive decrease until D20, regardless of the pregnancy status. Although no significant group by time interaction was detected, further analyses indicated a lower *IFNAR I* abundance in pregnant heifers on D20 when compared to the non-pregnant. Visually, it was also possible to observe a decrease on IFNARs abundance in both PMNs and PBMCs after D16, even though this response was not significant. The reasons why *IFNAR* expression oscillates throughout pregnancy and the estrous cycle have not yet been elucidated, but a negative feedback was reported after increased IFNT stimuli (TOJI et al., 2017). It was possible to conclude that IFNARs are not up-regulated in pregnant females, but more studies are necessary to elucidate if the negative feedback of IFNT concentrations on IFNARs abundance could result in a lower expression of these transcripts in pregnant females.

We also hypothesized that IFNARs response would be more prominent in PMNs than in PBMCs. Toji et al. (2017) reported no differences on the abundance of these transcripts between these immune cell groups, but this analysis was performed only on day 21 of gestation. To test this hypothesis, the expression of these genes in both immune cell types was compared only in pregnant heifers. No difference on *IFNAR I* expression between PMNs and PBMCs was observed, but a decrease on *IFNAR II* abundance was detected on D18 and D20 in PMNs. Thus, IFNT receptors response is not more prominent in PMNs, but there is an earlier decrease of *IFNAR II* in this cell group. Even noticing that the responsiveness of the cells to IFNT was not correlated to the expression levels of their receptors, Toji *et al.* (2017) also reported greater sensitivity of PMNs to the conceptus. Although we verified some weak to moderate correlation between *MX1/MX2* and *IFNAR I/IFNAR II* in PMNs and PBMCs, a greater sensitivity in PMNs when compared to PBMCs was not confirmed. Rosenfeld *et al.* (2002) also found no differences in the expression of these receptors in the endometrium of ewes, but a preceding study (HAN et al., 1997) detected greater expression of *IFNAR I* and *II* in the endometrium of pregnant ewes at D15 and D16 of gestation. Although these results were reported in the uterus, the activation of ISGs in PMNs and PBMCs follows the same pattern (TOJI et al., 2017) through JAK-STAT pathway (BINELLI et al., 2001).

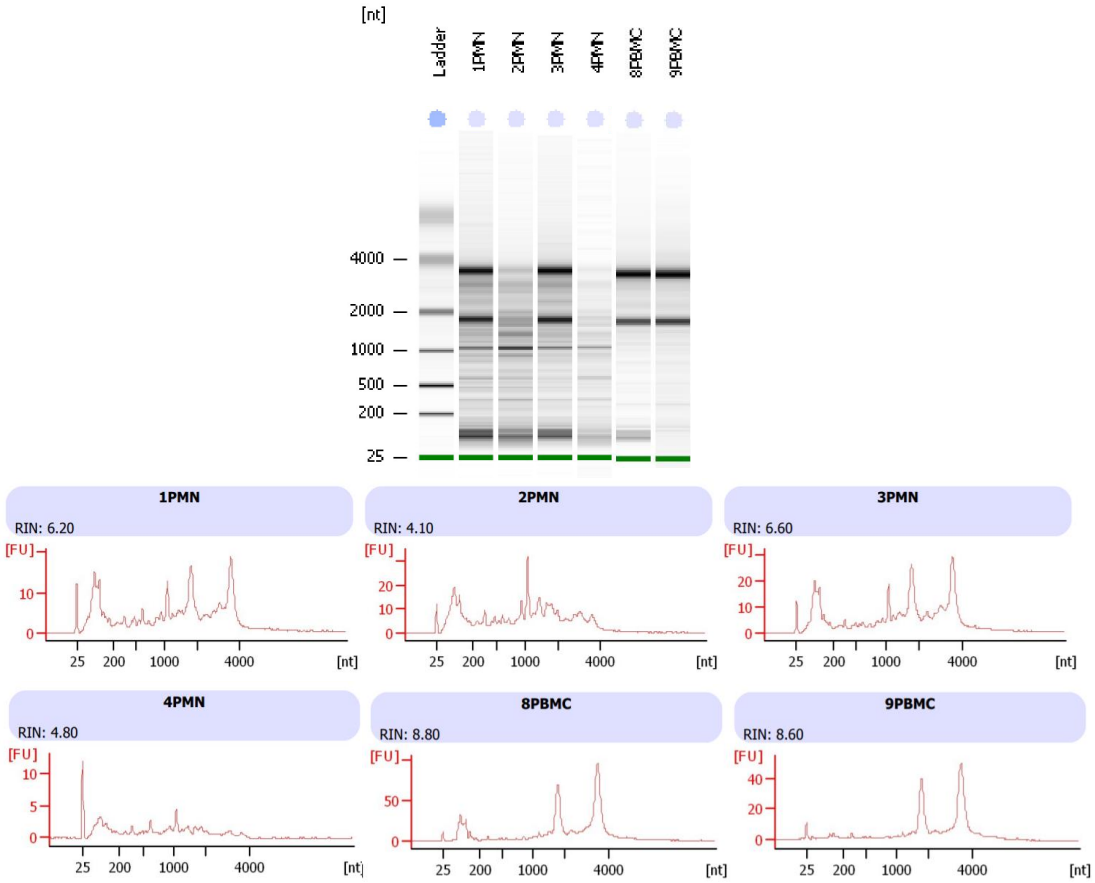
In the present study, we verified an overlap of gene expression data between pregnant and non-pregnant females, which was already reported by other authors (HAN et al., 2006; GREEN et al., 2010a). This overlap was observed even on days 18 to 20 of pregnancy, when greater IFNT concentrations are expected. One of the causes could be attributed to embryonic mortality, since all heifers were submitted to FTAI. Heifers diagnosed as non-pregnant could have experienced a gestational loss, which would cause an abnormal ISGs expression throughout the evaluated days (HAN et al., 2006; KOSE et al., 2016; SHEIKH et al., 2018). Other causes could be linked to the overlap in gene expression between the groups, such as the individual pattern release of IFNT from the conceptuses (THATCHER et al., 2001; CLEMENTE et al., 2011; BARNWELL et al., 2016), the response of ISGs to other type I interferons (SHAW et al., 2017; SHIROZU et al., 2017) and the parity status of the females (GREEN et al., 2010a; SOUMYA et al., 2017). This situation can significantly reduce the reliability of this method, especially when ISGs expression is evaluated within a day. Thus, to

improve the use of ISGs expression as pregnancy markers, better accuracies were obtained when the diagnosis was performed retrospectively, by considering the relative expression of each individual to a baseline sample collected at the day of insemination or prior to day 15 of pregnancy (HAN et al., 2006; GIFFORD et al., 2007; GREEN et al., 2010a; KIZAKI et al., 2013; PUGLIESI et al., 2014). Indeed, we noticed that all ISGs detected in PBMCs and *ISG15* and *OAS1* detected in PMNs were significantly greater on D18 and D20 when their expression was compared to D10.

In this study, we also verified an unusual decrease in the abundance of *ISG15* and *OAS1* transcripts between D0 and D10 in both PMNs and PBMCs, regardless of the pregnancy establishment. A similar response was reported by some authors (GREEN et al., 2010b; ZHAO et al., 2017), who described the expression of the same genes before FTAI. This finding could be associated with the response of immune cells to the greater circulating estradiol concentrations during the estrous period. Through its immunomodulatory effects, estradiol can alter the immune response, and studies (HAWK, 1983; ROTH et al., 1983) have shown that there is greater migration of leukocytes in the pro-estrous and estrous phases. Therefore, the upregulation of gene expression on D0 could be a result of increased leukocyte activity, which would be reduced on the luteal phase (LANDER CHACIN; HANSEN; DROST, 1990), and this could explain the decreased ISGs expression from D0 to D10. However, further studies are needed to investigate the role of ISGs at estrus and early diestrus phases in cattle.

In summary, our results suggest that PMNs do not respond earlier to the IFNT stimuli, having a similar pattern of *ISG15* and *OAS1* expression compared to PBMCs. Therefore, the expression of these genes can be evaluated in both PMNs and PBMCs for the detection of pregnancy, preferably between D18 and D20. The *MX1* and *MX2* are not suitable early pregnancy markers in PMNs. Expression of *IFNAR I* is similar between both immune cell types, but pregnancy establishment may strongly down regulate the expression of *IFNAR II* in PMNs on D18 and D20.

Figure 8 – Supplemental file. Agilent Bioanalyzer profiles of RNA extracted form PMNs and PBMCs.



Source: (MELO, G.D., 2019)
Legend: RIN: RNA Integrity Number.

3. CHAPTER 2: Applied use of interferon-tau stimulated genes expression in polymorphonuclear cells to detect pregnancy compared to other early predictors in beef cattle

3.1. INTRODUCTION

An early pregnancy diagnosis in beef and dairy cattle operations is fundamental to identify non-pregnant females and females undergoing pregnancy losses, allowing new chances of conception in a shorter interval (PUGLIESI et al., 2019). Currently, the most common method to diagnose pregnancy in cattle consists on the visualization of the embryo by transrectal ultrasonography. This method, however, is mainly executed from 28 to 40 days after breeding or artificial insemination (AI), which does not allow the pregnancy detection before estrus return.

In the last decades, other techniques have gained prominence on pregnancy diagnosis, and among them, the detection of structural and functional luteolysis in non-pregnant females is highlighted. The detection of structural luteolysis by Doppler ultrasonography (Doppler-US) has been used in beef cows 20-22 days after fixed time AI (FTAI) programs (PUGLIESI et al., 2014, 2018) with great accuracy and sensitivity. Functional luteolysis can be detected by the measurement of plasma progesterone (P4) concentrations (LAING; HEAP, 1971; SHEMESH; AYALON; LINDNER, 1973), as low P4 concentrations 18 to 24 days after breeding can accurately predict non-pregnancy (BALHARA et al., 2013). However, when Doppler-US and P4 methods were compared to the diagnosis on day 30 of gestation, a considerable percentage of false-positive results was reported (BALHARA et al., 2013; SIQUEIRA et al., 2013; PUGLIESI et al., 2014), since a functional corpus luteum (CL) at this period can be related to causes other than pregnancy establishment. Therefore, both methods identify the CL regression in non-pregnant females, but do not allow the detection of an embryo or any marker of its presence (GINTHER; SHRESTHA; BEG, 2010; PUGLIESI et al., 2013). Thus, the development of a method that identify pregnancy by detection of the conceptus or its markers could reduce the false positive results and improve the accuracy of pregnancy diagnostic methods within the first three weeks after FTAI.

As alternative methods to indirectly detect the conceptus presence, quantification of interferon-tau (IFNT) stimulated genes (ISGs) in peripheral blood leukocytes have been recently reported (GIFFORD et al., 2007; GREEN et al., 2010a; YOSHINO et al., 2018). In ruminants, IFNT is secreted by the conceptus in uterine environment, and participates in the process of maternal recognition of pregnancy by blocking the mechanisms involved in the pulsatile secretion of prostaglandin F2α (PGF2α) by the endometrium (WATHES; LAMMING, 1995). This blockage determines the maintenance of P4 secretion by the CL and, consequently, the establishment of gestation. The expression of ISGs was shown to be significant greater in pregnant females from D18-20 after FTAI in total leukocytes (HAN et al., 2006; GREEN et al., 2010a), peripheral blood mononuclear cells (PBMCs; GIFFORD et al., 2007; PUGLIESI et al., 2014) and polymorphonuclear cells (PMNs; YOSHINO et al., 2018; MELO et al., 2019). Previous studies (HAN et al., 2006; MATSUYAMA et al., 2012; KOSE et al., 2016; SHEIKH et al., 2018) also reported different profiles of ISGs expression in pregnant, non-pregnant and females undergoing pregnancy losses, suggesting that this difference could be used for prediction of embryonic losses, but there is still a lack of knowledge in this regard.

Circulating pregnancy associated glycoproteins (PAGs) concentrations have also been used as markers of pregnancy. The PAGs are members of the aspartic protease family, produced by binucleate trophoblast cells of the placenta (WOODING; ROBERTS; GREEN, 2005) and secreted into the uterine stroma, later reaching maternal circulation and detected after placentation onset (GREEN et al., 2005). Commercial ELISA assays for determination of PAGs provide a high accurate method for pregnancy detection when used in milk (RICCI et al., 2015) and blood samples 28 to 30 days after FTAI (POHLER et al., 2016a). Recent studies (REESE et al., 2019) demonstrated that day 24 of pregnancy can also be used for pregnancy diagnosis, as it is the first day in which a significant increase on PAGs concentrations was detected in suckled beef cows (POHLER et al., 2013). In addition, PAGs have also been pointed as a potential tool for the prediction of embryonic mortality, since circulating concentrations of these glycoproteins are reduced in *Bos Taurus* (GIORDANO et al., 2012; POHLER et al., 2013, 2016b) and *Bos indicus* cattle (POHLER et al., 2016a; FILHO et al., 2020) that underwent embryonic loss.

Thus, early identification of non-pregnant females, or females undergoing embryonic loss is important as it allows them to be quickly managed and reinserted into their respective reproductive programs, avoiding greater losses in terms of productivity and profit. Therefore, in the present study we aimed: 1) to evaluate the accuracy of ISGs abundance in PMNs on D20 post-FTAI as a pregnancy diagnosis method, and to compare this accuracy between beef cows and heifers; 2) to compare the effectiveness of the ISGs method with the evaluation of CL by Doppler-US and P4 concentrations on D20 and PAGs concentrations on D25; and 3) to evaluate the possibility of using ISGs abundance in PMNs as pregnancy loss predictors. We hypothesized that bovine conceptus can stimulate transcripts in immune cells that could serve as biomarkers of early gestation and predictors of pregnancy losses.

3.2. MATERIAL AND METHODS

3.2.1. Animals

The experiment was conducted at the research farm of the University of São Paulo in Pirassununga, SP, Brazil. Nelore cows at 35-45 days postpartum (primiparous, n=53; multiparous, n=91), weighing in average 501 kg (range: 389-650 kg), and Nelore heifers (n=103), weighing in average 350 kg (range: 284-386 kg), were used in this study. The animals were maintained on *Brachiaria brizantha* pastures, received mineralized salt and free access to water and were handled in accordance with the Institutional Committee for Ethics in Research of the University of São Paulo (CEUA-FMVZ number: 3554190717).

3.2.2. Experimental design

All animals were subjected to an estradiol (E2) and P4 based protocol for synchronization of ovulation and FTAI, as follows. On D-10, females received an intravaginal P4-releasing device (0.96 g, Progestar, Boehringer Ingelheim, Paulínia, SP, Brazil) along with the administration of 17β-Estradiol (5.5 mg, i.m., Betaproginn, Boehringer Ingelheim). Heifers also received a PGF2α analog (0.150 mg of D-cloprostenol, i.m., Croniben, Biogénesis Bagó). On D-2, the P4 device was removed and the animals received another treatment with a PGF2α analog (0.150 mg of D-

cloprostenol, i.m., Croniben, Biogénesis Bagó) and estradiol cypionate (1 mg, i.m., Croni-CIP, Biogénesis Bagó). On this day, heifers and cows with body condition scores <3 (1 to 5 scale; PFEIFER et al., 2017) also received equine chorionic gonadotropin (200 IU, i.m., Novormon, Zoetis, Campinas, SP, Brazil). On D0, all animals were artificially inseminated by two operators using thawed semen from eight Nelore sires. Ovulation after FTAI was not confirmed, but based on previous experiments using the same synchronization protocol, ovulation rates were expected to be 70%.

Pregnancy was assessed by ultrasound evaluations through the detection of a viable embryo or fetus (presence of embryonic membrane, fluid and heartbeat), respectively, on days 30 and 70 after FTAI. Pregnancy diagnosis on D30 was considered as the gold standard for comparison with the other methods. On D20, besides the evaluation of the CL blood perfusion by Doppler-US, pregnancy was also evaluated by the expression of ISGs (*ISG15* and *OAS1*) in PMNs and plasma P4 concentrations, while on D25, plasma PAGs concentrations were determined. After the detection of pregnancy losses on D70, the females were classified in one of the following four groups: 1) Pregnant (presence of a functional CL on D20 and a viable conceptus on D30 and D70); 2) Non-pregnant (non-functional CL on D20 and absence of a viable conceptus on D30); 3) CL-NP (presence of a functional CL on D20, but absence of a viable conceptus on D30) and 4) PL (Pregnancy loss; presence of a functional CL on D20, a viable conceptus on D30, but absence of a viable fetus on D70). In an attempt to detect the early embryonic losses, heifers and cows from the CL-NP group were divided in two subgroups: 1) functional CL on D20, but not on D25 (CL20-25) and 2) functional CL on D20 and D25, but not on D30 (CL25-30). The ISGs abundance, CL blood perfusion by Doppler-US and P4 concentrations on D20 and PAGs concentrations on D25 were compared between the subgroups.

For pregnancy prediction by the four early diagnostic methods proposed, the Doppler-US method was the only method used prospectively based on the presence of a functional CL on D20. The selected cutoff point (luteal blood perfusion ≤ 25%) for the Doppler-US method was used to identify the females with a non-functional CL and they were classified as non-pregnant, as previously determined in beef cows (PUGLIESI et al., 2014). For the other three methods, a cut-off point to identify pregnant and non-pregnant females was determined for each variable by a retrospective analysis using Receiver Operating Characteristic (ROC) curve. Therefore, the characteristics of accuracy and agreements among the methods were

compared to the gold standard method on D30 and between the methods on D20 and D25.

3.2.3. Ultrasound scanning

On D20, D25, D30 and D70 post-FTAI, the animals were evaluated by transrectal ultrasonography using a duplex ultrasound equipment (MyLab^{TM} Delta, Esaote, Italy) with a linear multi-frequency (3.5-7.5MHz) transducer in B-mode (RES-A, gain 50%, P 74mm, X/M, PRS 1) and color Doppler mode (gain 61%, PRF 730Hz, frequency 6,3 MHz, WF 4, PRS 3, PRC M/2). All evaluations were performed by a single operator. The B-mode evaluations were performed on D30 and D70 for pregnancy diagnosis. On D20 and D25, Doppler-US was also used for the assessment of luteal blood perfusion and detection of non-pregnant females that underwent luteolysis, as previously reported (GINTHER, 2007; PUGLIESI et al., 2014).

3.2.4. Blood collection

On D20, D25, D30 and D70, blood samples were collected in sodium heparinized tubes (BD Life Sciences, New Jersey, USA) from the jugular vein for PMN isolation or determination of plasma P4 or PAG concentrations. After collection and manual homogenization, samples were placed in chopped ice until its processing. For PMNs isolation, 25ml of blood were collected on D20. For measurement of PAGs concentrations, 10ml of blood were collected on D25. To measure P4 concentrations, 10ml of blood were collected on D20 and D25 from all the females, and on D30 and D70 from females diagnosed as pregnant on D30. Blood samples used for P4 and PAGs assays were centrifuged at 2500 x g for 15 minutes at 4ºC. The resulting plasma was decanted and stored at -20ºC in 1.5ml sterile microtubes until the assay was performed. The samples destined to PMNs isolation were submitted to the protocol right after animal handling.

3.2.5. Isolation of PMNs

PMNs were isolated by Ficoll® (Ficoll-Paque Plus, GE Healthcare, Chicago, IL, USA) gradient in a methodology adapted from (JIEMTAWEEBOON et al., 2011), as described in Melo et al., 2019 (unpublished data; submitted to Reproduction, Fertility and Development). Briefly, the whole blood was mixed with an equal volume of PBS and the solution was layered onto 15ml of Ficoll-Paque solution and centrifuged. After centrifugation, PBMC and plasma fractions were wasted and PMNs were washed with hypotonic distilled water and lysis solution until a clean pellet was obtained. The remaining pellet was stored at -80^{0}C in RNAse free tubes until RNA extraction. Purity of PMNs was checked immediately after the procedure by staining fresh isolates samples with the quick panoptic protocol. Samples were considered pure when 95% of the 200 counted cells were polymorphonuclear cells.

3.2.6. Assay of circulation progesterone concentrations

Plasma samples collected on D20, D25, D30 and D70 were assayed for P4 using a solid phase ^{125}P4 RIA kit following the manufacturer's instructions (ImmuChem Double Antibody, MP Biomedicals, Irvine, CA, USA). The intra-assay coefficient of variation (CV) was 6.1% and the inter-assay was 10%. Analyses were performed with fewer samples on D20 (n=99 heifers and 136 cows).

3.2.7. RNA extraction and cDNA synthesis

The isolated PMNs were thawed on ice and the RNA was extracted by a modified protocol using Trizol (Thermo Fisher Scientific, Waltham, USA) reagent associated with the DirectZol-RNA kit (Zymo Research, Irvine, USA), as described by Melo et al. (2019, unpublished data; submitted to Reproduction, Fertility and Development). Briefly, PMNs pellets were dissolved in Trizol, and the RNA content of each sample was pipetted after the addition of chloroform. The RNA washing procedures were performed in the columns provided by the kit, as indicated by manufacturer's instructions.

Total RNA samples from PMNs were treated with DNAse I (Life Technologies, Carlsbad, USA) for 15 min at room temperature in a 10µl reaction volume. The concentration of total RNA extracts was measured using a spectrophotometer (NanoVue, GE Healthcare, Chicago, USA). The isolated RNA (0.3µg) was subjected

to reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Life Technologies) according to the manufacturer's instructions, and the cDNA of each sample was stored at -20ºC until qPCR analysis.

3.2.8. Quantitative Polymerase Chain Reaction

Quantification of specific transcripts was performed by the real-time polymerase chain reaction (RT-qPCR) using SYBR Green (Life Technologies, Carlsbad, CA, USA), and the reactions were carried out using a Step One Plus apparatus (Life Technologies). The target (*ISG15* and *OAS1*) and reference genes (*GAPDH* and *ACTB*) were selected according to Melo et al. (2019), and the primer sequences are described on Table 4. The reactions were run in triplicates on a 96-well plate, which was sealed with a MicroAmp optical adhesive cover (Life Technologies) before its reading. The amplification data were extracted from the Step One Plus apparatus and each sample was analyzed through the LinReg PCR software for baseline correction, determination of qPCR efficiency and cycle quantification values (RUIJTER et al., 2009). Expression of each gene relative to the expression of the housekeeping genes was normalized by the comparative Ct method corrected for amplification efficiency (PFAFFL, 2001).

Table 4 - Forward (F) and reverse (R) primers sequences of target and reference genes analyzed using qPCR

Gene	GeneBank	Primer sequence	Reference
OAS1	NM_001040606.1	F: TAGCCTGGAACATCAGGTC R: TTTGGTCTGGCTGGATTACC	Shirasuna et al., 2012
ISG15	NM_174366	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT	Oliveira et al., 2008
GAPDH	NM_001034034.2	F: GCCATCAATGACCCCTTCAT R: TGCCGTGGGTGGAATCA	Araujo et al., 2016
ACTB	NM_173979.3	F: GGATGAGGCTCAGAGCAAGAGA R: TCGTCCCAGTTGGTGACGAT	Araujo et al., 2016

Source: (MELO, G.D., 2019)

3.2.9. Assay of circulating PAGs

Plasma samples collected on D25 were analyzed through an indirect sandwich ELISA assay to measure PAGs concentrations, as described by Green et al. (2005). Briefly, PAGs that were present in the plasma were trapped on the wells of a 96-well ELISA high affinity plate using a mixture of monoclonal antibodies (L4, J2 and A6). These antibodies were oriented in the wells after the plate was incubated overnight at 4°C with a sheep anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) and 0.1 M sodium bicarbonate (pH 9.5). After removing the anti-mouse antibody by inverting the plates and tapping them on a clean paper towel, the wells were filled with blocking solution and incubated at room temperature for one hour. The blocking solution was then removed, and the monoclonal antibodies were diluted in a TBST solution and added to the wells. After one-hour incubation at room temperature, the antibody solution was removed and TBST was added to the wells that would receive the samples and controls in order to prevent them from drying. Next, 100µl of plasma samples were added to the wells in duplicates. Twelve wells were used for the development of a standard curve by using serially diluted PAGs standards mixed with steer serum. The plates were incubated overnight at 4°C. On the following day, the plates were washed with wash buffer and the polyclonal antibody (Ab 63) was added diluted in TBST and steer serum. After one-hour incubation at room temperature, the plates were washed again, and a secondary antibody (goat anti-rabbit – Jackson ImmunoResearch) was added to the wells. Plates were incubated in room temperature for 30 minutes and washed. After washing all the plates, the Para-nitrophenyl phosphate (PNPP) substrate was added to each well, and the plates were kept covered from the light during 20 minutes until reading. The coefficient of variation (CV) intra-assay was 5.2% and the inter-assay was 11%.

3.2.10. Statistical analyses

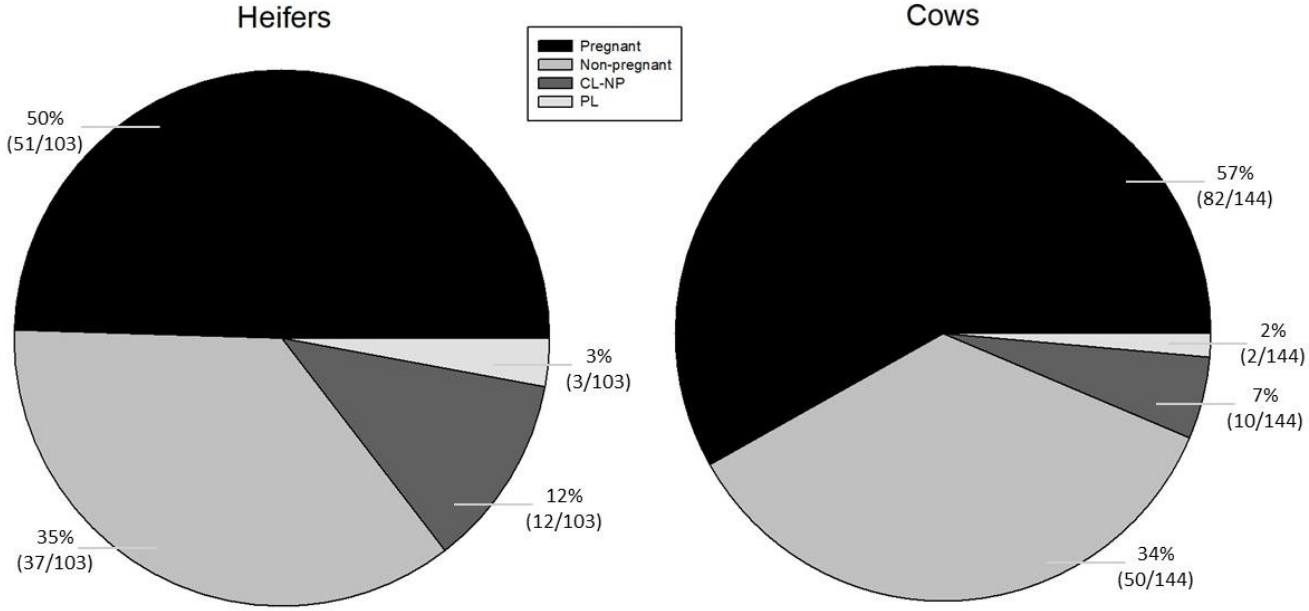
The CL blood perfusion, ISGs expression and PAGs concentrations were compared among groups by analysis of variance (ANOVA) using the PROC MIXED of SAS software (Version 9.2; SAS Institute) and considering the random effects of animals and fixed effects of group (pregnant, non-pregnant, CL-NP or PL), parity category (heifer or cow) and their interaction. Plasma P4 concentrations were analyzed by a split-plot ANOVA using the PROC MIXED of SAS software and considering the fixed effects of group, category, day (D20, D25, D30 and D70) and their interaction.

The data that were not normally distributed according to the Shapiro-Wilk test were transformed to logarithms or ranks. Pearson's correlation among ISGs expression, CL blood perfusion and P4 concentrations on D20 and PAGs concentrations on D25 was analyzed by the MedCalc software package (Version 19.1; MedCalc Software, Mariakerke, Belgium). In addition, accuracy of the pregnancy diagnosis methods by ISGs, PAGs, Doppler-US and P4 in comparison with B-mode ultrasound method on D30 (Gold Standard) was calculated by the number of false-negative (FN) and false positive (FP) observations, negative predictive value (NPV), positive predictive value (PPV), specificity and sensitivity, as previously described by Pugliesi et al. (2014). A cutoff value for ISGs expression, P4 and PAGs concentrations was determined through establishment of a ROC curve by using the MedCalc software. This software was also used to access the area under the curve (AUC) of each method. Agreements among ISGs expression, Doppler-US, P4 and PAGs concentrations were calculated through Kappa Statistic, as reported elsewhere (SIM; WRIGHT, 2005; PUGLIESI et al., 2014). Kappa values equals to 0 indicate no agreements other than what would be expected by chance. Kappa values ranging between 0.1 and 0.20 indicate a minimum agreement; between 0.21 and 0.40 indicate a reasonable agreement; between 0.41 and 0.60 indicate a moderate agreement; between 0.61 and 0.80 indicate a substantial agreement and between 0.81 and 1 indicate a perfect agreement.

3.3. RESULTS

Pregnancy was diagnosed by the detection of a viable embryo (D30) and viable fetus (D70), respectively, on 52% (54/103) and 50% (51/103) of the heifers; and 58% (84/144) and 57% (82/144) of the cows. The rate of detection of a function CL on D20 based on Doppler-US was 64% (66/103) for heifers and 65% (94/144) for cows. Therefore, the rate of animals bearing a functional CL on D20 but non-pregnant at D30 were reported at 12% (12/103) for heifers and 7% (10/144) for cows, and the rate of pregnancy losses between D30 and 70 were 3% (3/103) for heifers and 2% (2/144) for cows (Figure 9).

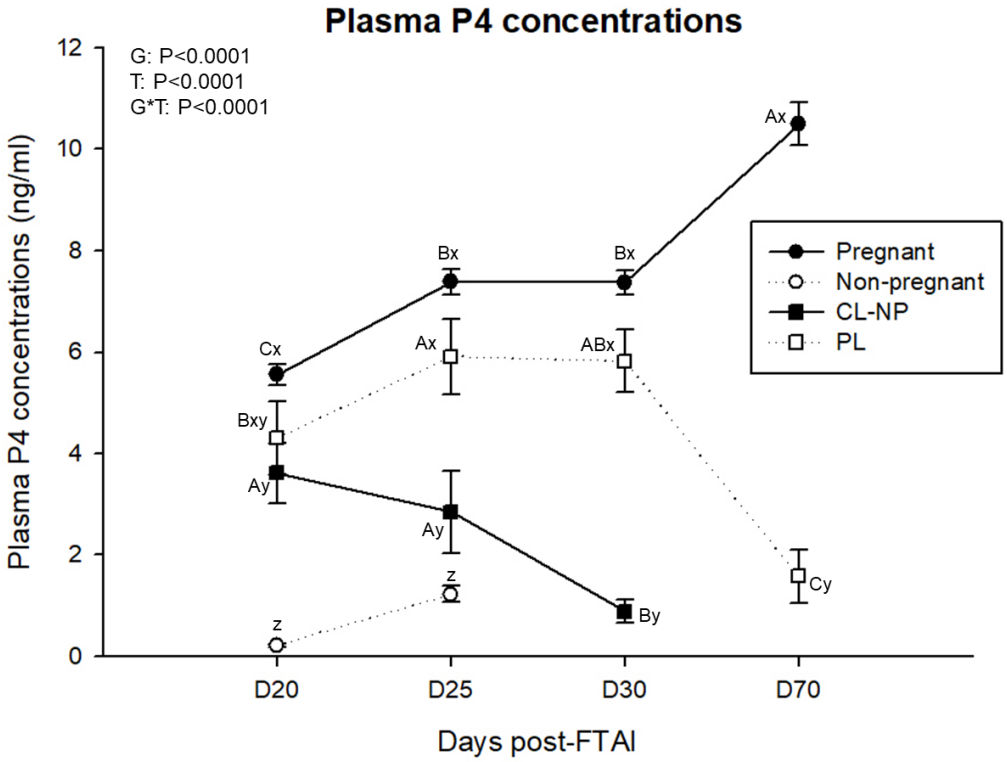
Figure 9 – Percentages and proportion of heifers and cows within each group.



Source: (MELO, G.D., 2019)

As shown in Figure 10, there were significant (P<0.05) effects of group, time and their interaction on plasma P4 concentrations, but a parity category interaction was not detected, so the results were combined for both categories (heifer and cow). The group by time interaction was represented by increased (P<0.05) P4 concentrations in the pregnant group from D25, reaching maximum concentrations on D70. In the PL group, there was also an increase (P<0.05) in P4 concentrations from D20 to D25, followed by a decrease (P<0.05) on D70. In the CL-NP group, P4 concentrations remained high (P<0.05) until D25, followed by a decrease (P<0.05) on D30. In the non-pregnant group, the plasma P4 concentrations were similar (P>0.1) between D20 and D25. Regarding the differences among groups, P4 concentrations were lower (P<0.05) in the non-pregnant group on D20 and 25 compared to the other three groups, and greater (P<0.05) in the pregnant and PL groups on D25 and D30 than in the CL-NP group. Finally, on D70, the P4 concentrations were greater (P<0.05) in the pregnant group compared to the PL group.

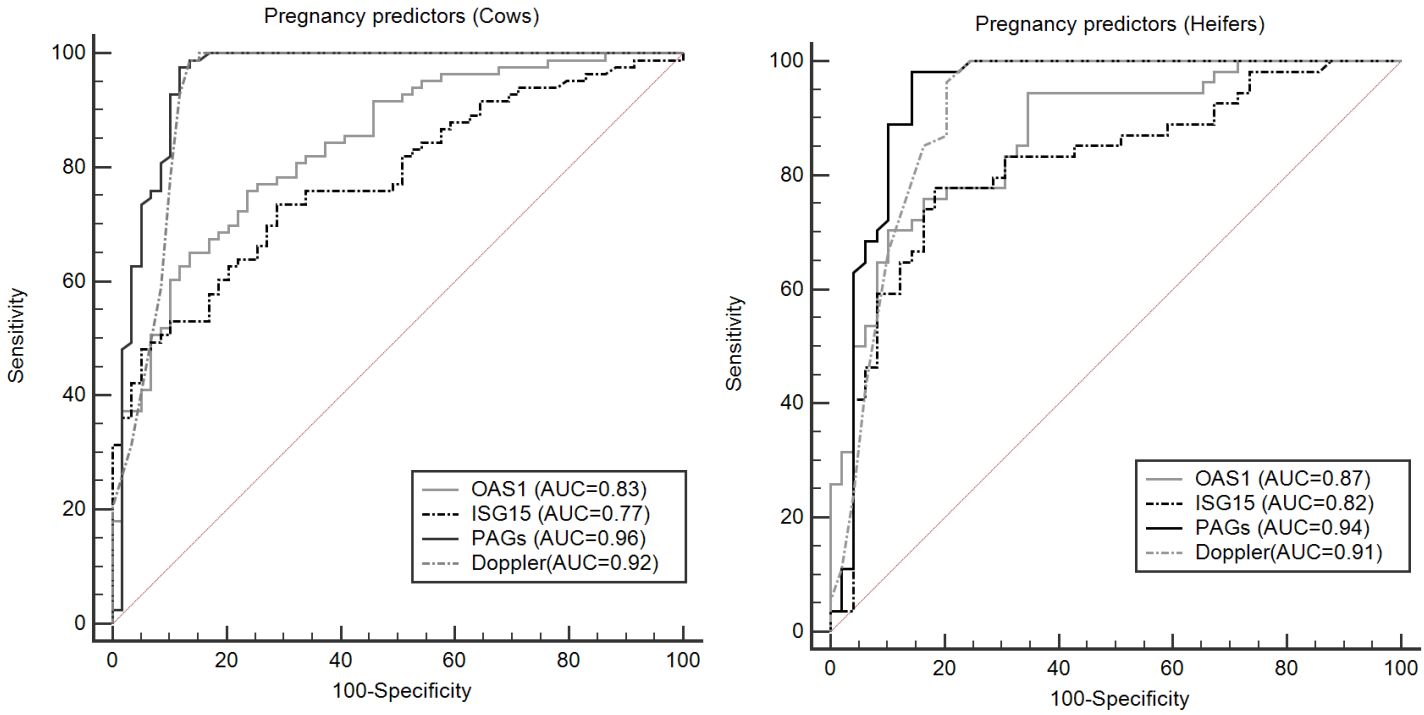
Figure 10 - Mean ± SEM plasma P4 concentrations (ng/ml) from D20 to D70 post-FTAI in pregnant (presence of a functional CL on D20 and a viable conceptus on D30 and D70; n=133), non-pregnant (non-functional CL on D20; n=87 CL-NP (presence of a functional CL on D20, but absence of a viable conceptus on D30; n=22) and PL (pregnancy loss; presence of a functional CL on D20, a viable conceptus on D30, but absence of a viable fetus on D70; n=5) beef heifers and cows.



Source: (MELO, G.D., 2019)
Legend: The main effects of group (G), time (T) and group by time interaction (G*T) are indicated. ABC; different letters within a group indicate a significant (P<0.05) difference between days post-FTAI. Xyz; different subscript letters indicate a significant (P<0.05) difference between groups within a day post-FTAI.

The analysis by the ROC curve indicated that plasma P4 concentrations on D20 are significant (P<0.05) predictors of pregnancy in heifers (AUC=0.90) and cows (AUC=0.94) (Figure 11). The ROC analysis also indicated a cutoff point of 1.25ng/ml for cows and 1.41ng/ml for heifers. When plasma P4 concentrations on D20 were compared to the diagnosis on D30, this method presented an accuracy > than 90% for heifers and for cows (Table 5). It also did not report any FN results, which lead to sensitivity and NPV of 100% for both categories. The number of FP was similar for heifers and cows (9/99 and 8/136, respectively), resulting in specificities of 80% for heifers and 85% for cows.

Figure 11 - Receiver operator characteristics (ROC) curves for plasma P4 concentrations, Doppler-US and ISGs on D20; and PAGs on D25 for heifers (n=103) and cows (n=144) compared to the diagnosis on D30.



Source: Source: (MELO, G.D., 2019)

Table 5 - Number of True-Positive (TP), True-Negative (TN), False-Positive (FP) and False-Negative (FN) results and Sensitivity (SENS), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (ACCU) for determining pregnancy status on D20 post-FTAI by Doppler-US, *ISG15*, *OAS1* or P4 and on D25 pos-FTAI by PAGs in heifers and cows.

Endpoint	Heifers					Cows				
	Doppler-US	*ISG15*	*OAS1*	P4	PAGs	Doppler-US	*ISG15*	*OAS1*	P4	PAGs
N	103	103	103	99	103	144	144	144	136	144
TP (N)	51	42	51	55	53	84	62	63	83	83
TN (N)	37	40	32	35	42	50	42	45	45	52
FP (N)	12	9	17	9	7	10	18	15	8	8
FN (N)	0	12	3	0	1	0	22	21	0	1
SENS (%)[a]	100	78	94	100	98	100	74	75	100	99
SPEC (%)[b]	75	82	65	80	86	83	70	75	85	87
PPV (%)[c]	81	82	75	86	88	89	78	81	91	91
NPV (%)[d]	100	77	91	100	98	100	66	68	100	98
ACCU (%)[e]	85	80	81	91	92	93	72	75	94	94

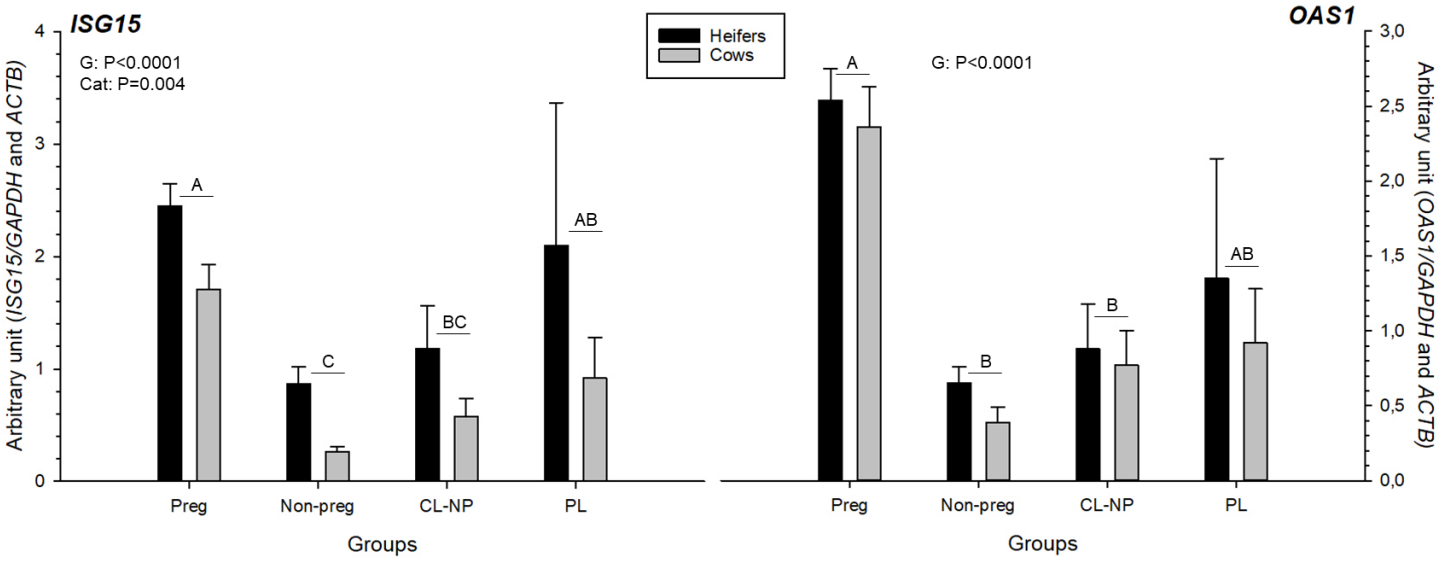
Source: (MELO, G.D., 2019)
Legend: [a] Sensitivity (probability that a test result will be positive when the cow is pregnant) = TP/(TP+FN).
[b] Specificity (probability that a test result will be negative when the cows is not pregnant) = TN/(FP+TN).
[c] PPV (probability that the cow is pregnant when the test is positive) = TP/(TP+FP).
[d] NPV (probability that the cow is not pregnant when the test is negative) = TN/(FN+TN).
[e] Accuracy = (TP+TN)/n.

The ROC analysis indicated that Doppler-US was a significant (P<0.05) predictor of pregnancy in heifers (AUC=0.91) and cows (AUC=0.92) on D20 (Figure 11). With regard to the performance parameters, when compared to the pregnancy diagnosis on D30 (Table 5), Doppler-US showed an accuracy of 85% for heifers and 93% for cows. Sensitivity and NPV was reported as 100% for both categories due to the absence of false negative results. The false positive results (12/103 for heifers and 10/144 for cows) culminated in specificity of 75% for heifers and 83% for cows.

As demonstrated in Figure 12, *ISG15* abundance in the pregnant group was 4-fold greater (P<0.05) than in the non-pregnant group, and 2.2-fold greater (P<0.05) than in the CL-NP group. For this gene, a parity category effect (P<0.05) was also detected, represented by a 1.5-fold greater abundance in heifers than cows. The *OAS1* abundance was only affected by the pregnant status, as indicated by the 4.8-fold

greater abundance in the pregnant group when compared to the non-pregnant, and 3-fold greater when compared to the CL-NP. Abundance of both genes in the PL heifers and cows remained intermediate and did not differ (P<0.1) from the other groups.

Figure 12 - Relative expression of *ISG15* and *OAS1* in PMNs from pregnant (Preg), non-pregnant (Non-preg), functional CL but non-pregnant (CL-NP) and pregnancy loss (PL) heifers (N=103) and cows (N=144) 20 days post FTAI.



Source: (MELO, G.D., 2019)
Legend: The main effects of group (G) and category (Cat) that were significant are shown. The means within a group with a different letter are different (P<0.05).

For *ISG15* and *OAS1*, the cutoff values were stablished through the ROC curve analysis. Due to the parity category effect reported on *ISG15* expression, a different cutoff value for this gene was selected for heifers (1.27 – arbitrary unit) and cows (0.31 – arbitrary unit), while for *OAS1*, the cutoff point was the same for both categories (0.53 – arbitrary unit). According to the ROC curve analysis (Figure 11), expression of *ISG15* and *OAS1* in PMNs are also significant predictors (P<0.05) of pregnancy in heifers (AUC=0.82 and 0.87, respectively) and cows (AUC=0.77 and 0.83, respectively). The accuracy (Table 5) was similar between the genes within each category (heifers: 80% and 81% for *ISG15* and *OAS1* respectively; cows: 72% and 75% for *ISG15* and *OAS1*, respectively). However, the *OAS1* expression in heifers had a lower number of FN (3/103), and consequently, greater NPV (91%) and sensitivity (94%) when compared to *ISG15*. For *ISG15*, there was a greater number of FN (12/103), which resulted in

lower NPV (77%) and sensitivity (78%) when compared to *OAS1*. However, the number of FP was greater for *OAS1* (17/103), which resulted in lower specificity for this gene (65%) when compared to *ISG15* (82%). The proportion of FP and FN results reported for ISGs expression in cows ranged from 18% to 33%, resulting in similar sensitivity (73.8% for *ISG15* and 75% for *OAS1*) and specificity (70% for *ISG15* and 75% for *OAS1*).

Due to the absence of FN results reporter by Doppler-US, a combined method between Doppler-US and ISGs was approached, characterized by the use of the ISGs method only in females with a functional CL on D20. Therefore, when *ISG15* and *OAS1* abundance were individually evaluated in these conditions (Table 6), the accuracy was improved for both genes and in both parity categories due to reduction of FP results and increased specificity (ISG15: 92% for heifers and 90% for cows; OAS1: 88% for heifers and 92% for cows). However, there was still a considerable number of FN results, except for OAS1 in heifers, which remained low (3/103). In an alternative attempt to reduce the FN results, both genes were combined in females with a functional CL (ISG15/OAS1; criteria: females with a functional CL were considered pregnant when the expression levels of at least one of the genes evaluated was greater than the predefined cutoffs). There was a slight increase on sensitivity (79%) and accuracy (83%) for the cows, but the FN results were still elevated (18/144) in this category.

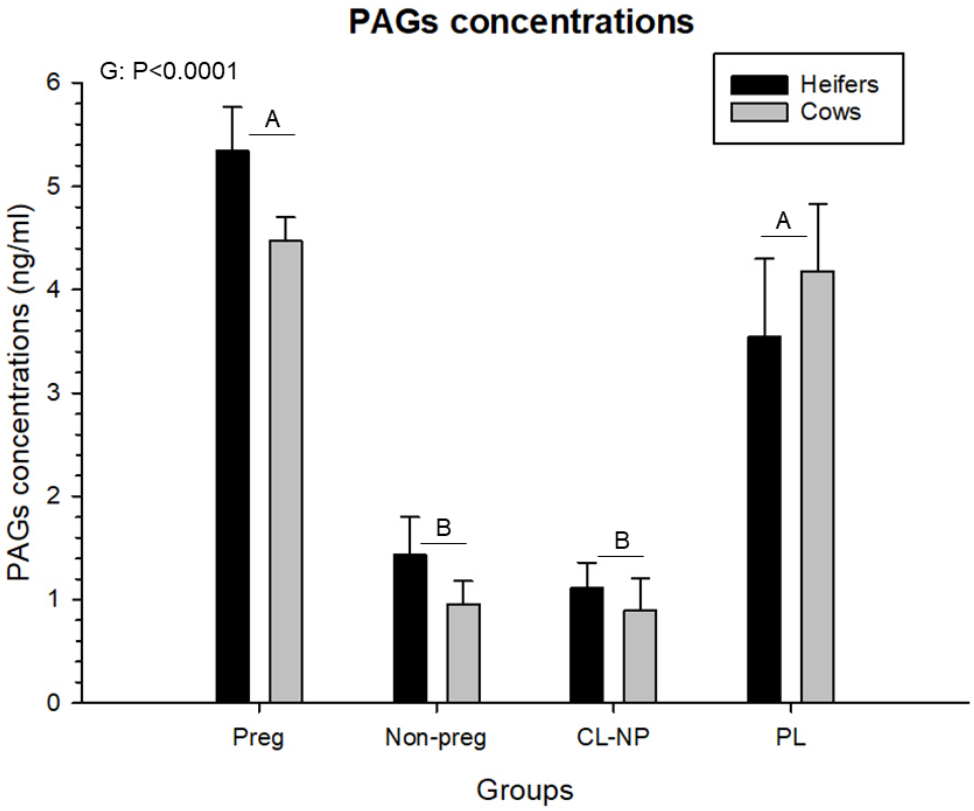
Table 6 - Number of True-Positive (TP), True-Negative (TN), False-Positive (FP) and False-Negative (FN) results and Sensitivity (SENS), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy (ACCU) for determining pregnancy status on D20 post-FTAI by *ISG15*, *OAS1* or *ISG15/OAS1* in heifers (N=103) and cows (N=144) with a functional CL.

Endpoint	Heifers with a functional CL			Cows with a functional CL		
	ISG15[a]	*OAS1*[a]	*ISG15/OAS1*[b]	*ISG15*[a]	*OAS1*[a]	*ISG15/OAS1*[b]
TP (N)	42	51	51	62	63	66
TN (N)	45	43	42	54	55	53
FP (N)	4	6	7	6	5	7
FN (N)	12	3	3	22	21	18
SENS (%)	78	94	94	74	75	79
SPEC (%)	92	88	86	90	92	88
PPV (%)	91	89	88	91	93	90
NPV (%)	79	93	93	71	72	75
ACCU (%)	84	91	90	81	82	83

Source: (MELO, G.D., 2019)
Legend: [a] Evaluation of *ISG15* and *OAS1* in females with a functional CL was performed by applying the predefined cutoffs only in females in which CL blood perfusion was > 25% on D20 post-FTAI.
[b] The combined use of both genes (*ISG15* and OAS1) was performed by considering the female as pregnant when the expression levels of at least one gene was greater than the predefined cutoffs

For PAGs concentrations, a difference between heifers and cows was not detected, but a significant (P<0.05) effect of group indicated 3 to 5-fold greater (P<0.05) concentrations in pregnant and PL females than in non-pregnant and CL-NP females (Figure 13). The ROC analysis indicated that the evaluation of PAGs concentrations can significantly (P<0.05) predict pregnancy in heifers (AUC=0.94) and cows (AUC=0.96), as demonstrated in Figure 11. The same analysis also indicated that the most suitable cutoff values to detect pregnancy in heifers and cows were, respectively, 1.49 and 1.36 ng/ml. By using these values to detect pregnancy, the diagnosis had an accuracy of 92% in heifers and 94% in cows when compared to the diagnosis performed on D30 (Table 5). This analysis also presented a low number of FP and FN results, thus providing good sensitivity (98% for heifers and 99% for cows) and specificity (86% for heifers and 86% for cows).

Figure 13 - Plasma PAGs concentrations (ng/ml) from pregnant (Preg), non-pregnant (Non-preg), functional CL but non-pregnant (CL-NP) and pregnancy loss (PL) heifers (N=103) and cows (N=144) 25 days post FTAI.



Source: (MELO, G.D., 2019)
Legend: The significant effect of group (G) was shown. The means within a group with a different letter are different (P<0.05).

As shown in Table 7, a positive significant (P<0.05) correlation was found among the characteristics evaluated on D20 and D25 when all groups were considered. The correlation coefficient (r), however, ranged from 0.3 to 0.6 for most of the comparisons, indicating that the correlations were weak or moderate, except for the significant (P<0.05) correlation found between *ISG15* and *OAS1* abundance for heifers and cows (r=0.8 to 0.9), and the expected significant (P<0.05) positive correlation found between P4 concentrations and CL blood perfusion for heifers and cows (r=0.7 to 0.8). When this analysis was performed only in pregnant females (Table 7), a significant (P<0.05) correlation in heifers was detected only between *ISG15* and *OAS1*, but for cows the correlation was significant (P<0.05) between ISGs and CL blood perfusion. A strong (r=0.8) significant (P<0.05) correlation was detected between *ISG15* and *OAS1* while, for the other significant comparisons, r was low (r=0.3 to 0.4).

Table 7 - Pearson's correlation coefficient (r) among the characteristics used for pregnancy prediction on D20 (plasma P4 concentrations, *ISG15* and *OAS1* expression in PMNs and CL blood perfusion[a] estimated by Doppler-US) and on D25 (CL blood perfusion[b] and plasma PAGs concentrations) post-FTAI in beef heifers and cows.

Endpoint	All groups				Pregnant on D30			
	Heifers		Cows		Heifers		Cows	
	r	P	r	P	r	P	r	P
CL blood perfusion[a] vs. P4	0.7	<0.05	0.8	<0.05	0.14	NS	0.07	NS
CL blood perfusion[a] vs. *ISG15*	0.4	<0.05	0.4	<0.05	0.2	NS	0.3	<0.05
CL blood perfusion[a] vs. *OAS1*	0.5	<0.05	0.6	<0.05	0.1	NS	0.4	<0.05
CL blood perfusion[b] vs. PAGs	0.5	<0.05	0.6	<0.05	0.3	NS	0.3	<0.05
P4 vs. *ISG15*	0.3	<0.05	0.3	<0.05	0.1	NS	0.01	NS
P4 vs. *OAS1*	0.4	<0.05	0.3	<0.05	0.03	NS	0.06	NS
P4 vs. PAGs	0.4	<0.05	0.5	<0.05	0.03	NS	0.01	NS
ISG15 vs. OAS1	0.8	<0.05	0.9	<0.05	0.8	<0.05	0.8	<0.05
ISG15 vs. PAGs	0.4	<0.05	0.3	<0.05	0.1	NS	0.1	<0.05
OAS1 vs. PAGs	0.5	<0.05	0.3	<0.05	0.1	NS	0.1	NS

Source: (MELO, G.D., 2019)
Legend: NS – Non-significant

Agreements between the results of different methods of diagnosis on D20 (ISGs, Doppler-US, P4), D25 (PAGs) and D30 (gold standard ultrasonography) were evaluated based on the previous defined kappa values in a scale of 0 (no agreement at all beyond chance) to 1 (perfect agreement). High levels of agreement were observed for heifers and cows between PAGs vs. Gold standard ultrasonography on D30, P4 vs. Gold standard and P4 vs. Doppler-US. Also, high levels of agreement were found for *ISG15* vs. *OAS1* and Doppler-US vs. Gold standard, but only for cows. For the other comparisons, substantial and moderate agreements were found, with exception of ISG15 vs. PAGs comparison in cows, in which the level of agreement was reasonable (Table 8).

Table 8 - Kappa values and level of agreement among the pregnancy diagnosis methods on D20 (plasma P4 concentrations, ISG15 and OAS1 expression in PMNs and Doppler-US[a]), D25 (Doppler-US[b] and plasma PAGs concentrations), and D30 (Gold standard method) post-FTAI in beef heifers and cows.

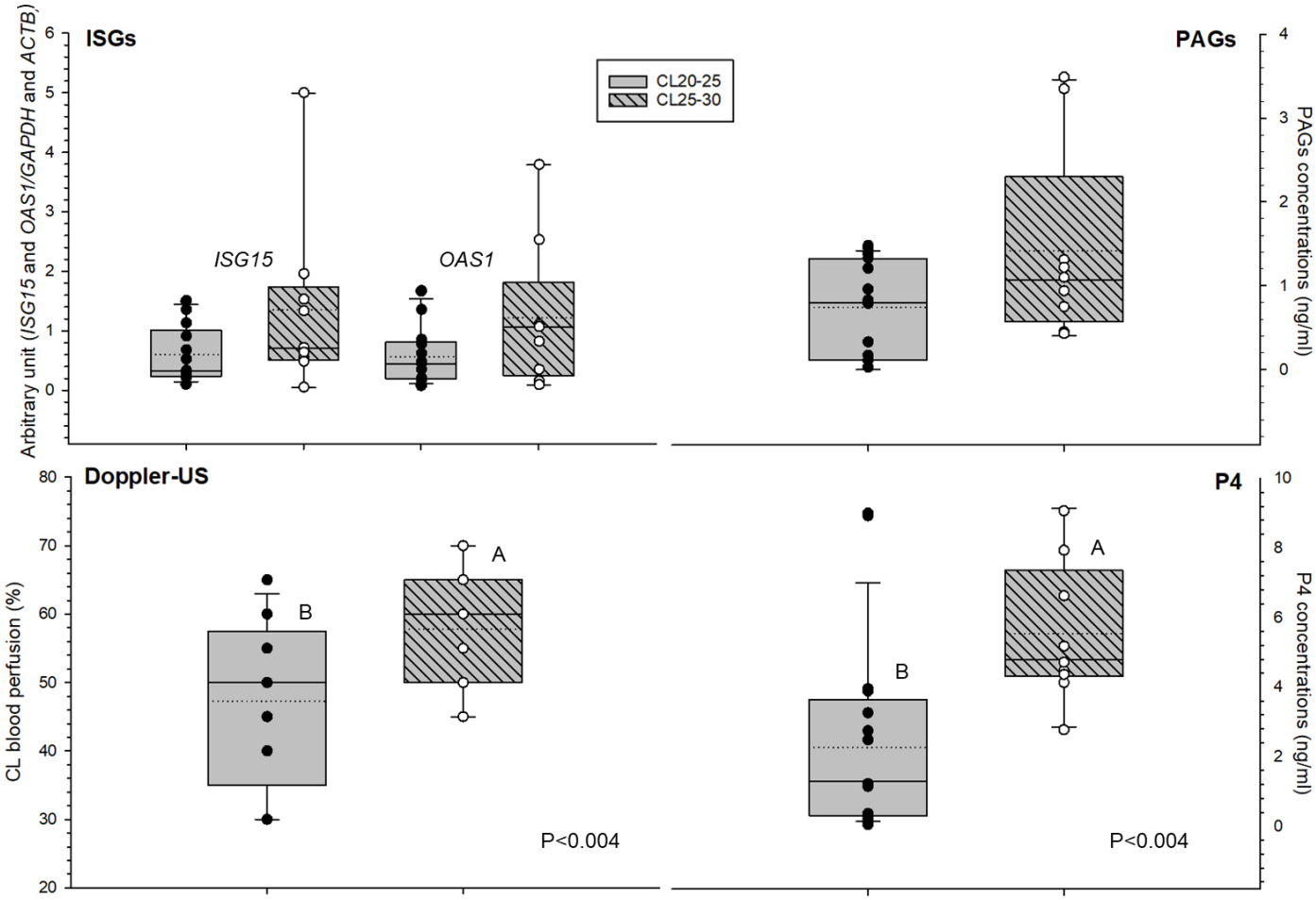
Endpoint	Heifers		Cows	
	Kappa	Level of agreement	Kappa	Level of agreement
Doppler-US[a] vs. P4	0.92	Perfect	0.92	Perfect
Doppler-US[a] vs. *ISG15*	0.5	Moderate	0.46	Moderate
Doppler-US[a] vs. *OAS1*	0.61	Substantial	0.51	Moderate
Doppler-US[b] vs. PAGs	0.65	Substantial	0.74	Substantial
P4 vs. *ISG15*	0.5	Moderate	0.49	Moderate
P4 vs. *OAS1*	0.57	Moderate	0.48	Moderate
P4 vs. PAGs	0.71	Substantial	0.74	Substantial
ISG15 vs. *OAS1*	0.63	Substantial	0.84	Perfect
ISG15 vs. PAGs	0.45	Moderate	0.36	Reasonable
OAS1 vs. PAGs	0.61	Substantial	0.44	Moderate
Doppler-US[a] vs. Gold standard	0.76	Substantial	0.85	Perfect
P4 vs. Gold standard	0.81	Perfect	0.86	Perfect
ISG15 vs. Gold standard	0.56	Moderate	0.44	Moderate
OAS1 vs. Gold standard	0.61	Substantial	0.51	Moderate
PAGs vs. Gold standard	0.84	Perfect	0.87	Perfect

Source: (MELO, G.D., 2019)

In an attempt to detect the early embryonic losses, heifers and cows from the CL-NP group were divided in two subgroups: 1) functional CL on D20, but not on D25 (CL20-25; n=13) and 2) functional CL on D20 and D25, but not on D30 (CL25-30; n=9). No differences (P>0.1) were found on ISGs expression and PAGs concentrations between the two subgroups evaluated, but the P4 concentrations and CL blood perfusion by Doppler-US on D20 were significantly (P<0.05) greater in the CL25-30 subgroup (Figure 14). We also verified the proportion of females in these subgroups that would be classified as pregnant or non-pregnant based on their ISGs expression on D20 and PAGs concentrations on D25. Through the ISGs method, using each ISG separately, 38% (5/13) of the females from the CL20-25 subgroup would be classified as pregnant by both ISGs, while for the CL25-30 subgroup, 56% (5/9) and 67% (6/9) would be classified as pregnant, respectively, for *ISG15* and *OAS1*. From those females considered pregnant by *ISG15* or *OAS1*, seven had the abundance of both genes combined (*ISG15* + *OAS1*) above the cutoff values, resulting in 32% of females (3/13 for CL20-25 subgroup and 4/9 for CL25-30 subgroup) that could have experienced a pregnancy loss between D20 and D30. By using PAGs, 0% (0/13) of

the females on the subgroup CL20-25 would be classified as pregnant, while on the CL25-30 subgroup, 22% (2/9) would be classified as pregnant. These 2 females (one heifer and one cow) were also identified as pregnant by ISGs on D20.

Figure 14 - Box plot representing ISGs expression (ISGs), CL blood perfusion (Doppler-US) and P4 concentrations (P4) on D20; and PAGs concentrations (PAGs) on D25 between females with a functional CL on D20, but not on D25 (CL20-25; n=13) and females with a functional CL on D20 and 25, but not on D30 (CL25-30; n=9).



Source: (MELO, G.D., 2019)
Legend: Mean – dotted line; median – continuous line

3.4. DISCUSSION

Evaluation of ISGs abundance in circulating immune cells was indicated as a potential marker for pregnancy diagnosis purposes in ruminants, as it indirectly signals the presence of the conceptus (YANKEY et al., 2001; PUGLIESI et al., 2014; MELO et al., 2019). Therefore, the development of an applied method using ISGs abundance to detect pregnancy or predict embryonic/fetal mortality compared to other early predictors could contribute to the improvement of beef cattle production systems. In the present study, we compared the use of ISGs abundance in PMNs on D20 post-FTAI with other methods of early pregnancy diagnosis performed on D20 or D25 post-FTAI in beef heifers and cows. This information could contribute to the choice of the most suitable method for each livestock system, as well as to the development of future research to explore the ISGs as markers of pregnancy and embryonic viability.

The abundance of ISGs in the granulocyte fraction (PMNs) was suggested in several studies to be more sensitive to the conceptus stimuli than in PBMCs (SHIRASUNA et al., 2012; KIZAKI et al., 2013; TOJI et al., 2017). Based on these studies and the results of a previous research from our group (MELO et al., 2019), we selected two classic ISGs in PMNs on D20 post-FTAI to diagnose pregnancy in beef heifers and cows. Our results indicated that a greater *ISG15* and *OAS1* abundance in PMNs on D20 of gestation is induced by the presence of a viable conceptus on D30, irrespective if the females experienced a pregnancy loss between D30 and D70. Also, these transcripts abundance was reduced in non-pregnant females on D30, regardless if they had a functional CL on D20 and D25. Previous studies also indicated a different expression of ISGs among females with different gestational statuses in dairy cows (HAN et al., 2006; MATSUYAMA et al., 2012; SHEIKH et al., 2018) and sheep (KOSE et al., 2016). The causes attributed to this finding may result from reduced IFNT secretion by females bearing a conceptus with impaired viability (HERNANDEZ-LEDEZMA et al., 1993; STOJKOVIC et al., 1995) and a positive correlation between IFNT concentrations and ISGs expression, as previously reported by Matsuyama et al. (2012); Shirasuna et al. (2012) and Sheikh et al. (2018). In the current study, the lower ISGs abundance in the CL-NP group could not be attributed only to reduced embryonic viability or development but also to females that failed to conceive.

The FP results between the diagnosis on D20-D22 and the detection of an embryo on D30 reported herein and in previous studies (SIQUEIRA et al., 2013;

PUGLIESI et al., 2014) are limiting factors when Doppler-US and P4 are used to diagnose pregnancy. These results could be related to late ovulation, long estrous cycles and embryonic mortality (POHLER et al., 2015; PUGLIESI et al., 2018). In this study, we verified that functional luteolysis in the CL-NP females was postponed, as indicated by the decreased plasma P4 concentrations after D25. Considering that pregnancy losses are more frequent until day 28 of gestation (SREENAN; DISKIN, 1986), we expected to find in the CL-NP group cases of early embryonic mortality. Therefore, the CL-NP females were divided in two subgroups according to the luteolysis detection. When both subgroups were compared, no differences on ISGs abundance or PAGs concentrations were observed. However, the CL function was already reduced on D20 in the CL20-25 subgroup, as indicated by lower P4 concentrations and CL blood perfusion. But, based on ISGs abundance (combined ISGs expression above the cutoff values), 32% (7/22) of the females diagnosed with a functional CL on D20 by Doppler-US were considered pregnant, which indicates that these animals experienced pregnancy losses until D30. From these females, 71% (5/7) could have suffered an embryonic loss between D20 and D25, as they were considered pregnant by all methods on D20, but not by PAGs concentrations on D25. Pregnancy losses before D25 would hardly be detected by PAGs concentrations, since in cattle, binucleate cells only appear around the third week of gestation (WALLACE et al., 2015). The remaining females with increased ISGs abundance (29%; 2/7) possibly experienced embryonic mortality between D25 and D30, as they were considered pregnant by all methods on D20 and D25. Thus, the majority of the females from the CL-NP group were animals that failed to conceive rather than experienced an early embryonic loss. In this regard, a recent study from our group (unpublished data) using beef heifers and cows synchronized for FTAI, but not inseminated, detected about 10-15% of females in which luteolysis was not detected up to 22 days after estrus.

Despite the small number of pregnancy losses between D30 and D70, the results obtained herein agree with others previously reported (MATSUYAMA et al., 2012; SHIRASUNA et al., 2012; SHEIKH et al., 2018), in which ISGs expression did not differ between pregnant females and those that experienced pregnancy losses after day 30 of gestation. These findings suggest that the events leading to embryo or fetus mortality could have compromised the conceptus after trophectoderm expansion, resulting in ISGs expression within normality on D20. Also, pregnancy losses can have

several causes other than conceptus' development and IFNT release, such as reduced P4 concentrations (SREENAN; DISKIN, 1983; DISKIN; MORRIS, 2008), a compromised uterine environment (SREENAN; BEEHAN, 1974; AYALON, 1978; HANSEN, 2002) and abnormal placenta function (REYNOLDS; REDMER, 1995).

Regardless of the gestational status, the *ISG15* abundance was increased in heifers when compared to cows. Increased ISGs relative levels in heifers and primiparous cows had already been reported in total leukocytes (GREEN et al., 2010a) and PBMCs (SOUMYA et al., 2017) of dairy females, but never in PMNs. The enhanced *ISG15* response in heifers could be attributed to the size of the conceptus, as heifers-derived conceptuses were reported to grow faster during the maternal recognition of pregnancy period than the ones derived from cows (BERG et al., 2010). Alternatively, the prior exposure of cows to the conceptus stimuli could reduce the response of ISGs on the subsequent exposure. However, further studies are needed to investigate the differences of IFNT stimuli in immune cells between nulliparous and parous females.

The effect of pregnancy status on PAGs in the current study was similar to that reported for ISGs, characterized by greater concentrations of this glycoprotein in the pregnant and PL groups. However, previous studies in dairy (HUMBLOT, 2001; BREUKELMAN et al., 2012; POHLER et al., 2016b) and beef cattle (PERRY et al., 2005; POHLER et al., 2013, 2016a; FILHO et al., 2020) described a significant difference in circulating PAGs concentrations between pregnant females and females that underwent pregnancy loss after days 25-39 of gestation. Nevertheless, our results corroborate with Reese et al. (2018), which described no differences in circulating PAGs concentrations on day 24 of gestation of dairy cows that maintained pregnancy until day 60 of gestation, and females that lost their pregnancy between day 31 and 60. Previous studies also reported an influence of parity on PAGs concentrations in beef cattle (LOBAGO et al., 2009; POHLER et al., 2016a; FILHO et al., 2020), but we could not detect any differences between cows and heifers.

With regard to the performance parameters, all the evaluated methods were classified as good predictors of pregnancy. Overall, Doppler-US, P4 and PAGs had better accuracies, reflected by the greater AUC values, a significant correlation and a

high level of agreement between the methods and the gold standard diagnosis on D30. The determination of CL blood perfusion and P4 concentrations allow the assessment of structural and functional luteolysis, respectively (SASSER; RUDER, 1987; PUGLIESI et al., 2014). Such methods are efficient because P4 concentrations in ruminants peak around day 14 of the estrous cycle, and when the conceptus is present, these concentrations remain elevated until day 21 or further (PARKINSON; TURVEY; JENNER, 1994). Additionally, circulating P4 concentrations are more correlated to CL blood perfusion than CL size during luteolysis in cattle (HERZOG et al., 2010; ROCHA et al., 2018). The high accuracy reported in both methods results from the absence of FN results, which culminated in a sensitivity of 100%. When these methods are applied in the field, especially in resynchronization protocols, the absence of FN results is advantageous because it prevents cows misdiagnosed as non-pregnant from receiving a PGF2α injection, which would induce a pregnancy loss (PUGLIESI et al., 2018; YOSHINO et al., 2018). Thus, the assessment of luteolysis is an excellent predictor of pregnancy, but it is not efficient in monitoring conceptus viability and predicting embryonic or fetal mortality, since reductions in circulating P4 concentrations only occur together or after embryonic death (POHLER et al., 2013, 2016b).

Although PAGs are less sensitive than Doppler-US and P4 in detecting non-pregnant females, this method still had a greater specificity due to the lower number of FP results. The reduced occurrence of FP was credited to the prediction of embryonic mortality by this method (POHLER et al., 2013, 2016b, 2016a; FILHO et al., 2020). In the present study, two out of 15 of the FP results were attributed to females that suffered from pregnancy losses between D25 and D30. The remaining FP results (13/15) resulted from failure in the technique, since they were attributed to non-pregnant females with low CL blood perfusion and P4 concentrations on D20, which is incompatible with pregnancy establishment. In cows, the FP results could have resulted from residual PAGs of the previous pregnancy, as PAGs have a long half-life in maternal circulation (GREEN et al., 2005; SILVA et al., 2007). In heifers, the greater PAGs concentrations in non-pregnant females could result from cross-reaction with other proteins, as suggested by Silva et al. (2007). The poor levels of agreement between ISGs and PAGs, in addition to low or non-significant correlation, emphasized that both methods represent a distinct signaling mechanism from the conceptus in different time points. That is, ISGs correspond to an indirect signaling mechanism to

the IFN concentrations, since the abundance of these transcripts are measured in peripheral blood leukocytes. On the other hand, PAGs are released by binucleate cells from the placenta and are directly measured in the bloodstream, representing a direct stimulus from the conceptus.

Despite being indicated as good predictors of pregnancy, *ISG15* and *OAS1* expression in PMNs had lower accuracy when compared to the other methods. When the ISGs expression was compared between the parity categories, greater accuracies were observed in heifers, which was also reported by Green et al. (2010a). Overall, the accuracy of the ISGs method was reduced due to the considerable number of FP and FN results, which led to lower sensitivity and specificity. Other studies (GREEN et al., 2010a; PUGLIESI et al., 2014; YOSHINO et al., 2018) evaluated the accuracy of this technique in peripheral blood leukocytes and reported similar results. Green et al. (2010a) registered that it was not possible to establish a suitable ROC curve for predicting pregnancy by ISGs expression in total leukocytes from dairy heifers and cows before day 20 of gestation. Pugliesi et al. (2014) described an accuracy close to 80% when this method was performed in PBMCs on day 20 of gestation of beef cows, but sensitivity ranged from 66 to 78%. Yoshino et al. (2018) reported in PMNs of dairy cows accuracies ranging from 57 to 86% between days 20 to 22 of gestation. In the present study, *OAS1* expression in heifers had the best performance due to the lowest amount of FN results, and when compared to the gold standard diagnosis on D30, the level of agreement was substantial. Interestingly, *OAS1* had the worst performance at Yoshino et al. (2018), which reported a sensitivity of only 25%. Authors have already stated that better accuracies on pregnancy diagnosis through ISGs expression are achieved when considering the relative expression of each female to a baseline sample collected before the maternal recognition of pregnancy period (HAN et al., 2006; GIFFORD et al., 2007; GREEN et al., 2010a, 2010b; KIZAKI et al., 2013; PUGLIESI et al., 2014). However, this would be an unfeasible sampling scheme for a practical situation in field conditions (GREEN et al., 2010b).

Considering the present and previous results indicating that Doppler-US is a highly effective tool for detecting non-pregnant females (100% sensitivity), and that its main limitation is the number of FP results, we combined this method with the expression of ISGs in PMNs. For this, the cutoff value of each gene individually or

together (*ISG15*, *OAS1* or *ISG15/OAS1*) was applied only in females with a functional CL on D20; while females with a non-functional CL on D20 were automatically classified as non-pregnant. Overall, the combined method increased the accuracy of the diagnosis due to the reduction in FP results, but the FN results were still numerous, especially in cows. The *OAS1* expression in heifers remained accurate and sensitive, and the combination between *ISG15* and *OAS1* in these females resulted in similar accuracy and sensitivity. Pugliesi et al. (2014) also reported a better accuracy (84%) when combining the use of two genes (*OAS1* and *MX2*) in females with a functional CL. Still, even combining both methods, FP and FN results were not mitigated, which in practical situations would cause financial losses to the producer, mainly due to the FN results. However, it is important to highlight that the ISGs method was performed 10 days earlier than the gold standard method. Therefore, the FP results resulted not only from failures in the technique, but also by early embryonic mortality cases, as indicated by the 32% of females that experienced a pregnancy loss between D20 and D30 in the CL-NP group.

In summary, we conclude that all the approached methods in this study are good predictors of pregnancy, but better accuracies were obtained by Doppler-US, and P4 and PAGs concentrations. Doppler-US and P4 can be performed with similar accuracy five days earlier than PAGs, but only Doppler-US provides an accurate real-time diagnosis. The expression of *ISG15* was more pronounced in heifers than in cows, and although both ISGs reported good accuracies, its applicability in the field is still not ideal due the number of FN results. In addition, neither ISGs nor PAGs were able to predict pregnancy losses after D30. Finally, our model indicated that about one third of the non-pregnant females with a functional CL on D20 experienced embryonic losses between D20 and D30.

4. FINAL CONSIDERATIONS

The IFNT was indicated as a potential marker for pregnancy diagnosis purposes in ruminants, as it signals the presence of the conceptus. The response of PMNs to this glycoprotein was also reported in many studies to happen earlier and more intensely when compared to PBMCs. Considering that most of the results are described in dairy cows, and the constant pursuit for a more accurate and early pregnancy detection method, we aimed to validate the use of ISGs abundance in PMNs in a practical scenario.

Even with several reports describing a different response to the IFNT stimuli between PMNs and PBMCs, in the present study we were no able to find a significant difference in the expression of ISGs between the cell groups. Contrary to our hypothesis, the response of PMNs to the conceptus was similar to that described in PBMCs, which leads us to believe that both cell groups can be used with similar effectiveness for pregnancy diagnosis. One of the caveats in both experiments was the PMNs isolation technique, which guaranteed good cell purity, but the long cell isolation process could have impaired the integrity of the RNA. For the development of future studies using the abundance of ISGs in PMNs, we suggest the improvement of the cell isolation technique, aiming faster isolation protocols in order to reach better mRNA integrity.

Despite being classified as good pregnancy predictors on D20, the use of ISGs for pregnancy diagnosis still needs to overcome some barriers for a practical application in the field. First, the considerable number of FP, and especially FN results, significantly reduces the accuracy of the technique. In practical situations, misdiagnosis can damage the farmer, and from a research perspective, it can compromise the results and lead to incorrect conclusions. Secondly, it is a costly and time-consuming technique. Currently, even if the diagnose is performed on D20 post-FTAI, technicians will spend some time from cell isolation until qPCR, which would not justify the use of this technique instead of others. Finally, ISGs expression in cows is not as marked as the expression in heifers, which make the use of this method in cows even less accurate. However, the Doppler-US method on D20 post-FTAI provides real-time results with greater accuracy, and later methods such as PAGs measurement are highly efficient and have greater potential for predicting gestational losses.

Still, even with so many obstacles, we believe that research using the ISGs as pregnancy markers should be incentivized, as these genes respond to a direct stimulus from the conceptus during early pregnancy. Technology is continually evolving, and more sensitive and fast techniques are already available, such as gene expression analyses through digital PCR, or more efficient manners to isolate cells. Moreover, ISGs abundance in total blood fraction could be explored, since we concluded that the cell fraction does not affect the expression of these genes. Other routes can also be investigated, such as the identification of new ISGs or other early pregnancy induced transcripts.

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ATTACHMENT

ATTACHMENT A – Proof of submission of the manuscript in the journal Reproduction, Fertility and Development

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Reproduction, Fertility and Development

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