DIEGO ANGELO SCHMIDT POIT

Influence of sub-clinical endometritis on early pregnancy predictors and pro-inflammatory cytokines in circulating immune cells in dairy cows

Pirassununga
2021

DIEGO ANGELO SCHMIDT POIT

Influence of sub-clinical endometritis on early pregnancy predictors and pro-inflammatory cytokines in circulating immune cells in dairy cows.

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

Department:

Animal Reproduction

Area:

Animal Reproduction

Advisor:

Prof. Guilherme Pugliesi, Ph.D.

Pirassununga

2021

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T. 4142
FMVZ

Poit, Diego Angelo Schmidt
Influence of sub-clinical endometritis on early pregnancy predictors and pro-inflammatory cytokines in circulating immune cells in dairy cows / Diego Angelo Schmidt Poit. – 2021.
61 f. : il.

Título traduzido: A influência da endometrite subclínica nos preditores precoces de gestão e citocinas pró-inflamatórias nas células imunes circulantes em vacas de leite.

Dissertação (Mestrado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Reprodução Animal, Pirassununga, 2021.

Programa de Pós-Graduação: Reprodução Animal.
Área de concentração: Reprodução Animal.
Orientador: Prof. Dr. Guilherme Pugliesi.

1. Endometrite subclínica. 2. ISGs. 3. Citocinas pró-inflamatórias. 4. Preditores precoces de gestação. I. Título.

Ficha catalográfica elaborada pela bibliotecária Camila Molgara Gamba CRB 7070-8, da FMVZ/USP.

Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "Influência da endometrite sub-clínica sobre a resposta de células imunes circulantes a presença do concepto em vacas de leite", protocolada sob o CEUA nº 1202270819 (ID 007223), sob a responsabilidade de **Guilherme Pugliesi** *e equipe; Diego Angelo Schmidt Poit* - 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 27/11/2019.

We certify that the proposal "Influences of subclinical endometritis on immune cell response in pregnant dairy cows", utilizing 80 Bovines (80 females), protocol number CEUA 1202270819 (ID 007223), under the responsibility of **Guilherme Pugliesi** *and team; Diego Angelo Schmidt Poit* - 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 11/27/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 11/2019 a 02/2020 Área: Reprodução Animal

Origem:	Animais de proprietários						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	15 a 60 meses	N:	80
Linhagem:	Holandesa			Peso:	330 a 630 kg		

Local do experimento: As atividades de campo será realizada em uma propriedade privada localizada na região de Pirassununga, porém, devido ao fato de não se ter iniciado o experimento, não sabemos qual será a propriedade em questão, porém o termo de consentimento será assinado pelo Médico Veterinário responsável, assim como pelo proprietário da fazenda. As análises laboratoriais, que serão realizadas no Laboratório de Fisiologia e Endocrinologia Molecular (LFEM).

São Paulo, 25 de novembro de 2021

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

Author: POIT, Diego

Title: **Influence of sub-clinical endometritis on early pregnancy predictors and pro-inflammatory cytokines in circulating immune cells in dairy cows**

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

Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Eu gostaria de dedicar essa dissertação primeiramente aos meus familiares, Rovaldo, Sara, Isabela e Matheus, os quais sempre me apoiaram muito em todas as decisões durante essa e todas as etapas percorridas. E ainda, a mina avó D. Rosa, que mesmo não estando presente, foi a pessoa mais importante durante toda vida.

ACKNOWLEDGEMENTS

Primeiramente, gostaria de agradecer a Deus, por proporcionar todos esses momentos maravilhosos, dos quais passei, até chegar a esse dia, tão esperada defesa de mestrado.

A minha avó, D. Rosa Angelo, que mesmo não estando mais entre nós, foi a pessoa mais importante em nossas vidas, tenho certeza que essa mulher sempre esteve nos guiando e protegendo de onde quer que ela esteja. Minha mãe e irmã, Sara Laine e Isabela, que muitas vezes fica difícil diferenciar a função de cada uma, porém, sem sombras de duvidas são as mulheres mais importantes para mim.

Ao Rovaldo e Matheus, dois serem bem complicados as vezes, sendo meu Pai e Cunhado, que, no entanto, foram os pilares mais firmes que me mantinham sustentados, para sempre seguir em frente. Sou imensamente grato a vocês.

Aos meus primos e tios da Família Poit, que sempre se mostraram presentes.

Aos irmãos mais doidos que a vida me deu, os ex e atuais moradores da Rep. Tente, que sempre foram uma segunda família, dando força e apoio total aos meus afazeres. Dentre eles, tenho o prazer de citar três nomes: Danilo Brito Bambil, Pedro Dibo e Renan Perego, que mesmo em minhas crises de querer desistir do curso e de tudo, essas "bestas" aí nunca permitiram, todos estarão sempre em meu coração.

Aos "Míticos": Gorgonzola, Parangole, Bronha, James e Frango. Esses amigos fizeram minha vida universitária e pessoal, algo muito melhor e muito mais divertida.

E ainda, três republicas que são muito especiais e que hoje fazem parte ativamente da minha vida Rep. Caipira, Rep. Boemia e Rep. Tcheca, obrigado por tudo e eu amo muito vocês.

Aos outros doidos de Nova Europa, os Milks Aventureiros: Victor, Smigol, Chaulin, Leo, Vardo, Pé redondo, Jeferson, Boi, Iago, Luzão, Vi,

e perdão se esqueci alguns nomes, mas, vocês participaram de tudo durante toda a minha vida.

Minhas famílias de plantão, Tia Thalita, Tio Fabio, Vi e Dudu; Tia Mima, Tio Regi e Renan. Ainda Bassin, Lari e Matheus.

Ao LFEM, por todo o apoio fornecido durante esse período de mestrado, Igor, Thiago, Danilo, Adomar, Amanda, Karine, Ana, Cecília, Gilmar, Isabela, que foram essenciais tanto no apoio emocional quanto de trabalho. No geral, vocês também se tornaram uma família para mim.

Ainda, a Gabi, Priscila e Giulia pelas idas a fazenda para concluir o experimento, ajudando em todas as tarefas necessárias. E ainda a todos os membros da Fazenda Rio Doce e ... pela oportunidade de realizar os experimentos a campo.

Ao meu orientador Guilherme Pugliesi, não tenho nem palavras para descrever o quanto sou grato a ele, pela dedicação, compreensão e muita paciência, você sempre foi fonte de admiração para todos nos.

Aos membros do VRA, Harumi e Clayton, por sempre estarem presentes quando eu precisei, muito obrigado.

Gostaria de agradecer a fundação CAPES, pelo auxílio financeiro durante esse período.

Obrigado a todos, por me ajudar a chegar a esse momento tão importante na minha vida.

RESUMO

POIT, D. A. S. **A influência da endometrite subclínica nos preditores precoces de gestão e citocinas pró-inflamatórias nas células imunes circulantes em vacas de leite.** 2021. 61 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2021.

Resumo

O desenvolvimento de técnicas capazes de diagnosticar a gestação antes do retorno do estro em vacas leiteiras é essencial para melhorar a eficiência reprodutiva dos sistemas de produção. No presente estudo, nossos objetivos foram: 1) avaliar se a expressão de ISGs e citocinas pró-inflamatórias em células mononucleares do sangue periférico (PBMCs) é afetada em vacas leiteiras com SCE em comparação com vacas saudáveis; e 2) comparar a precisão dos preditores precoces de gestação (ISGs e US-Doppler) em animais com e sem SCE. Para tal, foram realizados dois experimentos, no Experimento 1, 30 vacas holandesas, que estavam no período de espera voluntária, entre 30 e 37 dias pós-parto, foram submetidas a um exame reprodutivo por palpação transretal e ultrassonografia, sendo que todas as vacas que não apresentaram qualquer sinal de doença uterina foram incluídas no estudo e foram submetidas a um protocolo de sincronização da ovulação (protocolo de Co-synch com algumas modificações). Sete dias depois (Dia 0; dia do cio esperado), a ocorrência de SCE foi diagnosticada pela contagem da proporção de células polimorfonucleares (PMN) em uma amostra de citologia uterina coletada pela técnica Cytobrush. Nos Dias 0 e 7 (diestro), foram coletadas amostras de sangue da cauda para isolamento de células mononucleares do sangue periférico (PBMCs) e determinação da expressão gênica por RT-PCR. Amostras de 6 animais com SCE (SCE) e 6 animais saudáveis (NUD) foram selecionadas para a análise de expressão genica. Já no Experimento 2, foram utilizadas 50 vacas Holandesas, que estavam entre 40 e 55 dias pós-parto, com objetivo de avaliar a expressão dos genes alvos, assim como da perfusão sanguínea luteal em animais que foram diagnosticados com SCE no início do protocolo de inseminação artificial de tempo fixo (IATF). No dia -10 (D0, o dia da IATF) os animais foram submetidos a um exame reprodutivo e para aqueles detectados sem qualquer sinal de doença uterina, submetidos a técnica do Cytobrush como descrito em Experimento 1. No D21 após o protocolo de IATF, os animais foram avaliados por ultrassonografia transretal em

modo Doppler colorido para avaliar a perfusão sanguínea no corpo lúteo e uma amostra de sangue da cauda foi coletada para isolamento das PBMCs. No D32, um diagnóstico confirmatório de prenhez foi feito por ultrassonografia modo-B. Animais com ≥ 5 % do PMN no Exp 1 e ≥ 5,5% no Exp 2 foram considerados com SCE, e aqueles com ≤ 3% do PMN no Exp 1 e ≤ 2% na Exp 2 foram considerados sem doenças uterinas (grupo NUD). Assim, no Exp 2, 29 vacas holandesas, foram alocados nos seguintes grupos: animais saudáveis e gestantes (NUD-P; n=7); com SCE e gestantes (SCE-P; n=4); saudáveis e não gestantes (NUD-NP; n=8); e com SCE e não gestantes (SCE-NP; n=10). Foi avaliada a expressão gênica nas PBMCs por RT-PCR de ISGs (*ISG15, OAS1, MX1* e *IFI6*) e citocinas pró-inflamatórias (*IL1-β, TNF-α* e *IFN-γ*). No Exp. 1, a expressão *ISG15, MX1, IFI6, TNF-α* e *IFN-γ* não foi alterada pela presença de SCE ou momento da avaliação (Dia 0 ou 7; P >0,1). Entretanto, a expressão de *OAS1* e *IL1-β* nas PBMCs foi maior no dia 7 que no dia 0 (P=0,02). No Exp. 2, nenhum efeito significativo (P>0,1) do status gestacional, ocorrência da SCE, ou sua interação foi observada na expressão de *OAS1, MX1, IFI6* e *IFN-γ* (P >0,10). A abundância da *ISG15*, *TNF-α* e *IL1-β* foi maior (P=0,008, P=0,05 e P=0,06, respectivamente) nos animais gestantes em comparação aos não gestantes. Para a perfusão sanguínea luteal, apenas um efeito significativo da gestação (P=0,01) foi observado, sendo maior nos animais gestantes. Em conclusão, a expressão de *OAS1* e *IL1-β* é mais elevada durante o diestro em vacas de leite, independentemente da presença da SCE e mesmo na ausência do concepto. Por outro lado, a expressão de *ISG15, MX1, IFI6, TNF-α* e *IFN-γ* não sofrem alteração tanto pela ocorrência da SCE ou fase do ciclo estral. A expressão de *ISG15, IL1-β* e *TNF-α* no dia 21 pós-IATF é maior em vacas gestantes que não gestantes, independentemente da ocorrência da SCE. A abundância de *ISG15* é um bom preditor precoce de gestação, independentemente da ocorrência da SCE. Já a perfusão luteal e a abundância de *OAS1*, são bons preditores precoces da gestação apenas em vacas sem SCE. A abundância de *MX1 e IFI6* não se mostraram bons preditores aos 21 dias após a IATF.

Palavras-chave: Endometrite subclínica. ISGs. Citocinas pró-inflamatórias. Preditores precoce de gestação.

ABSTRACT

POIT, D. A. S. **Influence of sub-clinical endometritis on early pregnancy predictors and pro-inflammatory cytokines in circulating immune cells in dairy cows.** 2021. 61 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2021.

ABSTRACT

In the present study, our objectives were: 1) to evaluate if the expression of ISGs and proinflammatory cytokines in peripheral blood mononuclear cells (PBMCs) is affected in dairy cows with SCE compared to healthy cows; and 2) to compare the accuracy of early pregnancy predictors (ISGs and US-Doppler) in animals with and without SCE at the begging of FTAI protocol. For this , two experiments were desingned, in the Experiment 1, 30 Holstein cows, in the voluntary waiting period, between 30 and 37 days postpartum, were subbmetted a reproductive examination by transrectal palpation and ultrasonography, and all cows with no sign of uterine disease were included in the study and were subjected to an ovulation synchronization protocol (Co-synch protocol with some modifications). Seven days later (Day 0; day of expected estrus), the occurrence of SCE was diagnosed by counting the proportion of polymorphonuclear cells (PMN) in a uterine cytology sample collected by the Cytobrush technique. On days 0 and 7 (diestrus), tail blood samples were collected for isolation of peripheral blood mononuclear cells (PBMCs) and determination of gene expression by RT-PCR. After exclusion of some animals, only 12 cows were used for gene expression analysis., being 6 animals with SCE (SCE) and 6 healthy animals (NUD). Experiment 2, 50 Holstein cows that were between 40 and 55 days postpartum were used in order to evaluate the expression of target genes as well as luteal blood perfusion in animals that were diagnosed with SCE at the beginning of the fixed-time artificial insemination (FTAI) protocol. On day -10 (D0, the day of IATF) animals were subjected to a reproductive examination and for those detected without any sign of uterine disease, subjected to the Cytobrush technique as described in Exp 1. On day 21, after the IATF protocol, animals were evaluated by transrectal ultrasonography in color Doppler mode to assess blood perfusion in the corpus luteum and a tail blood sample was collected for PBMCs isolation and gene expression by RT-PCR. On day 32, a confirmatory diagnosis of pregnancy was made by B-mode ultrasonography to identify the presence of a viable embryo with heartbeat. After exclusion and

confirmatory diagnosis of pregnancy, they proceeded to gene expression and luteal perfusion analyses. Then, 29 Holstein cows, were allocated to the following groups, 1) healthy and pregnant animals (NUD-P; n=7), 2) with SCE and pregnant (SCE-P; n=4), 3) healthy and non-pregnant (NUD-NP; n=8) and 4) co SCE and non-pregnant (SCE-NP; n=10). Animals with ≥ 5 % of PMN at Exp 1 and ≥ 5.5% at Exp 2 were considered with SCE, and those with ≤ 3% of PMN at Exp 1 and ≤ 2% at Exp 2 were considered without uterine disease (NUD group). The target genes selected for both experiments were ISGs (*ISG15, OAS1, MX1* and *IFI6*) and pro-inflammatory cytokines (*IL1-β*, *TNF-α* and *IFN-y*). *ISG15, MX1, IFI6*, *TNF-α*, and IFN-γ expression was not altered from the presence or absence of SCE, time of assessment (Day 0 or 7), or interaction between them (P >0.1). However, a significant effect was detected on the expression of OAS1 and IL1-β in PBMCs, being greater on Day 7 than Day 0 (P=0.02). In Exp. 2, no significant effect (P>0.1) of gestational status, occurrence of SCE, or their interaction was observed on the expression of *OAS1, MX1, IFI6*, and *IFN-γ* (P >0.10). However, the abundance of *ISG15* and *TNF-α* showed a significant effect of gestational status being higher in gestating animals compared to nonpregnant animals (P=0.008 and P=0.05, respectively), while the abundance of *IL1-β* showed a tendency to have a higher expression in gestating animals than nonpregnant animals (P=0.06). For luteal blood perfusion, only a significant effect of gestation (P=0.01) was observed, being higher in gestating animals. In conclusion to the results of Experiment 1, the gene expression of *OAS1* and *IL1-β* are higher during the diestrus period in dairy cows, regardless of the presence of SCE and even in the absence of conception, while the expression of *ISG15*, *MX1*, *IFI6*, *TNF-α* and *IFN-γ* are not altered either by the occurrence of SCE or by the moment of the evaluation. Gene expression of *ISG15,IL1-β* and *TNF-α* are greater in pregnant cows on D21 after FTAI. The abundance of *ISG15* is a good early predictor of pregnancy regardless of the occurrence of SCE, while the abundance of *OAS1* and CL blood perfusion are good predictor of pregnancy in cows without SCE. However, the abundance of *MX1* and *IFI6* were not good predictors at 21 days after FTAI.

Keywords: Sub-clinical endometritis. ISGs. Pro-inflammatory cytokines. Early pregnancy predictors.

LIST OF FIGURES

LIST OF TABLES

SUMÁRIO

1. GENERAL INTRODUCTION

Brazil has a prominent position in global milk production, being the fifth largest producer in the world (CONAB, 2018), reaching 33.8 billion liters of milk in the year 2018, a fact that represents an increase of 1.16% over the previous year, reaching a national productivity greater than 2000/cow/year (IBGE, 2019). These senary show that dairy producers in the country are seeking better productivity in their herds, and one of the main aspects that contribute to improving productivity and profitability in dairy production is reproductive efficiency (Walsh et al., 2011).

Despite this improvement, there are still major challenges related to reproduction, because, according to Santos et al. (2004), lactating cows lose about 60% of pregnancies after conception, a fact that leads to economic losses to dairy producers. So far, uterine diseases are associate with impairs on reproduction outcomes, lower milk production, greater risk of culling and several costs for the farmers (Lima, F.S. 2020). Sub-clinical endometritis (SCE), is an uterine disease characterized by the infiltration of polymorphonuclear neutrophils (PMN) in the endometrium, without clinical signals (Sheldon et al., 2006). Therefore, PMN counting in the uterine lumen and diagnosis of SCE can be performed by taking a cytological sample using Cytobrush technique (Kasimanickam et al., 2005). An infiltration of leucocytes, can generate an inflammatory process inducing an upregulation of pro-inflammatory cytokines (Th1 response), leading in failures of pregnancy establishment (Galvão et al., 2011; Kasimanickam et al., 2014; Kim et al., 2014). Thus, dairy and beef cows at early postpartum, with an uterine infiltration of PMN indicated by the endometrial smears, have a lower pregnancy rate (Santos et al., 2009; Kasimanickam et al., 2004). Another factor important for the establishment of pregnancy in ruminants is maternal immune response to the presence of a viable conceptus (Ott, 2019). The rejection of the semi-

allogeneic conceptus is prevented due to immunological alterations of maternal cells, which drive the cytokine balance towards the anti-inflammatory (Th2) pathway, decreasing the expression of the pro-inflammatory cytokines (Wegmann et al., 1993; Oliveira et al., 2013; Morelli et al., 2015).

Still, early identification of animals that failed to conceive can increase the economic return associated with reproductive efficiency, increasing the profitability of breeders (Fricke, 2002). Therefore, the development of techniques capable of predicting pregnancy diagnosis before the return of estrus in dairy cows is essential to improve the reproductive efficiency of production systems (Pohler et al., 2016a). Early methods to predict pregnancy in cattle, have been widely studied in the last decades, and the evaluation of the corpus luteum functionality by Doppler ultrasonography and the measurement of genes stimulated by Interferon-tau (IFNt) have been promising methods.

In ruminants, IFNt is the main molecule responsible of the regulation of maternal regulation of pregnancy, inhibiting the mechanism of luteal regression, throughout of prevent the pulsatile prostaglandin F2α and acts in the immune maternal regulation, allowing the semi-allogenic conceptus growth (Spencer et al., 2007; Antoniazzi et al., 2013; Hansen et al., 2017). The IFNt is a glycoprotein produced and secreted by the mononuclear cells of the trophectoderm, as early as day 7 of conceptus development (Sponchiado et al., 2017). Interestingly, the IFNt can stimulate the gene expression of some targets, in peripheral blood immune cells, such as, interferon-stimulated gene 15 ubiquitin-like modifier (*ISG15*), MX dynamin like GTPase (MX) 1, MX2, and 2'-5'-oligoadenylate synthetase 1 (*OAS1*), referred as interferon-stimulated genes (ISGs). The use of expression of several ISGs in immune blood cells as a method for early diagnosis of pregnancy in cattle has reached a good accuracy, as soon as 20 days of

pregnancy (Pugliesi et al., 2014; Pohler et al., 2016b; Yoshino et al., 2017). However, Campos et al., (2018) and Fernandes et al., (2019) recently indicated that the abundance of the ISGs are affected in dairy and heifers with induced inflammation. Campos et al. (2018) reported a greater expression of *ISG15*, and *MX2* in the endometrium on day 19 of pregnancy; whereas, Fernandes et al. (2019) indicated a reduced expression of *ISG15,* in the endometrium, on day 15 of pregnancy.

Alternatively, evaluation of luteal blood perfusion by Doppler ultrasonography allows identification of luteal regression, which became an applied method to detect non pregnant animals nears to the expected time of estrous return (Siqueira et al., 2013; Pugliesi et al., 2014; 2019). This method had been performed in beef and dairy herds, resulting in a re-insemination as early as 22 days after fixed time artificial insemination (FTAI) (Pugliesi et al., 2019; Motta et al., 2020). In beef cattle, the use of Doppler ultrasonography to detect pregnancy status on day 20 after FTAI reaches an accuracy greater from 90% (Pugliesi et al., 2014; Dalmaso et al., 2018); while, in dairy cows the accuracy was 75% (Siqueira et al., 2013). One explanation for this lower accuracy in dairy cattle could be the greater embryonic mortality in dairy lactating females associated with greater occurrence of uterine diseases, as SCE, at early postpartum, when compared with beef cows.

Nevertheless, studies are necessary to understand the influence of uterine inflammation, caused by the SCE, on the early pregnancy predictors, such as gene expression of ISGs, cytokines and CL blood perfusion, which could compromise the use of biomarkers to predict pregnancy and result in a reduced accuracy of early pregnancy methods.

2. HYPOTHESES

We aimed with this study to test the following hypotheses:

1) The expression of ISGs is affected either by the occurrence of SCE or by the estrous cycle phase, even in the absence of the conceptus;

2) Pregnant animals would present a reduced expression of pro-inflammatory cytokines in circulating immune cells in comparison to non-pregnant animals;

3) The use of ISG expression as pregnancy predictor at 21 days of pregnancy is less effective in animals that presented SCE at the begging of FTAI protocol.

3. OBJECTIVES

In the present study, our objectives were:

1) To evaluate if the expression of ISGs and pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) is affected in dairy cows with SCE compared to healthy cows;

2) To compare the accuracy of pregnancy early predictors (ISGs and CL blood perfusion) on day 21 after insemination in animals with and without SCE.

4. INFLUENCE OF SUB-CLINICAL ENDOMETRITIS ON EARLY PREGNANCY PREDICTORS AND PRO-INFLAMMATORY CYTOKINES IN CIRCULATING IMMUNE CELLS IN DAIRY COWS

ABSTRACT

This study was performed to evaluate the influence of subclinical endometritis (SCE) and pregnancy status on expression of genes related to pregnancy and inflammation in circulating immune cells in dairy cows. In Experiment 1, Holstein cows, between 30 and 37 days post-partum were submitted to a reproductive exam by transrectal palpation and ultrasonography. All cows considered without any signal of uterine diseases were included in the study (n=30) and were submitted to a synchronization of ovulation protocol. Seven days later (Day 0; day of the expected estrus), the occurrence of SCE was diagnosed by counting the proportion of plymorphonuclear cells in a uterine cytological sample collected by cytobrush technique. On Days 0 and 7 (diestrus), blood samples were collected from the tail for isolation of peripheral blood mononuclear cells (PBMCs) and determination of gene expression in healthy cows or with SCE (n=6/group). In Experiment 2, 50 Holstein cows, between 40 and 55 days postpartum were submitted on D-10 (D0, the day of FTAI) to a reproductive exam and those detected without any signal of uterine disease had uterine samples collected for SCE diagnosis. Twenty-one days after FTAI (D21), animals were evaluated by transrectal ultrasound in color Doppler mode to assess blood perfusion in the corpus luteum and a blood sample from tail was collected for PBMCs isolation and gene expression by RT-PCR. On D32, a confirmatory pregnancy diagnosis was performed. Thus, cows were classified into 4 groups, healthy pregnant (n=7), pregnant with SCE (n=4), healthy non-pregnant (n=8), and non-pregnant with SCE (n=10). The target genes selected for both experiments were ISGs (*ISG15*, *OAS1*, *MX1* and *IFI6*) and pro-inflammatory cytokines (IL1b, *TNF-α* and IFN-y). In Exp. 1, expression of *ISG15*, *MX1*, *IFI6*, *TNF-α* and *IFN-γ* did not differ (P>0.1) between SCE and NUD cows and was not affected by the time of evaluation (Days 0 or 7). However, a significant (P=0.02) a greater expression of *OAS1* and *IL1-β* in PBMCs was observed on Day 7 than Day 0. In Exp. 2, no significant effects (P>0.1) of pregnancy status, SCE occurrence, or their interaction were observed on *OAS1*, *MX1*, *IFI6* and IFN-γ expression. However, *ISG15* abundance was 2.5-fold greater (P=0.0008), *IL1-β* tended (P=0.06) to be 2.4-fold greater and *TNF-α* was 2.2-fold greater (P=0.05) in

pregnant than non-pregnant cows, regardless the SCE presence. For luteal blood perfusion, only a significant effect of pregnancy (P=0.01) was observed. In conclusion, the abundance of *ISG15*, *MX1*, *IFI6*, *TNF-α* and *IFN-γ* were not affected by SCE presence, moment of estrous cycle (estrus or diestrus) in dairy cows. The *OAS1* and *IL1- β* are transcripts upregulated in PBMCs during diestrus of dairy cows, regardless of SCE occurrence. The pro-inflammatory cytokines in PBMCs on D21 after FTAI are not affected by SCE occurrence, but *IL1- β* and *TNF-α* are upregulated in pregnant animals. The abundance of *ISG15* on D21 is a good predictor of pregnancy on D32, regardless of SCE presence, while, *MX1* and *IFI6* are not accurate predictors. In addition, the accuracy of using *OAS1* abundance or corpus luteum blood perfusion as pregnancy predictors was reduced by the SCE occurrence.

4.1 INTRODUCTION

Recent studies have been developed to define a method for early diagnosis of pregnancy in cattle, through the expression of interferon-tau-stimulated genes (ISGs) in immune blood cells (Pugliesi et al., 2014; Pohler et al., 2016b; Yoshino et al., 2017). Interferon-tau (IFNt) is a cytokine produced by the trophectoderm, released in the uterus, which induces the expression of ISGs in various tissues, such as the endometrium (Forde et al., 2011) and in immune blood cells (Han et al., 2006; Pugliesi et al., 2014). The IFNt plays a fundamental role in the maternal recognition of pregnancy in ruminants (Rashid et al., 2018), indirectly preventing the regression of the corpus luteum (CL), by inhibiting the pulsatile frequency of prostaglandin $F_{2\alpha}$. Conceptus signals drives an immune response toward to pregnancy establishment, decreasing the pro-inflammatory (Th1) and inducing an anti-inflammatory (Th2) response. Rashid et al., (2018), reported that gene expression of *IL1- β* and *TNF-α,* both Th1 cytokines, was significant lower in cows with multiple embryos on day 7 of pregnancy.

Pregnancy establishment in cattle can be affected by several factors. The uterine diseases lead to decreased fertilization and development of the concept in dairy cows (Ribeiro et al., 2015). Among these diseases, we can highlight subclinical endometritis (SCE), also referred to as cytological endometritis, which is characterized by leukocyte infiltration in utero, mainly of polymorphonuclear cells (PMNs), without the manifestation of clinical symptoms (Esposito et al., 2014; Wagener et al., 2017). As well as other uterine diseases, SCE causes harmful effects on reproductive performance (Molina-Coto and Lucy, 2018). Salasel et al. (2010) report that 52.7% of animals that underwent more than one artificial insemination to become pregnant had this disease during early postpartum period. The SCE can be diagnosed by

determination of the proportion of PMNs in cells collected in the uterine lumen by the cytobrush technique (Ghasemi et al., 2012). The proportion of PMN to classify an animal with SCE ranges between 5 and 18% and is dependent on the phase of postpartum period (Wagener et al., 2017).

The inflammatory process generated by uterine diseases also leads to increased expression of pro-inflammatory cytokines, such as the tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ) and interleukins in the blood (Galvão et al., 2012) and uterus (Galvão et al., 2011; Kasimanickam et al., 2014; Kim et al., 2014). Thus, inflammation of the endometrium caused by the SCE is associated with embryo implantation failures, leading to losses during the maternal recognition process of pregnancy (Kasimanickam et al., 2014). Despite not having evaluated animals with SCE, Ribeiro et al. (2016), highlighted that those cows with retained placenta and puerperal metritis, had a decreased expression of *ISG15* in peripheral blood immune cells on day 19 of pregnancy, compared to pregnant cows that were considered healthy. The presence of those diseases leads to a smaller conceptus, which potentially result in reduced capacity of conceptus to secrete IFNt compared to those in healthy animals (Ribeiro et al. 2016). In contrast, induction of an inflammatory process through intramammary (Campos et al. 2018) or systemic (Fernandes et al., 2019) infusion with lipopolysaccharide resulted in greater expression of ISGs (*ISG15*, *MX1* and *MX2*) in the endometrium of cows between 15 and 19 days of pregnancy.

Therefore, the occurrence of SCE would be a relevant factor that compromise the accuracy of using ISGs expression as a pregnancy diagnostic method in cattle. The proportion of false results using several different ISGs is still no adequate for use as a method with accuracy and feasibility before day 20 of pregnancy (Melo et al., 2020; Ferraz et al., 2021). Great part of this false positive results, could be consequence of

early embryonic mortality or induction of ISGs by infections or other stimulus that induce IFN expression, as SCE (Melo et al., 2020). The IFNt receptor (IFNAR) is a non-selective receptor and is stimulated by any type 1 interferon, as those produce by several inflammatory processes (Takino et al., 2016). But, so far, there is no evidence that animals presenting an inflammatory uterine disease, as the SCE, may stimulate the expression of ISGs, even without the presence of the conceptus.

In the present study, our objectives were: 1) to evaluate if the expression of ISGs and pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) is affected in dairy cows with SCE compared to healthy cows; and 2) to compare the accuracy of pregnancy early predictors in animals with or without SCE. Therefore, we tested the following hypotheses: 1) the expression of ISGs is affected either by the occurrence of SCE or by the estrous cycle phase, even in the absence of the conceptus; 2) pregnant animals would present a reduced expression of pro-inflammatory cytokines in comparison to non-pregnant animals; and 3) the use of ISG expression as pregnancy predictor at 21 days of pregnancy is less effective in animals that presented SCE at the begging of FTAI protocol.

4.2 MATERIALS AND METHODS

4.2.1 Animals and experimental design

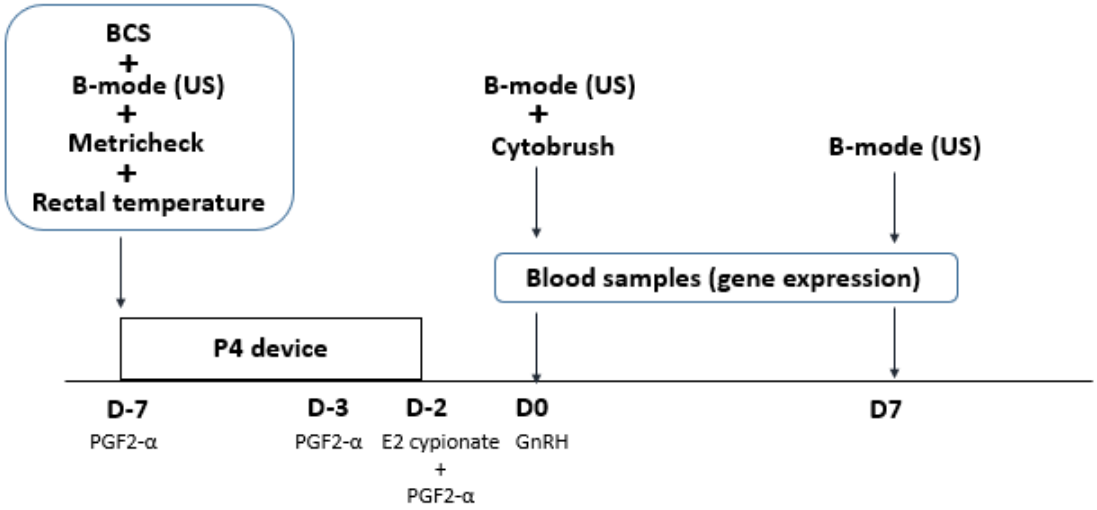
The experiments were approved by the ethics committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol 3851080519).

Experiment 1

Experiment 1 was carried out at a commercial farm, located at Itobi, SP- Brazil, from July/2020 to September/2020. Holstein cows (n=30) on the voluntary wait period (VWP), between 30 and 40 days postpartum, mean of DIM 33.1 ± 0.4, with BCS ranged from 2.5 to 4 (3.1 ± 0.7) and with milk yield 20.5 ± 0.6 kg/day, were used. The animals were housed on compost barn and milked thrice daily, and were fed a total mixed ration (TMR) twice daily that consisted of corn silage as forage, with addition of a corn and soybean meal-based concentrate. The TMR was formulated to meet or exceed minimum nutritional requirements for lactating dairy cows (NRC, 2001). All animals were submitted to Metricheck device evaluation, transrectal ultrasonography and rectal temperature on day -7 (D-7; D0 = day of expected estrus), for clinical endometritis diagnosis. The Metricheck evaluation was performed as described previously by Mcdougall et al. (2007).

The animals were submitted to a pre-synchronization protocol on a random day of estrous cycle. On D−7 an intravaginal P4-releasing device (Sincrogest®, Ourofino Saúde Animal, Brazil) and an intra-muscular single dose of a prostaglandin analogue (0,526mg of sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal) was administrated. After five days, the intravaginal device was withdrawn, and another dose of prostaglandin was administrated, followed by an injection of estradiol cypionate (1mg of estradiol cypionate, SincroCP®, Ourofino Saúde Animal). On D0 (day of expected estrus), animals received an intramuscular injection of GnRH analogue (10 µg of buserelin acetate; Sincroforte®, Ourofino Saúde Animal) to induce the ovulation, then, were submitted to Cytobrush technique for diagnose of SCE. Blood samples from the coccygeal vessels for gene expression were collected on days D0 (expected estrus) and D7 (diestrus).

Figure 1. Schematic diagram of the experimental design of experiment 1.



Source: (POIT, D. A. S., 2021)
Notes: Schematic diagram of the experimental design. At the enrollment (D-7), all animals had their BCS scored, ranged 1-5, and they were submitted to ultrasonography (B-mode), Metricheck device and rectal temperature (°C), however, only cows with no signals of uterine disease were included. Then, Pre-sync protocol (P4 and estradiol bases) were applied in these animals. Cytobrush to diagnose SCE were performed on D0, and blood samples for relative expression of targets were collected on D0 and D7 of the experiment.

Transrectal ultrasonography exams were performed to confirm the presence of a dominant follicle and CL, respectively, on D0 and D7. Only cows considered clinical healthy, and with presence of an ovarian dominant follicle (follicle with >10 mm in diameter in the absence of a CL) on D0 and presence of CL on D7 were included. Three animals with clinical endometritis, one with follicular cist and four which had no CL presence on D7 were identified and excluded for the subsequent procedures. On D0, the remaining 22 Holstein dairy cows were classified based on the PMN counting in the cytological samples collected by the Cytrobush technique. For classification of SCE occurrence, only cows with ≥ 5.5 % of PMN were considered with SCE (SCE group; n=6) and those with ≤ 3.0% of PMN were considered without any uterine disease (NUD group; n=6).

Experiment 2.

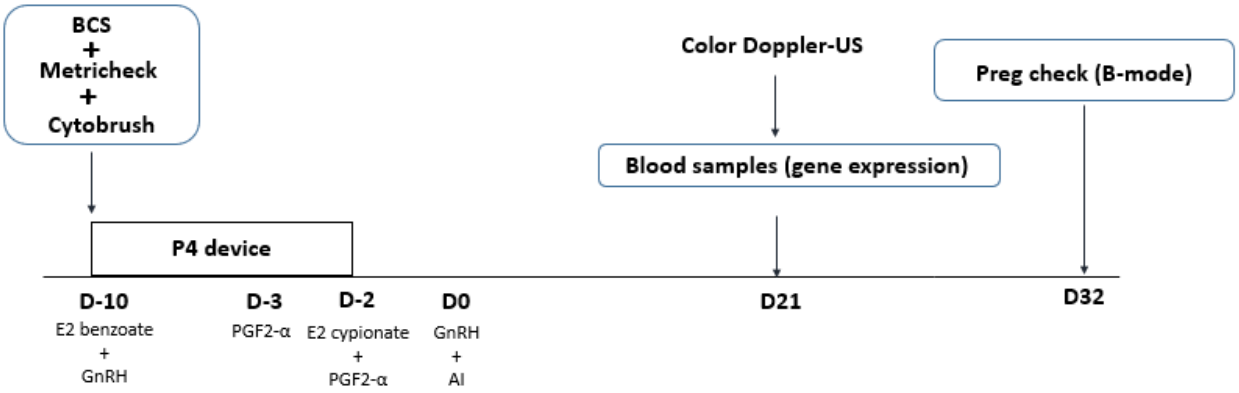
The experiment was carried out at a commercial farm, located in Arceburgo, MG-Brazil. At the enrollment, 50 Holstein dairy cows between 40 and 55 days post-partum (DIM: 44.02 ± 3.72), with BCS ranged from 2.5 to 3.75 (2.97 ± 0.12) and with milk yield 24.3 ± 0.8 kg/day were used. Cows were housed on compost barn and milked thrice daily, and were fed a TMR twice daily that consisted of corn silage and alfalfa silage as forage, with addition of a corn and soybean meal-based concentrate. The TMR was formulated to meet or exceed minimum nutritional requirements for lactating dairy cows (NRC, 2001).

Cows were submitted to Metricheck device evaluation, on day -10 (D-10; D0=FTAI) for clinical endometritis diagnosis. The Metricheck evaluation was performed as described in the experiment 1. Animals were excluded when: Metricheck score ≥ 3, diagnosed with any clinical disease, problems with calving or had another insemination throughout the experimental period. Nineteen animals were excluded because had Metricheck score greater than 2 (n=2) or had any failure during sample collections or evaluations (n=17).

The remaining cows (n=31) were submitted to a FTAI protocol with progesterone and estradiol basis. On day -10 (D-10, D0=FTAI), an intravaginal P4-releasing device (Fertilcare 1200®, MSD, São Paulo, Brazil) with an intramuscular injection of estradiol benzoate, doses of 2 mg (Sincrodiol®), followed by an intra muscular injection of GnRH (10 µg of gonadorelin acetate; Fertagyl®, MSD, São Paulo, Brazil) was administrated. A prostaglandin analogue (0.526mg of sodium cloprostenol; Sincrocio®) was administrated twice with 24 hours apart (D-3 and D-2). Also, on D-2, the intravaginal device was withdrawn, followed by an injection of estradiol cypionate (1mg of estradiol cypionate, SincroCP®). On D0, day of FTAI, an intra muscular injection of GnRH (10

µg of gonadorelin acetate; Fertagyl®) was administrated, to induce the ovulation, then, the animals were inseminated.

Figure 2. Schematic diagram of the experimental design of experiment 2.



Source: (POIT, D. A. S., 2021)
Notes: Schematic diagram of the experimental design. At the enrollment (D-7), all animals had their BCS scored, ranged 1-5, and they were submitted to ultrasonography (B-mode), Metricheck device and rectal temperature (°C), however, only cows with no signals of uterine disease were included. Then, Pre-sync protocol (P4 and estradiol bases) were applied in these animals. Cytobrush to diagnose SCE were performed on D0, and blood samples for relative expression of targets were collected on D0 and D7 of the experiment.

The SCE presence was diagnosed by performing the Cytobrush technique before the beginning of the FTAI protocol on D-10. For classification of SCE occurrence, considering that cows were at a later postpartum period than in the Exp 1, only cows with ≥ 5% of PMN were considered with SCE and those with ≤ 2.0% of PMN were considered without any uterine disease. Doppler ultrasonography (Doppler-US) and blood samples collection for gene expression were performed on D21 after FTAI. The final pregnancy diagnosis was performed on D32, under the presence of a viable embryo with heartbeats. Therefore, after pregnancy diagnosis, the animals were allocated in four experimental groups: Non-uterine disease and Pregnant (NUD-P;

n=7); Non-uterine disease and Non-Pregnant (NUD-NP; n=8); SCE and Pregnant (SCE-P; n=4); and SCE and Non-Pregnant (SCE-NP; n=10).

4.2.2 Cytobrush technique

Cytobrush was performed, for both experiments as previously detailed by Cardoso et al., (2017). The content present in the brush was passed to a microscope slide (26x76x1 mm), by a careful rolling of the brush over the surface of the slide, and then, the slides were stained by the rapid Panotic staining kit (Laborclin, Pinhais, Paraná, Brazil) for visualization in the optical microscope. For the determination of SCE, the proportion of PMNs in the sample was determined based on counting of approximately 200 cells.

4.2.3 Ultrasound scanning

In the experiment 1, the uterine illness evaluation and ovary structures detection (CL or dominant follicle), were performed using a B-mode instrument (DP-50 vet, Mindray, USA) with a linear multi-frequency (3.5-7.5 MHz) transducer in B-mode (gain 71%, frequency 7.5 MHz, P 74 mm, IP 4, depth 55 mm).

In the experiment 2, the cows were evaluated by transrectal ultrasonography using a duplex ultrasound equipment (ExaPad mini, IMV imaging, USA) with a linear multi-frequency (3.5 e 7.5 MHz) transducer in B-mode (gain 70%, depth 60 mm, Dynamic Range 70 dB) and color Doppler mode (gain 61%, PRF 1000 Hz, frequency 7.1 MHz, WF 2). The examination of the luteal area with color Doppler signals of blood perfusion (%) at each exam was determined as previously described by Rocha, et al. 2018. Cutoff point (luteal blood perfusion ≥ 25%) for the Doppler-US method was used to identify the females with a functional CL and they were classified as pregnant.

4.2.4 Isolation of PBMCs

Blood samples collected for PBMCs (≈30 mL) were submitted to a Ficoll gradient protocol, as described by Jiemtaweeboon et al. (2011). After collection, the whole blood was mixed with an equal volume of PBS, and the solution was layered onto 15 mL of Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden), placed in a 50 mL conical tube, and then centrifuged at 1,100×g for 30 min 20 °C to obtain the buffy coat. After the gradient formation, the buffy coat was utilized for PBMCs isolation, then, PBMCs were subject to successive lyses steps with hypertonic solutions and then PBS restored the isotonicity. resulting pellet was stored in a 1.5 mL conical tube at−80 °C until RNA extraction. To check the purity of the PBMCs, freshly isolated samples of each cell type were placed on a slice and stained with fast panotic method for morphological identification of cells by light microscopy under 400× magnification. The purity>95% for all samples.

4.2.5 RNA extraction, cDNA synthesis and RT q-PCR

Isolated PBMCs were submitted to RNA extraction using the Trizol® Kit (Invitrogen, Paisley, UK) as recommended by the manufacturer, according to the manufacturer's instructions.

For the synthesis of cDNA, firstly, the gene DNA will be removed using DNase and the TURBO DNA-free kit (Ambion, Austin, TX, USA), and a microgram of total RNA will be transcribed into cDNA using random primers and a reverse transcription kit High Capacity (Applied Biosystems, Foster City, CA, USA). RT-qPCR quantification was performed using the Power Mix SYBR Green PCR Master Mix (Thermo Fisher Scientific) and the ABI7300 real-time PCR system (Applied Biosystems). Two

reference genes with the most stable expression were used this specific cell type, GAPDH and PPIA. The expression of the target genes (*ISG15*, *OAS1*, *MX1*, *IFI6*, TNF-α, *IFN-γ* and IL-1β), were carried out using the comparative method (ΔΔ) CT as described by Pfaffl (2001), being normalized in relation to the two reference genes mentioned above. The following primers of *ISG15, OAS1* and *MX1* were taken from Pugliesi et al., (2014); *IFI6* from Rocha et al., (2020); *IL1-β* and *IFN-γ* from Talukder et al., (2018) and *TFN-α* from Rashid et al., (2018). The primers of housekeeping genes, were previously described by Melo et al., (2020) and Rocha et al., (2020).

Table 1. Forward (F) and reverse (R) primers sequences of target and reference genes analyzed in the experiment 1 and experiment 2, using qPCR.

Target	Gene Bank Number	Foward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ISG15	NM_174366	GGTATCCGAGCTGAAGCAGTT	ACCTCCCTGCTGTCAAGGT
OAS1	NM_001040606.1	TAGCCTGGAACATCAGGTC	TTTGGTCTGGCTGGATTACC
MX1	NM_173940.2	GTACGAGCCGAGTTCTCCAA	ATGTCCACAGCAGGCTCTTC
IFI6	NM_001075588.1	TGCTCTCCTCCAAGATACGGT	CAGAAGCTCGAGTCGCTGTT
IL1-β	NM_174093.1	AATCGAAGAAAGGCCCGTCT	ATATCCTGGCCACCTCGAAA
IFN-γ	NM_174086	AGCTCTGAGAAACTGGAGGACT	TGGCTTTGCGCTGGATCT
TFN-α	NM_173966.3	CAAAAGCATGATCCGGGATG	TTCTCGGAGAGCACCTCCTC
GAPDH	NM_01034034.2	GCCATCAATGACCCCTTCAT	TGCCGTGGGTGGAATCA
PPIA		GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT

4.2.6 Statistical Analyses

The statistical analysis of the gene expression was performed using ANOVA, considering the random effect of the cow and the fixed group effects (with or without subclinical endometritis), day (experiment1) or pregnancy status (experiment 2) and its possible interactions, using the PROC mixed software of the SAS (Version 9.2; SAS Institute). The variables were tested for normality of residues and homogeneity of variances and when it did not occur the data were transformed to Log or ranked. In

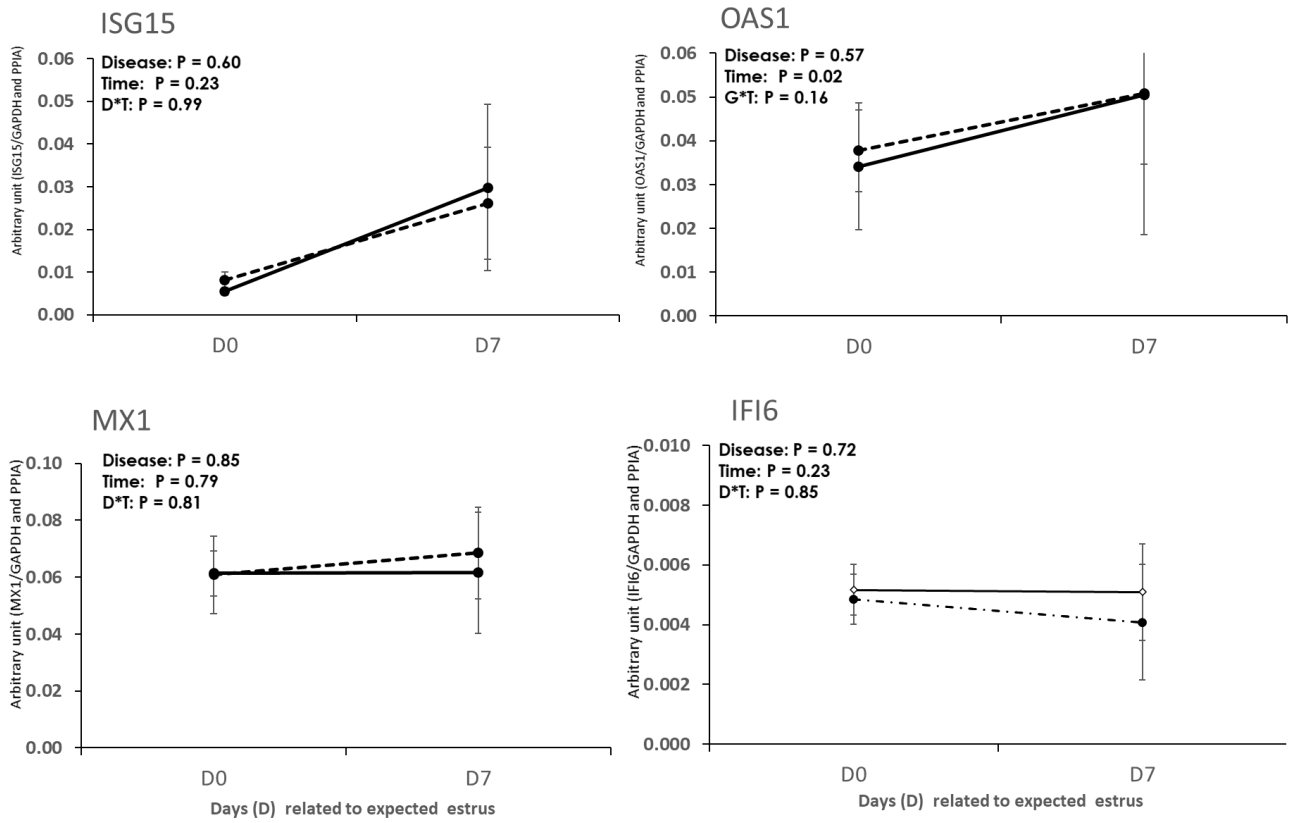
experiment 2, the accuracy of the pregnancy diagnosis methods by ISGs and Doppler-US on D21 and comparison with B-mode ultrasound method on D32 (Gold Standard) was calculated by specificity and sensitivity, as previously described by Pugliesi et al. 2014. A cutoff value for ISG expression and luteal blood perfusion was determined through establishment of a ROC curve by using MedCalc software. This software was also used to determine the area under the curve (AUC) of each method. Were considered as significant difference when $P \leq 0.05$, and as trend when it is between $0.05 < P < 0.10$. Data were expressed as mean ± standard error.

4.3 RESULTS

4.3.1 Experiment 1

The averaged mean of PMN proportion (%) of cytological samples in the NUD and SCE groups were, respectively, 2.1 ± 0.3 and 9.7 ± 3.6. The expression of ISGs is shown in Figure 3. For expression of *ISG15, MX1* and *IFI6,* a significant effect of SCE presence, time of sampling or an interaction of SCE and time of evaluation were not observed ($P > 0.1$). However, the abundance of *OAS1*, was significantly ($P=0.02$) affected by the time of sampling, as indicated by the 1.4-fold greater expression on D7 compared to D0.

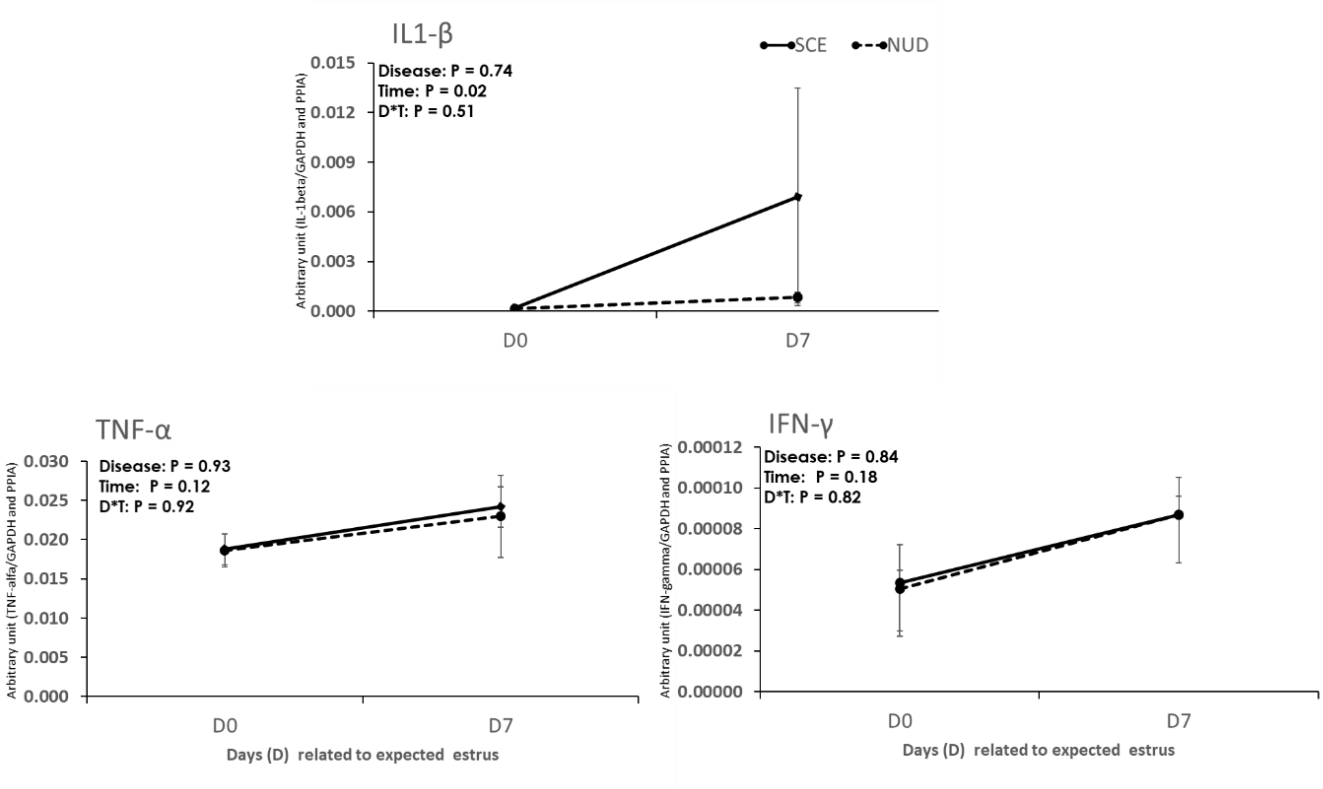
Figure 3. Relative expression of ISGs (*ISG15*, *OAS1*, *MX1* and *IFI6*) in PBMCs on D0 (day of expected estrus) and D7 in dairy cows with subclinical endometritis (SCE) or considered healthy (NUD).



Source: (POIT, D. A. S., 2021)
Notes: Relative expression of ISGs (*ISG15*, *OAS1*, *MX1* and *IFI6*) in PBMCs on D0 and D7 from subclinical endometritis (SCE; n=6) and non-uterine disease (NUD; n=6) dairy cows. The main effects of disease (D), time (T) and their interaction (D*T) were considered significant difference when, $p < 0.05$.

Gene expression of the proinflammatory cytokines is shown in Figure 4. For *TNF-α* and *IFN-γ*, a significant effect of SCE presence or an interaction of SCE and time of sampling were not detected. However, for *IL1-β,* a significant (P=0.02) effect of time of sampling was detected. The abundance of *IL1-β* was 19.3- fold greater on D7 compared to D0.

Figure 4. Relative expression of proinflammatory cytokines (*IL1-β*, *TNF-α* and *IFN-γ)* in PBMCs on D0 (day of expected estrus) and D7 in dairy cows with subclinical endometritis (SCE) or considered healthy (NUD).



Source: (POIT, D. A. S., 2021)

Notes: Relative expression of proinflammatory cytokines (IL1-β, TNF-α and IFN-γ) in PBMCs on D0 and D7 from subclinical endometritis (SCE; n=6) and non-uterine disease (NUD; n=6). The main effects of disease (D), time (T) and their interaction (D*T) were considered significant difference when, $p < 0.05$.

4.3.2 Experiment 2

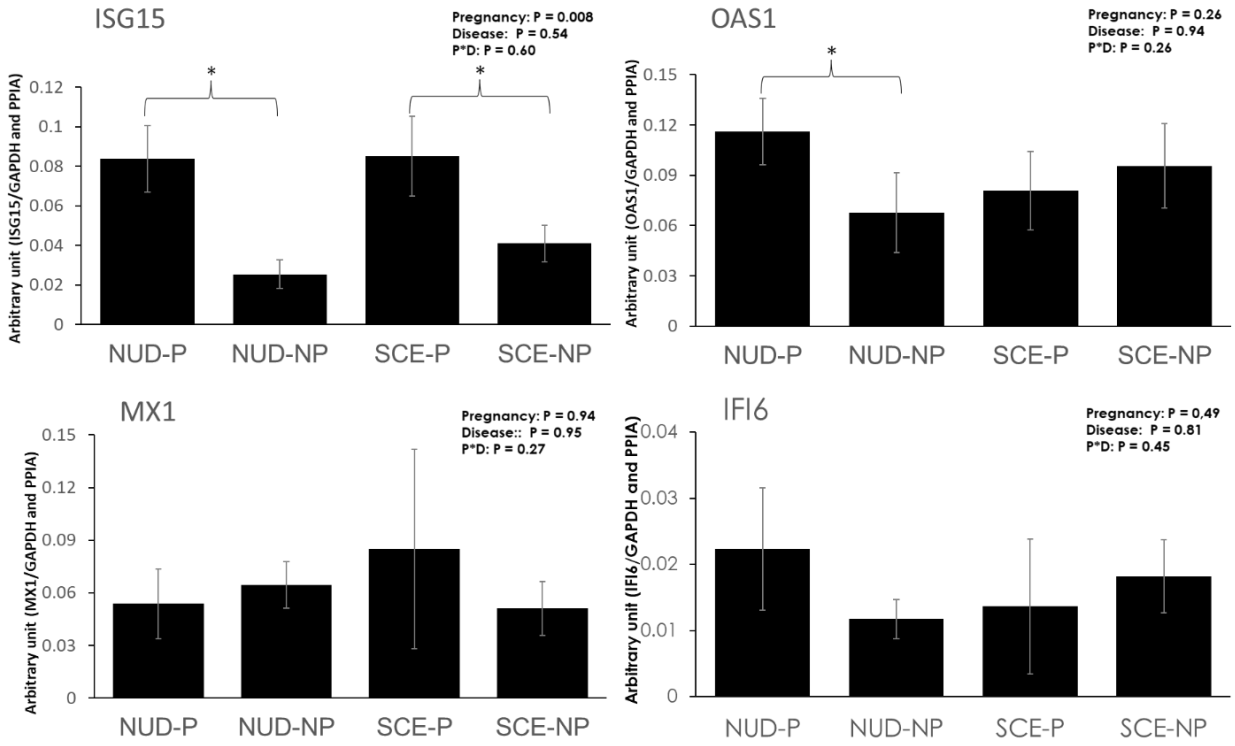
Proportion of PMN at beginning of the FTAI protocol

The averaged mean of PMN proportion (%) of cytological samples for NUD-P, NUD-NP, SCE-P and SCE-NP were, respectively, 1.7 ± 0.2; 1.5 ± 0.2; 6.2 ± 0.8 and 8.2 ± 1.1. The proportion of PMN did not differ ($P > 0.1$) between pregnant and non-pregnant animals in NUD or SCE groups.

Gene expression in PBMCs on D21 after FTAI

While the *ISG15* abundance was 2.47-fold greater (P<0.001) for pregnant cows, regardless the SCE presence, the abundance of the other ISGs (*OAS1*, *MX1* and *IFI6*) was not affected by pregnancy status nor SCE presence (Figure 5). Although a significant interaction of pregnancy status by SCE presence was not detected on gene expression for any ISG, when the pregnant and non-pregnant cows were compared separately in each SCE class (NUD or SCE), the *OAS1* expression in pregnant cows from the NUD class was 1.72-fold greater (P < 0.1) than non-pregnant cows, but did not differ (P > 0.1) between pregnancy status in the SCE class.

Figure 5. Relative expression of ISGs (*ISG15*, *OAS1*, *MX1* and *IFI6*) in PBMCs on day 21 after FTAI on pregnant and non-pregnant, with and without SCE in dairy cows.

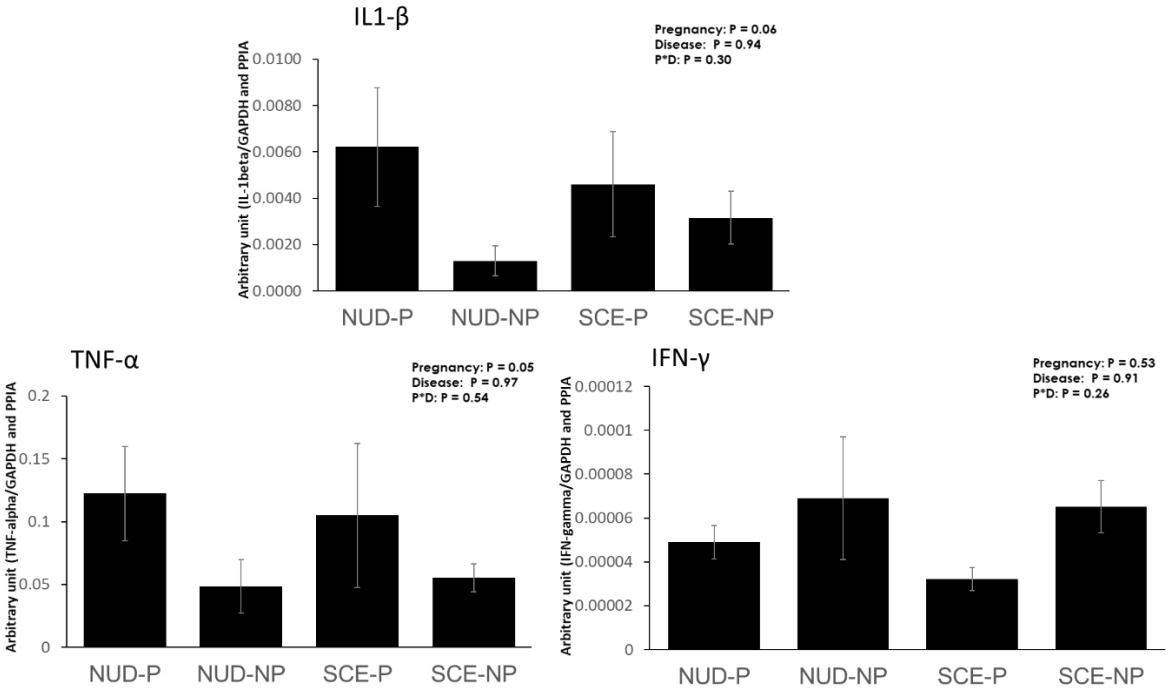


Source: (POIT, D. A. S., 2021)

Notes: Relative expression of ISGs (ISG15, OAS1, MX1MX1 and IFI6) in PBMCs on D21 post FTAI from non-uterine disease and pregnant (NUD-P; n=7); non-uterine disease and non-pregnant (NUD-NP; n=8), sub-clinical endometritis and pregnant (SCE-P; n=4), sub-clinical endometritis and non-pregnant (SCE-NP; n=10) cows. The main effects of disease (D) and pregnancy (P) that were significant are shown. An asterisk (*) indicates a statistical difference between the means between the groups in each class o SCE (P < 0.05).

Gene expression of pro-inflammatory cytokines is shown in Figure 6. A significant effect of SCE presence on gene expression was not detected for any cytokine. The abundance of *TNF-α* had no effect of pregnancy, SCE presence or an interaction of pregnancy status by SCE presence. Nevertheless, gene expression of *IL1-β* tended to be greater (2.39 - fold) in pregnant than non-pregnant cows, regardless the SCE presence; whereas, the abundance of *TNF-α* was significant greater in pregnant than non-pregnant (2.21- fold).

Figure 6. Relative expression of proinflammatory cytokines (*IL1-β*, *TNF-α* and *IFN-γ*) in PBMCs on day 21 after FTAI on pregnant and non-pregnant, with and without SCE in dairy cows.
.



Source: (POIT, D. A. S., 2021)

Notes: Relative expression of proinflammatory cytokines (*IL1-β*, *TNF-α* and *IFN-γ*) in PBMCs on D21 post FTAI from non-uterine disease and pregnant (NUD-P; n=7); non-uterine disease and non-pregnant (NUD-NP; n=8), sub-clinical endometritis and pregnant (SCE-P; n=4), sub-clinical endometritis and non-pregnant (SCE-NP; n=10).

Correlations between gene expression and PMN

When Pearson correlations were determined among ISG and cytokines (Table 1), ten significant (P < 0.05) and two approached significant (P < 0.1) positive correlations were detected in the variables evaluated on D21 post FTAI. Half (6 of 12) of the significant or approached significant correlations were moderate (0.6 < r < 0.8); whereas five were weak (r< 0.6) and only one strong correlation (r > 0.8) was observed. There was no significant correlation detected between PMN proportion with any target gene evaluated. Between the ISGs, significant correlations were observed for *OAS1* vs *ISG15*, *IFI6* vs *ISG15*, *IFI6* vs *OAS1* and *IFI6* vs *MX1*. A significant or approached significant correlations were observed among all proinflammatory cytokines. Regarding the correlations between the ISGs and proinflammatory cytokines, a significant correlation was detected for *ISG15* and *OAS1* with *IL1-β* and *TNF-α*.

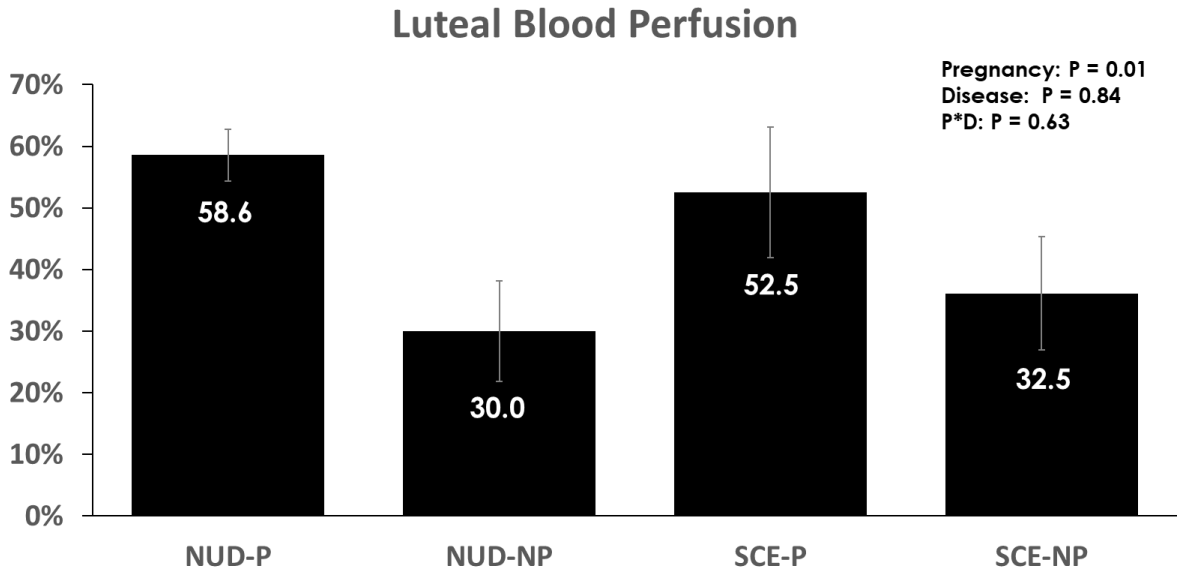
Table 2. Pearson's correlation coefficient (r) among the PMN proportion in the uterine cytological sample at the beginning of FTAI protocol and abundance of transcripts in PBMCs on 21 days post-FTAI in dairy cows.

Endpoint	Gene *vs* PMN		Endpoint	Between ISGs or Cytokines		Endpoint	ISGs *vs* Cytokines	
	r	P		R	P		R	P
ISG15 vs PMN	0.02	NS	*ISG15 vs* OAS1	**0.65**	**0.001**	*ISG15 vs* IL1- *β*	**0.48**	**0.01**
OAS1 *vs* PMN	0.03	NS	*ISG15 vs MX1*	0.26	NS	*ISG15 vs* TNF-*α*	**0.56**	**0.002**
MX1 vs PMN	-0.12	NS	*ISG15 vs IFI6*	**0.40**	**0.03**	*ISG15 vs* IFN-*γ*	0.14	NS
IFI6 vs PMN	0.05	NS	OAS1 *vs MX1*	0.25	NS	OAS1 *vs* IL1- *β*	**0.45**	**0.01**
IL1- *β vs* PMN	0.22	NS	OAS1 *vs IFI6*	**0.39**	**0.04**	OAS1 *vs* TNF-*α*	**0.65**	**0.0001**
TNF-*α vs* PMN	0.17	NS	*MX1 vs IFI6*	**0.74**	**<0.0001**	OAS1 *vs* IFN-*γ*	**0.32**	**0.09**
IFNγ *vs* PMN	0.09	NS	TNF-*α vs* IL1- *β*	**0.82**	**<0.0001**	*MX1 vs* IL1- *β*	-0.18	NS
			IFN-*γ vs* IL1- *β*	**0.35**	**0.07**	*MX1 vs* TNF-*α*	-0.24	NS
			IFN-*γ vs* TNF-*α*	**0.55**	**0.002**	*MX1 vs* IFN-*γ*	-0.32	NS
						IFI6 vs IL1- *β*	-0.17	NS
						IFI6 vs TNF-*α*	-0.05	NS
						IFI6 vs IFN-*γ*	0.17	NS

CL blood perfusion

Results of CL blood perfusion determined by Doppler-US is shown in Figure 7. As expected, the pregnancy status had influence on CL blood perfusion. The CL blood perfusion on D21 post FTAI was greater in pregnant than non-pregnant cows (56.4% *vs.* 31.4%), regardless the SCE presence.

Figure 7 – Mean ± SEM of luteal blood perfusion using Doppler-US (%) on day 21 after FTAI on pregnant and non-pregnant, with and without SCE in dairy cows.



Source: (POIT, D. A. S., 2021)
Notes: Mean ± SEM of luteal blood perfusion using Doppler-US on D21 post FTAI from non-uterine disease and pregnant (NUD-P; n=7); non-uterine disease and non-pregnant (NUD-NP; n=8), sub-clinical endometritis and pregnant (SCE-P; n=4), sub-clinical endometritis and non-pregnant (SCE-NP; n=10).

Accuracy of pregnancy predictors

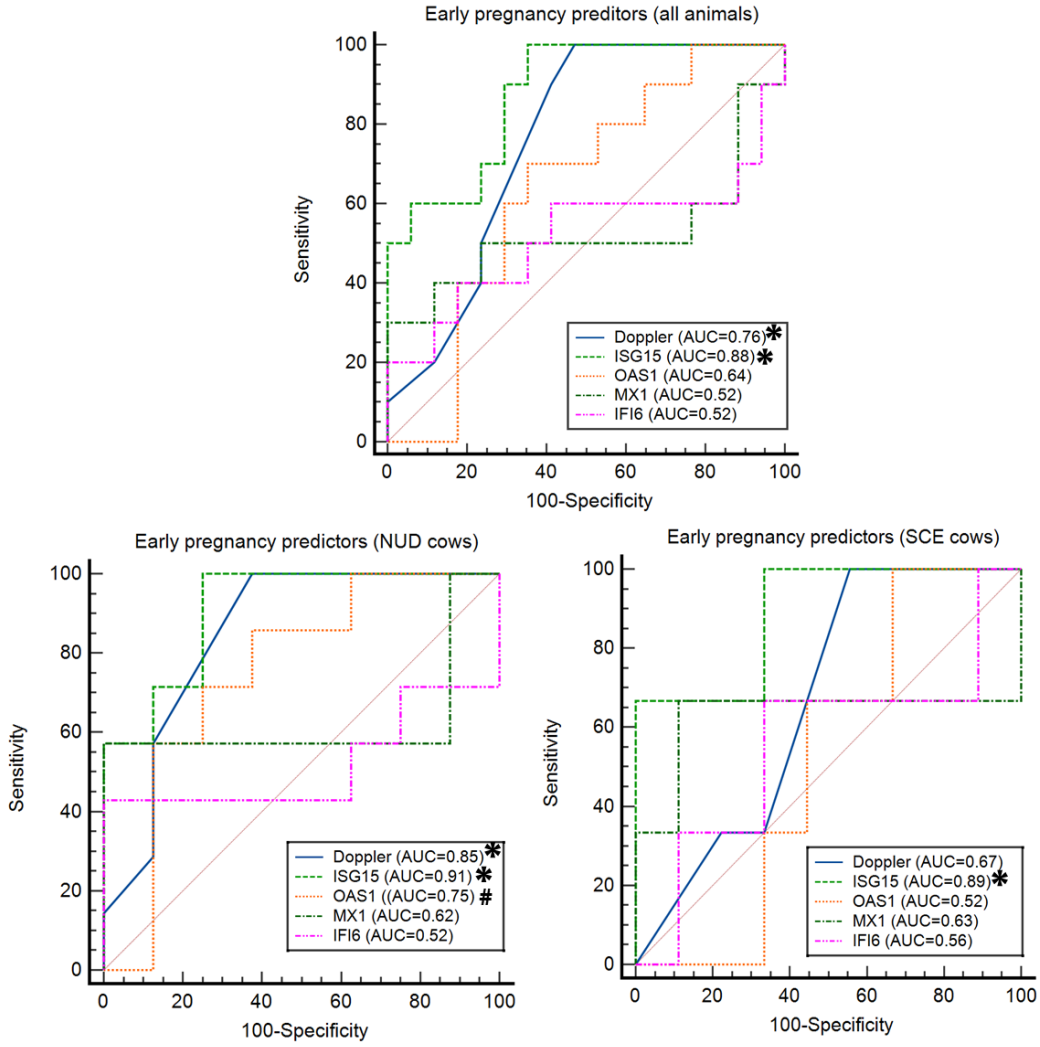
According to the ROC curve analysis (Figure 8), when the gene expression of ISGs were used for prediction of pregnancy on D32 post FTAI, only the abundance of *ISG15* were detected as a significant predictor ($P < 0.05$). For exploration of the influence of SCE presence on accuracy of using ISGs as pregnancy predictors in dairy cows, ROC curves were also generated for NUD or SCE cows apart (Figure 8). Therefore, when analyzed separately (Table 3), the *MX1* and *IFI6* resulted in low sensibility and were still not significant predictors of pregnancy ($P > 0.1$) in SCE and NUD cows; whereas

ISG15 was still considered a significant pregnancy predictor (P < 0. 0001) in NUD or SCE cows. However, the specificity of *OAS1* increased when analyzed for NUD cows separately (Table 3), which resulted in an approached significant (P = 0.07) method of prediction of pregnancy in NUD cows, but not significant in SCE cows. Also, the evaluation of CL blood perfusion on D21 post-FTAI was also considered as a good predictor, regardless the SCE presence. However, when analyzed separately of NUD or SCE cows, the sensibility and specificity for CL blood perfusing, by Doppler-US method were reduced in SCE cows (Table 3), resulting in a significant predictor of pregnancy only in NUD animals.

Table 3. Sensitivity (SENS) and Specificity (SPEC), for determining pregnancy status on D21 post-FTAI by Doppler-US, ISG15, OAS1 MX1 and IFI6 gene expression in PBMCs compared to diagnosis on D28 post-FTAI in dairy cows.

Target	All animals			NUD cows			SCE cows		
	SENS (%)	SPEC (%)	P Value	SENS (%)	SPEC (%)	P Value	SENS (%)	SPEC (%)	P Value
Doppler	100	52.9	**0.01**	100.0	62.5	**0.001**	80.0	44.4	NS
ISG15	100	61.0	**<0.0001**	100.0	75.0	**<0.001**	66.7	100	**0.01**
OAS1	72.7	61.1	NS	85.7	62.5	0.07	100.0	30.0	NS
MX1	30.0	100	NS	57.1	100.0	NS	66.7	80.0	NS
IFI6	45.5	100.	NS	42.9	100.0	NS	75.0	70.0	NS

Figure 8 – ROC curve analyses of early pregnancy predictors on day 21 after FTAI in dairy cows.



Source: (POIT, D. A. S., 2021)

Notes: Receiver operator characteristics (ROC) curves for Doppler-US and ISGs on D21 post FTAI for dairy cows (n =30) compared to the diagnosis on D28. An asterisk (*) indicates a significance ($P < 0.05$) and Hash mark (#) indicates tendency ($0.05 \geq P \geq 0.10$) of the evaluated methods as a pregnancy predictor on D21.

4.4 DISCUSSION

The development of accurate methods, which can diagnose early pregnancy or pregnancy failures, improves reproduction performance and promotes economic gains on dairy farms (Fricke et al., 2016). Yet, the factors that compromises the accuracy of ISGs as novel pregnancy biomarkers are still unclear. In the present study, our

objectives were evaluated if the SCE presence could affect the accuracy of early pregnancy predictors and modulation of pro-inflammatory cytokines gene expression in PBMCs. The herein results indicate, for the first time, that the SCE presence at postpartum period in dairy cattle does not affect the abundance of transcripts for ISGs and pro-inflammatory cytokines in PBMCs, but *OAS1* and *IL1-β* is modulated during diestrus of dairy cows, regardless of SCE occurrence. Also, at day 21 of pregnancy, most of the present pro-inflammatory cytokines evaluated are upregulated by presence of conceptus.

The SCE is an uterine disease with no presence of clinical signals, but is associated with impairments on reproductive performance and is usually diagnosed by PMN counting in uterine samples collected by cytobrush techniques (Kasimanickam et al., 2004). Madoz et al. (2013), using grazing dairy cows, suggested that the cutoff for % of PMN using the cytobrush method to determine animals with SCE has to be reduced according to the postpartum period. These authors indicated a cutoff for diagnosing SCE of PMN ≥ 6% in animals between 34 - 47 days after calving, and PMN ≥ 4% in animals between 48 - 55 days postpartum. In the present study, we used two different cutoffs to classify animals with SCE according to the different DIM interval. In the Experiments 1 and 2, the cutoff to classify SCE and the DIM interval, were, respectively: 5.5% and 30-40 days; and 5% and 40-55 days. For a better evaluation of the influence of SCE presence, we also considered a maximal % of PMN as a cutoff point for considering a cow as not having with uterine disease (healthy animals). Therefore, the model used in the present study might let a better identification of the SCE effects during early pregnancy as the cows with intermediate % of PMN were not considered.

Our first hypothesis that expression of ISGs is affected by the SCE occurrence or by the estrous cycle phase, even in the absence of the conceptus was not whole supported. The SCE had no effect on abundance of ISGs (*ISG15*, *OAS1*, *MX1* and *IFI6*) in PBMCs during estrous cycle (expected estrus or diestrus) or at day 21 of pregnancy. Results from non-inseminated cows (experiment 1) shows that, among the ISGs only the abundance of *OAS1* was affected by the time of sampling, where, animals had a greater gene expression (1.4-fold) under diestrus than estrus phase. The *OAS1* gene is involved in the arachidonic acid metabolism, altering the PGF2α secretion in the endometrial epithelium, which prevents the luteolisys (Schmitt et al., 1993). Therefore, the upregulation of *OAS1* gene in cows under high circulating P4 on D7 after expected estrus, could be involved in the maintenance of CL function to achieve successful pregnancy. In addition, the abundance of *IL1-β* was also upregulated at the diestrus phase. In contrast with our results, Talukder et al. (2018), reported in circulating PMN, that *IL1-β* expression did not differ between day 1 (after expected estrus) and day 7 in non-inseminated cows. Therefore, the upregulation in *IL1-β* expression in circulating immune cells may be stimulated by circulating progesterone, but only after it reaches the maximal concentrations during estrous cycle (after 10 days post estrus); however, the type of immune cells (PMN vs PBMCs), moment of sampling (early or late diestrus) and breed of the animals need to be considered in the mechanism involved in upregulation in *IL1-β* during diestrus.

Expression of ISGs in PBMCs and CL blood perfusion by Doppler-US, have been recently used to predict pregnancy in dairy and beef cows around day 20 of pregnancy (Pugliesi et al.,2014; Melo., et al 2020). In the present study, we evaluated the influence of SCE presence on early pregnancy predictors on D21 after FTAI in dairy cows submitted to the first service after calving. The novel results pointed that only

ISG15 expression on PBMCs and CL blood perfusion were affected by pregnancy status, regardless the SCE presence. That is, pregnant cows had *ISG15* abundance about 2.5 times greater and CL blood perfusion about 1.8 times greater than non-pregnant cows. Similarly, Ribeiro et al., (2016), reported that pregnant cows with no uterine disease diagnosed before insemination had the abundance of *ISG15* in the endometrium, on D19 after insemination, 2.4-fold greater than open cows. However, when evaluating cows diagnosed with retained placenta or clinical metrits before AI, no difference on gene expression of *ISG15* was detected between pregnant and non-pregnant cows. These authors suggests that the capacity of the embryo of those cows to secrete IFN-t, was affected by the uterine disease. A major difference in the previous reports with the present study that may explain the inconsistency in the results is that the SCE is a uterine disease with lesser inflammatory response and uterine injuries compared to retained placenta or clinical endometritis.

Previous reports in beef cattle (Green et al., 2010; Pugliesi et at., 2014; Yoshino et al., 2018; Rocha et al., 2020), indicate that gene expression of *IFI6* and *MX1* in PBMCs, are greater in pregnant animals when compared to non-pregnant on day 20 after FTAI. Those results, are not in agreement with the present study, as the abundance of *IFI6* and *MX1* was not affected by the pregnancy status. Although expression of *MX1* was not correlated with *ISG15* or *OAS1*, *MX1* had a high positive correlation with *IFI6* (r=0.74). The *IFI6* was also positively correlated with the other two ISGs evaluated on D21 post-FTAI. Therefore, these novel results pointed that *IFI6* and *MX1* expression are correlated, but apparently the conceptus stimulus on expression of both genes in dairy cattle are attenuated. The MX1 has been indicated as a classic ISG over the last two decades (Gifford et al., 2007; Green et al.,2010;), but its use as a pregnancy marker is doubtful as Myxovirus genes (*MX1* and *MX2*) and are important

immune genes that help mammals defend mammals against a broad range of viral infections (Hicks et al., 2003). Although the physiological role of IFI6 has not been fully characterized, it plays a critical role in immunomodulation and an antiapoptotic function in the mitochondria (Cheriyath et al.,2011). Therefore, considering that all animals were between 40 and 55 DIM, other factors related to immunomodulation and lactation peak during early postpartum might have affected the expression of these ISGs. Differences of breed, parity order and day of sampling between the previous studies performed in *Bos indicus* beef cattle and the present study have also to be considered on interpretation of the stimulus of conceptus presence on expression of *MX1* and *IFI6;* however, further studies are indicated to understand the pregnancy stimulus on those targets in *Bos taurus* dairy cows.

The SCE-induced local inflammation results in an increased expression of pro-inflammatory cytokines on the uterus and blood (Galvão et al., 2012; Galvão et al., 2011; Kasimanickam et al., 2014; Kim et al., 2014). Also, these cytokines lead to increase pregnancy losses during maternal recognition of pregnancy (Kasimanickam et al., 2014). The novel results of the present study describe that SCE has no influence in the expression of pro-inflammatory cytokines (*IL1-β*, *TNF-α* or *IFN-γ*) in PBMCs during estrous cycle or early pregnancy establishment. However, the abundance of *IL1-β*, was affected by the time of sampling, as its expression was 19.3-fold greater on D7 compared to D0. Contrary to the present results, Galvão et al. (2011), reported an increase on *IL1-β* expression in the endometrium tissue of cows with PMN ≥ 10% in uterine cytological samples on week 5 and 7 of postpartum. The difference between results of Galvão et al. (2011) and the present study may be related to that we used a lower cutoff value for classification of SCE occurrence or that we evaluated the expression in circulating immune cells instead of in the uterine environment. In this

regard, the inflammatory process associated to SCE is manly local, which could not modulate the circulating immune cells. However, Fischer et al. (2010) and Kasimanickam et al. (2014) reported, in cows with SCE presence, a greater gene expression of *IL1-α*, *IL1-β*, and *TNF-α* in the endometrial tissue, and also an increase of haptoglobin, an acute phase protein induced by inflammation process, in the blood.

Our hypothesis that pregnant animals would present a reduced expression of pro-inflammatory cytokines in circulating immune cells in comparison to non-pregnant animals was not supported. This assumption was considered based on previous studies (Roberts, 2007; Yang et al., 2014; Rashid et al., 2018), reporting that IFNt not only acts as blocking the luteolytic cascade, but also has an immunomodulatory function, mainly curbing immune cells to a Th2 response (anti-inflammatory). Rashid et al. (2018) reported a decreased expression of *IL1- β* and *TNF-α* in PBMCs cultured and treated with uterine flushing collected from multiple embryos, on day 7 of pregnancy. Unexpectedly, and in the opposite direction, the abundance of *TNF-α* and *IL1-β* were upregulated in the PBMCs on day 21 of pregnancy in the present study. In addition, significant positive correlations between *TNF-α and IL1- β* (r=0.82) and between both cytokines *with ISG15* and *OAS1* (r>0.45) were observed. Also, our results were not in accordance of Yang et al. (2016), where, on day 18 of pregnancy, pregnant cows had significantly lesser abundance of *IFN-γ* in PBMCs compared to non-pregnant cows. However, recent results from Mohapatra et al. (2020), on day 18 of pregnancy in crossbred dairy cows, indicated a greater abundance of *IFN-γ* and *TNF-α* expression in PBMCs of pregnant cows compared to non-pregnant. The higher gene expression of *TNF-α* might be implicated for decreasing the $PGF_{2\alpha}$ secretion and increasing prostaglandin E_2 production, a luteotropic prostaglandin, playing a crucial role in the establishment and maintenance of pregnancy (Szostek et al., 2014;

Sakumoto et al., 2014). Therefore, the previous described immunomodulatory effect of IFNt toward an anti-inflammatory response, may be restricted to the early phases of pregnancy related to immune tolerance to the embryo up its hatching (Rashid et al., 2018). The novel results of expression of pro-inflammatory cytokines in PBMCs on the third week of pregnancy indicates that those cytokines are important factors for pregnancy establishment after the semi-allogenic embryo starts to express major histocompatibility complex. Thus, our running hypothesis, is that after a period to recognize the semi-allogenic embryo, the adaptative maternal immune system is modulated and shifted from a Th2 to Th1 response, enhancing the cascade of pro-inflammatory cytokines.

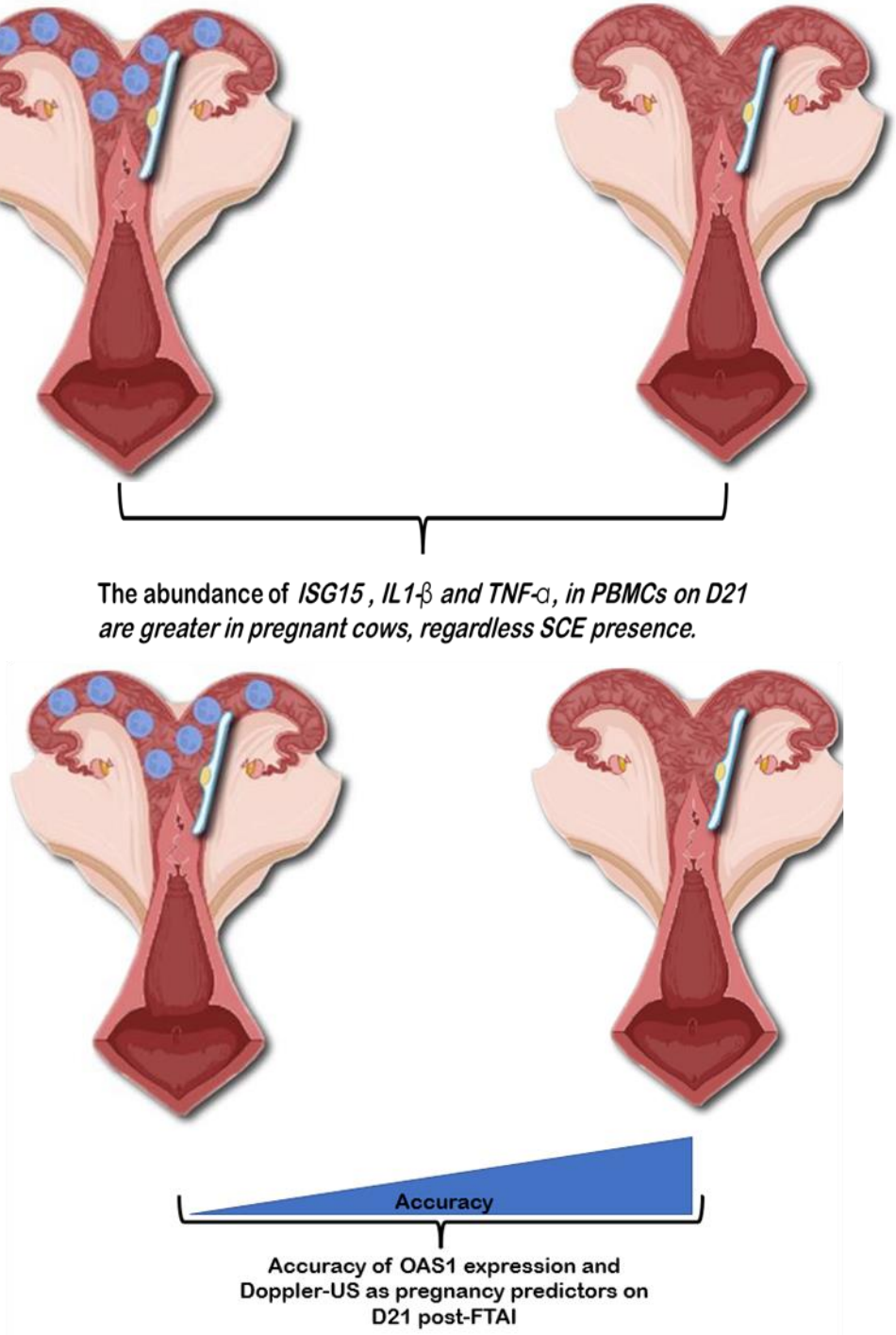
The accuracy of most of early pregnancy predictors herein evaluated were reduced when SCE was diagnosed at beginning of FTAI protocol. That is, the ROC curve analyses indicated that all of early pregnancy predictors were not effective in animals with SCE for diagnosing pregnancy on D21 after FTAI. In addition, the abundance of *ISG15* proved to be a highly accurate method to predict pregnancy, independently of SCE presence. Dalmaso de Melo et al. (2020) using beef cows and heifers, indicate that the abundance of *ISG15*, *OAS1* as well as, CL blood perfusion, using, Doppler-US, were good predictors of pregnancy on day 20 after FTAI. The *ISG15* and *OAS1* are classical ISGs and the gene expression of these targets in PBMCs, are widely used as early pregnancy diagnose in ruminants. The *ISG15* has a pivotal function for maintenance of pregnancy across all mammalian species, through involvement in glycosylation, and it also covalently conjugates with different proteins preventing the spread of threats (Hansen and Pru, 2014). Interestingly, in the present study, the CL blood perfusion, using Doppler-US method was a good predictor only when applied in healthy animals, reaching 85% accuracy. Similarly, the *OAS1* abundance tended to

accurately predict the pregnancy only in healthy animals. A previous report (Parent et al., 2002) indicated that animals diagnosed with SCE had a dys-regulation of PGE_2 synthases in the bovine endometrium, reducing the secretion of PGE_2, which have a crucial role in the CL formation. Also, it is known, that the *OAS1* gene plays a crucial role in the CL survival (Schmitt et al., 1993). Thus, the lower accuracy of CL blood perfusion, using Doppler-US, in animals with SCE at the begging of FTAI protocol, could be related to changes in PGE_2 concentrations or further factors involved in reduction of CL blood perfusion. However, further investigations are required to understand the effect of SCE on other early pregnancy predictors and how the anti- and pro-inflammatory cytokines are modulated throughout the first three weeks of pregnancy.

4. CONCLUSIONS

In conclusion, gene expression of *ISG15*, *MX1*, *IFI6*, *IFN-γ* and *TNF-α* in PBMCs are not affected by SCE occurrence or estrous cycle phase in dairy cows during wait voluntary period; however, *OAS1* and *IL1-β* are upregulated at diestrus, regardless SCE presence. The *MX1* and *IFI6* are not effective ISGs for use as early pregnancy markers in dairy cows with or without SCE at the beginning of FTAI protocol, as its expression is not powerfully stimulated in pregnant animals. On the other hand, *ISG15* shows to be the most efficient marker in PBMCs for predicting pregnancy at 21 days post-FTAI, regardless the occurrence of SCE. Also, the efficacy of *OAS1* and Doppler-US as pregnancy predictors was reduced by the SCE occurrence. Finally, pregnant animals have a greater expression of *IL1-β* and *TNF-α* on day 21 after FTAI, which could indicate a swift in the maternal immune response to the conceptus toward to a Th1 response in the third week of gestation.

Figure 9. Illustrative scheme of the main results obtained in the present study.



Source: (POIT, D. A. S., 2021)

Notes: Illustrative scheme about the main results obtained in the present study. The first scheme above shows that the abundance of *ISG15, IL1-β,* and TNF-α, were affect by the pregnancy regardless of SCE presence. While, the scheme below, shows the different accuracy of the abundance of *OAS1* and CL blood perfusion, in pregnant animals with and without SCE.

5. FINAL CONSIDERATIONS

Reproduction efficiency is one of the main problems affecting dairy herds in Brazil. Therefore, the development of early and accurate methods for pregnancy diagnosis close to the expected time of return to estrus may improve the detection of early pregnancy failures. The use of ISGs and CL as early pregnancy methods has been gradually studied, aiming to allow the improvement of reproductive programs in dairy and beef cattle operations. Uterine diseases are one of the main causes of conception failures in dairy cows. However, we can highlight SCE, which is an extremely important uterine disease that leads to reproductive problems, such as decreased conception rate and increased number of inseminations in dairy cows.

The results obtained in the present study, shows that the SCE have no influence in the abundance of ISGs and pro-inflammatory cytokines in circulating immune cells in dairy cows during the wait voluntary period (up to 60 days post-partum). Therefore, further studies using another type of cells or tissue such as endometrial tissue for determination of ISGs and cytokines, may be helpful to achieve a better knowledge of the inflammatory process generates by SCE in the uterus and its influence on pregnancy establishment.

Furthermore, the observed greater abundance of *IL1-β* and *TNF-α* in pregnant animals, open new theories of the role of cytokines in the pregnancy establishment, around the implantation period, as much as, the differences of cytokines profiles, that occurs throughout the pregnancy. Finally, the present study demonstrated, that the SCE can decrease the accuracy of some early pregnancy predictors, such as, gene expression of *OAS1* and CL blood perfusion. These founds, could generates further insights, about news roles of ISGs in the pregnancy establishment, eighter on the

prevention in the spread of threats or in the CL maintenance, and how it could suffer impairs in dairy and beef cows under uterine diseases.

REFERENCES

ANTONIAZZI, A.Q. et al. Endocrine delivery of interferon Tau protects the corpus Luteum from Prostaglandin F2 Alpha-Induced Luteolysis in Ewes1. Biol Reprod. 2013;88(6):88. http://dx.doi.org/10.1095/biolreprod.112.105684. PMid:23616594.

BAZER, F.W.; SPENCER, T. E.; OTT, T. L. Interferon tau: A novel pregnancy recognition signal. AJRI 1997; 37:412-420 0 Munksgaard, Copenhagen.

BENZAQUEN, M. E. et al. 2007. Rectal temperature, calving-related factors, and the incidence of puerperal metritis in postpartum dairy cows. J. Dairy Sci. 90:2804–2814.

CAMPOS, C. C. et al. Intramammary infusion of lipopolysaccharide promotes inflammation and alters endometrial gene expression in lactating Holstein cows. J. Dairy Sci. 101:1–16, 2018.

CARDOSO, B. et al. Cytobrush: A tool for sequential evaluationof gene expression in bovine endometrium. Reprod Dom Anim. 2017;52:1153–1157. https://doi.org/10.1111/rda.13037.

CHERIYATH, V.; LEAMAN, D. W.; BORDEN, E. C. Emerging roles of FAM14 family members (G1P3/ISG 6–16 and ISG12/IFI27) in innate immunity and cancer. J Interf Cytokine Res (2011) 31:173–81. doi: 10.1089/jir.2010.0105.

DALMASO, M. G. et al. Applied use of interferon-tau stimulated genes expression in polymorphonuclear cells to detect pregnancy compared to other early predictors in beef cattle. Theriogenology 2020;152:94–105.

DE BOER, M.W. et al. Invited review: Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. J Dairy Sci 2014;97: 3983-99.

ESPOSITO, G. et al. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim. Reprod. Sci. 144(3-4):60-71. doi:10.1016/j. anireprosci.2013.11.00.

FERGUSON, J. D. et al. Round table discussion: body condition of lactating cows. Agric. Practice, 15(4):17-21, 1994.

FERNANDES, A.C.C. et al. Effect of repeated intravenous lipopolysaccharide infusions on systemic inflammatory response and endometrium gene expression in Holstein heifers. J. Dairy Sci. 102:1–13, 2019.

FISCHER, C. et al. Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. Reprod. Fertil. Dev. 22:818–29, 2010.

FLINT, A. P. F.; GUESDON, F. M. J.; STEWART, H. J. Regulation of trophoblast interferon gene expression. Mol. Cell. Endocrinol. 100, 93–95, 1994.

FORDE, N.; LONERGAN P. Interferon-tau and fertility in ruminants. Reproduction. 2017;154(5):F33-43. http://dx.doi.org/10.1530/REP-17-0432. PMid:28887326.

FORDE, N. F. et al. Conceptus-Induced Changes in the Endometrial Transcriptome: How Soon Does the Cow Know She Is Pregnant?. Biology of Reproduction, Volume 85, Issue 1, 1 July 2011, Pages 144–156.

FRICKE, P. M. 2002. Scanning the future—Ultrasonography as a reproductive management tool for dairy cattle. J. Dairy Sci. 85:1918– 1926.

FRICKE, P. M. et al. Methods for and implementation of pregnancy diagnosis in dairy cows. Vet Clin Food Anim 2016;32:165–80.

GALVAO, K. N. et al. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. Theriogenology 2011;76:290–9.

GALVAO, K. N. et al. Evaluation of cytokine expression. by blood monocytes of lactating Holstein cows with or without pospartum uterine disease. Theriogenology, v. 7, p. 356-372, 2012.

GHASEMI, F. et al. 2012. Proinflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis. Theriogenology 78:1538–1547. https: / / doi .org/ 10 .1016/ j .theriogenology .2012 .06 .022.

GIFFORD, C. A. et al. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. J Dairy Sci 2007;90:274-80.

GILBERT, R. O. et al. Prevalence of endometritis and its effects on reproductive performance of dairy cows Theriogenology, 64, 1879–1888.

GREEN, J. C. et al. Measurement of interferon-tau (IFN-tau) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18-20d after insemination in dairy cattle. Anim Reprod Sci 2010;121:24-33.

HAN, H. et al. Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. J Endocrinol. 2006;191:505-512.

HANSEN, T. R.; PRU, J. K. ISGylation: a conserved pathway in mammalian pregnancy. Posttranslational Protein Modifications in the Reproductive System, pp. 13–31, 2014.

HANSEN, T. R.; SINEDINO, L. D. P.; SPENCER, T. E. Paracrine and endocrine actions of interferon tau (IFNT). Reproduction. 2017;154(5):F45-59. http://dx.doi.org/10.1530/REP-17-0315. PMid:28982937.

HICKS, B. A. et al. Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J. Anim. Sci. 81, 1552–1561, 2003.

IBGE. Instituto Brasileiro de Geografia e Estatística. Censo Agropecuário. 2018. Disponível em: https://agenciadenoticias.ibge.gov.br/agencia-sala-de-imprensa/2013-agencia-de-noticias/releases/25482-ppm-2018-rebanho-bovino-diminui-e-produtividade-nacional-de-leite-ultrapassa-2-mil-litros-por-animal-ao-ano. Acesso em: 20 de outubro de 2021.

JIEMTAWEEBOON, S. et al. Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. Reproductive Biology and Endocrinology, v. 9, p. 10, 2011.

KASIMANICKAM, R. et al. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows Theriogenology 2004, 62, 9–23.

KASIMANICKAM, R.; KASIMANICKAM, V.; KASTELIC, J. P. Mucin 1 and cytokines mRNA in endometrium of dairy cows with postpartum uterine disease or repeat breeding. Theriogenology 2014;81:952–958. e2.

KIM, I. H. et al. Inflammatory cytokine concentrations in uterine flush and serum samples from dairy cows with clinical or subclinical endometritis. Theriogenology 2014;82:427–32.

LIMA, F. S. Recent advances and future directions for uterine diseases diagnosis, pathogenesis, and management in dairy cows. Anim Reprod. 2020;17(3):e20200063. https://doi.org/10.1590/1984-3143-AR2020-0063

MCDOUGALL, S.; MACAULAY, R.; COMPTON, C. Association between endometritis diagnosis using a novel intravaginal device and reproductive performance in dairy cattle. Anim. Reprod. Sci. 2007; 99: 9–23.

MELO, G. D. et al. Applied use of interferon-tau stimulated genes expression in polymorphonuclear cells to detect pregnancy compared to other early predictors in beef cattle. Theriogenology, v. 152, p. 94- 105, 2020.

MIWA, N. et al. IDO expression on decidual and peripheral blood dendritic cells and monocytes/macrophages after treatment with CTLA-4 or interferon-γ increase in normal pregnancy but decrease in spontaneous abortion. Mol. Hum. Reprod. 11, 865–870, 2005.

MOLINA-COTO R.; LUCY M. C. Uterine inflammation affects the reproductive performance of dairy cows: A review. Agron. Mesoam. 29(2):449-468. Mayo-agosto, 2018.

MOTTA, I. G. et al. Increased pregnancy rate in beef heifers resynchronized with estradiol at 14 days after TAI. Theriogenology, New York, v. 147, p. 62-70, 2020. Disponível em: < http://dx.doi.org/10.1016/j.theriogenology.2020.02.009 > DOI: 10.1016/j.theriogenology.2020.02.009.

MORELLI, S. S. et al. The maternal immune system during pregnancy and its influence on fetal development. Res. Rep. Biol. 6, 171–189, 2015.

SANTOS, N. R. et al. Postpartum endometrial cytology in beef cows. Theriogenology. 2009 Mar 15;71(5):739-45. doi: 10.1016/j.theriogenology.2008.09.043. Epub 2008 Nov 6. PMID: 18992934.

SCHMITT, R. A. et al. Uterine cellular changes in 2′ , 5′ -oligoadenylate synthetase during the bovine estrous cycle and early pregnancy. Biol. Reprod. 48, 460–466, 1993.

SUNIL, K. M. et al. Implantation associated changes in expression profile of indoleamine-2, 3-dioxygenase 1, Th1-Th2 cytokines and interferon-stimulated genes on neutrophils and peripheral blood mononuclear cells of crossbred cows. Journal of Reproductive Immunology, Volume 142, 2020,103188.

OLIVEIRA, L. J. et al. Characterization of the Th profile of the bovine endometrium during the oestrous cycle and early pregnancy. PLoS One 8:e75571, 2013.

PFAFFL, M.W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research, v. 29, p. 2002, 2001.

Ott TL. Symposium review: Immunological detection of the bovine conceptus during early pregnancy[*]. J Dairy Sci. 2019;102(4):3766-77. http://dx.doi.org/10.3168/jds.2018-15668. PMid:30712941.

PARENT, J. et al. Expression of microsomal prostaglandin E synthase in bovine endometrium: Coexpression with cyclooxygenase type 2 and regulation by interferon-τ. Endocrinology 2002;143: 2936-43.

POHLER, K. G. et al. Circulating concentrations of bovine pregnancyassociated glycoproteins and late embryonic mortality in lactating dairy herds. Journal Dairy Science, v.99, p. 1–11, 2016a.

POHLER, K. G. et al. Past, present and future of pregnancy detection methods. Applied Reproductive Strategies in Beef Cattle- Des Moines, Iowa – September, 7-8, 2016b.

PRISCILA, A. F. et al. Feasibility and accuracy of using different methods to detect pregnancy by conceptus-stimulated genes in dairy cattle, JDS Communications, Volume 2, Issue 3, 2021, Pages 153-158, ISSN 2666-9102, https://doi.org/10.3168/jdsc.2020-0062.

PUGLIESI, G. et al. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: How early can we detect pregnancy? Biol Reprod. 2014;91:95.

RASHID, M. B. et al. Evidence that interferontau secreted from Day-7 embryo in vivo generates anti-inflammatory immune response in the bovine uterus. Biochem Biophys Res Commun. 2018; 500:879-884.

RIBEIRO, E. S. et al. Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. J. Dairy Sci. 99:2201-20. doi:10.3168/jds.2015-10337, 2015.

ROBERTS, R. M. Interferon-tau, a Type 1 interferon involved in maternal recognition of pregnancy. Cytokine Growth Factor Rev. 18, 403–408, 2007

ROCHA, C. C. et al. Ultrasonography-accessed luteal size endpoint that most closely associates with circulating progesterone during the estrous cycle and early pregnancy in beef cows. Animal Reproduction Science, Amsterdam, Elsevier, v. 201, p. 12-21, 2019. Disponível em: < http://dx.doi.org/10.1016/j.anireprosci.2018.12.003 > DOI: 10.1016/j.anireprosci.2018.12.003.

ROCHA, C. C. et al. Conceptus-modulated innate immune function during early pregnancy in ruminants: a review. Anim Reprod. 2021;18(1):e20200048. https://doi.org/10.1590/1984-3143-AR2020-0048.

SAKUMOTO, R.; HAYASHI, K. G.; TAKAHASHI, T. 2014. Different expression of PGE synthase, PGF receptor, TNF, Fas and oxytocin in the bovine corpus luteum of the estrous cycle and pregnancy. Reprod. Biol. 14, 115–121.

SALASEL, B.; MOKHTARI, A.; TAKTAZ, T. 2010. Prevalence, risk factors for and impact of subclinical endometritis in repeat breeder dairy cows. Theriogenology 74, 1271-1278.

SHELDON, I. M. et al. 2006. Defining postpartum uterine disease in cattle. Theriogenology 65:1516–1530.

SIQUEIRA, L.G. et al. Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination. J Dairy Sci. 2013 Oct;96(10):6461-72. doi: 10.3168/jds.2013-6814. Epub 2013 Aug 16. PMID: 23958005.

SMITH, B. I.; RISCO C. A. Management of periparturient disorders in dairy cattle. Vet. Clin. North Am. Food Anim. Pract.21:503–521, 2005.

SPONCHIADO, M. et al. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLoS One. 2017;12(4):23. http://dx.doi.org/10.1371/journal.pone.0175954. PMid:28423001.

SZOSTEK, A.Z. et al. Ovarian steroid-dependent tumor necrosis factor-α production and its action on the equine endometrium in vitro. Cytokine 67, 85–91, 2014.

TAKINO, T. et al. Hagiwara Change in the responsiveness of interferon-stimulated genes during early pregnancy in cows with Borna virus-1 infection. BMC Veterinary Research, v.12, p.253, 2016.

WAGENER, K.; GABLER, C.; DRILLICH, M. A review of the ongoing discussion about definition, diagnosis and pathomechanism of subclinical endometritis in dairy cows.

Theriogenology 94:21–30. https: / / doi .org/ 10 .1016/ j .theriogenology .2017 .02 .005, 2017.

WEGMANN, T. G. et al. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol. Today 14:353–356, 1993.

WALSH, S. W.; WILLIAMS, E. J.; EVANS, A. C. A review of the causes of poor fertility in high milk producing dairy cows. Animal Repoduction Science, v. 123 (3-4), p. 127-138, 2011.

YANG, L. et al. Changes in expression of Th1 and Th2 cytokines in bovine peripheral blood mononuclear cells during early pregnancy. Indian J. Anim. Res. 50, 466–470, 2016.

YANG, L. et al. Maternal immune regulation by conceptus during early pregnancy in the bovine. Asian J. Anim. Vet. Adv. 9, 610–620, 2014.

YOSHINO, H. et al. A predictive threshold value for the diagnosis of early pregnancy in cows using interferon-stimulated genes in granulocytes. Theriogenology, v. 107, p. 188-193, 2017.