UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

THE ROLE OF IL-12 IN THE PATHOGENESIS OF ENDOMETROSIS IN MARE

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MAFALDA RAMOS ALVES RAIO E GUERREIRO

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2024

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Assinatura: Mafalda Raio Guerreiro.

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“I am building a house
where the floor is made up of strength,
where the walls are crafted of ambition,
where the roof is a masterpiece of forgiveness.
I am building myself.”

- Noor Unnahar

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Resumo

O PAPEL DA INTERLEUCINA-12 NA PATOGENESE DA ENDOMETROSE NA ÉGUA

A endometrose equina é uma doença uterina de carácter degenerativo e crónico. É caracterizada pela acumulação de fibras de colagénio que se desenvolvem ao redor das glândulas endometriais e no estroma, em que os processos envolvidos podem ser ativos ou inativos. A gravidade da fibrose endometrial é classificada de acordo com quatro categorias: categoria I (sem fibrose), seguida por IIA, II B e III, que indicam fibrose e inflamação leve, moderada e grave. A endometrose contribui significativamente para a infertilidade das éguas, resultando em perdas financeiras para os proprietários de cavalos em todo o mundo, incluindo em Portugal. Embora a patogénese da endometrose seja multifatorial, este estudo investiga especificamente a potencial influência da interleucina (IL)-12. Num estudo recente, que se concentrou no perfil transcriptómico do endométrio de éguas, foram demonstradas alterações na transcrição de genes associados à sinalização e produção de interleucina (IL) -12 por fibroblastos em diferentes estágios da endometrose, identificando a IL-12 como um potencial ator no desenvolvimento de fibrose endometrial. A interleucina 12 é uma citocina pró-inflamatória sintetizada e liberada por macrófagos, monócitos, células dendríticas e células B, que participa nas respostas imunitárias celulares e humorais. Esta citocina é constituída pelas subunidades IL-12a (IL-12p35) e IL-12b (IL-12p40), ligadas covalentemente. Os objetivos do presente estudo foram: (i) avaliar a transcrição de mRNA das subunidade da IL-12 e seus receptores (IL-12p35, IL-12p40, IL-12Rβ1 e IL-12Rβ2), em endométrios (n = 77) das categorias I, IIA, IIB e III na fase lútea média (MLP) e na fase folicular (FP) do ciclo éstrico, bem como (ii) o efeito da IL-12 na transcrição do mRNA de marcadores fibróticos, tais como: o colagénio (COL1A1 e COL3A1), a fibronectina (Fn1), a lisil oxidase-like 2 (Loxl2), a α-smooth muscle actin (α-Sma), as metaloproteinases de matriz (MMP) 2, 3 e 9, e os inibidores teciduais de metaloproteinases (TIMP) 1 e 2 em fibroblastos endometriais (n=6); e (iii) o efeito da IL-12 na proliferação dos fibroblastos (n=6). Para a medição da transcrição de mRNA dos componentes e recetores da IL-12, foi realizado qPCR em amostras de endométrio. A avaliação da transcrição dos marcadores de fibrose foi realizada por qPCR em explantes de endométrio após tratamento com IL-12 (10 ng/ml) durante 48 e 96h. Nos endométrios classificados como categoria IIB, a transcrição de IL-12Rβ2 revelou-se aumentada na fase lútea, comparando com a fase folicular, e o tratamento de 96h com IL-12 aumentou a transcrição de mRNA de COL1A1, COL3A1, α -SMA, MMP3 e -9 nos fibroblastos endometriais provenientes de culturas in vitro ($p<0.05$). Além disso, a proliferação de fibroblastos diminuiu em resposta ao tratamento com IL-12 após 96h ($P<0.001$). Os resultados do presente estudo indicam que a IL-12 tem um impacto direto na transcrição de marcadores fibróticos em fibroblastos endometriais e na sua proliferação. Por conseguinte, propomos uma correlação entre a IL-12 e a endometrose através do aumento dos componentes da MEC e da transcrição de MMP associada à fibrogénese.

Palavras-chave: endometrose; endométrio; interleucina 12; égua

Abstract

THE ROLE OF INTERLEUKIN-12 IN THE PATHOGENESIS OF ENDOMETROSIS IN MARE

Equine endometrosis is a chronic degenerative uterine condition. It is characterized by fibrotic processes developing around the endometrial glands and stroma, in which the involved processes can be active or inactive. The severity of endometrial fibrosis is graded according to four categories: Category I (no fibrosis), followed by IIA, IIB, and III, which indicate mild, moderate, and severe fibrosis and inflammation. Endometrosis is a significant contributor to mare infertility, resulting in financial loss for horse owners globally, including those in Portugal. Although the pathogenesis of endometrosis is multifactorial, our study specifically investigated the potential role of interleukin (IL)-12. In a recent study, which focused on transcriptomic profiling of mare endometrium, changes in the expression of genes associated with interleukin (IL)-12 signaling and production in fibroblasts at different stages of endometriosis were demonstrated, identifying IL-12 as a potential player in the development of endometrial fibrosis. Interleukin 12 is a proinflammatory cytokine synthesized and released by macrophages, monocytes, dendritic cells, and B cells that participates in both cellular and humoral immune responses. This cytokine is composed of two covalently linked subunits (IL-12a(p35) and IL-12b(p40)). The purpose of the current study was to: (i) evaluate the mRNA expression of *IL-12* subunits (*IL-12p35, IL-12p40*) and its receptors (*IL-12Rβ1* e *IL-12Rβ2*), in mare endometria (n=77) from categories I, IIA, IIB, and III at the mid-luteal phase (MLP) and follicular phase (FP) of the estrous cycle; as well as (ii) the effect of *IL-12* on the mRNA expression of fibrotic markers, such as: *collagen COL1A1* and COL3A1, *fibronectin (FN1)*, *lysyl oxidase-like 2* (*LOXL2)*, α-smooth muscle actin (*α-SMA*), *matrix metalloproteinases* (*MMP*) *2*, *3* and *9*, *and tissue inhibitor of metalloproteinases* (*TIMP*)*1* and *2* in endometrial fibroblasts (n=6); and (iii) the effect of IL-12 on fibroblast proliferation (n=6). Real-time PCR was performed on endometrial samples to determine mRNA expression of IL-12 components and receptors. The transcriptional evaluation of fibrotic markers was performed by qPCR in endometrial explants after the treatment with IL-12 (10 ng/ml) for 48 and 96h. In Category IIB endometria, the mRNA expression of *IL-12Rβ2* was up-regulated in the MLP, compared to FP of the estrous cycle, and IL-12 treatment increased *COL1A1*, *COL3A1*,*α-SMA, MMP3,* and *-9* mRNA expression in mare endometrial fibroblasts cultured *in vitro* after 96h ($p<0.05$). Moreover, fibroblast proliferation was decreased in response to IL-12 treatment after 96h ($P<0.001$). The findings from the current study indicate that IL-12 has a direct impact on the expression of fibrotic markers in endometrial fibroblasts and fibroblast proliferation. It suggests a correlation between IL-12 and endometrosis development through the increased ECM components and MMP expression associated with fibrogenesis.

Keywords: endometrosis; endometrium; interleukin-12; mare

Resumo alargado
O PAPEL DA INTERLEUCINA-12 NA PATOGÉNESE DA ENDOMETROSE NA ÉGUA

Atualmente, sabe-se que 45% da mortalidade humana em todo o mundo é atribuída a doenças de origem fibrótica, como é o caso da fibrose pulmonar, renal e até do miocárdio. Embora existam algumas diferenças na etiologia e na apresentação clínica da fibrose nos diferentes tecidos/ órgãos, são conhecidas muitas semelhanças nos mecanismos moleculares e celulares envolvidos, assim como os processos e interações celulares que ocorrem durante os processos fibróticos. Contudo, os mecanismos que explicam o desenvolvimento destas doenças estão ainda longe de ser totalmente compreendidos. Apesar da fibrose ser uma causa conhecida de mortalidade, os tratamentos propostos têm demonstrado eficácia limitada. Há assim uma crescente necessidade de encontrar terapias anti-fibróticas eficazes, sendo para isso crucial compreender como se inicia o processo de fibrose, se e como pode ser revertido, e identificar potenciais estratégias de tratamento. Portanto, a descrição precisa e a elucidação dos processos e vias moleculares subjacentes que contribuem para o início e progressão da fibrose podem fornecer a base para o desenvolvimento de tratamentos novos e eficazes para a fibrose em diferentes tecidos e órgãos.

As doenças fibróticas partilham uma característica comum: acumulação descontrolada e progressiva de componentes da matriz extracelular nos órgãos afetados, causando a sua disfunção e falência final. A endometrose equina é, assim, uma alteração fibrótica degenerativa e crónica do endométrio que se manifestada pela deposição excessiva de componentes da matriz extracelular (MEC). A fibrose progressiva do estroma do tecido resulta em alterações na estrutura e função uterinas com consequentemente redução da fertilidade nas éguas. As alterações podem ser divididas em histológicas e do microambiente uterino. As alterações histológicas que caracterizam a endometrose são a deposição excessiva de colagénio em redor das glândulas endometriais, formando os “ninhos glândulares”, e na lâmina própria do epitélio endometrial e membrana basal do endométrio. O microambiente uterino, por sua vez, fica alterado por diminuição da secreção das glândulas endometriais e por alteração da contratilidade do miométrio, resultando num ambiente uterino hostil para a sobrevivência de um embrião.

No conjunto de fatores envolvidos na fisiopatologia desta doença destacam-se as células inflamatórias, as citoquinas por estas produzidas, os miofibroblastos, as NETs e as MMPs. Quando estamos perante uma inflamação ocorre recrutamento de células para o local do estímulo/lesão, as quais não só vão fagocitar bactérias e debris celulares como também libertar citoquinas pro-inflamatórias que recrutam outras células de defesa. Os mecanismos de resolução de lesão num tecido são fundamentais se ativos por tempo limitado, mas problemáticos se se perpetuarem. Uma inflamação crónica com persistente recrutamento de

células inflamatórias para o endométrio conta com a participação de diversos grupos de mediadores inflamatórios, sendo um deles as interleucinas. A interleucina-12 é secretada por monócitos, macrófagos e células dendríticas e a sua principal atividade biológica passa por iniciar a diferenciação de linfócitos T naïve em células Th1 e promoção da secreção de interferão-gama, uma citoquina pró-inflamatória. Por sua vez, os miofibroblastos estão entre as principais células intervenientes na patogénese desta doença já que são capazes de produzir grandes quantidades de componentes da MEC. A sua ação é fundamental no restabelecimento da homeostase dos tecidos lesionados, no entanto, quando se prolonga no tempo, os seus mecanismos de ação perpetuam-se havendo excessiva deposição de componentes da MEC, especialmente colagénio.

Na sequência do estudo da análise transcriptómica do tecido endometrial por Szostek-Mioduchowska, cujos resultados demonstraram alterações génicas associadas à transcrição da IL-12, o presente estudo visou determinar a transcrição das subunidades da IL-12 (IL-12p35 e IL-12p40) e dos seus recetores (IL-12Rβ1 e IL-12Rβ2) em éguas classificadas em diferentes categorias de endometrose e determinar o efeito da IL-12 na remodelação da MEC e na diferenciação de miofibroblastos e na capacidade de proliferação dos fibroblastos endometriais. Para a medição da transcrição de mRNA dos componentes e recetores da IL-12, foi realizado qPCR em amostras de endométrio. A avaliação do efeito da IL-12 na remodelação da MEC foi feita através da determinação da transcrição de marcadores de fibrose selecionados (Colagénio tipo 1 e 3, fibronectina, alfa-smooth muscle actin, lysyl oxidase-like 2, metaloproteinases de matiz 2, 3 e 9, e os inibidores de metaloproteinases de matriz 1 e 2) em fibroblastros endometriais in vitro após tratamento com IL-12 (10 ng/ml) durante 48 e 96h, recorrendo a qPCR. A determinação da proliferação dos fibroblastos foi feita através do método BrdU, após tratamento *in vitro* dos fibroblastos com IL-12 (10 ng/ml) durante 48 e 96h.

A presença das diferentes subunidades de IL-12 e do seu recetor foi determinada nas diferentes categorias de endométrio equinos classificados de acordo com o sistema de classificação de Kenney e Doig. Contudo, não foram encontradas diferenças significativas na transcrição das subunidades IL12p35, IL12p40 e do recetor IL-12Rβ1 para qualquer categoria de endométrio durante a fase folicular e lútea. No entanto, os resultados demonstraram um aumento na transcrição do mRNA do recetor IL-12Rβ2 no endométrio de categoria IIB durante a fase lútea média em comparação com a fase folicular do ciclo éstrico ($P<0.01$). Para além disso, os resultados sugerem que IL-12 está associada a um aumento da transcrição de componentes da MEC como Loxl2 ($P<0.01$), α-SMA e Col1a1 ($P<0.05$) e Col3a1, Mmp3 e Mmp9 ($P <0.01$) pelos fibroblastos endometriais e a uma inibição da proliferação de fibroblastos endometriais in vitro após 96h de incubação ($P<0.001$).

O objetivo deste estudo foi explicar o papel da IL-12 na progressão da fibrose endometrial equina recorrendo a amostras endometriais e a culturas in vitro de fibroblastos. A experiência 1 demonstrou pela primeira vez que as subunidades p35, p40 da IL-12 e seus recetores estão presentes tanto no endométrio equino saudável (Cat. I) como no endométrio com diferentes graus de fibrose (Cat. IIA, IIB e III). No entanto, o aumento da transcrição do recetor IL-12Rβ2 na categoria IIB na fase lútea em comparação com a folicular tem pouco significado, sendo necessárias posteriores análises visando realizar a quantificação de proteína através de Western Blot, ou até mesmo abordagem através da imunolocalização.

Os resultados da experiência 2 demonstraram que a IL-12 tem um impacto direto na transcrição de mRNA de marcadores de fibrose em fibroblastos endometriais e na proliferação de fibroblastos de maneira dependente do tempo. Os resultados indicam que a IL-12 tem um efeito estimulante na transcrição de mRNA de fatores-chave envolvidos na patogénese da endometrose, incluindo colágenio, LOXL-2, α-SMA, e as MMP-2 e -9, podendo contribuir para a excessiva deposição de componentes da MEC, que está na base da patogénese desta doença. Além disso, sugere também que a IL-12 tem impacto na transcrição da MMP, que não só degrada o colagénio, mas também possui propriedades pró-fibróticas. Com isto, os resultados obtidos em estudos *in vitro* mostram que a IL-12 tem um efeito estimulador da transcrição dos componentes da MEC, sugerindo um papel pró-fibrótico dessa citoquina.

A experiência 3, que visou determinar o impacto da IL-12 na proliferação dos fibroblastos endometriais *in vitro,* demonstrou que esta citocina teve um efeito inibidor da proliferação destas células. Contudo, tendo em conta os resultados da experiência 2, seria expectável que a acompanhar um aumento da produção de componentes da MEC tivessemos um aumento da proliferação, sendo por isso os resultados desta experiência ambíguos, não nos permitindo aferir com certeza qual o impacto da IL-12 na proliferação das principais células que constituem o tecido conjuntivo.

Até à data, nenhum estudo determinou o efeito da IL-12 na patogénese da endometrose, portanto os nossos resultados fornecem dados úteis sobre esta doença, que é uma das principais causas de infertilidade e de morte embrionária na égua. Além disso, este estudo elucida a potencial participação da IL-12 em patologias associadas à fibrose em vários órgãos humanos, aproximando-nos da compreensão dos mecanismos subjacentes. Os dados deste estudo sugerem que a IL-12 possa ter um papel na progressão da endometrose em éguas, afetando diretamente a deposição de MEC e a regulação de MMPs em fibroblastos, bem como a diferenciação de fibroblastos em miofibroblastos.

Palavras-chave: endometrose; endométrio; interleucina 12; égua

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AA - antibiotic antimycotic

AI - artificial insemination

APC - antigen-presenting cell

CL - corpus luteum

COL - collagen

COL1 - collagen type 1

COL3 - collagen type 3

ECM - extracellular matrix

FN - fibronectin

FP - follicular phase

HA - hyaluronic acid

hPDLFs – human periodontal ligament fibroblasts

IL - interleukin

IL-1β - interleukin 1 beta

IL-6 - interleukin 6

IL- 8 – interleukin 8

IL-12 – interleukin 12

IFN – interferon

IFN-γ - interferon gamma

IRF - interferon regulatory factors

IUF - intra uterine fluid

JAK - Janus kinase

LAP - latency-associated peptide

LOXL2 - lysyl oxidase like-2

MMP - matrix metallopeptidase

MMP-2 - matrix metallopeptidase 2

MMP-3 - matrix metallopeptidase 3

MMP-9 - matrix metallopeptidase 9

MLP - mid-luteal phase

MΦ – macrophage

NETs – neutrophil extracellular traps

NF-kB - nuclear factor kappa light chain enhancer of activated B cells

NK - natural killer

PBE - post-breeding *endometritis*

PDGF - platelet-derived growth factor

PG - prostaglandin

PGF-2α - Prostaglandin 2α

PMN - polymorphonuclear neutrophils

ROS – reactive oxigen species

STAT - signal transducer activator of transcription

SSc – systemic sclerosis

TGF- transforming growth factor

TGF-β1 - transforming growth factor beta 1

Th - T helper

TIMP - tissue inhibitors of metalloproteinases

TIMP-1 - tissue inhibitor of metalloproteinase 1

TIMP-2 - tissue inhibitor of metalloproteinase 2

TLR - toll-like receptor

TNF-α - tumor necrosis factor-alpha

TYK - tyrosine kinase

α-SMA – alpha - smooth muscle actin

Traineeship Report

Although my curricular internship took place in the second semester and was focused on research about equine reproduction, I used the first semester to get in contact with other fields of Veterinary Medicine that I have an interest in pursuing: equine clinics and reproduction, farm animals clinics and reproduction, and small animal clinics.

Curricular Internship: Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (Olsztyn, Poland)

This dissertation is based on my curricular internship that took place at the Department of Reproductive Immunology and Pathology, at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland, from the 1st of March of 2023 to the 30th of June of 2023. During this period, I worked under the supervision of Dr. Anna Szóstek-Mioduchowska and was included in the daily activities of the Department of Reproductive Immunology and Pathology. I had the opportunity to conduct my own experiments. The first month was focused on learning the basic tasks in the laboratory, such as autoclaving the material, getting familiar with the instruments, preparing gel for electrophoresis, Western blotting, measurement of total protein (BCA method), and evaluation of cell culture viability resorting to MTT method. During the other 3 months, I had the opportunity to assist the Ph.D. student Magda Słyszewska in the scope of her doctoral project, participated in the activities, and went through daily tasks of monitoring growing fibroblast and macrophage cell cultures, isolation of blood cells collected from selected mares, treatment, and observation of cells in culture, evaluation of cell proliferation and viability, and to conduct my study.

During my time at the Institute, I attended weekly meetings of the Szóstek-Mioduchowska research group. The aim of these meetings was to introduce crucial scientific themes to my co-workers. This task helped me substantially in improving my knowledge on laboratory techniques, while expanding my understanding of immunology, reproduction, tissue fibrosis, molecular biology, and public speaking skills.

Moreover, the results I obtained during my internship formed the basis of a conference abstract entitled “Rola interleukiny 12 w patogenezie endometrosis u klaczy”/ The role of interleukin 12 in the pathogenesis of endometrosis in mare” Mafalda Guerreiro, Magda Słyszewska, Agnieszka Sadowska, Graca Ferreira-Dias, Ana Catarina Tores, Anna Szóstek-Mioduchowska. The abstract was presented at the Forum Hipologiczne on 21-22 September 2023 in Racot, Poland.

Extracurricular Externship

During the first semester, I have spent my time exploring other veterinary fields: equine and farm animal ambulatory clinics, and small animal clinics. First, I joined Dr. Mariana Carido for a month (October of 2022), during which I was enrolled in her daily routine in the scope of her ambulatory clinic, in the district of Lisbon. I was able to assist her during reproductive procedures in several species (equine, ovine, bovine, caprine), such as artificial insemination, uterine lavage, transrectal and transabdominal ultrasonography, estrous synchronization, and, in less frequency, routine procedures (vaccinations, deworming) and emergencies.

In addition, during November, followed the daily casuistic at Pet24 Veterinary Hospital, located in Alfragide, Lisbon. During this externship, it was possible for me to get in touch with routine procedures in companion animals: vaccination and deworming programs, follow-up patients with chronic illnesses (Mainly Chronic kidney disease and Diabetes Mellitus), assist several surgeries, help with hospitalized animals in the basic duties (e. g. medication, walking, physical examinations, feeding), and joined ultrasound appointments. More than teaching me the basics in this field, this externship helped me understand how companion animal clinics work and the difficulties that come with it.

At last, from the 6th of January to the 20th of February, I was enrolled in the Hospital Escolar Veterinário – Faculdade de Medicina Veterinária, Universidade de Lisboa, Lisbon,during which period I mostly assisted in the emergency room and imaging diagnostic service. I had the chance to be present in critical situations and increase my knowledge about how to deal with them, while I was following the X-rays and Tomography appointments whenever feasible.

I - Introduction

Endometrosis, which is considered the major cause of mare's infertility, is clinically described as fibrotic and degenerative changes in the endometrium, manifested by fertility loss and embryonic death, and histologically as endometrial periglandular and/or stromal fibrosis (Kenney 1978; Doig et al. 1981). Endometrosis is characterized by a pathological deposition of collagen in the endometrial lamina propria around the endometrial glands, stromal cells and under the basement membrane of the epithelium (Kenney 1978). While the cause of this condition remains unknown, prior research suggests that advanced age, multiple pregnancies, and recurring inflammation or infection in the uterus affect the development of this degenerative condition (Kenney and Ganjam 1975). Endometrosis, which is considered the major cause of mare's infertility, is clinically described as fibrotic and degenerative changes in the endometrium, manifested by fertility loss and embryonic death, and histologically as endometrial periglandular and/or stromal fibrosis (Kenney 1978; Doig et al. 1981). Endometrosis is characterized by a pathological deposition of collagen in the endometrial lamina propria around the endometrial glands, stromal cells and under the basement membrane of the epithelium (Kenney 1978). While the cause of this condition remains unknown, prior research suggests that advanced age, multiple pregnancies, and recurrent inflammation or infection in the uterus affect the development of this degenerative condition (Kenney and Ganjam 1975).

The process of fibrosis is linked to many different cell types, including fibroblasts, epithelial cells, endothelial cells and immune cells, which produce cytokines that promote fibrosis. Fibrosis is a complicated event that is driven by several cytokines and growth factors that contribute to its development over time. It is impossible to attribute its development as the response to a single factor. Instead, it results from the combined effect of several factors. Cytokines appear to play a vital role in the pathogenesis of endometrosis. Identifying the role of specific cytokines is crucial for finding appropriate treatment, as a thorough understanding of the causes is necessary. So far, the role of transforming growth factor-β1 (Szóstek-Mioduchowska et al. 2019, 2020), interleukin IL-1β (Szóstek-Mioduchowska et al. 2019), and IL-6 (Szóstek-Mioduchowska et al. 2019), as well as IL-4, IL-13, and IL-17 (Szóstek-Mioduchowska et al. 2023) was described. OBJ OBJ The recent study, which is focused on transcriptomic profiling of mare endometrium, revealed changes in the expression of genes associated with "interleukin (IL)-12 signaling and production in macrophages (MΦ)" at different stages of endometrosis, identifying IL-12 as another potential player in the development of endometrial fibrosis (Szóstek-Mioduchowska et al. 2023). Thus, this dissertation is focused on IL-12, a cytokine that gives its name to the family it is part of, the Interleukin 12 family, together

with interleukin-23, interleukin-27, and interleukin-35. Interleukin-12 is mainly produced by monocytes, MΦs, dendritic cells, and B cells ￼(Trinchieri et al. ￼.

The purpose of this study is to explore the effect of IL-12 in the development and progression of endometrosis. The study determined the expression of IL-12 subunits and receptors in endometrial samples from the mid-luteal and follicular phases of the estrous cycle, classified into 4 categories (Kenney and Doig 1986). Additionally, to assess the effects of this cytokine on extracellular matrix (ECM) turnover, we examined the mRNA expression of fibrotic markers in *in vitro* cultured endometrial fibroblasts and its impact on fibroblast proliferation.

II – Literature Review

1. Endometrosis

Endometrosis is the term given to the condition characterized by chronic degenerative *endometritis* associated with decreased fertility and early embryonic loss (Kenney, 1992). The first approach to chronic endometrial histological changes was led by Kenney (1978). The term endometrosis was introduced by Kenney (Allen 1993) to define changes in the mare uterus previously referred to as chronic degenerative *endometritis*. Endometrosis is characterized by fibrotic and degenerative changes in the mare endometrium including endometrial periglandular and/or stromal fibrosis and glandular alterations, such as cystic dilation, atrophy, or hypertrophy of the epithelium (Kenney 1978; Doig et al. 1981). The histological structural changes in the endometrium are described in Table 1. Equine endometrosis results in both histological changes and alterations to the uterine microenvironment (Hofmann et al. 2009; Lehmann et al. 2011). These alterations encompass changes in the endometrial expression of steroid hormone receptors (Hoffmann et al. 2009a), endometrial protein, specifically uteroglobin, uterocalin, calbidin, glycogen (Hoffmann et al. 2009b) synthases of prostaglandin (Szóstek et al. 2012), cytokines (Szóstek et al. 2013) at the different stages of endometrosis.

Additionally, myometrium alterations have also been described during endometrosis. During this condition, the muscle layer of the uterus appears to have atrophy of the smooth muscle fascicles and cells, along with fatty degeneration of atrophic myocytes and increased deposition of collagen and elastin fibers in between the smooth muscle bundles (Hanada et al. 2014). Moreover, recent studies described that there are alterations in the transcriptomic profile of mare myometrium at different stages of endometrosis (Drzewiecka et al. 2023).

According to the degree of endometrial structural changes, endometria can be divided into four different categories: I, IIA, IIB, and III (Kenney and Doig 1986). In category I endometrial structure remains intact and without inflammation and fibrosis, expecting a foaling rate of 80-90%. Categories IIA, IIB, and III include endometria with mild, moderate, and severe fibrosis and inflammation. The expected foaling rate for category IIA is 50-80%, for IIB is 10-50%, and for III is 10% (Kenney and Doig 1986). Thus, this condition has a negative impact on the economy of the horse-breeding industry.

Category	**Histological structural changes in** endometrium
I	Normal to healthy, active and well distributed glands, no to little inflammatory cells
IIA	Mild, scatted inflammation and fibrosis; lack of glandular nests; slight to moderate inflammatory changes, lymphatic lacunae, partial endometrial atrophy
IIB	Moderate scatted inflammation and fibrosis, 2-4 fibrotic, 2-4 fibrotic nests of gland, inflammatory and lymphatic changes are widespread, diffuse and moderately severe
III	Dilated glands surrounded by layers of fibrotic cells, 5 or more fibrotic nests, diffuse and severe inflammatory changes, severe lymphatic lacunae

Table 1 – Endometrial histological changes in different endometria Category (Kenney and Doig 1986).

Currently there is no available effective therapy to treat endometrosis in mares. For the last three decades, a number of therapies have been proposed. For example, Ricketts (1985) suggested mechanical curettage as a method to improve the foaling rate. In his study, this method increased by 60% the pregnancy rate, even though it was ineffective in older mares. Another treatment proposed by Ley and colleagues (1989) showed a decrease in the infiltration of chronic inflammatory cells and in the periglandular fibrosis in 30% of mares, by administrating intra-uterine dimethyl sulfoxide, although the foaling rates remained the same. Other therapeutic possibility includes the use of kerosene uterine instillation (Bracher et al., 1991). Nevertheless, uterine infusion of Kerosene did not change the endometrial classification of mares (Podico et al. 2020). Stem cells have been also proposed as another approach to treat endometrosis (Mambelli et al. 2013; Mambelli et al. 2014; Falomo et al. 2015; Alvarenga et al. 2016). Mambelli and colleagues reported for the first time that, through uterine instillation, stem cells can be incorporated and widely distributed in the uterus of mares with endometrosis (Mambelli et al. 2013). Thus, mesenchymal stem cells seem to be a promising new therapeutic approach for endometrial regeneration, but further studies must be carried out.

The etiology and pathogenesis of endometrosis remain unclear and the description of processes underlying the initiation of endometrosis in mares would help to develop a strategy for its effective treatment.

1.1. Pathogenesis of endometrosis

The pathophysiology of endometrosis remains unclear, although age, repeated pregnancies, parturition, chronic inflammation, and endocrine problems were indicated as factors that seem to influence the genesis and severity of this condition (Kenney and Ganjam 1975). However, another study observed that maiden mares developed advanced endometrosis even though their endometria had not been in contact with semen, or experienced Post-breeding endometritis (PBE), foaling, or post-partum uterine involution (Ricketts and Alonso, 1991). The retrospective study conducted by Ebert et al. (2014) examined 9120 biopsies submitted for routine diagnostic evaluation to determine the age-related incidence of endometrosis in mares. The results of the study indicated that the incidence was 32% in mares aged 5 years and under, 66% in those aged 6–10 years, 84% in those aged 11–15 years, 90% for mares aged 16–20 years, and 92.5% in those over 20 years old. Additionally, genetic predisposition should be considered as a potential cause of endometrosis (Oddsdittor 2008). Likely, a genetic predisposition might disrupt the endometrial resolution, inflammation, and repair mechanisms, triggering a continuous activation of collagen synthesis in response to mediators released by local and infiltrating cells (Oddsdittor 2008).

The use of transcriptomic analysis helped to fill gaps in our understanding of the nature of endometrial fibrosis and identify novel pathways and regulators involved in the development of endometrosis. Recently, Szóstek-Mioduchowska and colleagues (2023) compared the transcriptome profiles of mare endometrium classified as categories I, IIA, and IIB. The significant differences in the expression of 230 genes (58 up-regulated and 172 down-regulated), and 1101 genes (598 up-regulated and 503 down-regulated) were observed in categories IIA and IIB, compared to category I endometria, respectively. The results showed that the inflammatory and metabolic alterations in the endometrium are typically found in mild and moderate stages of endometrosis and that cytokines secreted by inflammatory cells possibly influence the expression of genes associated with ECM remodelling (Szóstek-Mioduchowska et al. 2023). Moreover, in the mild stage of endometrosis, differentially expressed genes (DEGs) were annotated among others to the metabolism of reactive oxygen species (ROS), connective tissue quantities, wound healing, and pulmonary fibrosis idiopathic signaling pathways when compared to category I endometria. In the moderate stage of endometrosis compared to category I, DEGs are noted among other things, fibrosis, cellular death, cellular homeostasis, and mitochondrial dysfunction remodeling (Szóstek-Mioduchowska et al. 2023).

Recent studies have shown also that microRNAs (miRNAs) are important regulators of many cellular processes and functions in the pathogenesis of fibrosis. Szóstek-Mioduchowska

and colleagues identified 1, 26 and 5 differentially expressed miRNAs (DEmiRs), respectively, in the IIA (mild fibrosis), IIB (moderate fibrosis) and III (severe fibrosis) groups compared to the I (no fibrosis) endometrial group. Functional enrichment analysis revealed that DEmiRs target genes involved in the mitogen-activated protein kinase (MAPK), Hippo and phosphoinositide-3-kinase (PI3K)-Akt signaling pathways, focal adhesion and extracellular matrix-receptor interaction what can suggest that miRNA have a regulatory role in the development of endometrosis (Wójtowicz et al. 2023).

1.1.1. Uterine defense mechanisms and post-breeding *endometritis*

Over time, animal evolution has been accompanied by the adaptation of organisms to risk factors through the development of defense mechanisms at different levels, for example, in infection prevention. Physical barriers are the first protection in preventing the entrance of microorganisms through the skin and mucosal surfaces, in gastrointestinal, respiratory, and urogenital tracts (Carneiro and Junqueira 2013a; 2013b). Furthermore, enzymes, fatty acids, and oils in the skin, bacteriolytic enzymes, mucopolysaccharides, and antibodies in the mucosal surface inhibit microorganisms' growth when they cross the physical barrier (Reed et al. 2004).

The first line of defense comprises the physical barriers, such as the external genitalia, the closed cervix, the mucous flow from the uterine body through the cervix to the exterior, and uterine contractions (Wira et al. 2011). All these factors are under the influence of specific hormones, such as prostaglandin 2 alpha (PGF2α), oxytocin, and progesterone. The second line of defense includes the innate immune response, involving inflammatory cells, such as neutrophils, MΦs, and monocytes, and the later defense is the adaptative immune response, mainly carried by lymphocytes through the production of immunoglobulins (Katila and Ferreira-Dias 2022).

In 2006, Troedsson stated that, in the first hours after breeding, the physiological establishment of a transient breeding-induced endometritis is considered a normal event in mare reproductive system. This transitory inflammation appears to have an effective role in the removal of bacteria, spermatozoa and debris introduced in the uterus during AI or mating (Troedsson 2006). The physical clearance of the uterine content is crucial to prevent the accumulation of fluid with bacteria, semen, or other exogen products that might increase the probability of *endometritis* (Evans et al., 1986), especially in the first 24-48 hours after mating or artificial insemination. It results from the mechanical pathway from the uterine lumen, through the cervix, towards the exterior, and the innate immune response that is triggered by the contact of exogenous materials with the endometrium, generating a neutrophilic migration to the uterus (Kotilainen et al., 1994). Besides the described above, PGF2α also contributes to generating uterine contractility, leading to its clearance from fluids, debris, and bacteria

(Gastal et al. 1998). In addition, it has already been proved that mucus provides a physical barrier impairing microorganisms' invasion and leading later to their elimination (Sweeney 1989), as well as beating cilia and cervical endometrium folds that seem to be adjuvant in the mucociliary apparatus, promoting microcurrents that direct the uterine content through the exterior (Ginther 1992).

Many authors have suggested that inflammatory infiltration during *endometritis* relates to the development of endometrosis (Kenney and Doig 1986; Flores et al. 1995). As is the case of a study showing that 10 out of 20 mares with experimental-induced *endometritis*, experienced a temporary metabolic activation of the fibrotic stromal cells. On the other hand, the same study stated that mares with endometrosis have a higher risk of developing *endometritis* (Hoffmann et al. 2009a), suggesting a correlation between these two conditions. The activation of inflammatory cells stimulates the release of pro-inflammatory mediators, neutrophil extracellular traps (NETs), and the activation of pro-fibrotic mechanisms, which progressively leads to the establishment of a fibrotic endometrium. Consequently, the uterus becomes more susceptible to infection, turning the uterine milieu hostile to sperm, leading eventually to a decrease in fertility rates (Katila and Ferreira-Dias 2022).

1.1.2. Cellular and molecular mechanisms of pathogenesis of fibrosis

Fibrosis refers to the excessive deposition at the site of injury of ECM components, such as collagen (COL), mainly COL1, but also fibronectin (FN), and hyaluronic acid (HA), in response to the persistent influence of pro-fibrotic mediators, for example, transforming growth factor (TGF)-β1, prostaglandins (PG), cytokines, and enzymes found in NETs (Hoffmann et al. 2009a; Skarzynski et al. 2020).

After a uterine intervention or agression, the wound-healing process takes place, and it is usually characterized by the sequential, but overlapping, stages of recruitment of inflammatory cells, the release of fibrogenic cytokines, and finally, the activation of collagen-producing cells (Lee and Kalluri 2010). However, the inflammatory response can be both beneficial and detrimental, because it repairs the injury, but can also lead to progressive fibrosis if it becomes uncontrolled, resulting in loss of tissue normal function (Schrier 2007), since the excessive deposition of ECM components destroys normal tissue architecture (Eckes et al. 2000).

During the early wound-healing process, it is suggested that a variety of cells interact and influence each other, among them are fibroblasts, polymorphonuclear neutrophils (PMN), MΦs, and myofibroblasts (Reinke and Sorg 2012). In the first place, the inflammatory stimulus activates the complement cascade, which causes an increase of C3b and C5, leukotrienes, and PG, responsible for the recruitment of PMN into the uterus (Canisso et al. 2020). When

these cells arrive at the site of injury, they phagocytize bacteria and cell debris, degranulate, releasing ROS, hydrolytic enzymes and other antimicrobial proteins/peptides, chemokines, cytokines, and lipid mediators (Sheshachalam et al. 2014). Among the products released by PMNs and MΦs, pro-inflammatory cytokines are critical to the development of the disorder, such as interleukin (IL)-1β, interferon (IFN), IL-8, IL-6, and tumor necrosis factor (TNF)-α, since they not only recruit more inflammatory cells (monocytes, MΦs, lymphocytes) but also stimulate the release of profibrotic cytokines, for example, TGF-β1, IL-10, and monocyte chemoattractant protein (MCP)-1, and transcription factor nuclear factor kappa B (NF-kB). In addition, TGF-β1 stimulates the differentiation of tissue fibroblasts into myofibroblasts (Szóstek-Mioduchowska et al. 2019b).

Neutrophils, the most abundant leukocytes in the blood, are triggered by pathogens and destroy them by phagocytosis, degranulation, and release of NETs (Borregaard 2014; Scapini and Cassatella 2014). NETs are complexes of chromatin filaments and antimicrobial molecules, like histones and proteases, and their purpose is to impair the dissemination of bacteria or other pathogenic agents (Ravindran et al. 2019). However, after they complete their purpose, if neutrophils do not undergo apoptosis and/or NETs are not eliminated by MΦs, there is an increased contribution to the perpetuation of the lesion due to continuous NETs influence (Martin et al. 2003). The NET removal is managed by the action of DNAse I, which first breaks NETs into smaller fragments, and by MΦs, responsible for phagocytizing the fragments (Ravindran et al. 2019). Uncontrolled NETs formation has been linked to the development of several pathological conditions (Martin et al. 2003), like non-infectious diseases, for example in human rheumatoid arthritis (Wang et al. 2018), diabetes mellitus (Fadini et al. 2016), and cancer (Houghton et al. 2010). In 2014, Rebordão et al. associated NETs components, such as elastase, myeloperoxidase, and cathepsin G with the increased *in vitro* production of COL1 in equine endometrial explants (Rebordão et al. 2014), previously referred to as the main collage type produced during endometrosis. In addition, a study in human patients with lung fibrosis showed that neutrophils stimulate fibroblasts activation and differentiation (Chrysanthopoulou, et al. 2014), meaning that these cells can also indirectly promote fibrogenesis by stimulating the most important cells in the synthesis of ECM, myofibroblasts (Tomasek et al. 2002).

Myofibroblasts and fibroblasts are crucial to physiological tissue homeostasis, but their activation can also be involved in pathological processes (Hinz et al. 2007). Fibroblasts are stromal cells that provide mechanical support and fill wounds or damaged tissues (Atamas 2002). These cells interact with others resorting either to cytokines or to cell surface proteins, are affected by systemic stimuli (i.e. ischemia, particles, serotonin), and participate in ECM remodeling (Atamas 2002). It was thought that myofibroblasts differentiated exclusively from fibroblasts when stimulated by TGF-β1. However, other studies have proved that

myofibroblasts can originate from other cell lines, for example, pericytes, adipocytes, and epithelial and mesenchymal cells (reviewed by Zent and Guo 2018). Myofibroblasts, also called “activated fibroblasts”, are modified fibroblasts with *de novo* α-smooth-muscle actin (α-SMA) expression, a smooth muscle protein that forms contractile fibers. This feature gives the cells the ability to contract, promoting wound contraction (Zent and Guo 2018).

Additionally, myofibroblasts synthesize ECM components, such as COL I, FN, and matrix metalloproteinase (MMPs) (Darby et al., 1990; Skalli et al., 1986), promoting lesion filling. Therefore, in the early stage of the wound repair process, these activated fibroblasts have a protective role by promoting wound closure by cell contraction feature and ECM component synthesis at the site of injury. However, when their action fails to cease and becomes prolonged it results in the progressive establishment of a fibrotic disease (Jun and Lau 2018) (**Figure 2**). The mechanism by which myofibroblasts' action becomes persistent remains unclear, thus further investigation is needed to be fully understood.

The protein TGF-β1 not only is responsible for myofibroblast differentiation, but also increases fibroblast ECM components secretion, and stimulates fibroblast proliferation as was determine in mare endometrial fibroblasts (Szóstek-Mioduchowska et al. 2019b). In endometrosis, the endometrial presence of α-SMA positive cells is almost exclusively restricted to the fibrotic loci and dilated cystic glands (Walter et al. 2001; Hoffmann et al. 2009a). Additionally, Szóstek-Mioduchowska and colleagues in 2019 associated the presence of myofibroblasts with the severity of equine endometrosis by showing that α-SMA expression is up-regulated in the most severe stage of the condition compared to the initial stage. The lack of information about myofibroblasts activity inhibitors that could be a potential control factor for fibrosis seems to be a limitation to the treatment of endometrosis.

In physiological situations, monocytes are recruited to the tissues to reestablish the steady state of resident MΦs and in inflammatory scenarios to differentiate into MΦs or dendritic cells (Gordon and Taylor 2005). Macrophages are present in every tissue and participate in the innate and humoral immune response by phagocytizing pathogens and presenting the antigen to active T cells (Jensen et al. 2012). When a tissue is injured, the tissue-resident MΦ population is complimented by many monocytes that are recruited from the blood via chemokine gradients and various adhesion molecules, often exceeding the tissue-resident population of MΦs (Galli et al. 2011). In response to growth factors and cytokines released in the local tissue microenvironment, the recruited and resident MΦ populations proliferate and suffer significant phenotypic and functional changes (Jenkins et al. 2011; Jenkins et al. 2013).

There are two known MΦ sub-populations or phenotypes: the “classically” activated, pro-inflammatory, or type 1 (Mϕ1), and the “alternatively” activated, reparative, or type 2 (Mϕ2) (Landén and Li 2016). It is thought that the severity of fibrosis might be connected to the predominant MΦ phenotype and to the persistence of inflammatory insult (Wynn and Ramalingam 2012; Cao et al. 2014). MΦs are also responsible for producing many MMPs, such as MMP-1, -7, -8, -9, and -12, and their suppressors, tissue inhibitors of MMPs (TIMPs). Recently, it was suggested that the ratio and the action-activated MΦs type 1 (pro-inflammatory) and MΦs type 2 (reparative) play an important role in processes related to the development of fibrosis (Galli et al. 2011; Wynn and Vannella 2016). For example, in the kidneys, the dominant action of MΦ2 could have a pro-fibrotic effect through the secretion of pro-fibrotic factors TGF-β1 and Galactin-3, leading subsequently to renal fibrosis (Vernon et al. 2010). Furthermore, given that MΦs can assume intermediate phenotypes, and continuously develop and change, the resulting populations are heterogeneous and perform a variety of physiological roles. However, remains unknown the mechanism by which MΦs phenotypes are defined (Ross et al. 2021).

1.1.3. Extracellular matrix components

Extracellular matrix constitution mainly includes fibers, among them collagen, fibronectin, elastin, proteoglycans, glycoproteins, and polysaccharides, such as hyaluronic acid (HA). Collagen is the most abundant protein in ECM because provides structural support to the cells (Cui et al. 2017). In healthy tissue, homeostasis is maintained by the existing balance between the degradation and production of ECM components, especially collagen. There are 28 known types of collagens, characterized by having three amino acid chains, which are divided into 4 groups depending on their structure, distribution, function, and chemical composition (Ricard-Blum 2011a). Whereas collagen 3 (COL3), typically with a small diameter, is the predominant type in reticular fibers and it forms a net in organs under constant physiological changes in size and volume, as it happens in the uterus during the estrous cycle, collagen 1 fibers are thicker and stronger, which provide a stronger resistance to the tissue (Junqueira and Carneiro 2013a). Thus, in the healthy equine endometrium, COL3 is the predominant type, but during endometrosis, COL3 is gradually replaced by COL1, leading to endometrial fibrotic changes (Masseno 2012). The mechanisms involved in normal tissue composition maintenance are susceptible to change with the influence of cytokines, growth factors, MMPs, and other components including *lysyl oxidase-like 2* (LOXL2), because of its influence on extracellular matrix remodeling and stability (Smith-Mungo and Kagan 1998).

Matrix metalloproteinases are calcium- and zinc-dependent proteases crucial to physiological processes, such as tissue growth and regeneration, by degrading and removing

ECM from the tissue (Vu and Werb 2000; Nagase et al. 2006). This family of endopeptidases comprises 25 members that can be secreted or membrane-bound enzymes, and function as crucial molecules in the maintenance of ECM and in tissue repair (Giannandrea and Parks 2014). Although MMPs have been mostly related to ECM turnover by degrading its components, these proteins also mediate immunologic processes, including cell migration, leukocyte activation, antimicrobial defense, and chemokine processing (Gill and Parks 2008; Manicone and McGuire 2008). The spectrum of functions of the different MMPs is wide, meaning that MMPs can function in a way that does not overlap with the role of other MMPs (Gill et al. 2010). The expression of extracellular matrix components is influenced by the action of immune cells (neutrophils, eosinophils, lymphocytes, MΦs, mast cells), especially collagens. In turn, these cells, resorting to pro-fibrotic cytokines, influence the expression of MMPs and their inhibitors (TIMPs) in mares endometrium (Woodward and Troedsson 2015; Szóstek-Mioduchowska et al. 2019a; Szóstek-Mioduchowska et al. 2019b). Most of these metalloproteinases are secreted as pro-enzymes and then activated by other mediators (i.e. MMPs, plasmin, IL-1β, and TNF-α) (Visse and Nagase 2003; Walter et al. 2005). The different MMPs are divided into groups with similar functions. Among these groups are collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), and stromelysins (MMP-3 and -10) (Visse and Nagase 2003).

MMP-2, also known as gelatinase A, and MMP-9, or gelatinase B, are the most known among these groups of proteins given their ability to degrade type IV collagen in the basement membrane and in the collagen fibrillar (Nagase et al. 2006; Aresu et al. 2011). In addition, MMP-2 was proven to have antifibrotic properties in the liver (Onozuka et al. 2011; Radbill et al. 2011), and kidney (Takamiya et al. 2013). On the other hand, MMP-3, also known as stromelysin 1, not only acts on the degradation of ECM components, but also activates many proMMPs, among them proMMP-1, which means that MMP-3 is essential to the activation of the active form of MMP-1 (Visse and Nagase 2003).

In humans, MMPs participate in several physiological processes, including angiogenesis, ovulation, embryogenesis, endometrial cycle changes, and extracellular matrix remodeling (Nagase et al. 2006). The different types of MMPs and TIMPs, control the COL deposition and ECM remodeling, whilst, according to human studies, cytokines modulate MMP activity (Singer et al. 1999). In addition, Szóstek-Mioduchowska and colleagues showed that TGF-β1 regulates endometrial ECM remodeling by modulating MMPs and TIMPs activity on fibroblasts and epithelial cells (Szóstek-Mioduchowska et al. 2020).

On the other hand, dysregulated expression of MMPs has already been associated with many pathological processes, such as fibrosis, weakening of ECM, or tissue destruction (Di Nezza et al. 2002; Amălinei et al. 2010). For example, TGF-β1 and platelet-derived growth factor (PDGF) play a pro-fibrotic role and activate fibroblasts, which secondarily control ECM

turnover by regulating the balance of MMPs and their tissue inhibitors (Wynn and Barron 2010). For this reason, regulatory mechanisms are necessary to keep homeostasis in ECM, and this is achieved mostly by the action of TIMPs (Gomez et al. 1997), but recently have been reported several other proteins capable of inhibiting MMPs, as it is the case of β-amyloid inhibiting MMP-2 (Higashi and Miyazaki 2003).

Matrix metalloproteinases and TIMPs can be present in various ratios according to the individual health condition. The influence of MMPs in different pathological processes including osteoarthritis and laminitis, it was already demonstrated in various studies and reviewed by Clutterbuck et al. in 2010. Compared to healthy human cartilage, where the ECM turnover is well established by the predominance of TIMPs compared to MMPs, in equine osteoarthritis, joint cartilage has a reversed ratio of MMPs and TIMPs, being MMPs more abundant than TIMPs (Dean et al. 1989), resulting in excessive ECM degradation. In another study, led by Walter in 2005, the importance of MMP-2 was the subject of a study comparing healthy mares with mares diagnosed with endometrosis, in which endometrial biopsy samples were analyzed by immunohistochemistry technique. The results showed a significant abundance of MMP-2 in the mares diagnosed with endometrosis. These mechanisms need further research in order to be better described, and in the future, the management of MMP, through the modulation of their expression, in pathological processes might be a useful tool to find an efficient treatment for the conditions/diseases in which these proteins are involved, for example, endometrosis (Clutterbuck et al. 2010).

In addition, it is important to mention that lysyl oxidase (LOX), as a family of proteins involved in maintaining ECM integrity and tensile strength by establishing the covalent crosslinking between other molecules, for example, collagen and elastin (Trackman 2016), and in a lower scale, management of gene transcription and cell signaling modulation (Barker et al. 2012). This family includes the proteins: LOX, LOXL1, LOXL2, LOXL3, and LOXL4, and some of them have already been associated with several diseases. In human systemic sclerosis, the levels of LOX in the skin and in the blood were shown to be increased (Chanoki et al. 2006; Rimar et al. 2014). Cheng and colleagues demonstrated that LOX expression was higher in mice with bleomycin-induced pulmonary fibrosis, while the inhibition of LOX expression or activity resulted in a reduction of collagen deposition and alleviated the fibrosis (Cheng et al. 2014). Furthermore, in 2016 Liu and colleagues reported that, in patients with liver fibrosis, the inhibition of LOX contributed to a quicker reversal of the ECM deposition in the tissue (Liu et al. 2016). Although the mechanism by which LOX affect on ECM deposition remains unclear and needs further investigation to be better understood, this family of proteins has already been suggested, as a novel therapeutic target in fibroproliferative disorders (Nguyen et al. 2021).

1.1.4. Cytokines

Cytokines, a heterogeneous group of polypeptide mediators produced almost exclusively by the immune cells, are involved in the inflammatory process by mediating the recruitment and differentiation of the immune system cells and other cells (Holtmann and Resch 1995). These proteins bind to specific receptors in the target cells and generate a biological response, mediating cellular activity. In the pathogenesis of endometrosis, it is important to mention some cytokines due to their fundamental role in the process, among them there is TGF-β1, IL-1β, IL-6, TNFα, IL-10, NF-kB, and MCP-1. The two main cytokine functions relevant to endometrosis is the pro-inflammatory, e.g. IL-1β, IFN, IL-8, IL-6, TNFα, and the pro-fibrotic, such as TGF-β1, IL-10 (Katila and Ferreira-Dias 2022).

The pro-fibrotic cytokine, TGF-β1, a mediator produced by Mϕ, neutrophils, platelets, and fibroblasts, has been referred to as a fibrotic stimulant since it regulates the differentiation and activity of myofibroblasts (Desmoulibre et al. 1993). The stimulant influence of TGF-β1 in the activation of resident fibroblasts into differentiated myofibroblasts was shown by an *in vitro* study in which, after endometrial treatment with TGF-β1, the expression of ECM components, such as COL1, COL3, and FN, and alpha-smooth muscle actin (α-SMA) were upregulated (Szóstek-Mioduchowska et al., 2020; Szóstek-Mioduchowska et al., 2019). Additionally, TGF-β1 was proven to affect MMPs and TIMPs expression in equine fibroblasts and epithelial cells.

Previous studies showed that IL-1β inhibits collagen production in human dermal fibroblasts (Bhatnagar et al. 1986; Heino and Heinonen 1990). According to Szóstek-Mioduchowska and colleagues, IL-1β and IL-6 directly affect ECM, MMP, and TIMP expression in mares' endometria, and the effect depends on the Category the endometria is classified in (Szóstek-Mioduchowska et al. 2019a). The results of their study in 2019 showed that IL-1β led to an increase in the expression of MMP2 and TIMPs and that IL-6 influence caused an up-regulation of MMP2 and MMP3, but a down-regulation in MMP9 (Szóstek-Mioduchowska et al. 2019a). The described changes in MMPs and TIMPs expression suggest a major influence of these two cytokines in ECM turnover, being possible that the MMPs up-regulation occurs as a cellular response to excessive ECM production caused by the cytokines IL-1β and IL-6 and may be responsible for intracellular mechanisms that alter endometrial cells and possibly related to fibrogenesis (Szóstek-Mioduchowska et al. 2019a).

A variety of cytokines are involved in the development of fibrotic disorders by recruiting other cells, modulating their activity, and stimulating the production of more mediators. However, there is a lack of information about the role of many of them in the progression of endometrosis. Nevertheless, a recent study, which focused on transcriptomic profiling of mare endometrium showed a link of fibrosis to the inflammatory response, which can be a result of cellular infiltration by MΦs, Th1 and Th2 activation pathways, IL-17 signaling pathways, and fMLP

signaling in neutrophils (Szóstek-Mioduchowska et al. 2023). Moreover, in this study, changes in the expression of genes that were associated with "interleukin (IL)-12 signaling and production in MΦs" at different stages of endometrosis, identified IL-12 as another potential player in the development of endometrial fibrosis (Szóstek-Mioduchowska et al. 2023).

2. Interleukin 12

2.1. Interleukin 12 family

Interleukin 12 cytokine family is characterized by its heterodimeric cytokines, each of them composed of an alpha-chain and a beta-chain, formed by monomers (Vignali et al. 2008), and it includes the cytokines IL-12, IL-23, IL-27, and IL-35 (Collison et al. 2007). The IL-12 family members are primarily produced by dendritic cells through the Toll-like receptor (TLR) ligands (Goriely and Goldman 2008) and by MΦs (Vignali and Kuchroo 2012).

This family has a particularity called chain-sharing, given that the same chain can be part of different interleukins (Collison and Vignali 2008; Jones and Vignali 2011). For example, the subunit p40 is the β-chain of both IL-12 and IL-23, and p35 is present in IL-12 and IL-35, as their α-chain. This is also the case with the receptors: IL-12 shares its receptor subunits with IL-23 and IL-35 receptors (Collison and Vignali 2008; Delgoffe et al. 2011). The members of the IL-12 family bind to five pairs of receptor chains, IL-12Rβ1, IL-12Rβ2, IL-23R, gp130, and WSX-1, mediating biological activities through Janus kinases (JAKs) and JAK-STAT, especially STAT4, pathways (Trinchieri et al. 2003). Additionally, recent studies have already stated that each component can be secreted alone or in combination with others and even act autonomously as a monomer or as a homodimer (Stumhofer et al. 2010).

Among the elements of the IL-12 family, there is a similarity in their structure, but their biological activities are very different. While IL-12 has a pro-inflammatory function by enhancing T helper 1 differentiation (Hsieh et al. 1993a), IL-23 has a pro-stimulatory activity (Langrish et al. 2004) through the promotion of Th17 responses and activation of memory T cells (Zelante et al. 2007). On the other hand, IL-27 and IL-35 were shown to be immunoregulatory cytokines (Stumhofer and Hunter 2008; Vignali et al. 2008), respectively through inhibition of IL-2 signaling, Th17 cells antagonization and regulatory T cells activity stimulation (Tregs) (Villarino et al. 2003), and, through the stimulation of Tregs and regulatory B cells and the suppression of T cell responses (Wang et al. 2014). However, the function of each element of this family remains ambigous, for example, a study proved that IL-27 can also exhibit pro-inflammatory influence by enhancing Th1 cell differentiation activity (Pflanz et al. 2002), and another one that the development of a Th1 or Th17 subset is influenced by the balance in IL-12 and IL-23 production by dendritic cells (Goriely and Goldman 2008).

2.2. Interleukin 12: function, structure, and receptors

Interleukin 12 is formed by subunits p35 (α-chain) and p40 (β-chain) which are covalently linked (Trinchieri et al. 2003) (Figure 4), and is primarily produced by monocytes, MΦs, dendritic cells, and B cells (Trinchieri et al. 2003; Thompson and Orr 2018). It binds to the receptors IL-12Rβ1 and IL-12Rβ2, and, in turn, Jak kinases - Janus kinases 2 (JAK2) and Tyrosine kinase 2 (TYK2) - are activated, leading to the receptor phosphorylation, which allows it to bind STAT4 proteins (Bacon et al. 1995) (Fig.1).

Interleukin 12 overall function bridges the early innate immune response and the later antigen-specific adaptive immunity (Hamza et al. 2010), having typically a proinflammatory function. This cytokine amplifies the inflammatory signals by stimulating the natural killer cells (NK) and T cells to produce interferon-gamma (IFN-γ), it also enhances NK cell's cytotoxicity by acting as a chemotactic (Allavena et al. 1994) but its principal role is the promotion of the naïve T cells differentiation into effectors Th1 cells, enhancing the cellular immune response (Gately et al. 1998). In turn, Th1 cells produce IL-2 and IFN-γ, both mediators capable of stimulating the proliferation and activation of CD8+ cytolytic cells, NK cells, and MΦs. As positive feedback, the produced IFN-γ stimulates additional APCs to produce more IL-12 (Lederer et al. 1996), which is crucial to avoiding the deficiency of IL-12 to be established due to the presence of certain pathogens (Reiner et al. 1994; Müller et al. 2001). Thus, a great number of IL-12 effects have been associated with IFN-γ secretion, making this cytokine the main agent in IL-12 biological effects (Coughlin et al. 1998). Being interleukin 12 a proinflammatory cytokine involved in the differentiation of naive T cells into Th1 cells (Hsieh et al. 1993b), it is possibly related to the pathogenesis of fibrosis, which is the result of a chronic inflammatory condition. IL-12 inhibits the establishment of Th2-type cytokine responses and adaptive immunity, especially responses involving immunoglobulins G and E (Gately et al. 1994). However, under some experimental conditions, IL-12 was shown to be also capable of potentiating Th2 responses (Schmitt et al. 1994; Wynn et al. 1995).

On the contrary, some published studies have been reporting evidence of IL-12 anti-fibrotic and antitumoral properties, respectively in human bleomycin-induced pulmonary fibrosis (Keane et al. 2001) and in murine mammary carcinoma (Coughlin et al. 1998). In this first study, Keane and colleagues not only showed that intraperitoneal IL-12 administration reduced the fibrosis level in the mice lungs, but also that this decrease is caused by the subsequent higher intrapulmonary IFN-γ levels. Furthermore, the antitumor response of IL-12 was also associated with IFN-γ production. This cytokine stimulates numerous mechanisms including slowing cellular proliferation (Boehm et al. 1997), induction of nitric oxide (NO) production (Xie

et al. 1993), and angiogenesis inhibition (Voest et al. 1995), that are thought to improve tumor regression (Coughlin et al. 1998).

The induction of IFN-γ by IL-12 is exacerbated by a strong synergistic effect with other IFN-gamma inducers, particularly IL-2, phorbol diesters (Yang et al.1999) and IL-18 (Coughlin et al. 1998). Besides having a stimulatory effect on MΦs increasing their ability to kill a variety of intracellular and extracellular bacteria (Murray 1990), IFN-γ also potentiates NO and IDO influence on antigen-presenting cells, decreasing the number of effector T cells, and suppressing IL-17 mediated inflammatory events (Goriely and Goldman 2007).

The IL-12 expression is up-regulated by the transcription factors interferon regulatory factors (IRFs), which are included eight members (1 to 8). Among the identified members the IRFs 1, 2, 5, 7, and 8, are the ones involved in the p35 and p40 transcription (Goriely and Goldman 2008). Moreover, deficiency in IL-12 or IL-12R genes was proven to cause an increase in infectious disease susceptibility (Altare et al. 1998; Wu et al. 2000). On the other hand, to prevent overexpression of IL-12, a negative regulation is needed. For example, IL-10, produced by Th2, is a critical inhibitor of IL-12 production (D'Andrea et al. 1993; Aste-Amezaga et al. 1998) through the decrease of NFkB and AP-1 activation (Rahim et al. 2005) and the association of IL-12p40 promoter with RNA polymerase (Zhou et al. 2004). IL-12 is also inhibited by TGF-β (Branton and Kopp 1999), IL-11, IL-13, INF-α/β (Cousens et al. 1997; McRae et al. 2000), measles receptor CD46 and cholera toxin (Karp et al. 1996). The sequence of the p40 chain has homology to the extracellular domain of the IL-6 receptor (IL-6R) α-chain and the ciliary neurotrophic factor (Gearing and Cosman 1991).

There is a lack of information about the role of IL-12 in the genesis and progression of fibrosis in animal tissues. However, a human study published in 2006 showed that the level of IL-12 in blood serum increased in idiopathic pulmonary fibrosis (IPF) patients as compared to the control group (Tsoutsou and Koukourakis 2006). Another example is an experiment using myocardial cells, in which the decrease in IL-12p35 showed higher levels of cardiac mitochondrial ROS and calcium ion overload, leading subsequently to worsened cardiac dysfunction, and increased cardiac fibrosis in 25-month-old aging mice (Ye et al., 2020). Given the particularities already described, and studies described in mice, it is hypothesized that a synthetic analog of recombinant interleukin 12 (rIL-12) may contribute as a therapeutic tool to some tumors and infectious diseases (Brunda et al. 1993).

In conclusion, the role of IL-12 in the pathogenesis of fibrosis and its direct effect on fibroblasts not being well established yet and the chain-sharing properties of the IL-12 family are the major limitations in the understanding this cytokine's role in fibrotic conditions, such as endometrosis. Thus, the purpose of this study is to determine the role of IL-12 in the processes associated with the development of endometrosis in mares, and we hypothesize that this cytokine takes part in the pathogenesis of this condition by acting on the expression of ECM components, MMP, myofibroblast differentiation and on fibroblast proliferation.

3. Aims and Objectives

Interleukin 12 is a proinflammatory cytokine, and fibrosis is the end result of chronic inflammatory reactions. The level of IL-12 in blood serum increased in idiopathic pulmonary fibrosis (IPF) patients, as compared to the control group (Tsoutsou et al. 2006). The role of IL-12 in the pathogenesis of fibrosis and its direct effect on fibroblast is not established yet. However, the transcriptomic analysis of mare endometrial tissue revealed alterations in gene expression linked to "interleukin (IL)-12 signaling and MΦ" throughout various stages of endometrial pathology, proposing that IL-12 might also participate in the pathogenesis of endometrial fibrosis (Szóstek-Mioduchowska et al. 2023). Thus, we put forward the hypothesis that IL-12 takes part in the processes associated with the development of endometrosis in mare. Thus, the three main aims of this study were:

Aim 1. The determination of the transcription of endometrial IL-12 subunits and their receptor in mare at different stages of endometrosis.

Aim 2. The effect of IL-12 on ECM remodeling, and myofibroblast differentiation.

Aim 3. The effect of IL-12 on endometrial fibroblast properties.

III - Experimental work

Material and methods

Uteri used in these experiments (n=89 in total; n=77 in study 1, n=6 in study 2 and n= 6 in study 3) were collected *post-mortem* from cyclic mares, at a local abattoir (Rawicz, Poland), between April and June. Mares used in the experiment were normally cycling cold-blooded, weighing 500 ± 100 kg, and the age range between 2 and 20 years old. Ethical approval was not required for the animal study under local legislation and institutional requirements, since the animals were slaughtered to obtain meat as part of routine breeding as slaughter animals. Based on an official government veterinary inspection of the mares and on health history, they were considered clinically healthy. The uteri collection was done within 5 minutes *post-mortem*. The phases of the estrous cycle were identified based on the macroscopic evaluation of ovaries: mares whose ovaria contained at least one follicle >35mm in diameter and the absence of an active corpus luteum (CL) were assigned to the follicular phase; mares whose ovaries presented a well-developed CL, associated with follicles with 15-20 mm in diameter, were considered to be in the mid-luteal phase (Szóstek et al. 2013).

The collected endometrial tissue, from the active site of the ovary, was divided into two parts, one was put in 4% paraformaldehyde for histological analysis after hematoxylin-eosin staining and the other part was kept in *RNAlater* (#AM7021; Invitrogen, Burlington, ON, Canada) for gene expression. The horns of the uteri (n=12) were placed in cold sterile physiological saline with 0.01% of antibiotic antimycotic (AA) solution (A5955, Sigma-Aldrich) and kept on ice during the transportation to the lab. After hematoxylin-eosin staining, endometria were retrospectively assigned to categories I, IIA, IIB, or III, based on Kenney and Doig classification (Kenney and Doig 1986).

1.1. Isolation and culture of fibroblasts

The fibroblasts were isolated from mare endometrium category I, cultured as described recently (Szóstek-Mioduchowska et al. 2019), with some modifications. First, the uterine lumen was flushed with a 50 mL solution of sterile Hanks' balanced salts (HBSS; H1387; Sigma-Aldrich) containing 0.01% of AA solution, repeating the process two times. Then, in order to expose the endometrial surface, the uterine horn was cut open with scissors, and endometrial strips were excised from the myometrium layer with a scalpel and cut into very small pieces (1-3mm^3). The tissue was digested in 100 ml of sterile HBSS containing 0.05%, (w/v) collagenase I (C2674, Sigma-Aldrich), 0.005% (w/v) DNAse I (11284932001; Roche), 0.01% AA, and 0.1% (w/v) bovine serum albumin (BSA; A9418, Sigma-Aldrich), for 45 minutes at 37ºC, followed by filtration through the 100 µm, and 70 µm strainers to remove the undigested tissue fragments, and centrifugation at 200 x g for 10 min at 4ºC. The resulting cell pellet was

gently mixed with 1 ml of Red Blood Cell Lysing Buffer Hybri-Max™ (R7757; Sigma-Aldrich), to lyse red blood cells, then washed two times with HBSS supplemented with antibiotics and 0.1% (w/v) BSA resorting to centrifugation (4ºC, 200 x g, 10 min). The final pellet of endometrial cells was resuspended in FBM™ Basal Medium (CC-3131, LONZA) supplemented with FGM™-2 SingleQuots™ supplements. The cells were counted using a Burker chamber. The viability of fibroblasts was determined using the trypan blue exclusion test and it was higher than 95%.

The cells were seeded separately at a density of 5×10^5 viable cells/ml and incubated at the conditions of 38.0 °C and 5% CO_2 humidified atmosphere. The medium was changed 3h after seeding to purify the fibroblast population, since after this period fibroblasts had already attached selectively to the bottom surface of the well, which means that when removing the medium, we are eliminating all cell types that are not fibroblasts, for example, epithelial and endothelial cells. The medium was changed every second day until the cells reached the anticipated confluence. The microscopic view of the fibroblasts in the *in vitro* culture is represented in Figure 5. The fibroblast homogeneity was confirmed using immunofluorescent staining for vimentin based on the protocol described recently [(Szóstek-Mioduchowska et al. 2021); data not shown]. The purity of fibroblast after isolation was around 96%.

Upon reaching 90% confluency, the cells were trypsinized and cryopreserved using the method previously described by Szóstek et al. in 2012. The fibroblasts were rinsed twice with sterile PBS, followed by the addition of 0.025% trypsin and EDTA solution (Sigma Aldrich, Madison, USA). After a period of 0.5 to 1 minute, the enzyme solution was removed, and the fibroblasts were incubated at 38°C for 1 to 5 minutes. To inhibit trypsin activity, DMEM supplemented with 10% FCS and 1% antibiotics and antimycotic was added. Fibroblasts were washed through centrifugation (7 minutes at 100g) before cryopreservation. Following trypsinization, the fibroblasts were centrifuged and suspended in 1 mL of cryoprotectant comprising 7% DMSO (Sigma Aldrich, Madison, USA), 43% FCS, and 50% DMEM. The cells were suspended at a density of $1x10^6$ viable cells/mL and the mixture was carefully transferred to a cryotube before being gradually frozen to a temperature of -80°C.

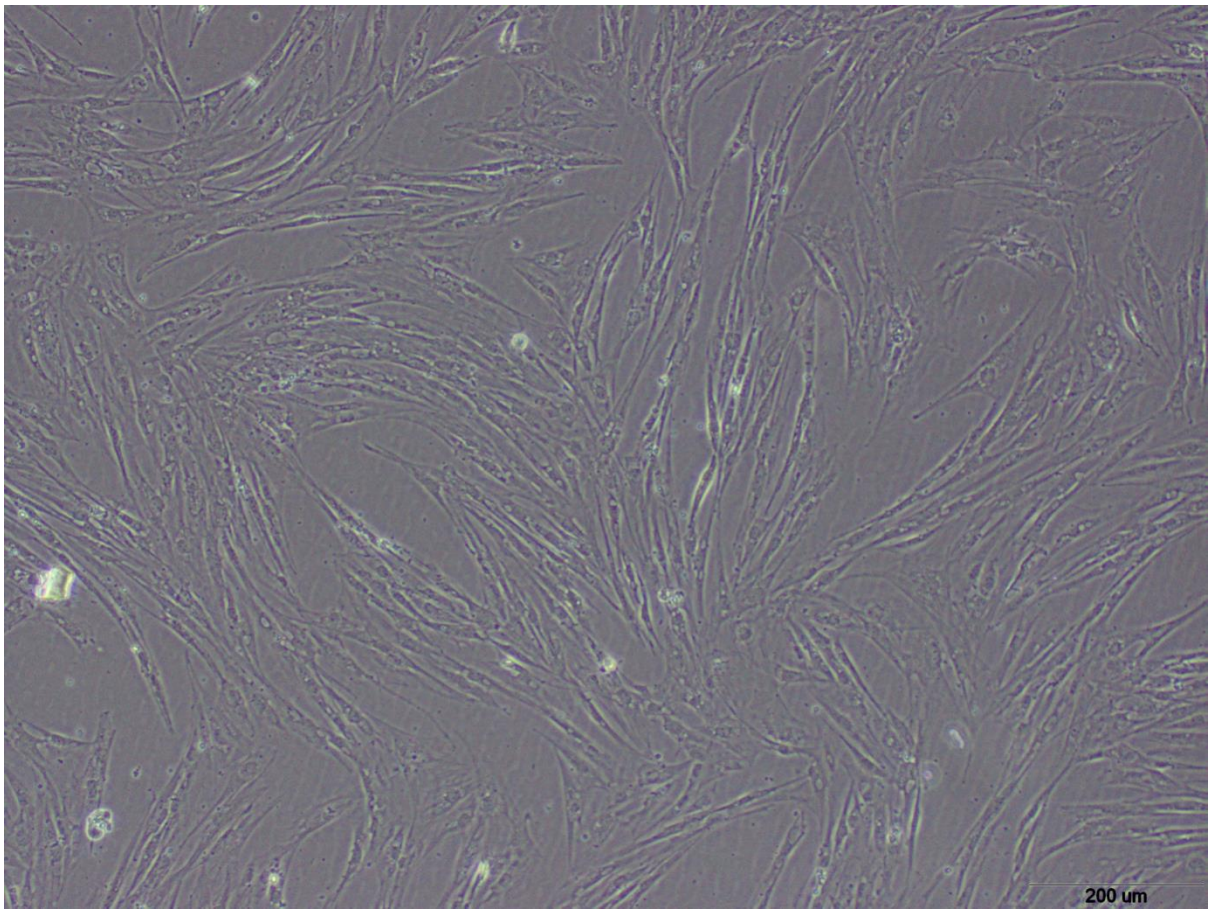

Figure 1- Morphology of fibroblasts culture in vitro.

1.1.1. The determination of endometrial IL-12 subunits and their receptor mRNA expression at different stages of endometrosis in mare

The endometrial samples were obtained from mares in the mid-luteal phase (n=38) and follicular phase (n=39) of the estrous cycle. The endometria in the follicular phase were classified according to Kenney and Doig (1986): 10 from category I, 10 from category IIA, 9 as category IIB, and 10 as category III. In the mid-luteal phase, 10 were classified as category I, 10 as category IIA, 8 as category IIB, and 9 as category III. The mRNA transcription of IL-12 subunits (IL-12α or IL-12p35; IL-12β or IL-12p40) and their receptors (IL-12Rβ1, IL-12Rβ2) was measured using qPCR. For this experiment, endometrial tissue stored at -80ºC were used. mRNA extraction protocol (described in 1.2.) was carried out followed by reverse transcription and finally qPCR.

1.1.2. The effect of IL-12 on ECM remodeling, and myofibroblast differentiation

Fibroblasts (n=6, derived from category I endometria, in MLP) previously kept at -80ºC, were thawed and then seeded in a T75cm^2 tissue culture flask with FBM™ Basal Medium (CC-3131; Lonza) supplemented with FGM™-2 SingleQuotsTM (CC-4126, Lonza) supplements and ascorbic acid (100ng/ml; A4544; Sigma-Aldrich). When the culture reached 90% of confluence, the cells were trypsinized to the well to detach fibroblasts from the bottom surface as described (Szóstek et al. 2012). Then, the cells were resuspended and seeded at a density of 5×10^5 viable cells/ml on 24-well plates. When the seeded fibroblasts reached the desired 60% (for 96h IL-12 treatment) or 80% (for 48h IL-12 treatment) confluence, the culture medium was replaced with the starvation medium: Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/Ham's F-12; D2906; Sigma-Aldrich) supplemented with 0.01% of AA solution, ascorbic acid (100ng/ml) and 0.1% (w/v) BSA, and the cells were incubated at 38ºC in 5% of CO2. After the starvation, cells were treated with IL-12 (10ng/ml; BON OPUS #CM39) for 48h and 96h. The dose of IL-12 was chosen based on a previous study (Miao et al. 2017). After the treatments, the cells were dispersed with 1 mL TRI Reagent and stored at -80ºC for subsequent RNA extraction and qPCR. Using qPCR, the mRNA expression of *COL1A1, COL3A1, FN1, LOXL2, α-SMA, MMP-2, MMP-3*, and *MMP-9*, *TIMP-1* and *-2* was determined.

1.1.3. The effect of IL-12 on endometrial fibroblast proliferation

The effect of IL-12 on the proliferation of equine endometrial fibroblasts was determined by cell proliferation ELISA, using the BrdU (colorimetric) kit (11647229001; Roche) according to the manufacturer's instructions. The assay is based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis in replicating cells. Fibroblasts derived from category I endometria, in MLP (n=6) previously kept at -80ºC, were thawed, cultured (T175 Cell Culture Flask; culture medium: FBMTM basal medium supplemented with FGMTM-2 SingleQuotsTM supplements and ascorbic acid) to 90% confluence and trypsinized (Szóstek et al. 2012). For the target experiment, cells were seeded in 96-well plates (1 × 105 cells/ 100 µL culture medium). After pre-culture, i.e. when 80-85% confluence was reached, the cells were treated with IL-12 (10 ng/ml) for 48 or 96 hours (n=6 for each time point; treatments within specific experiments were performed in triplicate). Two hours before the end of the culture, BrdU labelling solution was added at 10% of the culture medium volume (10 µl). After removal of the labelling medium and drying of the labelled cells, the plates were stored at +4°C for up to one week. The cells were then fixed, incubated with anti-BrdU antibody (Anti-BrdU-POD working solution; 90 min, RT), washed three times with washing solution (PBS) and

incubated with substrate solution for 20 min in the dark. The absorbance of the samples was measured spectrophotometrically (370 nm).

1.2. RNA extraction and cDNA synthesis

For experiment 1, total RNA was extracted using mirVana™ Isolation Kit (AM1560, AM1561) according to the manufacturer's instructions. First, lysis solution and miRNA Homogenate Additive are added to the endometrium samples, followed by the addition of acid phenol: chloroform extract solution to remove the other cellular components leaving, almost exclusively, a semi-pure RNA sample. The result solution was washed 3 times, using ethanol and a glass-fiber filter, and finally eluted in nuclease-free water at 95ºC.

For experiment 2, total RNA was isolated with Total RNA Mini (Cat. Number. 031-100; AandA Biotechnology) according to the manufacturer's instructions. Briefly, the first step included adding fenozol to inactivate endogenous RNases and incubating for 5 min at 50ºC, followed by adding chloroform and next, isopropanol. The final steps include sequences of washing and filtrations using A1 wash solution and minicolumns, ending with nuclease-free water.

For cDNA synthesis, Dnase I (AMPD-1; Sigma-Aldrich) was used according to the manufacturer's directions for eliminating genomic DNA from RNA samples before qPCR. RNA was dissolved in nuclease-free water, then it was added to each sample DNA wipeout buffer containing Dnase I and 10x Reaction buffer in 1:1 proportion (DNase 1 Amplification Grade, AMPD1-1KT, 051M6157, Sigma Aldrich), and incubated at room temperature for 15 min. Next, a stop solution (DNase 1 Amplification Grade, AMPD1-1KT, 051M6157, Sigma Aldrich) was added, and the samples were incubated for 10 min in a thermal cycler at 70ºC. Using a ThermoScript RT-PCR System reverse transcription of the RNA (1μg) was carried out according to the manufacturer's directions (no. 11146-016; Invitrogen). The thermal profile for cDNA synthesis was as follows: 25ºC for 10 min, then 37ºC for 120 min, and 85ºC for 5 min, and the resulting samples were stored at –20ºC until being used later.

1.3. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Real-time PCR was performed on 7900HT Fast Real-Time PCR System using TaqMan Universal Master Mix II (4440049; Applied Biosystems, Foster City, CA, USA) with 384-well plates. All samples were run in duplicates. For measurements of mRNA expression of *COL1A1, COL3A1, Fn1, LOXL2, α-SMA, MMP-2, MMP-3*, and *MMP-9*, *TIMP-1* and *-2* Single Tube TaqMan Gene Expression Assays (Life Technologies Thermo Fisher Scientific) were used. The Category numbers and information about probes are described in Table 2 and 3.

Based on the information given by NormFinder software, the most adequate reference genes were selected, *SDHA* and *HPRT* (Andersen et al. 2004). These reference genes were found to have the most stable expression across the endometrosis categories and in the fibroblast culture, so their gene expression was used to normalize the results.

The reaction mixture for the qPCR assay comprised 5 µL TaqMan Universal PCR Master Mix, 0.5 µL TaqMan probe, 3 µL DNA (10 ng), and 1.5 µL nuclease-free water for a final volume of 10 µL. As a negative control, nuclease-free water instead of template cDNA was used. cDNA amplification was performed under the following conditions: initial denaturation for 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The data were analyzed using the method described previously (Zhao and Fernald 2005). The relative concentration of mRNA (R0) for each target and reference gene *SDHA* and *HPRT* was calculated using the equation $R0 = 1/ (1 + E)^{Ct}$, in which E is the average gene efficiency and Ct is the cycle number at the threshold. The relative gene expression was calculated as $R0_{target\ gene}/R0_{reference\ gene}$ and was expressed in arbitrary units.

Probe	Cat. No
12p35	Ec03468747_m1
12p40	Ec03468777_m1
IL-12Rβ1	Ec07087595_m1
IL-12Rβ12	*Ec07013802_m1*

Table 2: Category numbers of probes used in Experiment 1 (Single Tube TaqMan Gene Expression Assays (Life Technologies Thermo Fisher Scientific));

Probe	Cat. No	Probe	Cat. No
Col1a1	Ec03469676_m1	*α-SMA*	Ec07040967_m1
Col3a1	Ec03469743_m1	*Mmp-2*	Ec03469995_m1
Fn1	Ec03470760_m1	*Mmp-3*	Ec03468676_m1
Loxl2	Ec06966406_m1	*Mmp-9*	Ec03469193_m1
Timp-1	Ec03468772_m1	*Timp-2*	Ec03470558_m1
SDHA	Ec03470487_m1	HPRT	Ec03470217_m1

Table 3: Category numbers of probes used in Experiment 2 (Single Tube TaqMan Gene Expression Assays (Life Technologies Thermo Fisher Scientific)).

1.4. Statistical Analysis

Data values are shown as mean ± SD, and the results were considered significant at $P<0.05$. For each analysis, the Gaussian distribution of results was tested using the D'Agostino and Pearson normality test (GraphPad Software version 9; GraphPad, San Diego, CA). Whenever the assumptions of normal distribution were not met, nonparametric statistical analyses were done. In Experiment 1, the significant differences were determined by a two-way ANOVA followed by Bonferroni multiple comparison test. Differences in gene expression in myometrium in Kenney and Doig's category I, II A, IIB, and III endometria within the mid-luteal and follicular phase of the estrous cycle, and between those groups were assessed. In Experiments 2 and 3, the significant differences were determined by the nonparametric Mann-Whitney U test.

IV – Results

1. The determination of IL-12 subunits and their receptor mRNA transcription in the endometrium at different stages of endometrosis in mare

The presence of different subunits of IL-12 and their receptors were determined in the different Kenney and Doig equine endometria (**Figure 2: A, B, C, D**). Nevertheless, no differences were found for IL12p35, IL12p40, and IL-12Rβ*1* transcripts for either endometrial category during the follicular and luteal phase (**Figure 2: A, B, C**). However, in category IIB endometrium, *IL-12Rβ2* mRNA transcription was up-regulated in the mid-luteal phase (MLP) compared to the follicular phase (FP) of the estrous cycle (**Fig. 2D**; P<0.05).

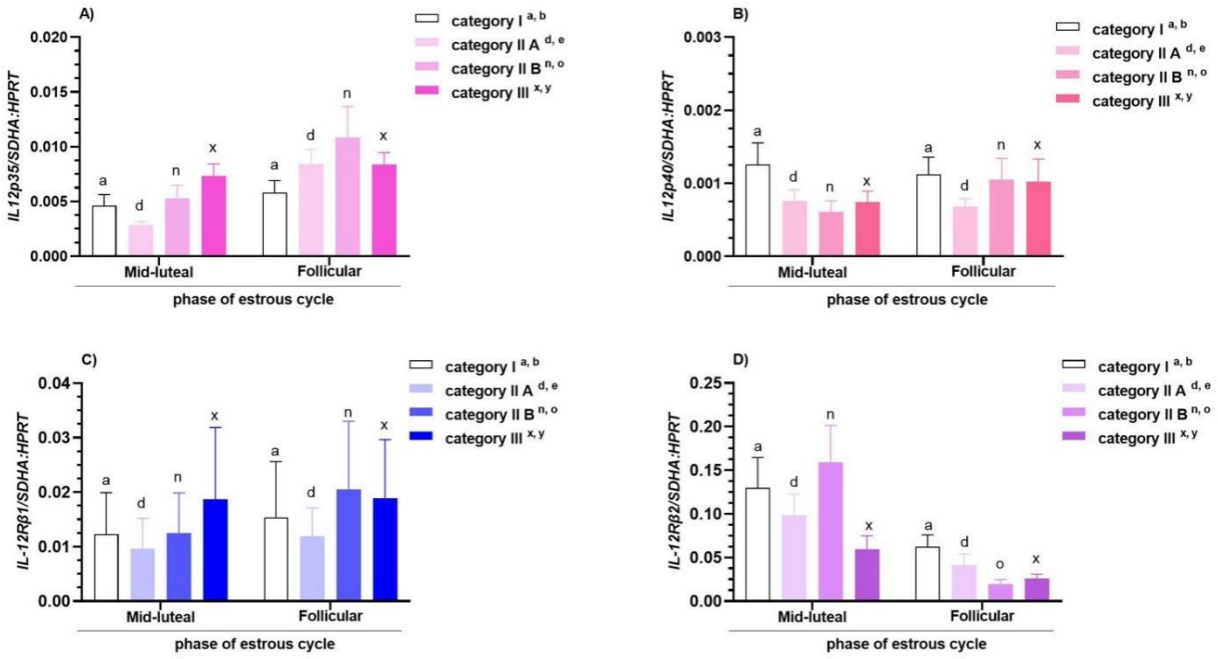

Figure 2: Endometrial mRNA transcription of *IL12p35*, *IL12p40*, *IL12Rβ1, and IL12Rβ2* at different stages of mare endometrosis. **(A)** *IL12p35* **mRNA transcription, (B)** *IL12p40* **mRNA transcription, (C)** *IL12Rβ1* **mRNA transcription, and (D)** *IL12Rβ2* **in the mid-luteal phase (MLP)** and follicular phase (FP) of the estrous cycle **at different stages of** mare endometrosis **(Kenney and Doig's *endometrium* categories I, IIA and IIB and III).** The superscript letters indicate statistical differences between MLP and FF in Kenney and Doig's category I; category IIA; category IIB; and category III endometria.

2. The effect of IL-12 on ECM remodeling, and myofibroblast differentiation

Interleukin 12 at a dose of 10ng/ml increased *COL1A1* and *COL3A1* mRNA transcription after 96h culture (Fig. 3A and 3B; P<0.05 and P<0.01, respectively), and *LOXL2* mRNA transcription after 48h (Fig. 4B; P<0.01;) in endometrial fibroblasts cultured *in vitro*. Additionally, IL-12 at a dose of 10ng/ml up-regulated *α-SMA* (Fig. 5; P<0.05), *MMP*3 (Fig. 6B; P<0.01), and *MMP9* mRNA transcription after 96h in endometrial fibroblasts cultured *in vitro* (Fig. 6C; P<0.01).

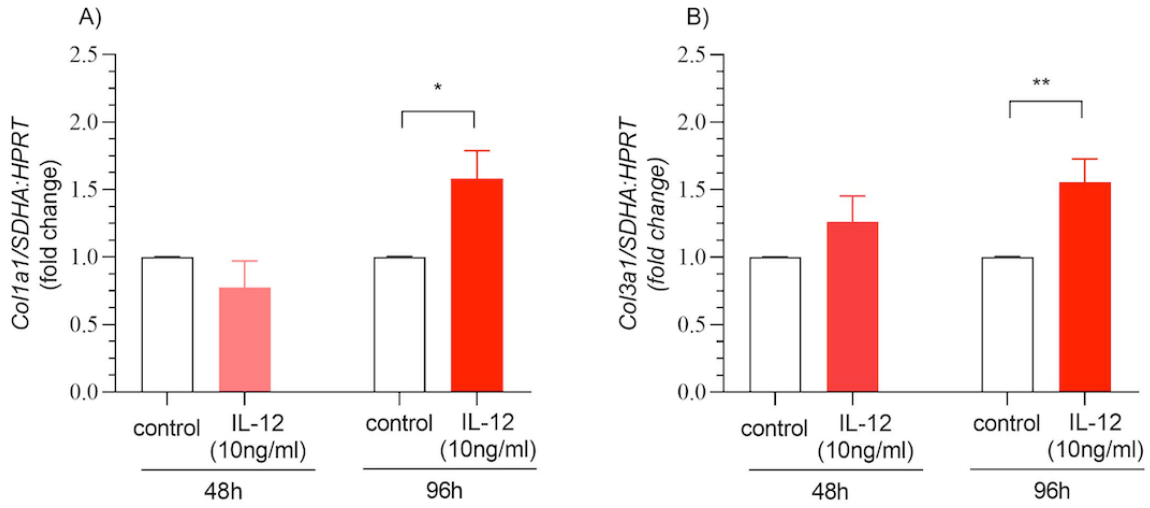

Figure 3: The effect of IL-12 at different treatment times (48 and 96h), on *COL1A1* and *COL3A1* mRNA expression in equine endometrial fibroblasts. (A) ***COL1A1*** mRNA transcription**, (B) *COL3A1*** mRNA transcription in endometrial fibroblasts cultured *in vitro* (n=6) **for 48 and 96h.** Asterisks indicate statistical differences between respective control and treatment groups (*P<0.05; **P<0.01)**.** All values are expressed as a fold change**. *COL1A1* -** Collagen type 1 alpha 1**; *COL3A1* -** Collagen type 3 alpha 1**.**

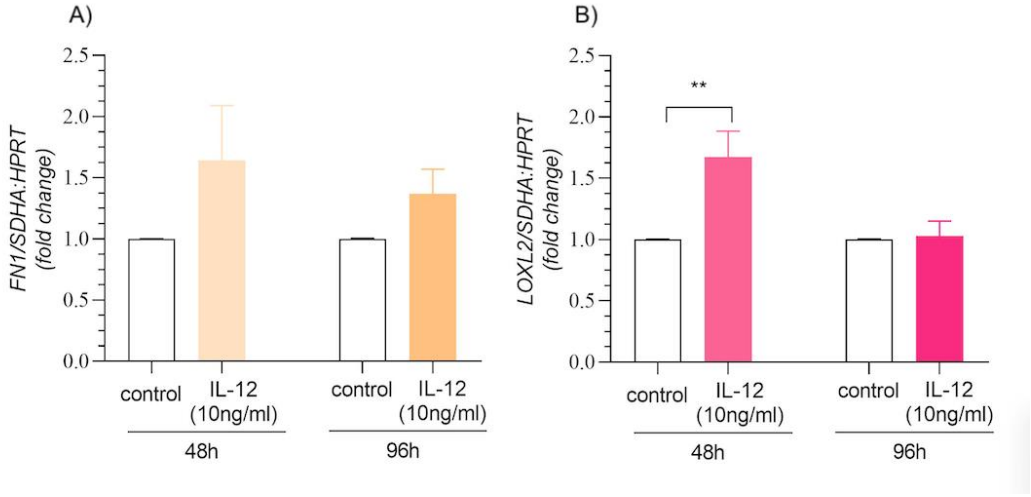

Figure 4: The effect of IL-12 at different treatment times (48 and 96h), on *FN1* and *LOXL2* mRNA expression in equine endometrial fibroblasts. (A) ***FN1*** mRNA transcription**, (B) *LOXL2*** mRNA transcription in endometrial fibroblasts cultured *in vitro* (n=6) **for 48 and 96h.** Asterisks indicate statistical differences between respective control and treatment groups (*P<0.05; **P<0.01)**.** All values are expressed as a fold change**. FN1 –** fibronectin 1**; LOXL2 –** Lysyl oxidase like-2.

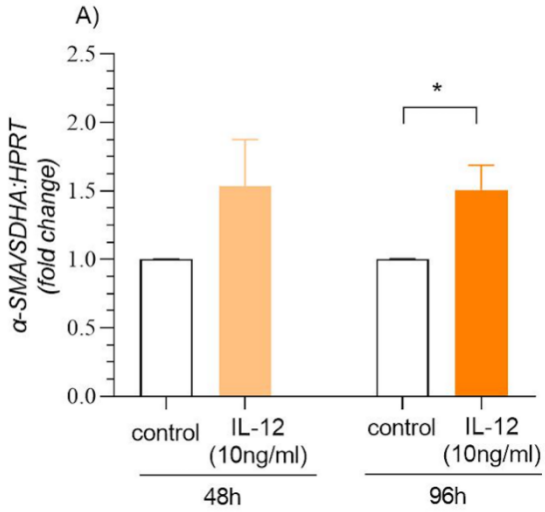

Figure 5: The effect of IL-12 at different treatment times (48 and 96h), on α-SMA mRNA expression in equine endometrial fibroblasts. (A) ***α-SMA*** mRNA transcription in endometrial fibroblasts cultured *in vitro* (n=6) **for 48 and 96h.** Asterisks indicate statistical differences between respective control and treatment groups (*P<0.05; **P<0.01)**.** All values are expressed as a fold change**. *α-SMA* –** alpha smooth muscle actin.

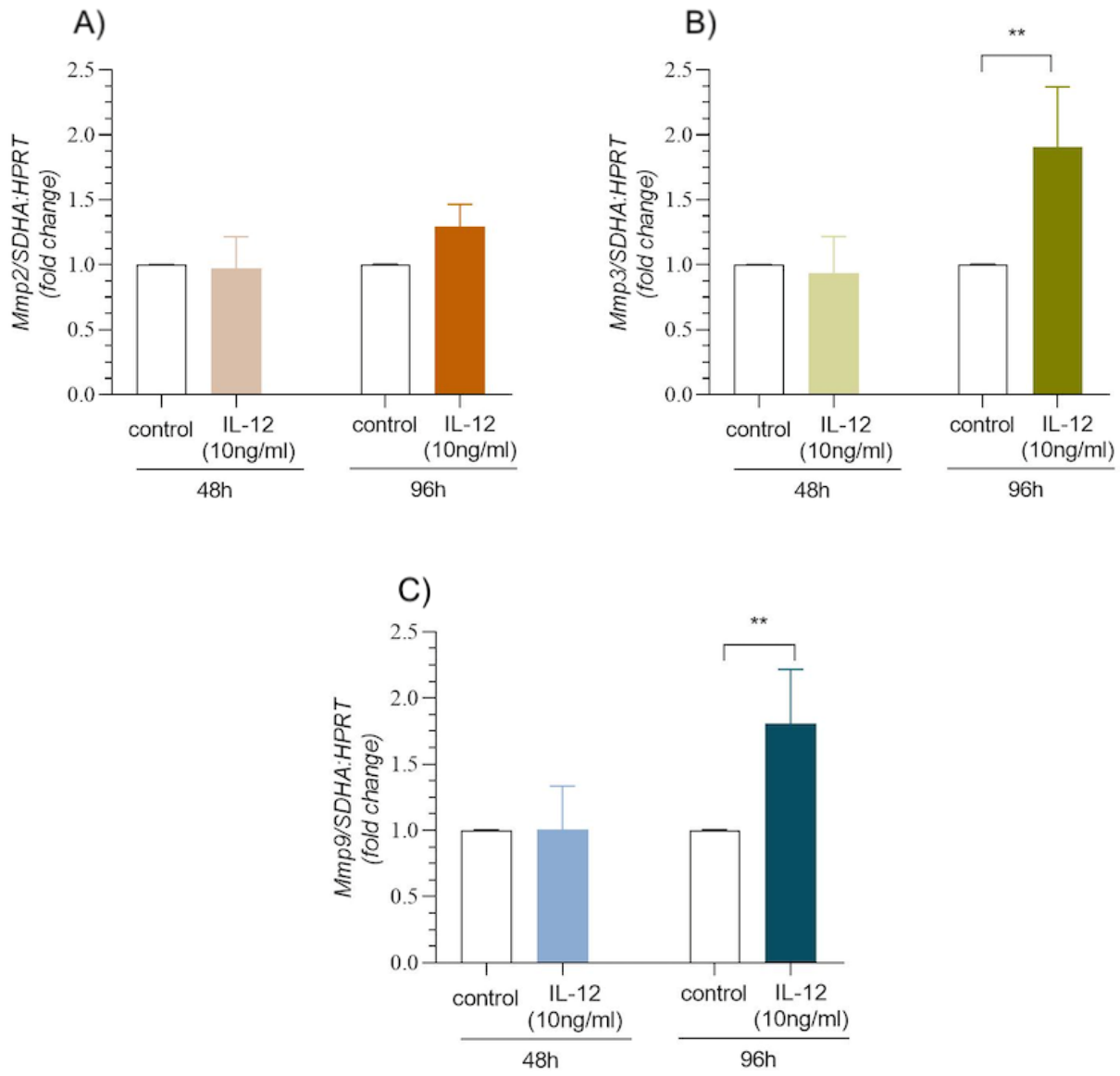

Figure 6: The effect of IL-12 at different treatment times (48 and 96h), on MMP-2, MMP-3, and MMP-9 mRNA expression in equine endometrial fibroblasts. (A) ***MMP2*** mRNA transcription, (B) MMP3 mRNA transcription, (C) **MMP9** mRNA transcription in endometrial fibroblasts cultured *in vitro* (n=6) **for 48 and 96h.** Asterisks indicate statistical differences between respective control and treatment groups (*P<0.05; **P<0.01)**.** All values are expressed as a fold change**.** ***MMP2* –** Matrix metalloproteinase 2; **MMP3** – Matrix metalloproteinase 3; **MMP9** – matrix metalloproteinase 9.

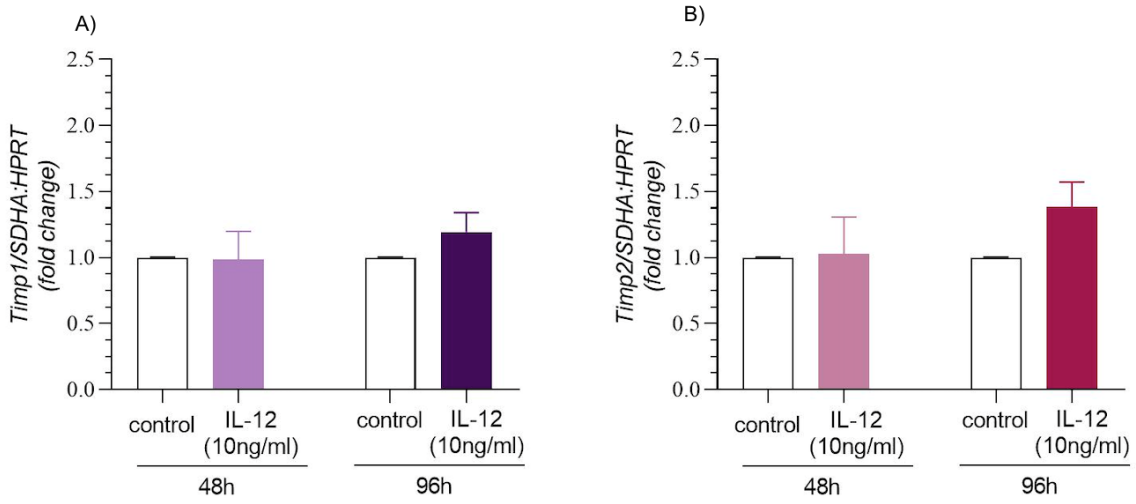

Figure 7: The effect of IL-12 at different treatment times (48 and 96h), on TIMP1 and TIMP2 mRNA expression in equine endometrial fibroblasts. (A) ***TIMP1*** mRNA transcription, (B) ***TIMP2*** mRNA transcription in endometrial fibroblasts cultured *in vitro* (n=6) for 48 and 96h. Asterisks indicate statistical differences between respective control and treatment groups (*P<0.05; **P<0.01)**.** All values are expressed as a fold change**.** ***TIMP1* –** Tissue inhibitor of matrix metalloproteinase 1; ***TIMP2* –** Tissue inhibitor of matrix metalloproteinase 2;

3. The effect of IL-12 on endometrial fibroblast proliferation

Interleukin 12 at a dose of 10ng/ml decreased the proliferation of endometrial fibroblasts cultured *in vitro* after 96 h of treatment (Figure 8; P<0.001).

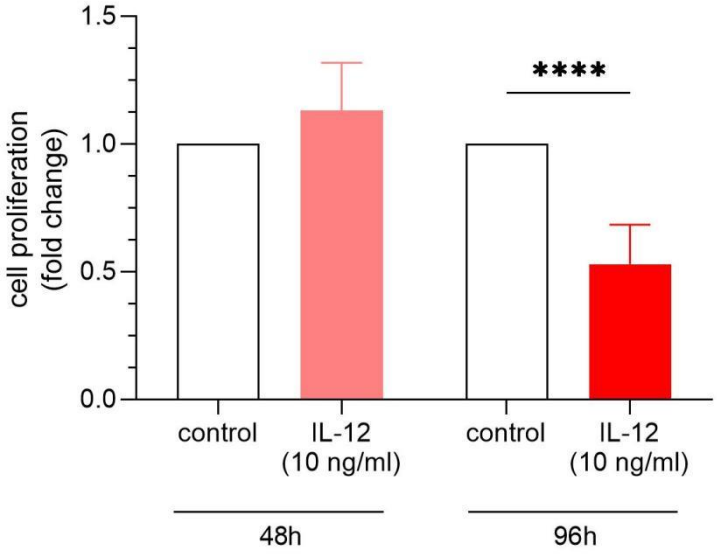

Figure 8: The effect of IL-12 at a dose of 10 ng/ml on fibroblast proliferation after 48 and 96h of treatment. All values are expressed as a fold change. Asterisks indicate statistical differences between groups (*P<0.001).

V - Discussion

When discussing endometrial fibrosis in mares, it should also be noted that approximately 45% of human mortality worldwide has been connected to fibrotic disorders, such as fibrosis in the heart, lungs, kidneys, peritoneum, and liver (Wynn 2004). Even though there are some differences in the etiology and clinical presentation of fibrotic condition in different organs, many similarities in the underlying molecular pathways, processes, and cellular interactions that occur during the fibrotic processes have been described. The causative mechanisms are far from being fully understood, fibrotic disorders share a common feature: uncontrolled and progressive accumulation of ECM components in affected organs causing their dysfunction and ultimate failure (Rosenbloom et al. 2017). Similarly, to what researchers described on equine endometrial fibrosis, human fibrotic diseases also rely on the function of myofibroblasts (Kendal and Feghali-Bostwick 2014). Despite fibrosis being a recognized cause of mortality, current treatments have shown limited efficacy (Finnerty et al 2021). Therefore, since there is a great need for effective anti-fibrotic therapies, it is crucial to understand how the fibrosis process starts, whether and how it can be reversed, and to identify potential treatment strategies. Therefore, the accurate description and elucidation of the underlying molecular processes and pathways that contribute to the onset and progression of fibrosis may provide the basis for the development of new, effective treatments for fibrosis in different tissues and organs.

Previously, the horse was addressed as a desirable model for research in human diseases, such as metabolic syndrome, melanoma, musculoskeletal diseases, autoimmune uveitis, and asthma (Smith et al. 2014). In mares, endometrosis occurs naturally as a pathological process, unlike experimentally induced endometrial fibrosis in laboratory animals. In addition, the availability of study material is not limited, the collection of material is not highly invasive, and tissue can be obtained repeatedly from the same individual or from the slaughterhouse. Research into the mechanisms involved in tissue fibrosis is necessary because knowledge of changes in cell differentiation and remodeling of the ECM may help to understand its pathogenesis and may contribute to the development of new treatments for this condition.

Even though the pathogenesis of endometrosis remains poorly understood, by now researchers suggest that this condition results from persistent inflammatory processes that lead to a subsequent exaggerated synthesis of ECM (Katila and Ferreira-Dias 2022). Endometrial fibrosis is characterized by excessive deposition of ECM components, in response to the persistent influence of pro-fibrotic mediators such as TGF-β1, prostaglandins, and cytokines (i.e. interleukins, and enzymes found in NETs) in mares (Szóstek-Mioduchowska et al. 2020). Interleukins might play a role in the onset and development of this condition, among them IL-12. To the best of our knowledge, the mRNA transcription of IL-12 subunits and receptor

components, as well as its influence on the expression of fibrotic markers and proliferation of fibroblasts, has not been documented in the mare endometrium. As described before in this work, interleukin 12 connects the innate immune response and the later antigen-specific adaptive response, by potentiating T naïve cell differentiation into Th1 cells, stimulates the production of several immune effector molecules by NK cells and T cells, and enhances the cytotoxicity of NK and cytotoxic T cells (Gately et al. 1998). When interpreting data on the impact of IL-12 on fibrotic markers in equine endometrial fibroblasts, it is important to note that there is limited research on the direct effect of this cytokine on fibroblasts derived from human tissue. The action of IL-12 has been associated with the development of several diseases, including psoriasis (Glowacka et al. 2010), rheumatoid arthritis (Hueber et al. 2010), and periodontal disorders (Sasaki et al. 2008; Miao et al. 2017). However, unpublished data by Wójtowicz et al. (2023) suggests that there is no anti-fibrotic action of INF-γ in equine endometrial fibroblasts and epithelial cells. This study investigated the effect of IFN-γ on mRNA transcription of selected fibrosis markers (COL1A1, COL3A1, Fn1, α-SMA, MMP2, -3, -9, TIMP1, -2) in fibroblasts and endometrial epithelial cells of mares. The results showed that IFN-γ treatment decreased *TIMP1* mRNA transcription in endometrial epithelial cells and increased *FN1* mRNA in endometrial fibroblasts. These findings did not confirm the antifibrotic action of IFN-γ in equine endometrosis. Thus, the antifibrotic effect of IL-12 through IFN-γ stimulation should be cautiously assessed.

The results of the current study showed no relevant differences in the mRNA transcription of *IL-12* subunits (*IL-12p35* and *IL-12p40*) and *IL-12Rβ1* in any of the four endometrial categories. However, in category IIB endometria, the *IL-12Rβ2* mRNA transcription was up-regulated during mid-luteal phase (MLP) compared to follicular phase (FP) of the estrous cycle. The absence of differences in the expression of IL-12 ligands can be caused by the fact that IL-12 is expressed by several types of immune cells such as monocytes, MΦs, and dendritic cells (Goriely and Goldman 2008), and in this study, we are considering the whole endometrial tissue, among them there are epithelial cells, fibroblast and endothelial cells. The heterogeneity of endometrial tissue may not reflect changes in the expression of IL-12 ligands when we consider the small population of immune cells expressing this cytokine compared to other cells in the endometrium. In addition, as described above, IL-12 family cytokines' can share their chains and receptors meaning that it is possible that these subunits interact independently with receptors and produce biological changes in immune cells (Collison and Vignali 2008). This particularity makes us believe that the results of this study might be ambiguous and the term "*IL-12 family members*" is possibly more correct than correlating all these findings exclusively with IL-12 itself. Moreover, there are only a few studies addressing IL-12 resulting in a lack of literature to fundament our study. Therefore, further studies need to be driven on IL-12 subunits and their receptors, specifically approaching it

through immunolocalization analysis and protein abundance of this cytokine to better understand its role in the development of fibrotic disorders, such as endometrosis.

Experiment 2 focused on the determination of the effect of IL-12 on the mRNA transcription of fibrotic markers such Col1 and Col3, FN1, LOXL 2, α-SMA, MMP-2, -3, and -9 as well as TIMP-1 and -2, in endometrial fibroblasts. These proteins can be interpreted as fibrotic markers since their impaired expression has been correlated with the development of fibrosis. In turn, fibroblasts are pivotal in the pathological accumulation of extracellular matrix (ECM) during fibrosis, and the cellular proliferation and differentiation that ensue in response to prolonged tissue injury and chronic inflammation. In general, fibroblasts consist of a heterogeneous population of stromal cells and are present in several tissues to provide structural support through the synthesis of ECM. The primary function of fibroblasts is to maintain the structural integrity of connective tissue, but in addition to producing ECM, fibroblasts have a number of other functions. Depending on the tissue, fibroblasts can present differences in their proliferation rate, collagen, and MMP production (Lindner et al. 2012), contractility, and immunomodulatory function (Brouty-Boyé et al. 2000). Additionally, besides contributing to the maintenance of homeostasis in adjacent cells, fibroblasts also participate in the orchestration of an inflammatory response (Van Linthout et al. 2014), having a crucial influence in the change from an acute situation to chronic persistent inflammation (Parsonage 2005).

Excessive accumulation of collagen and other ECM components characterizes fibrosis. Understanding the regulation of these processes during fibrosis pathogenesis is essential to comprehending the nature of this condition. Collagen is the most abundant group of proteins in the ECM, providing structural support, organization, and shape to the tissues, and interacts with other cells through various types of receptors regulating their proliferation, migration, and differentiation (Ricard-Blum 2011b). The increased deposition of collagen in the tissue has already been correlated to the establishment of fibrosis, and consequently the loss of normal function and structure of the tissues (Eckes et al. 2000; Schrier 2007). Many studies have already described this increased collagen deposition in the liver (Poynard et al. 2003), lungs (Keane et al. 2001), and kidney (Oldroyd et al. 1999) as the main histologic characteristics of fibrosis. In the healthy equine endometrium, COL3 is the predominant type. However, during endometrosis, COL3 is gradually surpassed by COL1, leading to endometrial fibrotic changes manifested namely by the change in tissue architecture, impaired function, and subsequently embryonic loss (Masseno 2012). Recent studies have shown that NET enzyme such as elastase, cathepsin-G or myeloperoxidase (Rebordão et al. 2021) and TGF-β1 increased transcription of *Col1* and *Col3* in endometrial tissue and fibroblasts, respectively (Rebordão et al. 2018) (Szóstek-Mioduchowska et al. 2019).

In the current study, IL-12 treatment did not affect *COL1A1* and *COL3A1* mRNA transcription after 48h but increased *COL1A1* and *COL3A1* mRNA transcription after 96h in equine endometrial fibroblasts. The time-dependent alteration in *COL1A1* and *COL3A1* mRNA transcription in response to IL-12 treatment may be related to the intricacies involved in synthesizing extracellular matrix components, particularly collagen fibers. The process of producing collagen fibers can assume different rates of synthesis which are determined by numerous factors, for example, cell density, however, protein production generally involves enormous cell stress (Schwarz 2015).

To the best of our knowledge, there is no study showing the direct effect of IL-12 on collagen expression in fibroblasts cultured *in vitro* derived from other tissue. Previous studies have demonstrated that IL-12 acts as an anti-fibrotic mediator by stimulating IFNγ production in Th1 cells (Borthwick et al. 2013). Although IL-12 appears to have no effect in the establishment of a chronic granuloma associated with schistosomiasis, the treatment with recombinant IL-12 resulted in reduced collagen deposition in the tissue (Wynn et al. 1994); and in bleomycin mouse models of lung fibrosis, the IL-12 treatment caused a significant reduction in hydroxyproline content of the lung, a major component of collagen fibers that is used as good fibrotic index measure (Keane et al. 2001). However, the results of the present study suggested that IL-12 might function as a stimulating factor in collagen deposition, which ultimately leads to the establishment of a fibrotic condition in the tissue, meaning that this cytokine might be related to the development of endometrosis in mare. Moreover, our findings provide alternative insights into the potential impact of IL-12 direct effects on ECM production in fibroblasts within the context of fibrosis, and require further investigation.

The enzyme lysyl oxidase-like 2 (LOXL2) is secreted into the ECM and promotes the crosslinking of collagen fibers and elastin through mediated oxidative deamination of lysine residues (López-Jiménez et al. 2017). *LOXL2* belongs to the Lysyl oxidase (LOX) family, which is a family of proteins whose function involves keeping ECM integrity and tensile strength by promoting the covalent crosslinking between other molecules (Trackman 2016), and on a lower scale, the management of gene transcription and cell signaling modulation (Barker et al. 2012). Similarly, in other experiments using human systemic sclerosis, the levels of LOX in the skin and in the blood serum were shown to be increased (Chanoki et al. 2006; Rimar et al. 2014), and in mice with bleomycin-induced pulmonary fibrosis, inhibition of LOX expression or activity resulted in a reduction of collagen deposition and alleviated the fibrosis (Cheng et al. 2014). In addition, liver fibrosis models were used to investigate the effect of the inhibition of LOX and the result showed a quicker reversal of the ECM deposition in the tissue (Liu et al. 2016). This family of proteins has already been suggested as a novel therapeutic target in fibroproliferative disorders (Nguyen et al. 2021). Our results showed that IL-12 increased the mRNA transcription of *Loxl2* after 96h. Thus, this enzyme was proven to participate in other fibrotic

disorders, and based on our results we suggest that LOXL2 might also play a relevant role in the development of endometrial fibrosis, even though further studies focused on the activity of this enzyme in the endometrium during homeostasis and when an inflammatory stimulus occurs are required to confirm our results.

Another fibrotic marker evaluated in this study was *α-SMA.* A-smooth muscle actin is expressed by myofibroblasts, which are an activated form of fibroblasts capable of producing ECM components in larger amounts, among them collagen I, extra-domain A containing Fibronectin (EDA-FN), and MMPs. In mares diagnosed with endometrosis, most of the *α-SMA-positive* cells are limited to the fibrotic loci and dilated cystic glands (Hoffmann et al. 2009a). However, Walter and colleagues showed that category IIA endometria, were presented with single *α-SMA-positive* cells near the glandular epithelium although it does not appear to be fibrotic, suggesting that myofibroblasts are involved in the early stages of endometrosis (Walter et al. 2001). Szóstek-Mioduchowska reported that the expression of *α-SMA* is up-regulated in the final stage of endometrosis compared to the initial stage (A. Z. Szóstek-Mioduchowska et al. 2019). The treatment of endometrial fibroblasts with TGF-β1 resulted in an up-regulation of α-SMA protein expression after 72h (Szóstek-Mioduchowska et al. 2019). However, to the best of our knowledge, there are no studies focusing on the direct effect of IL-12 on the expression of α-SMA in fibroblasts in mare or in other species. Our results showed that *α-SMA* mRNA transcription was up-regulated after 96h even though after 48h no changes were noticed. Our results suggested an increased expression in α-SMA, which can mean that IL-12 stimulate the activation of fibroblasts, leading to the differentiation of fibroblasts into myofibroblasts. It is possible that prolonged exposure to IL-12 directly contributes to the development and progression of endometriosis, as myofibroblasts excessively deposit extracellular matrix (ECM) components, including collagen fibers.

Metalloproteinases are mostly known for being involved in the ECM turnover by degrading its components, and their action is controlled by their tissue inhibitors, TIMPs (Nagase et al. 2006). There are many studies approaching MMP expression in different tissues however, it seems to be difficult to get to a consensus about the influence of these proteases in fibrogenesis since some of them seem to have pro-fibrotic roles in the tissues, but others seem to be anti-fibrotic. Furthermore, previous studies using targeted genes in mice showed evidence that individual MMP function do not always overlap the role of other MMPs (Gill et al., 2010). In general terms, MMPs not only participate in the ECM remodeling by degrading its components but also in the regulation of a variety of biological processes, particularly those associated with tissue repair, remodeling, and immunity (Giannandrea and Parks 2014). In a normal wound-healing situation the balance among MMPs is established and the homeostasis ends up being reassured. However, towards fibrosis disorders the expression of these

proteases might be up- or down-regulated, compared to the previous situation. Thus, assuming their versatile functionality, it has been suggested the importance of understanding how MMP activity is controlled since the balance between the different MMPs can shape the overall activity (reviewed by Giannandrea and Parks 2014). Changes in MMP expression might be due to an alteration in their transcription during biosynthesis, or caused by a direct enzyme activity, or even regards TIMP functions (Ra and Parks 2007). However, the role of IL-12 in the regulation of MMP and TIMP mRNA transcription in mare endometrial fibroblast have not been described. The results of our study showed and increased transcription of MMP-3 and MMP-9, leading us to believe that being MMPs involved in the degradation of the collagen, during a fibrotic disease these markers would be decreased. These findings reinsure the ambiguity of the mechanisms involved in the pathogenesis of endometriosis.

Metalloproteinase 2 acts on the last phase of collagen degradation and is mainly responsible for degrading type IV collagen and non-collagenous components of the ECM (Gomes et al. 2017). The concentration of MMP-2 was reported to be increased in category IIA endometria compared to category I in mares during FP (Szóstek-Mioduchowska et al. 2020). The results of our study showed that IL-12 did not affect *MMP2* mRNA transcription in endometrial fibroblasts. For the best of our knowledge, matrix metalloproteinase 2 was proven to have an antifibrotic role in the liver and kidney since previous studies showed that MMP2-knocked-out mice had an accelerated progression of diabetic nephropathy (Takamiya et al. 2013) and that in MMP2-deprived mice cholestatic liver fibrosis and toxin-induced fibrosis was exacerbated (Onozuka et al. 2011). Furthermore, Miao and colleagues showed, using models of human Periodontal Ligament Fibroblasts (hPDLFs), that, after IL-12 treatment, the mRNA expression and protein levels of MMP-2 were down-regulated (Miao et al. 2017). Thus, these findings culminate in the possibility of MMP2 having a negative influence on collagen deposition, although the mechanism involved remains unclear.

Matrix metalloproteinase 9, expressed by leukocytes, fibroblasts, epithelial cells and endothelial cells (Owen and Campbell 1999), mainly degrades the most abundant element of basement membranes, collagen, specially collagen type IV (Aresu et al. 2012), but also gelatin, and elastin, and is capable of releasing active TGF-β1, a pro-fibrotic cytokine, by proteolytic cleavage of latency-associated peptide (LAP) bound to TGF-β1 (Yu and Stamenkovic 2000). In addition, MMP-9 contain the particularity of a fibronectin-like region that promotes a stronger binding to their substrate (Fischer and Riedl 2019). Matrix metalloproteinase 3 has a broad of functions that goes from activating latent MMPs, such as MMP1, to degrading several ECM components for example fibronectin, laminin, gelatins, proteoglycans, and collagen types IV and IX (Sorsa et al. 2004). Transforming growth factor-beta 1 has been referred to as a key factor in endometrosis due to its variable functions influencing endometrial fibroblast proliferation, collagen synthesis, and myofibroblast differentiation (Szóstek-Mioduchowska et

al. 2019), but its pleiotropic effects range from influencing cell growth and differentiation to chemotaxis, apoptosis, and tumor suppression. Although both MMP-3 and MMP-9 were proven to contribute to tissue homeostasis by participating in their degradation and renewal, the over-expression of *MMP-3* and the downregulation of *MMP-9* have already been associated with the establishment of human periodontal disease (Miao et al. 2017). Compared with healthy human samples, in a patient with idiopathic pulmonary fibrosis (IPF), MMP-3, MMP-9, and TIMP-1 showed decreased expression levels in bronchoalveolar lavage fluid (BALF), but the forced vital capacity and six-minute walking distance showed no differences (Chuang et al. 2019). Another study reported that hPDLFs treated with IL-12 expressed significantly higher mRNA expression levels of *MMP-3* comparing to the non-treated group (Miao et al. 2017). Miao and colleagues also stated that NF-kB might be a crucial transcription factor whose IL-12-mediated MMP expression is dependent on, since its levels were shown to be significantly increased following the influence of IL-12 on hPDLFs. NF-kB is a protein transcription factor (Salminen et al. 2008) that participates in the regulation of innate immune responses (Baltimore 2009), through diverse intracellular pathways, and has been target of several studies on inflammatory disorders, and so far, it was reported as a transcription factor with regulatory influence on MMP expression (Westermarck and Kähäri 1999; Miao et al. 2017; Wu et al. 2014). The current study showed that IL-12 treatment increased *MMP-3* and *MMP-9* mRNA transcription after 96h in endometrial fibroblast, suggesting the involvement of IL-12 in the ECM turnover and possibly in the pathogenesis of endometrosis. However, further investigation should be conducted on the impact of IL-12 on metalloproteinases, given the intricate role that MMPs play in the pathogenesis of fibrosis.

Tissue inhibitors of matrix metalloproteinases is a family of four polypeptides capable of silencing MMPs catalytic activity through different mechanisms such as endocytosis, oxidative processes, and other inhibitors (Ra and Parks, 2007). In their review, Giannandrea and Parks, highlighted the possibility of an excess of metalloproteinases activity over the known controlling mechanisms being in the source of an imbalance of these proteases, and its possible association with fibrogenesis (Giannandrea and Parks 2014). The findings of our study suggested that IL-12 have no effect on the mRNA expression of TIMP-1 and TIMP-2. Similarly, the study driven by Miao and colleagues using a model of human periodontal disease showed that IL-12 treatment of hPDLFs did not lead to any changes in TIMP-1 and TIMP-2 expression levels (Miao et al. 2017). These findings might indicate that, although the results showed increased expression of MMP-3 and MMP-9, the control of these proteases relies on a vast type of mechanisms, and not exclusively on TIMPs. However, further investigations must be carried out to better explain the role of TIMPs in fibrogenesis.

Fibroblasts respond to the process of wound healing by proliferating and chemotaxing towards the tissue injury sites to reconstruct the extracellular matrix (ECM). Fibroblasts are responsible for the generation and secretion of all the components of the extracellular matrix. Thus, a higher rate of proliferation leads to an enhanced production of ECM necessary for the remodeling of the tissues. Nevertheless, if the healing process is not completely resolved, there is an excessive deposition of ECM leading to fibrosis. Several profibrotic factors impact fibroblast proliferation, not only by affecting ECM production or regulating MMP activity and myofibroblast differentiation. In mare endometrial fibroblast and tissue, transforming growth factor-β1 and IL-6, apart from the role on ECM, MMP, and TIMP expression, increase fibroblast proliferation (Szóstek-Mioduchowska et al. 2019, Szóstek et al. 2014). The present study reveals that treatment with IL-12 reduces the proliferation of endometrial fibroblasts after 96 hours. This indicates that the pro-fibrotic impact of IL-12 may be directly linked to the stimulation of ECM deposition in fibroblasts, rather than an effect on fibroblast proliferation.

Conclusion

The purpose of this study was to explain the role of IL-12 in the progression of equine endometrial fibrosis resorting to endometrial samples, and *in vitro* fibroblast culture. To the best of our knowledge, it was shown for the first time that IL-12 subunits p35, p40 and their receptors IL-12R$\beta 1$ and IL-12R$\beta 2$, are present in healthy (Cat I) and diseased equine endometrium. In fact, they were transcribed in endometria with mostly inflammatory processes (Cat IIA, and in others where fibrosis was predominant (Cat IIB and Cat III).

Our research found that IL-12 has a direct impact on mRNA transcription of fibrosis markers in endometrial fibroblasts and the proliferation of fibroblasts in a time-dependent manner. The results indicate that IL-12 has a stimulating effect on the mRNA transcription of key factors involved in the pathogenesis of endometrosis, including collagen, LOXL-2, and α-SMA. This may result in an excessive deposition of ECM components. Additionally, research indicates that IL-12 impacts the expression of MMP, which not only break down collagen but also possess profibrotic properties. These discoveries support the assertion that IL-12 can be classified as a profibrotic cytokine.

Until now, no studies have examined the effect of IL-12 in the pathogenesis of endometrosis, and our results provide useful data on this condition, which is a major cause of infertility and embryonic loss in mares. Moreover, this study elucidates the potential role of IL-12 in fibrosis-related pathologies across various human organs, bringing us closer to comprehending the underlying mechanisms. Specifically, our findings suggest that IL-12 may have a role in the progression of endometrosis in mares and fibrotic disorders in humans, by directly affecting ECM deposition and MMPs regulation in fibroblasts as well as myofibroblast differentiation from fibroblast.

VI - Bibliography

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VII – Attachments

Rola interleukiny 12 w patogenezie endometrosis u klaczy

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Endometrosis to chroniczne degenerujące włóknienie błony śluzowej macicy klaczy objawiające się nadmiernym odkładaniem składników macierzy pozakomórkowej (ECM). Postępujące włóknienie zrębu łącznotkankowego macicy prowadzi do zmian w strukturze i funkcji narządu, a w konsekwencji do obniżenia płodności klaczy. Wśród czynników wpływających na rozwoju endometrosis wymienia się m.in. przewlekły stan zapalny, w którym komórki odpornościowe wydzielają liczne cytokiny. Interleukina (IL)-12 jest cytokiną wydzielaną przez monocyty, makrofagi oraz komórki dendrytyczne. Jest heterodimerem zbudowanym z dwóch połączonych kowalencyjnie podjednostek – IL-12a (IL-12p35) oraz IL-12b (IL-12p40). Biologiczna aktywność IL-12 zależna jest od wiązania się cząsteczki z kompleksem receptora błonowego, zbudowanego z podjednostek: IL-12Rβ1 i IL-12Rβ2. Główną rolą IL-12 jest inicjowanie różnicowania naiwnych limfocytów T w kierunku limfocytów pomocniczych Th1 i promowanie wydzielania interferonu (IFN)-γ będącego cytokiną prozapalną aktywującą makrofagi typu 1. Makrofagi typu 1 wykazują działanie profibrotyczne w płucach i nerkach. Jednakże bezpośredni wpływ IL-12 na fibroblasty zrębu łącznotkankowego macicy jest do tej pory nieznany. **Celem badań** było określenie profilu ekspresji podjednostek IL-12 oraz jej receptora na poziomie mRNA w błonie śluzowej macicy klaczy w różnych stadiach endometrosis oraz zbadanie jej wpływu na ekspresję wybranych markerów włóknienia w fibroblastach zrębu *endometrium*.
W doświadczeniu 1. materiał do badań stanowiły *endometria* macic w kategorii I, IIA, IIB oraz III wg Kenneya i Doiga pobrane *post-mortem* od klaczy w środkowej fazie lutealnej (n=38) oraz fazie pęcherzykowej (n=40) cyklu rujowego. W badanej tkance określono profil ekspresji podjednostek IL-12 (*IL-12a* i *IL-12b*) oraz jej receptora (*IL-12Rβ1* i *IL-12Rβ2*) na poziomie mRNA w przebiegu endometrosis przy użyciu metody qPCR.
W doświadczeniu 2. materiał do badań stanowiły fibroblasty wyizolowane z *endometrium* w kategorii I pobranych od klaczy w środkowej fazie lutealnej (n=6). Wyizolowane fibroblasty traktowano IL-12 (10 ng/ml) przez 48 i 96 godzin. Określono wpływ IL-12 na ekspresję genów wybranych składników ECM (*Col1a1, Col3a1, Fn1*) oraz genów zaangażowanych w rozwój włóknienia (*Mmp-2, -3, -9, Timp-1, -2, Loxl2, αSMA*) przy użyciu metody qPCR. Ponadto, przy użyciu testu BrdU, zbadano wpływ IL-12 na poziom proliferacji fibroblastów.
Wykazano, że ekspresja *IL-12Rβ2* w *endometrium* kategorii IIB była wyższa w środkowej fazie lutealnej w porównaniu do fazy pęcherzykowej cyklu rujowego (P<0,01). Wykazano również, że po 48 godzinach hodowli, IL-12 zwiększyła ekspresję *Loxl2* (P<0,01), a po 96 godzinach zwiększyła ekspresję *αSMA i Col1a1* (P<0,05) oraz*Col3a1, Mmp3 i Mmp9* (P<0,01) na poziomie mRNA w fibroblastach w warunkach *in vitro*. Wykazano również, że IL-12 hamowała proliferację fibroblastów *endometrium* w warunkach *in vitro* (P<0,001).
Wzrost ekspresji podjednostki β2 receptora IL-12 w *endometrium* wskazuje na potencjalną rolę tej cytokiny w rozwoju włóknienia macicy klaczy. Ponadto, wyniki uzyskane w badaniach *in vitro* pokazują, że IL-12 wywiera stymulujący wpływ na ekspresję genów składników ECM, co sugeruje profibrotyczną rolę tej cytokiny.
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Attachment 1 – Publication of the abstract on a Polish conference (September 2023).