UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

MARE ENDOMETRIUM FIBROSIS: EPIGENETICS AND NOVEL FIBROSIS MARKERS

JOANA CABRAL DA GAMA DE ALPOIM MOREIRA

Orientadora: Professora Doutora Graça Maria Leitão Ferreira Dias

Co-Orientadoras: Doutora Maria Rosa Rebordão Cordeiro Simões Crisóstomo

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Ciências Biológicas e Biomédicas

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But we make a life by what we give”

Winston Churchill

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Titulo da dissertação: FIBROSE DO ENDOMÉTRIO DA ÉGUA: EPIGENÉTICA E NOVOS MARCADORES DE FIBROSE

Resumo

A endometrose é uma das principais causas de infertilidade em éguas e envolve a deposição excessiva de matriz extracelular (ECM) no endométrio, tal como o colagénio do tipo I (COL1), e do tipo III (COL3). Na fibrose existe um desequilíbrio entre as metaloproteinases (MMPs) e os seus inibidores tecidulares (TIMPs), resultando na desregulação entre a degradação e a deposição de colagénio. O factor transformador de crescimento β1 (TGF-β1) é um dos principais sinais pró-fibróticos na diferenciação dos miofibroblastos, identificados pela expressão da α-actina do músculo liso (α-SMA), promovendo a produção de colagénio. As alterações no fenótipo dos fibroblastos estão associadas a modificações epigenéticas. As alterações epigenéticas são reversíveis, sendo por isso extremamente promissoras para uso terapêutico. A análise da metilação do DNA é um dos métodos mais utilizados na detecção de alterações epigenéticas. Os objetivos deste estudo foram: (i) avaliar se o COL1, COL3 e hidroxiprolina poderiam servir como possíveis biomarcadores sanguíneos no diagnóstico de endometrose equina e na avaliação da fertilidade; (ii) avaliar uma possível correlação entre os níveis de mRNA das DNA metiltransferases (*DNMTs)* e dos *COL1A2* e *COL3A1* em diferentes categorias de endométrio (Kenney e Doig) e as fases do ciclo éstrico das éguas; (iii) avaliar a transcrição e a metilação do DNA em locais específicos do *COL1A1, COL1A2, COL3A1, MMP2, MMP9, TIMP1* e *TIMP2* em diferentes categorias de endométrio; (iv) investigar a regulação epigenética, *in vitro,* em fibroblastos endometriais tratados com TGF-β1 e o uso do agente epigenético, 5-aza-2'-desoxicitidina (5-aza-dC ou decitabina), em fibroblastos previamente expostos a TGF-β1. A concentração sérica de COL3 aumentou nas categorias de endométrio mais avançadas, e foi também maior nas éguas inférteis, quando comparada com as férteis. A sensibilidade e a especificidade do COL3 foram mais eficazes na diferenciação das éguas da categoria I, das éguas das restantes categorias. Apenas o COL3 demostrou ser um possível biomarcador de fibrose endometrial nas éguas. Em relação aos estudos epigenéticos, a *DNMT3B* aumentou com a categoria endometrial, independentemente da fase do ciclo éstrico. Ocorreu uma menor transcrição da *MMP2* e *MMP9*, concomitante com uma maior metilação do DNA, nas categorias de endométrio com maior grau de fibrose. O desmetilante, 5-aza-dC reduziu a expressão de colagénio (mRNA e proteína) nos fibroblastos endometriais tratados com TGF-β1. Uma abordagem diferente da endometrose equina, através da epigenética, pode constituir uma alternativa para um conhecimento mais aprofundado da sua fisiopatologia.

Palavras-chave: epigenética, endométrio equino, fibrose, biomarcadores, metilação do DNA

Thesis title: MARE ENDOMETRIUM FIBROSIS: EPIGENETICS AND NOVEL FIBROSIS MARKERS

Abstract

Endometrosis is a major cause of infertility in mares and involves the excessive deposition of extracellular matrix (ECM) in the mare's endometrium, such as collagen type I (COL1), and type III (COL3). In fibrosis there is an imbalance between metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), resulting in dysregulation between collagen degradation and deposition. Transforming growth factor β1 (TGF-β1) is one of the major pro-fibrotic signals for myofibroblast differentiation, identified by the expression of α-smooth muscle actin (α-SMA), promoting collagen production. Alterations in fibroblast phenotype are associated with epigenetic alterations in chromatin structure. Epigenetic changes can be reversed and therefore extremely promising for therapeutic use. DNA methylation analysis is one of the most used methods to detect epigenetic changes. The aims of this study were to: (i) evaluate if COL1, COL3 and hydroxyproline could serve as possible blood biomarkers of equine endometrosis diagnosis and fertility assessment; (ii) evaluate the existence of a possible correlation between mRNA levels of DNA methyltransferases (*DNMTs)* and *COL1A2* or *COL3A1* in different Kenney and Doig's endometrial categories and at different estrous cycle phases; (iii) evaluate the transcription and locus specific DNA methylation of *COL1A1, COL1A2, COL3A1, MMP2, MMP9, TIMP1*, and *TIMP2* in different mare endometrial categories; (iv) investigate the *in vitro* epigenetic regulation in endometrial fibroblasts challenged with TGF-β1 and to assess the use of the epigenetic modifier, 5-aza-2′-deoxycytidine (5-aza-dC or decitabine), on endometrial fibroblasts exposed to profibrotic TGF-β1. Serum COL3 concentrations increased with more advanced Kenney and Doig´s endometrial categories. Serum COL3 concentration was also higher in infertile mares, when compared to fertile mares. Both sensibility and specificity of COL3 was most effective to differentiate mares from category I from all other categories. Only COL3 proved to be a possible biomarker of mare endometrial fibrosis. Concerning epigenetic studies, *DNMT3B* increased with endometrial category grade, and it was independent of estrous cycle phase. There were lower transcription levels of *MMP2* and *MMP9*, simultaneously with higher DNA methylation percentage, in higher endometrial categories. The demethylating drug 5-aza-dC reduced the collagen expression (mRNA and protein) in TGF-β1 treated endometrial fibroblasts. A different approach via epigenetics may provide an alternative means to address mare endometrial fibrosis and a more in-depth knowledge of the pathophysiology of this condition.

Keywords: epigenetics, equine endometrium, fibrosis, biomarkers, DNA methylation

Título da dissertação: FIBROSE DO ENDOMÉTRIO DA ÉGUA: EPIGENÉTICA E NOVOS MARCADORES DE FIBROSE

Resumo alargado:

A endometrose é uma das causas de infertilidade em éguas com grande impacto económico. É caracterizada por fibrose periglandular do endométrio, comprometendo a integridade e a função das glândulas endometriais necessárias à sobrevivência do embrião e ao desenvolvimento placentário. Contudo, esta doença é um enigma quanto à patogénese e tratamento. A disposição periglandular dos miofibroblastos, associada à deposição de matriz extracelular (ECM), como o colagénio (COL), é uma característica fundamental da endometrose nas éguas. A acumulação de colagénio tipo I (COL1) e III (COL3) ocorre nos processos fibróticos, o que promove o desenvolvimento de tecidos cicatriciais. As metaloproteinases (MMPs) são endopeptidases importantes no processo da fibrose, tendo como seus inibidores, os inibidores tecidulares das metaloproteinases (TIMPs). As MMP-2 e a MMP-9 parecem estar envolvidas na endometrose. Uma característica fundamental da fibrose é o desequilíbrio entre MMPs e TIMPs, resultando na perda da homeostase entre fibrólise e fibrogénese. Há mais de 50 anos, que a maioria das alterações degenerativas características da endometrose é apenas diagnosticada pela avaliação histológica de biópsia endometrial. Embora considerado um método seguro e prático, além de ser invasivo, não fornece informações totalmente precisas. O desenvolvimento de técnicas adicionais e menos invasiva seria desejável. Por conseguinte, neste estudo colocou-se a hipótese de alguns componentes da ECM do endométrio da égua, como COL1, COL3 e hidroxiprolina (HYP), poderem servir como potenciais biomarcadores sanguíneos no diagnóstico de endometrose e prognóstico de fertilidade em éguas. Assim, foram realizadas biópsias uterinas e colheitas de sangue e posteriormente determinadas as categorias endometriais segundo Kenney e Doig e as concentrações de COL1, COL3 e HYP no tecido e no soro. Observou-se um aumento da concentração de COL3 nos endométrios de categoria III, quando comparada com os saudáveis (categoria I), tanto no tecido como no soro. Houve uma forte correlação entre a concentração de COL3 e a categoria endometrial. Um cut off the 60.9 ng/mL conseguiu diferenciar éguas de categoria I das restantes categorias com uma AUC (area sob a curva) de 0.90. Foi possível diferenciar as categorias I e IIA das categorias IIB e III, além da categoria III de todas as outras, mas com uma AUC inferior (0.85). Apenas o COL3 mostrou potencial como biomarcador de fibrose endometrial. Foi também avaliado se o COL3 poderia ser indicador de fertilidade. Foi recolhido sangue a 50 éguas, classificadas como férteis e inférteis no fim da época reprodutiva. A concentração de COL3 foi maior nas éguas inférteis e houve uma correlação fraca entre a concentração de COL3 e a infertilidade, sendo a AUC de 0.72

para diferenciar as éguas férteis das inférteis. O COL3 mostrou potencial como biomarcador de fibrose endometrial, e de fertilidade em éguas.

Novas descobertas referem um papel das modificações epigenéticas na progressão da fibrose, devido a alteração dos perfis de expressão génica. As modificações epigenéticas, alterações no genoma que não alteram a sequência do DNA, influenciam ou regulam a expressão dos genes e podem ser revertidas, sendo por isso extremamente promissoras para uso terapêutico. A metilação do DNA constitui uma importante modificação epigenética do genoma, sendo catalisada por DNA metiltransferases (DNMTs), tais como a DNMT1, DNMT3A e DNMT3B. A hipermetilação do promotor de um gene é reconhecida como um meio eficaz de repressão da transcrição do mesmo. Como os mecanismos epigenéticos parecem estar envolvidos na fibrose e são importantes na manutenção do estado activado dos miofibroblastos, colocou-se a hipótese que a endometrose da égua pudesse ser modulada por mecanismos epigenéticos. Para caracterizar a assinatura epigenética da fibrose endometrial em éguas, foi realizada uma primeira análise global da metilação do DNA em éguas com endométrio de diferentes categorias (Kenney e Doig), pela análise da transcrição das *DNMTs*, além dos genes *COL1A2* e *COL3A1*. A transcrição da *DNMT3B* aumentou nos endométrios de categoria III, em relação aos saudáveis. Não houve alterações na transcrição dos genes *COL1A2* ou *COL3A1*, entre as diferentes categorias de Kenney e Doig. A hipermetilação no endométrio de categoria III sugere um envolvimento epigenético no estabelecimento da fibrose endometrial. Uma vez que esta hipermetilação ocorreu sem aumento da transcrição dos genes do colagénio, poderá indicar que a regulação esteja a ocorrer nos genes anti-fibróticos e não nos genes pró-fibróticos.

Posteriormente, fomos avaliar em que genes poderia estar a ocorrer esta hipermetilação. A transcrição de alguns dos genes envolvidos na fibrose endometrial equina (*COL1A1, COL1A2, COL1A3, MMP2, MMP9, TIMP1* e *TIMP2*) foi analisada. Também foi avaliado se as alterações observadas na transcrição génica eram moduladas epigenéticamente, através da análise de metilação do DNA em locais específicos (por pirosequenciação bissulfito) nas regiões regulatórias dos genes escolhidos. Não houve alteração na transcrição dos genes do *COL1A1, COL1A2* ou *COL3A1* entre as categorias de Kenney e Doig. A transcrição da *MMP2* e *MMP9* diminuiu, e da *TIMP1* aumentou nos endométrios de categoria III em relação aos de categoria I. Relativamente à metilação, esta aumentou nos genes *COL1A1, MMP2* e *MMP9* nos endométrios de categoria III. O aumento da metilação dos genes da *MMP2* e *MMP9* ocorreu simultaneamente com a dimuição da transcrição dos mesmos, com o avançar da fibrose endometrial, sugerindo uma vez mais, um possivel envolvimento de mecanismos epigenéticos na regulação da fibrose endometrial nas éguas. Estes mecanismos epigenéticos parecem actuar através da hipermetilação de genes anti-fibróticos (*MMP2* e *MMP9)* em vez da hipometilação de genes pró-fibróticos.

O último estudo pretendeu avaliar o que se passava a nível celular. Na medicina humana, os fármacos epigenéticos têm sido usados principalmente no tratamento de doenças relacionadas com o cancro. Contudo, o seu uso na fibrose está a ser testado com resultados promissores. Neste âmbito, foi também levantada a hipótese de que os modificadores epigenéticos poderiam ter um potencial uso no tratamento da fibrose endometrial. Assim, o efeito *in vitro* do fármaco desmetilante, 5-aza-deoxycytidna (5-aza-dC, Decitabina) foi investigado para avaliar o envolvimento epigenético na regulação dos fibroblastos endometriais equinos. Foi realizada a cultura de fibroblastos endometriais de égua. Os fibroblastos foram posteriormente tratados com TGF-β1 e/ou 5-aza-dC, durante 48h. A transcrição dos genes *COL1A1*, *COL3A1, a-SMA* e *DNMT3A* aumentou após o tratamento com TGF-β1, bem como a expressão proteica do COL1 e COL3. Após o tratamento com 5-aza-dC durante 48h, dos fibroblastos previamente tratados com TGF-β1, ocorreu uma redução da transcrição do *COL1A1, COL3A1* e *DNMT3A*, mas não do *α-SMA*. Também houve diminuição da expressão proteica do COL1 e COL3. A hipermetilação (aumento do *DNMT3A*) e o aumento da expressão do COL1 e COL3, observados com a administração de TGF-β1, foram reduzidos após tratamento deste grupo com 5-aza-dC. Como tal, postulou-se que mecanismos epigenéticos parecem estar envolvidos na regulação, *in vitro,* dos fibroblastos endometriais equinos.

Como não existe um tratamento eficaz para resolver a fibrose endometrial da égua, é imperativo procurar uma terapia válida/competente. É fundamental a investigação de novas abordagens, como a epigenética, em relação à estratégia terapêutica, bem como o conhecimento mais detalhado dos mecanismos fisiopatológicos envolvidos na endometrose em éguas. Ao investigar o envolvimento epigenético na formação da fibrose endometrial na égua, este trabalho inédito pretendeu contribuir não só para o avanço do escasso conhecimento científico nesta área, mas também para melhorar a eficiência reprodutiva dos equinos. Além disso, esta nova abordagem apresentada neste estudo poderá também contribuir para a compreensão de outras doenças fibróticas nos animais.

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List of Abbreviations

Acetyl-CoA	acetyl-coenzyme A
AdoMet	S-adenosyl-L-methionine
AML	acute myeloid leukaemia
ANOVA	analysis of variance
Ap	activator protein
ASC	adipose stem cells
AUC	area under the curve
5-Aza	5-azacytydine; azacytidine; 5-AZC
5-aza-dC	5-azacytidine-2'- deoxycytidine; decitabine; DAC
BER	base excision repair
BSA	bovine serum albumin
caC	carboxylcytosine
CAL	calcium-binding protein
CAT	cathepsin G
ChIP-seq	chromatin immunoprecipitation sequencing
CMML	chronic myelomonocytic leukaemia
COL	collagen
COL1	collagen type I
COL1A1	collagen type 1 α1 gene
COL1A2	collagen type 1 α2 gene
COL3	collagen type III
COL3A1	collagen type 3 α1 gene
CpG	cytosine phosphodiester guanine
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPR-Cas	CRISPR-associated protein
CTCL	cutaneous T-cell lymphoma
CV	coefficient of variation
dCas	dead CRISPR-associated protein
DNA	deoxyribonucleic acid
DNMT	deoxyribonucleic acid methyltransferase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	desoxirribonuclease
ECM	extracellular matrix

EDTA	ethylenediaminetetraacetic acid
EHV	equine herpesvirus
ELA	elastase
ELISA	enzyme-linked immunosorbent assay
ENCODE	encyclopedia of DNA elements
ERK	extracellular signal-regulated kinase
EU	European Union
FC	formylcytosine
FDA	food and drug administration
FP	follicular phase
H3K4me2/3	histone 3 lysine 9 di-and trimethylation
HAT	histone acetyltransferase
HCL	hydrochloric acid
HCP	high-CpG-density promoter
HDAC	histone deacetylase
HKMT	histone lysine methyltransferase
hmc	hydroxymethylcytosine
HMT	histone methyltransferase
IFN	interferon
IL	interleukin
IPF	idiopathic pulmonary fibrosis
JH	Jeju horses
JmjC	Jumonji C
Kaiso	POZ/BTB family protein, ZBTB33
KDM	lysine histone demethylase
KMT	lysine histone methyltransferase
LCP	low-CpG-density promoter
lncRNA	long non-coding ribonucleic acid
LP	luteal phase
MEK	mitogen-activated protein kinase kinase
MAPK	mitogen-activated protein kinase
mc	methylcytosine
MBD	methyl CpG binding domain
MDS	myelodysplastic syndromes
MeCP	methyl CpG binding protein
Miravirsen	miR-122 inhibitor
miRNA	micro ribonucleic acid

MMP	matrix metallopeptidase
modENCODE	model organism encyclopedia of DNA elements
MRG-110	miR-92a inhibitor
mRNA	messenger ribonucleic acid
MRPL32	Mitochondrial ribosomal protein L32
MSCs	mesenchymal stem cells
ncRNA	non-coding ribonucleic acid
NF-kB	nuclear factor kappa B
NETs	neutrophil extracellular traps
NSG	next generation sequencing
P4	progesterone
PCR	polymerase chain reaction
PG	prostaglandin
PGE2	prostaglandin E_2
PGF2α	prostaglandin F2α
PI3K	phosphatidylinositol-3 kinase
PTCL	peripheral T-cell lymphoma
qPCR	quantitative polymerase chain reaction
RG-125	miR-103/107 inhibitor
ROC	receiver operating characteristic curve
SAHA	suberoylanilide hydroxamic acid, vorinostat
SAM	s-adenosylmethionine
SDHA2	succinate dehydrogenase complex flavoprotein subunit A, mitochondrial
SEM	standard error of the mean
SGI-110	guadecitabine
SIRT	sirtuin
SMAD	Sma and Mad related family
Sp	specificity protein
SUV	suppressor of variegation
TALEN	transcription activator-like effector
TET	ten-eleven translocation protein
TF	transcription factor
TGF	transforming growth factor
TH	Thoroughbred horses
TIMP	tissue inhibitor of metallopeptidase
TMB	tetramethylbenzidine substrate
TRANSFAC	TRANScription FACtor database

UC	uterocalin
UF	uteroferrin
UG	uteroglobin
UHFR	ubiquitin-like, containing PHD and RING finger domains
USA	United States of America
ZBTB	zinc finger and BTB domain containing
ZNF	zinc finger protein
α-SMA	α smooth muscle actin

CHAPTER I – General Introduction and Objectives

1. General Introduction

Endometrosis, a term first introduced by Kenney in 1992, is one of the causes of infertility in mares, especially in older mares (Buczkowska et al. 2014) and has a large economic impact on the horse breeding history (Schöniger and Schoon 2020). Endometrosis is mainly characterized by periglandular fibrosis of the endometrium (Schoon et al. 1992; Schoon et al. 1997), which compromises the integrity and function of the endometrial glands required for embryo survival in the preimplantation period and for placental development (Gray et al. 2001). However, this condition is still a puzzle regarding its pathogenesis and treatment. Periglandular arrangement of myofibroblasts, associated with the deposition of extracellular matrix (ECM), such as collagen (COL), is a cardinal feature of endometrosis in mares (Walter et al. 2005). Bochsler and Slauson (2002) stated that the deposition of collagen types I (COL1) and III (COL3) occurs in fibrotic processes, which promotes the development of cicatricial tissues with major tensile strength. Metalloproteinases (MMPs) are a family of extracellular endopeptidases (Ra and Parks 2007) that are important factors in the process of fibrosis. Data concerning MMP expression in equine endometrial fibrosis are limited but MMP-2 and MMP-9 (now called 72 kDa gelatinase and 92 kDa gelatinase, in the horse), seem to be involved in this process (Aresu et al. 2012; Centeno et al. 2018; Crociati et al. 2019; Szóstek-Mioduchowska et al. 2020). The endogenous inhibitors of MMPs are tissue inhibitors of metalloproteinases (TIMPs) and neutralize the activity of MMPs. A key feature of fibrosis is the imbalance between MMPs and TIMPs resulting in the loss of the homeostasis between fibrolysis and fibrogenesis (Hemmann et al. 2007).

Currently (and over 50 years) most degenerative changes typical for endometrosis can only be diagnosed through the histological evaluation of an endometrial biopsy (Ricketts and Alonso 1991; Aresu et al. 2012; Kenney 1978; Kenney and Doig 1986; Flores et al. 1995; Ricketts and Barrelet 1997; Katkiewicz et al. 2007; Zajac et al. 2008; Schlafer 2007; Snider et al. 2011; Hanada et al. 2014). Even though it has been considered as a safe, practical, and especially useful method (Kenney 1972; Ricketts and Barrelet 1997), it does not provide a 100% accurate information. The search for an additional and less invasive technique would be desirable and therefore, in the first part of this study it was hypothesized that some ECM components of mare endometrosis, such as COL1, COL3 and hydroxyproline, could serve as potential blood biomarkers for the diagnosis of endometrial fibrosis and fertility prognosis in mares.

Novel findings implicate a role for epigenetic modifications contributing to the progression of fibrosis by alteration of gene expression profiles (Neary et al. 2015; Bergmann and Distler 2017; Avci et al. 2022; Yang et al. 2022). Epigenetic modifications, heritable changes in the genome that do not alter the DNA sequence, influence, or regulate gene expression (Egger et al. 2004; Waterland 2006; Berger et al. 2009). Epigenetic changes, unlike genetic alterations, can be reversed (Handy et al. 2011; Neary et al. 2015) as thus extremely promising for therapeutic use (Bates 2020). DNA methylation constitutes a major epigenetic modification of the genome and is essential for cellular reprogramming, tissue differentiation, and normal development related to many biological processes including gene expression regulation. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) such as DNMT1, DNMT3A, and DNMT3B (Okano et al. 1998; Cheng and Roberts 2001; Robertson 2002). Hypermethylation of a gene promoter has long been well recognized as an efficient means of repressing transcription (Fuks 2005; Mohn et al. 2008). As epigenetic mechanisms seem to be involved in fibrosis and play an important role in maintaining the activated state of myofibroblasts (Hinz et al. 2007; Helling and Yang 2015; Neary et al. 2015; Moran-Salvador and Mann 2017; O'Reilly 2017; Gibb et al. 2020), it was hypothesized that mare endometrosis might be modulated by epigenetic mechanisms. To characterize the epigenetic signature of mare endometrosis, a first global DNA methylation analysis was performed in mares with different endometrial categories, through DNMTs transcription analysis. As hypermethylation of a gene promoter is recognized as a mean of repressing transcription, we analysed the transcription of some of the genes involved in equine endometrosis. Then, it was evaluated if the observed changes in gene transcription were epigenetic modulated, through locus specific DNA methylation analysis (by bisulfite pyrosequencing) of the regulatory regions of the chosen genes.

In human medicine, epigenetic drugs have been used mainly in the treatment of cancer related diseases, however, their use in fibrosis is being tested with promising results (Duong and Hagood 2018; Gorica et al. 2022; Vichaikul et al. 2022). Within this scope, it was also hypothesized that epigenetic modifiers, could be of potential use for the treatment of endometrial fibrosis. As such, the *in vitro* effect of the anti-methylating drug decitabine, was investigated to evaluate if it could reduce the increased collagen production in TGF-β1 treated mare endometrial fibroblasts.

As no effective treatment is available to address mare endometrial fibrosis, it is imperative to search for an effective therapy. Not only the pursue of new approaches such as epigenetics is needed regarding therapeutic strategy, but it is also necessary for a better knowledge of the pathophysiological mechanisms involved in mare endometrosis. By understanding the epigenetic involvement in endometrial fibrosis formation in the mare, this work will contribute not only to the advancement of scientific knowledge, but also to improve the reproductive efficiency of horses, representing an enormous economic advantage for the

breeding industry. Moreover, this new approach presented in this study may also contribute to help understanding other fibrotic disorders of animals.

2. Objectives

The main objectives of this work were, as follows:

(1) To identify possible blood biomarkers as early predictors of equine endometrosis diagnosis and fertility assessment.

The developed work was published in *Animals*:

Alpoim-Moreira J, Fernandes C, Rebordão MR, Costa AL, Bliebernicht M, Nunes T, Szóstek-Mioduchowska A, Skarzynski DJ, Ferreira-Dias G. 2022. Collagen Type III as a Possible Blood Biomarker of Fibrosis in Equine Endometrium. *Animals*; 12(14):1854. doi: 10.3390/ani12141854.

(2) To evaluate the epigenetic involvement through DNMTs gene expression in mares with different endometrium categories and at different estrous cycle phases.

The developed work was published in *Reproduction of Domestic Animals*:

Alpoim-Moreira J, Fernandes C, Rebordão MR, Amaral A, Pinto-Bravo P, Bliebernicht M, Skarzynski DJ; Ferreira-Dias G. 2019. Collagens and DNA methyltransferases in equine endometrosis. Reprod Dom Anim. 54:46–52. doi:10.1111/rda.13515

(3) To evaluate the transcriptomic pattern of some of the most relevant genes (*COL1A1, COL1A2, COL3A1, MMP2, MMP9, TIMP1*, and *TIMP2*) involved in mare endometrosis. Secondly, to evaluate if the observed changes in gene transcription were epigenetically modulated, through locus specific DNA methylation analysis of the regulatory regions of those genes.

The developed work was published in *Frontiers in Veterinary Science*:

Alpoim-Moreira J, Fernandes C, Pimenta J, Bliebernicht M, Rebordão MR, Castelo-Branco P, Szóstek-Mioduchowska A, Skarzynski DJ, Ferreira-Dias G. 2022. Metallopeptidades 2 and 9 genes epigenetically modulate equine endometrial fibrosis. *Frontiers in veterinary science*, *9*, 970003. https://doi.org/10.3389/fvets.2022.970003

4) To evaluate the effect of the anti-methylating drug decitabine (5-aza-dC) on endometrial fibroblasts exposed to profibrotic TGF-β1.

The developed work was published in *Animals*:

Alpoim-Moreira J, Szóstek-Mioduchowska A, Słyszewska M, Rebordão MR, Skarzynski DJ, Ferreira-Dias G. 5-Aza-2'-Deoxycytidine (5-Aza-dC, Decitabine) Inhibits Collagen Type I and III Expression in TGF-β1-Treated Equine Endometrial Fibroblasts. Animals (Basel). 2023 Mar 30;13(7):1212. doi: 10.3390/ani13071212.

1. Equine endometrial fibrosis

Fibrosis is characterized by an accumulation of extracellular matrix (ECM) proteins (Iredale et al. 2013). In fibrotic tissues, myofibroblasts are the leading contributors to the excessive production of ECM proteins, such as collagen (COL) and fibronectin, regardless of the cause (Zeisberg and Kalluri, 2013). The expression of α-smooth muscle actin (α-SMA) is the defining feature of the myofibroblast (Phan 2002; Hinz et al. 2007). When a tissue is injured, fibroblasts adopt a myofibroblast phenotype and promote wound repair, through their contractility and secretion of ECM proteins (Hinz et al. 2007). In fibrosis, the accumulation of myofibroblasts and ECM production becomes exuberant, parenchymal cells are replaced by myofibroblasts and changes to the microvasculature result in reduced perfusion and infiltration of mononuclear cells. This ECM accumulation impairs organ function due to hardening of the tissue (Zeisberg and Kalluri 2013; Dowson and O´Reilly 2016).

Endometrosis is a severe and complex fibrotic disease of the mare endometrium (Hoffmann et al. 2009a) that often leads to subfertility and infertility, causing significant economic loss in the horse breeding industry (Hoffmann et al. 2009a; Aresu et al. 2012; Buczkowska et al. 2014). Moreover, this fibrotic condition in the mare is considered irreversible (Kenney 1992; Hoffmann et al. 2009a; Aresu et al. 2012), limiting clinicians regarding treatment options. Numerous factors, such as age, multiple pregnancies, parturition, repeated endometritis, impaired uterine clearance, and endocrine problems seem to play a major role in the onset and severity of endometrosis (Doig et al. 1981; Ricketts and Alonso 1991; Hoffmann et al. 2009a; LeBlanc and Causey 2009; Buczkowska et al. 2014; Reilas et al. 2016). Endometrosis is characterized by endometrial periglandular fibrosis associated with dysfunction of affected glandular epithelial cells, and where lymphocytes, plasma cells and macrophages infiltrate the endometrium (Doig et al. 1981; Walter et al. 2001; Ganjam and Evans 2006; Hoffmann et al. 2009b). In this condition the stromal cells and/or collagen present a concentric disposition around the affected endometrial glands and under the basement membrane of the surface epithelium (Kenney 1978; Schöniger and Schoon 2020).

The term endometrosis was first introduced by Kenney in 1992 based on specific histopathologic alterations of the equine endometrium (Kenney 1992). In the late 70s, Kenney developed a three-category grading classification (category I, II and III) of the mare endometrium based on histopathological alterations, to estimate mare's fertility rate (Kenney, 1978). Later, in 1986 a more detailed classification was introduced by Kenney and Doig (1986), grading the equine endometrium in four categories (category I, IIA, IIB and III), mainly depending on the

degree of inflammation and fibrosis in the endometrium, as described in Table 1. More severe and widespread alterations in the mare endometrium correspond to higher endometrial category and to a lower probability of carrying a foal to term (Kenney and Doig 1986).

Table 1: Kenney and Doig's (1986) classification system for histopathological changes in equine endometrium

Category	Structural changes in endometrium	% altered glands	Expected foaling rate (%)
I	Normal and healthy, active, and well distributed glands, little to no inflammatory cells	-	80-90%
IIA	Mild, scattered inflammation and fibrosis around individual branches, lack of glandular nests, slight to moderate inflammatory changes, lymphatic lacunae, partial endometrial atrophy	10-35%	50-80%
IIB	Moderate scattered inflammation and fibrosis, 2-4 fibrotic nests, inflammatory and lymphatic changes are widespread, diffuse and moderately severe	35-60%	10-50%
III	Dilated glands surrounded by layers and fibrotic cells, 5 or more fibrotic nests, diffuse and severe inflammatory changes, severe lymphatic lacunae	>60%	10%

This was later modified by Schoon (Schoon et al. 1992; Schoon et al. 1997). Currently endometrosis is defined as active or inactive periglandular and/or stromal endometrial fibrosis including glandular alterations within fibrotic foci (Hoffman et al. 2009a). Single glands and/or glandular nests may be affected in equine endometrosis (Kenney 1978; Schöniger and Schoon 2020). Endometrosis is classified as active, when stromal cells around the endometrial glands are oval in shape, the cytoplasm is pale and depict ovoid hypochromatic nuclei. In contrast, when the endometrosis is inactive, the periglandular stromal cells are spindle-shaped with elongated hyperchromatic nuclei (Hoffmann et al. 2009a). In equine patients, endometrosis is further classified as either destructive (degeneration and necrosis of glandular epithelial cells) or non-destructive (intact epithelial cells) forms of fibrosis (Lehmann et al. 2011; Schöniger and Schoon 2020). According to Hoffmann and co-authors (2009a), destructive endometrosis is characterized by an overwhelming number of myofibroblasts, marked epithelial expression of vimentin, excessive accumulation of ECM, glands destruction and/or cystic dilations and alteration of the basal lamina. Moreover, mares suffering from destructive endometrosis, have a higher frequency of endometritis (Hoffmann et al. 2009a). The distinction between active and inactive, destructive, or non-destructive endometrosis, is mainly based on the pathological

characteristics of stromal cells involved in the fibrotic foci (Hoffmann et al. 2009a; Lehmann et al. 2011; Trundell 2022).

It has been referred that the degree of endometrosis increases with age, independently of the number of foalings (Ricketts and Alonso 1991). According to several authors endometrosis is more associated to age than parity, since aged maiden mares that were never bred also developed endometrosis (Doig et al. 1981; Ricketts and Alonso 1991; Hoffmann et al. 2009a). The cyclicity of mares, which is regulated by seasonal endocrine changes appear to have no effect on the endometrosis process (Hoffman et al. 2009b).

At the initial stage of endometrosis, fibroblasts differentiate into myofibroblasts, which are responsible for collagen fiber synthesis, ECM deposition, and resulting in endometrial fibrosis (Raila 2000; Hoffmann et al. 2009b; Szóstek-Mioduchowska et al. 2019a). Endometrosis is characterized by abound fibrosis, lack of cilia and cell boundaries, and cells present more degenerative structures and few organelles (Ferreira-Dias et al. 1994, 1999). These histological changes result in decreased pregnancy rates in the mare (Kenney 1978; Liepina and Antane 2010). In a healthy endometrium, the first collagen to be synthesized is COL3, which in turn is gradually replaced by COL1 following the development of fibrotic lesions (Sephel and Woodward 2001; Masseno 2009; Costa 2015). The predominance of COL type has been controversial. According to Lunelli and co-workers (2013), the endometrial samples of categories I and II had a predominance of COL3. In another study, both COL1 and COL3 were increased in equine category III endometrium when compared to category I (Alpoim-Moreira et al. 2022a). However, COL1 predominates relative to COL3 in mare endometrial periglandular fibrosis (Porto 2006; Pinto-Bravo et al. 2018). In addition, in inactive and/or destructive endometrosis COL1 fibers were prevalent, while COL3 fibers were predominant in active and/or non-destructive endometrosis with the simultaneous presence of periglandular myofibroblasts (Costa 2015).

Endometrosis has a wide range of reproductive implications. In endometrosis, endometrial vascular alterations, such as angiosis and elastofibrosis of arteries, veins and lymphatic vessels in the uterine wall and lymphatic lacunae can also be observed (Kenney et al. 1978; Oikawa et al. 1993; Snider et al. 2011; Hanada et al. 2014). Furthermore, the elastofibrosis of arteries, veins and lymphatic vessels in the uterine wall increases with age and is closely related to the progression of endometrial fibrosis (Hanada et al. 2014). Angiosclerosis occurs in two thirds of the mares with endometrosis (Hoffmann et al. 2006), since fibrotic lesions of inactive and inactive destructive endometrosis were related to severe vascular fibrotic lesions it was suggested that they trigger endometrosis (Masseno 2012). Furthermore, periglandular fibrosis and cystic dilated glands in the endometrium have been associated with fluid accumulation in the uterine lumen and ageing of mares (Kenney 1986; Reilas et al. 1997; Ozgen et al. 2002; Woodward et al. 2012). In addition, mares with a higher Kenney and Doig's category grade are more prone to fluid accumulation inside the uterine lumen after insemination

(Woodward et al. 2012). Chronic inflamed endometria predispose to persistent uterine infection (LeBlanc and Causey 2009; LeBlanc 2010) as occurs in prolonged post-breeding endometritis and may lead to an increase in endometrial fibrosis (Reilas et al. 2016). Thus, a link between inflammation and fibrosis is suggested. The periglandular deposition of collagen in the endometrium contributes for the formation of fibrotic nests, which may impair glandular flow secretion (Walter et al. 2001; Hoffmann et al. 2009b). Indeed, it was recently demonstrated by proteomic analysis of uterine lavage fluid that the secretion of essential proteins is affected in mares with endometrosis, due to endometrial glandular function impairment (Diel de Amorim et al. 2020). Also, the decreased number and area of healthy glands results in a deficient nutrient exchange between the placenta and the conceptus, and therefore may hinder its viability (Kenney 1978; Gray et al. 2001; Allen and Wilsher 2009). Therefore, all these endometrial alterations may result in pregnancy failure, delayed placental development, retarded fetal growth or abortion (Kenney 1978; Hoffmann et al. 2009a; Lehmann et al. 2011).

1.1. Pathophysiology

An initial insult to the endometrium, whether bacteriologic in nature or not, which triggers the start of a complex pathophysiologic process, ending in endometrosis seems to be consensually accepted by many authors (Szóstek et al. 2013; Amaral et al. 2018; Rebordão et al. 2018; Szóstek-Mioduchoswka et al. 2019; Rebordão et al. 2019). The relationship between endometrial fibrosis, aging, and infertility has been firmly set. However, the etiology of endometrosis and chronic inflammation of the endometrium stands controversial (Kenney 1978; Hoffmann et al. 2009; Aresu et al. 2012; Hanada et al. 2014; Katila and Ferreira-Dias 2022). An initial epithelial alteration and activation with a partial thickening of the affected parts of the basal lamina of the mare endometrium has been reported as a possible mechanism for endometrosis pathogenesis (Hoffman et al. 2009). There is a temporary activation of fibrotic stromal cells via profibrotic growth factors and cytokines that are released by inflammatory cells, after experimentally induced bacterial endometritis (Hoffman et al. 2009). The prevention of epithelial cell activation and the synthesis of profibrotic growth factors can only be achieved by an intact basal lamina (Streuli et al. 1993).

Some pathways have been studied in equine endometrial fibrosis pathogenesis, such as cytokines (Skarzynski et al. 2020), enzymes found in neutrophill extracellular traps (NETs) (Rebordão et al. 2018), prostaglandins (PG) (Rebordão et al. 2019; Szóstek-Mioduchowska et al. 2020b; Rebordão et al., 2021), transforming growth factor (TGF) β1 (Szóstek-Mioduchowska et al. 2019a), metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Walter et al. 2005; Oddsdóttir et al. 2008; Aresu et al. 2012; Centeno et al. 2018; Szósteck-Mioduschoswka et al. 2019b), interleukins, such as IL-1α, IL-1β, IL-6, IL-10 (Szóstek et al. 2013; de Holanda et al. 2019; Szóstek-Mioduchowska et al. 2019a; Szóstek-Mioduchowska et al.

2019b), uterine enzymes such as, uteroglobulin (UG), uterocalin (UC), calbindinD9k (CAL) and uteroferin (UF) (Stewart et al. 2000; Hoffmann, 2006; Hoffmann et al. 2009b; Ellenberger et al. 2008; Lehmann et al. 2011) and nuclear factor-kB (NF-kB) and its activation pathways (Jasiński et al. 2022).

Endometrial inflammatory cells produce pro-fibrotic cytokines, chemokines, interleukins, growth factors, and other proteins, which induce differentiation of fibroblasts into myofibroblasts and trigger fibrogenesis with COL production. The pro-fibrotic cytokines, synthetised by endometrial neutrophils, eosinophils, lymphocytes, macrophages, and mast cells, seem to influence metalloproteinases (MMPs) and their tissue inhibitors (TIMP) in mare endometrium (Szóstek-Mioduchwoska et al. 2019b; Skarzynski et al. 2020) indirectly impairing the ECM homeostasis (Crociati et al. 2019; Diel de Amorim et al. 2020), which in turn might predispose to fibrogenesis.

Polymorphonuclear neutrophils have an important function in controlling infections, fighting bacteria that cause endometritis by phagocytosis, and entrapping bacteria by neutrophil extracellular traps (NETs) (Rebordão et al. 2014a; Rebordão et al. 2014b; Rebordão et al. 2018). However, if the inflammation/infection becomes chronic, they may also have a detrimental effect by releasing their content that can modify the endometrium and likely contribute to chronic degenerative changes, culminating in fibrosis (Hoffmann et al. 2009a; Lögters et al. 2009; Reilas et al. 2016; Morris et al. 2020). *In vitro* studies have demonstrated that NETs components myeloperoxidase, elastase, and cathepsin G increase the transcript levels of COL1 and COL3, TGF-β1 and TIMPs and that is dependent on the stage of the estrous cycle and Kenney and Doig's endometrial category (Rebordão et al. 2014a; Rebordão et al. 2018). In equine endometrosis, there is an altered synthesis of prostaglandins and mRNA transcription of prostaglandin synthases (Szóstek et al. 2012). Also, other *in vitro* studies, demonstrated that decreased prostaglandin E_2 (PGE_2) production (an anti-fibrotic PG), together with increased in COL1 mRNA level by mare endometrium, may establish a pathway to endometrial fibrosis (Rebordão et al. 2019). Changes in PGF2α mediators in endometrial explants *in vitro* challenged with NET enzymes were also depicted, suggesting the PGF2α pathway as a putative additional mechanism of fibrogenesis in the mare's endometrium (Rebordão et al. 2021). Despite regulation of cell growth, development, and tissue remodelling, TGF-β1 is also involved in the pathogenesis of equine endometrosis. However, the role of TGF-β1 in the equine endometrosis pathogenesis has not been widely investigated. This cytokine plays an important function in fibroblast activation and differentiation into myofibroblast and induces the expression of genes for ECM components including COL1 (Ueha, et al. 2012; Zeisberg and Kalluri 2013; Seki and Brenner 2015). *In vitro* treatment of equine endometrial fibroblasts with TGF-β1 raised the expression of ECM components and α-SMA transcripts through its effect on MMPs and TIMPs (Szóstek-Mioduchwoska et al. 2019a¸ Szóstek-Mioduchwoska et al. 2020b). Thus, TGF-β1 may

influence the severity of endometrosis (Gamjam and Evans 2006; Cardoso et al. 2015; Szóstek-Mioduchwoska et al. 2019a; Szóstek-Mioduchwoska et al. 2019b). Matrix metalloproteinases are a family of extracellular endopeptidases (Ra and Parks 2007) that are important factors in the process of fibrosis but, are also involved in regulating the activity of a range of immunoregulatory molecules (Giannandrea and Parks 2014). Data concerning MMP expression in equine endometrial fibrosis are limited, but MMP-2 and MMP-9 (now called 72 kDa gelatinase and 92 kDa gelatinase, in the horse), appear to be involved in this process (Aresu et al. 2012; Centeno et al. 2018; Crociati et al. 2019; Szóstek-Mioduchowska et al. 2020a). MMP-2 and MMP-9 denature collagens and other ECM substrates (Vandooren et al. 2013; Djuric and Zivkovic 2017). Tissue inhibitors of metalloproteinases neutralize the activity of MMPs. The imbalance between MMPs and TIMPs is a key feature of fibrosis resulting in the loss of the homeostasis between fibrolysis and fibrogenesis (Hemmann et al. 2007). Since MMP-9 might be a pro-fibrotic mediator, as a potential activator of TGF-β1, it can be indirectly involved in myofibroblast formation (Giannandrea and Parks 2014). Also, MMP-2 and -9 seem to be involved in the inflammatory response and collagen remodelling during acute equine endometritis and contribute to the establishment of endometrial fibrosis (Oddsdóttir et al. 2008). Recently, *in vitro* studies in equine endometrium demonstrated that mediators of inflammation, such as interleukins, TGFβ1 and prostaglandins, influence the expression of MMPs (Szóstek-Mioduchowska et al. 2019b, 2020a, 2020b), which vary with the degree of endometrosis (Szóstek-Mioduchowska et al. 2020a).

In equine endometrosis, the expression of interleukins (IL) is up-regulated and IL6, IL1α, IL1β, and IL10 have been linked to inflammatory cells (lymphocytes, neutrophils, eosinophils), and to histopathological lesions of the endometrium (Szóstek et al. 2013; de Holanda et al. 2019; Szóstek-Mioduchwoska et al. 2019b).

Several researchers examined alterations in the secretion pattern of uterine enzymes, namely uteroglobin, uterocalin, calbindinD9k, uteroferrin, and their potential role in the pathogenesis of endometrosis (Stewart et al. 2000; Hoffmann, 2006; Ellenberger et al. 2008; Hoffmann et al. 2009b; Lehmann et al. 2011). It was shown in affected equine endometrial glandular epithelia, that uterocalin, uteroglobin and calbindinD9K expression was decreased, whereas uteroferrin expression was increased (Hoffmann, 2006). It was also observed a decrease in uterocalin expression in mares suffering from both destructive and non-destructive endometrosis (Hoffmann 2006). This suggests that the deviation of the protein pattern may be a possible cause for the reduced fertility in barren mares suffering from endometrosis (Hoffmann 2006).

It is believed that the cell metabolism regulator, nuclear factor-κB (NF-κB), and its activation pathways play a role in equine endometrosis pathogenesis. Furthermore, the transcription of endometrial steroid receptors (estrogen and progesterone) can be disrupted with

the degree of equine endometrosis and modulated by the NF-κB pathway depending on the estrous cycle phase (Jasiński et al. 2022).

1.1.1. Transforming growth factor β1 (TGF-β1)

Transforming growth factor β1 is regarded as one of the paramount pro-fibrotic signals for myofibroblast differentiation and promotes collagen production (mainly COL1 and COL3) as well as other ECM components. It is produced by fibroblasts, neutrophils, macrophages, and platelets and it also regulates tissue homeostasis and inflammatory responses, ECM deposition, and cell differentiation and growth (Yang et al. 2010; Ueha et al. 2012; Zeisberg and Kalluri, 2013; Seki and Brenner 2015). In the equine endometrium, the activity of TGF-β1 is associated with endometrosis (Gamjam and Evans 2006; Szóstek-Mioduchowska, 2019a). In other tissues, TGF-β1 induces differentiation of many cell types into myofibroblasts, which are characterized by α-SMA expression and an ability to deposit excessive amounts of ECM components (Smith et al. 2019). Moreover, aberrant expression of TGF-β1 after injury stimulates the expression of α-SMA (Rønnov-Jessen and Petersen 1993) and ECM formation (Hewitson et al. 2017) by fibroblasts, regardless of their origin (Zeisberg and Kalluri 2013). TGF-β family members, including TGF-β1 induce different signaling pathways, which are divided into Smad-dependent and Smad-independent ones. Mitogen-activated protein kinase kinase (MEK/ERK1/2) and phosphatidyl inositol 3 kinase (PI3K) are two pathways induced by TGF-β1 that promote the expression of COL1 and COL3 and ultimately fibrosis establishment (Seki and Brenner 2015). TGF-β1 also regulates micro ribonucleic acids (miRNAs) and other proteins, such as fibronectin and TIMP-1 and -3, during fibrosis. These proteins are responsible for the stable form of collagen, which is initially secreted as procollagen and later modified to tropocollagen before turning into fibrillar collagen, as they protect tropocollagen from being degraded. TGF-β is secreted in inactive form in the ECM as a latent complex (Annes et al. 2003) and is cleaved by MMP-2 and MMP-9. However, TGF-β1 may also induce the expression of MMP-9 through TGF-β receptor (Nakerakanti and Trojanowska 2012). The activation of the inactive form of TGF-β can occur by the action of several factors in the fibrotic tissues and, as such, these factors may have a preponderant function in fibrosis progression (Nakerakanti and Trojanowska 2012).

The active form of TGFβ is regulated by fibronectin that prevents its overproduction in the tissues (Kawelke et al. 2011). However, no differences in the expression of TGF-β1 mRNA levels in equine endometrium were found between endometrial categories (Cadario et al. 2002). Later it was suggested that TGF-β1 might be involved in equine endometrial fibroblast differentiation into myofibroblasts, as the concentration of TGF-β1 increased with the grade of endometrial fibrosis (Ganjam and Evans 2006). Nevertheless, a contradictory study has found that TGFβ expression was downregulated in stromal cells within fibrotic foci (Kiesow et al. 2011). A study by Szóstek-Mioduchowska and collaborators also described an up-regulation of α-SMA,

COL1, COL3 and fibronectin protein relative abundance in TGF-β1 treated equine endometrial explants (Szóstek-Mioduchowska et al. 2019a).

Inhibition of TGF-β might appear at first sight as a promising therapeutic, however it showed intolerable side effects due to its critical function in the maintenance of homeostasis (Ueha et al. 2012).

1.1.2. MMPs and TIMPs

Matrix metallopeptidases (MMPs)–are involved in fibrosis development (Robert et al. 2016; Djuric and Zivkovic 2017; Wang and Khalil 2018), but little is known regarding their regulation and involvement in equine endometrosis. They are the most important enzymes involved in ECM turnover and are secreted into the extracellular space as pro-MMPs or zymogens by several cell types (Nissinen and Kähäri 2014; Harvey et al. 2016). Several mechanisms such as gene transcription, protein production, pro-enzyme activation and activity inhibition regulate the activity of MMPs (Sternlicht and Werb 2001; Harvey et al. 2016). The gene expression of MMPs is induced by growth factors, cytokines, hormones, and interactions cell-cell and cell-extracellular matrix (Vandooren et al. 2013; Harvey et al. 2016). Signaling pathways, including mitogen-activated protein kinase (MAPKs), are involved in the regulation of MMP gene expression (Vandooren et al. 2013) and can promote or inhibit MMP transcription in a cell type dependent manner (Harvey et al. 2016). Posttranscriptional regulation includes messenger ribonucleic acid (mRNA) stability, protein translational efficiency, and regulation by miRNAs (Pardo et al. 2016; Djuric and Zivkovic 2017). After translation, MMPs are activated in the pericellular space by other MMPs or by serine proteases (Harvey et al. 2016). The MMP family comprises four classes of enzymes: collagenases, gelatinases, membrane type enzymes and stromelysins (Vandooren et al. 2013; Djuric and Zivkovic 2017). Besides cell proliferation, migration and differentiation, angiogenesis, apoptosis, and tissue repair (Wang and Khalil 2018), MMPs can degrade ECM contents. Since some MMPs are anti-fibrotic whereas others are pro-fibrotic (Giannandrea and Parks 2014), this might explain the conflicting results regarding the expression of MMPs in fibrotic processes. Furthermore, the action of MMPs is tissue dependent and, as such, their action may differ between distinct organs (Giannandrea and Parks 2014). Both MMPs and TIMPs play an important role in the regulation of ECM turnover. The activity of MMPs can be inhibited by TIMPs, which bind to the active site of the MMPs blocking their action of degradation of ECM components (Harvey et al. 2016; Djuric and Zivkovic 2017; Wang and Khalil 2018). The TIMP family comprises four types, TIMP-1, -2, -3 and -4, and they are capable of inhibiting over 20 MMMPs (Harvey et al. 2016). TIMP-1 is a specific inhibitor for MMP-9 (Vandooren et al. 2013), while TIMP-2 regulates MMP-2 activity (Giannandrea and Parks, 2014). TIMPs also have other functions, such as, cell growth promoting, anti-apoptotic, steroidogenic,

and antiangiogenic activities which are somewhat independent of MMP inhibition (Robert et al. 2016).

MMP-2 and MMP-9 are gelatinases that degrade collagens and other ECM components (Vandooren et al. 2013; Djuric and Zivkovic 2017) and seem to be involved in equine endometrosis (Aresu et al. 2012; Centeno et al. 2018; Crociati et al. 2019; Szóstek-Mioduchowska et al. 2020a). Walter et al. (2005), reported that the expression of MMP-2 was increased in fibrotic endometrial tissues, mainly in diestrus, and another study determined that MMP2 transcription was upregulated in endometrial fibrosis (Centeno et al. 2018; Centeno 2019). However, Aresu (2012) found no differences in MMP-2, MMP-9, MMP-14 (an enzyme capable to activate MMP-2 and MMP-9) and TIMP-2 expression between equine endometrial categories. Another study demonstrated an increase in MMP-2 in glandular epithelial cells of fibrotic endometrium, but a decrease in MMP-9 in vascular walls in fibrotic tissues (Porto et al. 2011). Therefore, it was proposed that this decreased expression of MMP-9 in vascular walls could be related to impaired angiogenesis, which would lead to a greater accumulation of fibrotic tissue (Porto et al. 2011). In another study, MMP-2 was also increased in stromal cells of all endometrial foci, and it was hypothesized this could be responsible for the progressive destruction of the glandular basal lamina (Kiesow et al. 2011). On the contrary, decreased transcript levels of MMP-2, MMP-14, and TIMP-2 and elevated transcription levels of MMP-9 were reported in fibrotic endometrium, when compared to healthy tissue (Falomo et al. 2015). In another study, it was observed a decrease in *TIMP2* and an increase in *MMP2* transcripts levels in cat III endometrium, when compared to cat I and II, in mares (Centeno 2019). Furthermore, in equine endometrial fibroblasts the expression of MMPs and TIMPs was altered after treatment with TGFβ1(Szóstek-Mioduchowska et al. 2020a).

1.2. Diagnosis of Endometrosis

1.2.1. Endometrial Biopsy

Evaluation of the degree of endometrial fibrosis is essential as fibrosis is irreversible, and if severe, it becomes responsible for decreased reproductive performance of the mare (Kenney and Doig 1986). Most degenerative changes paramount of endometrosis can only be diagnosed through the histological evaluation of an endometrial biopsy (Kenney 1978; Kenney and Doig 1986; Ricketts and Alonso 1991; Ricketts and Barrelet 1997; Katkiewicz et al. 2007; Zajac et al. 2008; Schlafer 2007; Snider et al. 2011; Aresu et al. 2012). Uterine biopsy is a standard and safe method to evaluate the endometrium, to grade it and most importantly to give the clinician a prognosis of likelihood of the mare to carry a pregnancy to term (Kenney 1978; Ricketts and Barrelet 1997). It is also the main (and only) procedure for histopathologic assessment of equine uterine health (Kenney et al. 1978; Snider et al. 2011) for over 40 years. Pregnancy is the only

known contraindication for the performance of the biopsy (Kenney and Doig 1986; Schlafer 2007; Snider et al. 2011). Depending on the degree of intensification of structural changes in the endometrium, such as the extent of inflammation, and/or fibrosis, lymphatic system and atrophic changes, Kenney and Doig (1986) assigned mare endometrium into four categories. Briefly the endometrium from category I presents a normal histology or with very mild, focal inflammation or fibrosis, category IIA shows mild to moderate inflammation and/or multifocal fibrosis with 1–3 layers of fibroblasts surrounding the endometrial glands; category IIB has moderate inflammation and/or multifocal–diffuse fibrosis with 4 or more layers of fibroblasts surrounding the endometrial glands; and category III corresponds to an endometrium with severe inflammation and/or extensive fibrosis. The endometrial category increases with more advanced histopathological changes in the endometrium and the prognosis of pregnancy and maintenance of pregnancy worsens (Kenney and Doig 1986). Although several amendments have been done to the histopathological classification of the endometrium (Schoon et al. 1997; Schoon and Schoon 2003; Hoffman et al. 2009a; Lehmann et al. 2011; Snider et al. 2011), the most widely and commonly used in equine practice is still Kenney and Doig's classification (1986). However, the limitations of Kenney-Doig system relate to its disregard of important histological findings, such as angiosis and endometrial maldifferentiation (Snider et al. 2011).

Even though mare endometrial biopsy is considered as safe and practical (Kenney 1978) and used as a routine standard procedure in the breeding examination of mares, there are some drawbacks. The endometrial biopsy classification may be biased since the interpretation of histopathological lesions is highly subjective and variable due to the different grading experience of pathologists/theriogenologists, heterogenous endometrial sampling site and tissue characteristics. Indeed, a study by Westendorf demonstrated a significant difference between individual pathologists grading tendencies, and the categories IIA and IIB were the ones that most varied (Westendorf 2022). Additionally, some researchers concluded that collection of an endometrial specimen from one uterine portion does not necessarily reflect the state of its remaining areas (Keller et al. 2006; Fiala et al. 2010). However, according to other researchers, a single biopsy represents very well the condition of the whole uterus if the biopsy material has proper size and shape, and it is prepared and evaluated in the appropriate way (Kenney 1978; Kenney and Doig 1986). Thus, caution should be taken when interpreting research data based on endometrial biopsy classification.

1.2.2. Uterine biomarkers

A biomarker is a biological indicator of a pathophysiological process or of a treatment response that can be objectively evaluated (Moore et al. 2007; Callif 2018; Taylor 2019). Besides their use for the diagnosis of a pathological condition, some biomarkers have prognostic value

or can predict the response to a certain treatment (Moore et al. 2007; Callif 2018; Taylor 2019). Currently histopathological examination of mare endometrium biopsy is the only available standardized scientific approach, although not perfect. Apart from microscopic examination of the biopsy material, immunohistochemical examination can also be performed (Schlafer 2007). Also, analyses of uterine lavage have been used in the identification of patterns of secretion of equine endometrial proteins in endometrosis affected mares (McDowell et al. 1987; Bader et al. 1997; Lehmann et al. 2011).

Hoffmann (2006) established histochemical staining procedures and immunohistochemical methods to characterize the secretory patterns of selected endometrial proteins in mares suffering from endometrosis. Uterocalin (UC), the most prominent progesterone dependent uterine derived protein is abundantly expressed during pregnancy (Ellenberger et al. 2008) and transports small hydrophobic molecules from the mare to the conceptus (Crosset et al. 1998; Suire et al. 2001). Uteroglobin (UG) is another progesterone dependent protein of the horse uterus that appears to protect the trophoblast from the immune system response of the mother (Miele et al. 1994) and also has anti-inflammatory and antichemotactic activities (Miele et al. 1994; Mukherjee et al. 1999). Uteroferrin (UF) is also found in uterine secretions from the pregnant mare, is involved in iron transport during pregnancy (Roberts et al. 1984; Ellenberger et al. 2008) and its secretion is induced by progesterone and synergistic effects of estrogen, released by the equine conceptus (Bazer et al. 1991). The calcium-binding protein calbindinD9k (CAL) is a small protein, which is expressed by the uterus (Inpanbutr et al. 1994) and its responsible for the transport of calcium from the apex of the glandular epithelia and from the blood to the lumen of the uterus (Wooding et al. 2000). Hoffman described a decreased expression pattern of uterocalin (UC), uteroglobin (UG) and CalbindinD9K (CAL) and an increased expression of uteroferrin (UF) in fibrotic endometria when compared with healthy endometrium (Hoffmann et al. 2009b). An investigation, by Lehman and collaborators, attempted to ascertain whether the degree of fibrosis and any obvious deviations of the endometrial protein patterns should be considered as additional parameters to the classification system for a more precise fertility prognosis (Lehmann et al. 2011). It was concluded that only uterogoblin and uterocalin should be utilized when trying to refine the endometrial biopsy classification for the mare population, as this study was not in agreement with previous work regarding uteroferrin and calbindinD9K (Hoffman et al. 2009b; Lehmann et al. 2011). It was also suggested that barren mares suffering from a moderate destructive endometrosis should be graded in category III, especially when obvious deviations in the protein expression pattern of UG and UC are observed.

These results indicate the necessity of a more detailed classification system for endometrosis and suggest that the degree of fibrosis, as well as immunohistochemical analysis, with a particular focus on UG and UC, for more precise fertility prognosis Kenney and Doig 1986;

Schoon et al. 1992). Furthermore, it was considered that some of the described molecular markers qualify as biomarkers and could lead to an objective measurement of endometrial dysfunction and might help to clarify the fertility prognosis for an individual mare (Schöniger and Schoon 2020).

The combination of immunohistochemistry staining, for vimentin and cytokeratin, with second harmonic generation, for detection of collagen without staining, seems to be useful for validation and further characterization of the routine hematoxylin-eosin, and may be used as a supporting aid for the diagnosis of endometrosis (Rojas et al. 2020). Rojas et al. (2020) used both techniques to evaluate the progression of equine endometrosis. Vimentin expression is indicative of the presence of fibroblasts and the absence or decrease in steroid receptors may cause changes in the function of endometrial glands. The vimentin and cytokeratin (an epithelial cell marker) expression differed with the degree of the endometrosis. Cytokeratin was detected in glands from healthy mares but was nearly absent with the progression of the condition. Vimentin was mainly detected in endometrial biopsies with absent or mild fibrosis. The progesterone receptor showed no correlation with endometrosis and the lymphocyte T specific CD3 marker was not detected in the more severe cases of endometrosis (Rojas et al. 2020).

As an alternative, and/or as a complementary diagnostic approach, the development of less invasive and more reliable techniques, such as blood biomarkers would be desirable (Katila and Ferreira-Dias 2022).

1.3. Treatment

For the time being there is no effective treatment for equine endometrosis and this narrows the therapeutic options for clinicians working in equine reproduction. The changes to the endometrium are considered by most authors to be irreversible. However, there is some evidence of treatments that include mechanical curettage or use of chemical agents such as kerosene, dimethyl sulfoxide (DMSO) and isotonic salts which may be beneficial (Keller et al. 2006).

The physical curettage applied to mares with various degrees of endometrosis combined with intrauterine administration of antibiotics to prevent infection, was proposed (Allen 1993). An improvement of endometrial biopsy grade was observed in 44% of mares, no effect was seen in 51% of the mares, while in 5% of mares it was worse, after the treatment. Also, no significant effect on the fertility rates was observed and the prognosis was worse in older mares, where curettage had no effect (Ricketts 1985). Several attempts to use kerosene intra-uterine had different outcomes. Improved fertility rates were observed after irrigating the uterus with 250-500 ml of kerosene, which induced uterine oedema resulting in expulsion of retained excretions from the uterine glands (Bracher et al. 1991). Other research demonstrated that the use of

kerosene therapy only had a short-term effect, as half of the pregnant mares, previously treated with kerosene, miscarriage later (Allen 1993). Most recently, intra-uterine kerosene therapy was re-evaluated, and no effect was observed on endometrial histopathology grade (Podico et al. 2020). The treatment with DMSO due to its anti-inflammatory properties was also suggested and undertaken to reduce fibrosis. Although the intrauterine administration of DMSO (10-30%) caused a reduction in chronic inflammatory cell infiltrates and periglandular fibrosis in 30% of the tested mares, the pregnancy rates did not improve when compared to saline-treated mares (Ley et al. 1989).

Treatment of equine endometrial fibrosis with mesenchymal stem cells (MSCs) was also attempted, due to their immunomodulation activity and capacity to regenerate tissues. Mesenchymal stem cells are heterogeneous stromal cells that have self-renewal capacities and differentiate into a wide variety of specialized cell types (Gulati et al. 2013). By producing bioactive mediators and adhesion molecules, MSCs reduce scar tissue formation and cell apoptosis, increase angiogenesis, and stimulate the intrinsic cell population to regenerate function (Gulati et al. 2013). The equine MSCs were incorporated in clusters in periglandular and glandular tissue, by a similar method to artificial insemination and were widely distributed in the uterus of mares with endometrosis, suggesting that MCSc proliferate within the endometrium (Mambelli et al. 2013). Another study, by the same group demonstrated that the intrauterine transplantation of equine adipose tissue-derived MSCs in mares with endometrosis induced a positive remodeling of the endometrial tissue until day 60. It was also described that the α-SMA expression, modulated by the MSCs, was no longer observed at day 7 in uterine glands, suggesting the use of MSCs as therapy in endometrosis (Mambelli et al. 2014). In contrast, notwithstanding the successful incorporation of adipose-derived stem cells in endometrial periglandular tissue and single glands, there was an increase in pro-inflammatory interleukins (Falomo et al. 2015). Other studies with MSCs successfully administered autologous bone marrow MSCs by endometrial injection, opening the clinical trials for the use of these stem cells (Alvarenga et al. 2016). The presence of MSCs were firstly identified in equine endometrium in 2017, offering a promising new therapeutically approach for endometrial regeneration (Rink et al. 2017). These cells were autologously infused in the healthy uterine horns of mares in early diestrus and were identified after 6, 12 and 24h in the uterine lumen, but not in the endometrial tissue, restraining the promising results (Rink et al. 2018). Moreover, a recent study did not support the use of MSCs for the treatment of age-related ovarian dysfunction in mares, as the intra-ovarian injection of MSCs from donors, did not improve ovarian function in aged mares (Grady et al. 2019).

2. Epigenetics in biological processes

Genetics is the study of heritable changes in gene activity or function due to the direct alteration of the deoxyribonucleic acid (DNA) sequence (Egger et al. 2004). In contrast, epigenetics studies heritable changes in genome function that do not modify the nucleotide sequence and have a prominent role in the regulation of gene expression (Waterland 2006; Jirtle and Skimer 2007; Berger et al. 2009; Handy et al. 2011; Neary et al. 2015). In 1940, Waddington proposed the existence of an epigenotype to explain certain aspects of development that were influenced by the environment and not the result of changes in the genotype (Waddington 1940) and introduced the term epigenetics in 1942 (Waddington 1942). However, the correlation between gene repression and differentiation was only established in 1980. Not all genes are expressed simultaneously by all cell types, even though virtually all cells in an organism contain the same genetic information (Gibney and Nolan 2010; Moore et al. 2013). The various gene expression profiles in many cells and tissues are mediated by epigenetic mechanisms (Barros and Offenbacher 2009; Gibney and Nolan 2010; Moore et al. 2013; Al Aboud et al. 2022). In mammals, DNA-based processes are highly regulated by epigenetic mechanisms that impact biology and chromatin transcriptional states. Epigenetic modifications often take place during the lifetime of an organism and if these alterations occur in germ cells they can be transferred to the next generation (Bird 2007; Chandler 2007; Plunk and Richards 2020).

Epigenetics comprise three main mechanisms: DNA methylation, histone modifications and non-coding RNAs, including long and short non-coding RNAs (Turner 2002; Egger et al. 2004; O'Reilly 2017, Loaeza-Loaeza et al. 2020). These mechanisms affect promoter function and gene transcription through a wide variety of interactions, allowing or restricting the access of transcription factors, transcriptional machinery, and epigenetic modifiers of DNA sequence, and therefore regulate all biological process in the body from conception to death (Delcuve et al. 2009; Miller and Grant 2013) These events that contribute to genome reorganization and cell differentiation are vital during early development and have now also been linked in disease pathogenesis (Egger et al. 2004¸ Dwivedi et al. 2011). In fact, epigenetic alterations may result in various disorders such as cancer (Moosavi and Ardekani 2016; Zhang et al. 2017; Han et al. 2019; Zhang et al. 2020) and fibrosis (Helling and Yan, 2015; Moran-Salvador and Mann 2017; O'Reilly 2017; Bartczak et al. 2020; McErlean et al. 2021; Shao et al. 2022). Epigenetic changes are responsible in determining which proteins are transcribed, by switching the genes on or off (Simmons 2008; Nightingale 2015), contributing to cell plasticity and therefore, to distinct phenotypes (Loaeza-Loaeza et al. 2020). Epigenetic mechanisms have some regulation steps that are accomplished by epigenetic writers (enzymes that establish DNA methylation or histone modifications by adding a methyl group), erasers (enzymes that remove these markers) and

readers (enzymes that bind to modifications and facilitate epigenetic activities), allowing the dynamic cell-specific gene expression (Schübeler 2015; Xu et al. 2017; Biswas and Rao 2018).

Epigenetics is also important for X-chromosome inactivation in female mammals, preventing females from having twice the number of X-chromosome gene products as males (Egger et al. 2004; Kalantry 2011). Moreover, the fact that some epigenetic marks can be reversible lead to increasing studies on epigenetic therapy (Kelly et al. 2010; Miranda-Furtado et al. 2019; Bates 2020; Licht and Bennett 2021).

2.1. DNA Methylation

Methylation of DNA is the process of adding a methyl group to a nucleotide within the DNA. This methyl group is commonly donated by S-adenosyl-L-methionine (AdoMet) and its transfer is facilitated through the action of the DNA methyltransferase (DNMT) family of enzymes (Okano et al. 1998; Cheng and Roberts 2001). In earlier *in vitro* studies on the composition and biochemical properties of nuclei acids, 5-methylcytosine (5mC) was discovered and it was described as an unknown element, in the DNA (Wheeler 1910). From studies in both vertebrates and plants (Vanyushin et al. 1970) emerged the first hint of 5mC in gene regulation (Holliday and Pugh 1975). The direct correlation between gene repression and differentiation was established in 1980 (Loaeza-Loaeza et al. 2020). This methyl group can be transmitted to daughter cells after cell division and can influence the binding of sequence-specific and methyl-binding proteins (Law and Jacobsen 2010). DNA methylation is now, one of the best-characterized epigenetic modifications, and it has a critical role in active and inactive chromatin equilibrium for gene expression control (Lee et al. 2014). It was accepted for several years that methylation played a crucial role in repressing gene expression, possibly by blocking the promoters at which activating transcription factors should bind (Turek-Plewa and Jagodzinski 2005). DNA methylation mediates gene expression and higher promoter methylation is correlated with low or no gene transcription (Suzuki and Bird 2008). Moreover, methylation near gene promoters varies considerably depending on cell type (Suzuki and Bird 2008). Gene silencing by DNA methylation is necessary to balance and regulate cell biological processes. DNA methylation increases the information contained in the DNA sequence, changing the functional status of a gene from active to inactive or vice versa (Lee et al. 2014; Schübeler et al. 2015). The pattern of DNA methylation in the genome changes during development, due to a dynamic process that involves both *de novo* DNA methylation and demethylation. Consequently, differentiated cells develop a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription (Moore et al. 2013). Therefore, the pattern of DNA methylation is an important component of the regulatory mechanisms of gene expression (Leonhardt et al. 1992; Liu et al. 1998; Okano et al. 1999; Plachot and Lelièvre 2004; Mann et al. 2007; Miranda and Jones 2007; Lee and Dunn 2008; Poplawski et al. 2008; Prokhortchouk and Defossez 2008).

The methylation of DNA occurs predominantly at CpG dinucleotides, where a cytosine and guanine are separated by a single phosphate (Gruenbaum et al. 1982; Bird 2002). The methylation occurs specifically on the 5th carbon atom of the cytosine ring (Meehan et al. 1992) and is then referred to as 5-methylcytosine (5mC). In the mammalian genome, 60-90% of CpG dinucleotides are methylated (Gruenbaum et al. 1981; Cross and Bird 1995), and are found in genome regions that are transcriptionally inactive and late replicating (Meehan and Stancheva 2001).

CpG islands are sequences of repeating C and G nucleotides with about 1000 base pairs in length that have a higher frequency of CpG sequences than the rest of the genome, and often are not methylated (Bird et al, 1985; Saxonov et al. 2006; Sharif et al. 2010; Deaton and Bird 2011). A defining feature of CpG islands is that they tend to escape DNA methylation whereas cytosines in the genome as a whole, and in repetitive DNA in particular, tend to be heavily methylated (Yoder et al.,1997). Almost half of the genes in humans (~40%) and mouse (~47%) have CpG islands in their promoters (Akan and Deloukas 2008), which strongly suggest a correlation between CpG islands and gene transcription in mammals (Sharif et al. 2010). In fact, 70% of CpG islands are located within gene promoters (Saxonov et al, 2006).

DNA hypermethylation is usually associated with gene repression (Fuks 2005; Mohn et al. 2008) and hypomethylation of DNA with increased gene expression (Karouzakis et al. 2009), however methylation does not always cause a change in expression (Suzuki and Bird 2008).

Although DNA is a considerably stable storage form of genetic information, chemical modification of DNA molecules can still occur. These modifications can seriously impair DNA and induce several DNA repair mechanisms to prevent the accumulation of mutations. Potential detrimental DNA modifications, spontaneous or mutagen-induced, usually include oxidation of bases, conversion of amine groups to diazo groups, formation of covalent adducts, deamination of cytosines or adenines, loss of purine bases to form apurinic sites, or generation of strand breaks (Chahwan et al. 2011). However, DNA methylation, a distinct group of modifications that occurs mainly within CpG dinucleotides in vertebrates (Bird 2002), does not trigger repair mechanisms. In fact, DNA methylation is essential for normal development, regulating tissue-specific gene expression and is also associated with genomic imprinting (Reik and Walter 2001) and inactivation of the X-chromosome (Wolf and Migeon 1982).

2.1.1. Location of DNA Methylation

The genomic region where DNA methylation occurs may influence gene activities based on the underlying genetic sequence (Moore et al. 2013). The frequency of CpG dinucleotides is extraordinarily low in the mammalian genome (Bird 1980). However, there are isolated sites that are noticeably CpG rich and are prominent landmarks in otherwise CpG poor areas of the genome (Sharif et al. 2010).

2.1.1.1. Intergenic Regions

Roughly around 45% of the mammalian genome comprises transposable and viral elements that are suppressed by bulk methylation (Schulz et al. 2006). Most of these elements are inactivated by DNA methylation or by mutations acquired in course of time due to the deamination of 5mC (Walsh et al. 1998). If these elements are expressed, their replication and insertion can result in gene disruption and DNA mutation, which may be potentially hazardous (Wu et al. 1997; Gwynn et al. 1998; Ukai et al. 2003). Therefore, one of the main roles of DNA methylation, within intergenic regions, is to inhibit the expression of potentially harmful genetic elements.

2.1.1.2. CpG Islands

CpG islands are stretches of DNA around 1000 base pairs long and they have a higher CpG density than the rest of the genome and are usually associated with gene promoters (near the transcription start sites), which are especially rich in CpG sequences. While 70-80% of CpG sites are methylated, the remaining unmethylated CpG sites assemble in a cluster which is recognized as CpG islands (Erlich et al. 1982; Ramsahoye et al. 2000; Bird 2002; Lister et al. 2009; Xin et al. 2011).

However, they are usually not methylated when located in the promoter regions (Bird et al. 1985; Saxonov et al. 2006; Irizarry et al. 2009; Sharif et al. 2010; Nishiyama and Nakanishi 2021) and are therefore favourable for gene expression (Sharif et al. 2010). Several of these hypomethylated regions of DNA modulate gene expression, such as promoters and enhancers (Nishiyama and Nakanishi 2021). Moreover, after analysing the unmethylated DNA and methylated CpG as ligands it was found that DNA methylation promotes the binding of many transcription factors (Yin et al. 2017).

In the human genome the promoters can be divided into two classes in respect to their CpG content: high CpG content (HCP) promoters and low CpG content (LCP). Both groups are highly conserved in vertebrates (Saxonov et al. 2006; Weber et al. 2007; Elango and Yi 2008). The HCP promoters constitute 72% of promoters and LCP 28%, the latest being characteristic of the overall genome (Saxonov et al. 2006). Most CpG islands, roughly 70%, lie in within gene promoters (Saxonov et al. 2006). Promoters with high CpG content normally have low methylation rates and are widespread in genes with higher expression which is the case of housekeeping genes that often have inserted CpG islands in their promoters (Gardiner-Garden and Frommer 1987). In fact, evidence from some studies advocates that non-methylated CpG sequences are more often connected with “house-keeping” genes than with tissue specific genes (Larsen et al. 1992; Ponger et al. 2001; Robinson et al. 2004). On the opposite, LCP promoters are usually methylated (both maintenance and *de novo* methylation) and occur in genes with lower or inhibited expression (Weber et al. 2007). The discreet differences that turn

out in loss of CpGs content are often noticed in LCP promoters due to the occurrence of mutations derived from transitions of methylated cytosines to thymine (5mC < T) (Weber et al. 2007). In promoters with intermediate CpG content the transcription is regulated by methylation and depends on tissue, differentiation, and cell cycle (Weber et al. 2007; Ball et al. 2009; Lienert et al. 2011). The CpGs elements have been evolutionarily preserved in an effort to modulate gene expression alterations between an embryonic state and a differentiated state (Robertson 2005).

CpG islands are enriched in nearly half of the promoters in humans and mice, suggesting an important role for CpG islands in mammalian transcriptional regulation (Akan and Deloukas 2008; Sharif et al. 2010). Remarkably, CpG islands accumulation at the transcription start sites seems to be a vertebrate specific genomic feature, suggesting a link between CpG islands and evolution (Sharif et al. 2010).

The location and preservation of CpG islands throughout evolution suggests that these regions have an important functional role in gene expression through chromatin structure regulation and transcription factor binding (van Eijk et al. 2012; Pervjakova et al. 2016). DNA is usually wrapped around histone proteins forming the nucleosomes. DNA is less accessible for gene expression when its association with histone proteins is tighter. Among other features, CpG Islands have the particularity of possessing less nucleosomes than other stretches of DNA (Tazi and Bird 1990; Ramirez-Carrozzi et al. 2009; Choi 2010). The few nucleosomes associated with CpG islands usually contain histones with modifications that are involved in gene expression promotion (Tazi and Bird 1990; Mikkelsen et al. 2007). Frequently, CpG islands lack common promoter elements such as TATA boxes, nevertheless 50% of CpG islands contain known transcription start sites (Carninci et al. 2006). CpG islands enhance the accessibility of DNA and promote the binding to transcriptional start sites possibly because the binding sites for transcription factors are rich in GC nucleosides. DNA methylation of CpG islands regulates gene expression during development and differentiation (Weber et al. 2007; Mohn et al. 2008; Meissner et al. 2008), and this is especially important for establishing imprinting (Zwart et al. 2001; Choi et al. 2005). As CpG islands are linked with gene expression regulation, it is possible that CpG islands might present tissue-specific patterns of DNA methylation (Moore et al. 2013). However, the role of CpG islands in regulating gene expression is still being unveiled. The methylation of CpG islands results in impaired binding of transcription factors, recruitment of repressive methyl-binding proteins and stable silencing of gene expression (Mohn et al. 2008). Nevertheless, CpG islands are hardly ever methylated, particularly those associated with gene promoters (Greally 2013; Pfeifer 2018).

2.1.1.3. Gene Body

The gene body is regarded as the region of the gene past the first exon. Methylation of the first exon causes gene silencing, as in promoter methylation (Brenet et al. 2011). However, some studies suggested that gene body methylation is linked to increased gene expression in dividing cells (Hellman and Chess 2007; Ball et al. 2009; Aran et al. 2011).

2.1.2. Mechanisms of DNA methylation

The enzymes that establish, remove, and recognize DNA methylation can be divided into three classes: writers, erasers, and readers. Writers are the enzymes are responsible for the addition of methyl groups onto cytosine residues. Erasers modify and remove the methyl group. Readers recognize and bind to methyl groups to finally regulate gene expression. Numerous proteins and mechanisms involved in DNA methylation have been identified due to several studies committed to unveil the epigenetic modifications during embryonic development (Moore et al. 2013). The methylation and demethylation of DNA is a stable inherited epigenetic modification in which the DNA sequence is not modified and still induces a change in gene expression (Ciechomska et al. 2014). DNA methylation occurs through the action of one of three enzymes called DNA methyltransferases (DNMT1, DNMT3A or DNMT3B) (Robertson 2002; Egger et al. 2004). The insertion of methyl groups alters DNA appearance and structure of DNA and changes the interactions of a gene with the machinery within a cell's nucleus, that is required for transcription. (Simmons 2008). Methylation and 5-methylcytosine (5MeC) formation results in repression of gene transcription by two possible mechanisms. The first is by direct interference through the action of 5MeC, that can hinder transcription factors from specifically bind to recognition sites in their respective promoters (Das and Singal 2004). In the second, gene silencing may occur by direct binding of specific repressor transcription factors to methylated DNA. These methyl-CpG-binding proteins (MeCPs) can regulate the expression of many genes through their interaction with methylated DNA and their association with histone modifying enzymes (Mann et al. 2010). The first two protein complexes found were MeCP1 and MeCP2, but subsequently many others were identified, such as methyl CpG binding domain 1 (MBD1), MBD2 and MBD4. The majority of these repressor protein complexes use their methyl CpG binding domain (MBD) motif to bind to methylated DNA (Das and Singal 2004).

All the aforementioned epigenetic proteins (readers, writers, and erasers) can be targeted by small-molecule inhibitors and therefore, are extremely promising regarding therapeutics (Ganesan et al. 2019)

2.1.2.1. Writers of DNA methylation – DNA methyltransferases (DNMTs)

DNA methyltransferases (DNMTs) play an essential role in DNA methylation and in the transcriptional regulation in the genome (Hermann et al. 2004). DNMTs regulate the dynamic

DNA methylation patterns and a thorough comprehension of these mechanisms is crucial as DNA methylation modulates gene expression in health and disease (Loaeza-Loaeza et al. 2020; Dhar et al. 2021). In humans and other mammals, there are mainly three catalytically active DNMTs: DNMT1, DNMT3A, and DNMT3B, that mediate DNA methylation (Zhang et al. 2020). DNMT1 is recruited to sites of DNA and is responsible for maintaining the already existing methylation, while DNMT3A and DNMT3B establish new methylation patterns, and are therefore referred to as *de novo* DMTs (Leonhardt et al.,1992; Liu et al. 1998; Okano et al. 1999; Plachot and Lelièvre 2004; Mortusewicz et al. 2005; Miranda and Jones 2007; Prokhortchouk and Defossez 2008; Zhang and Xu 2017). Additionally, DNMT3 and DNMT1 act synergistically, as there is evidence that methylation by DNMT3 stimulates DNMT1 to further methylate the DNA (Fatemi et al. 2002).

Abrogation of expression of these finely regulated enzymes results in aberrant methylation (Bestor 2000; Rhee et al. 2002). The precise regulation of genes through methylation varies from specific allele methylation patterns to differentially expressed genes between stem cells and adult cells (Loaeza-Loaeza et al.2020).

The three DNMT enzymes (DNMT1, DNMT3A and DNMT3B) have unique functions and expression patterns, although they share a similar structure with a large N-terminal regulatory domain and a C-terminal catalytic domain (Yen et al. 1992; Xie et al. 1999). Some studies have demonstrated that abnormalities in gene expression caused by alterations in DNMT activity and function, are closely associated with cancer development (Gao et al. 2008; Micevic et al. 2017). Also, other studies emphasized similarities between cancer development and prolonged fibrogenesis (Ding et al. 2006; Bechtel et al. 2010). Another study reported that hypoxia-induced pro-fibrotic changes were associated with global DNA hypermethylation, and increased expression of DNMT enzymes in rat cardiac tissue (Watson et al. 2016). A study by Neveu et al. 2015, demonstrated that DNMT activity was upregulated in mouse lung fibroblasts, through the action of TGF-β1.

Additionally, DNA methyltransferase DNMT3L, is a DNMT3-like protein that lacks a catalytic domain. DNMT3L can interact with DNMT3A and DNMT3B to improve their catalytic activity and positively regulate DNA *de novo* methylation (Jurkowska et al. 2008).

2.1.2.1.1. Structure and Function of DNMT1

DNMT1 is an abundant enzyme in somatic cells and binds to the newly synthesized DNA and accurately imitates the original methylation pattern present before DNA replication (Hermann et al. 2004). DNMT1 preferentially methylates hemimethylated DNA, unlike the other DNMTs (Pradhan et al. 1999; Ramsahoye et al. 2000). Therefore, the activity of DNMT1 is basically dependent on a hemimethylated substrate (Vilkaitis et al. 2005). For this reason, DNMT1 is known as the maintenance DNMT because it maintains the original pattern of DNA

methylation in a cell lineage. Additionally, DNMT1 can also repair DNA methylation (Mortusewicz et al. 2005). This enzyme is highly conserved in eukaryotes and is essentially expressed in dividing cells. DNMT1 comprises 1620 amino acids and 10 conserved motifs that are involved with its catalytic function (Ye et al. 2018). It has a large N-terminal domain with regulatory function and a smaller C-terminal catalytic domain related to DNA methyltransferase activity. The active centre of the C-terminal domain interacts particularly with a predilection 30–40-fold higher for hemi methylated DNA (Jeltsch 2006; Bashtrykov et al. 2012). In DNMT1 knockout mice it was demonstrated that this enzyme is necessary for correct embryonic development, genomic imprinting, and X-chromosome inactivation (Robertson 2001; Rountree et al. 2001). DNMT1 plays a vital role in cellular differentiation as well as in dividing cells (Moore et al. 2013). DNMT1 was the first of DNMTs to be identified with aberrant expression in human cancer (Kanai et al. 2003). Mutations in DNMT1 generally cause neurological diseases in humans (Klein et al. 2011). In Humans, DNMT1 is associated with early embryo implantation in the endometrium, where it is an important component of the DNA replication complex (Zhang et al. 2020). Regarding fibrosis, it was described that DNMT1 mediates anti-fibrotic Ras protein activator like 1 (*Rasal1)* gene methylation, in mouse kidney fibroblasts (Bechtel et al. 2010).

2.1.2.1.2. Structure and Function of DNMT3A and DNMT3B

DNMT3A and DNMT3B enzymes are responsible for *de novo* methylation (Hervouet et al. 2018) during early development because they can introduce methylation into naked DNA. They are very similar, particularly in their catalytic domain, sharing a homology of roughly 84% (Gowher et al. 2006). Nevertheless, their methylation mechanisms and nonredundant functions are distinct. While DNMT3A has a cooperative methylation mechanism, DNMT3B has a noncooperative methylation mechanism (Norvil et al. 2016; Rinaldi et al. 2016). The gene expression pattern is what primarily distinguishes DNMT3A from DNMT3B. DNMT3A is expressed nearly everywhere, whereas DNMT3B is poorly expressed by most of differentiated tissues (Xie et al. 1999). While DNMT3A consists of 26 exons/25 introns, codes for 912 amino acids and is found on the small arm of chromosome 2 at position 23.3, DNMT3B comprises 24 exons/23 introns, codes for 853 amino acids and is found on the long arm of chromosome 20 at position 11.21. Apparently, DNMT3A is necessary for normal cellular differentiation, while DNMT3B is necessary during early development (Okano et al. 1999; Iurlaro et al. 2017; Gagliardi et al. 2018). The slender structural differences between DNMT3A and DNMT3B are crucial to their differential methylation and biochemical interaction with the DNA strand. However, when overexpressed both DNMT3A and DNMT3B can methylate native and synthetic DNA without preference for hemi methylated DNA, unlike DNMT1 (Okano et al. 1999). The last member of the DNMT family is DNMT3L, a protein distinguished by the absence of the catalytic domain present in other DNMT enzymes (Aapola et al. 2000; Hata et al. 2002). Even though DNMT3L

has no catalytic function alone, it can associate with DNMT3A and DNMT3B and prompt their methyltransferase activity (Hata et al. 2002; Jia et al. 2007). DNMT3L is mostly expressed in early development and is essential for establishing maternal and paternal genomic imprinting, for methylating retrotransposons, and for compaction of the X chromosome (Hata et al. 2002; Webster et al. 2005; Zamudio et al. 2011). DNMT3L is predominantly expressed in early development and is limited to the germ cells and thymus in adulthood (Aapola et al. 2000).

2.1.2.2. Erasers of DNA methylation – Ten-eleven translocation (TET) proteins

Demethylation is a process in which a series of chemical reactions take place and where 5mC is further modified. This modification occurs through reactions such as deamination and/or oxidation that result in a product that is recognized by the base excision repair (BER) pathway and replace the modified base with naked cytosine, resulting in the demethylation of once methylated cytosines (Kholi and Zhang 2013; Neary et al. 2015). DNA demethylation is defined as one of two: passive or active. Passive DNA demethylation occurs in dividing cells. Although DNA methylation marks are quite stable and can be transmitted through generations, they are not perpetual and can be erased. DNMT1 actively preserves DNA methylation during cell replication however, its repression or dysfunction allows newly incorporated cytosine to remain unmethylated and therefore largely decreases the methylation level after each cell division (Valinluck and Sowers 2007). Active DNA demethylation needs enzymatic reactions to process the 5mC (and return it to a naked cytosine) and can occur in both dividing and nondividing cells (Oswald et al. 2000; Zhang et al. 2007). Additionally, several mechanisms of active DNA demethylation have been proposed. One is the chemical modification of 5mC at the amine group and methyl group sites. Another is found to be mediated by the ten–eleven translocation (TET) enzymes Tet1, Tet2, and Tet3 which are responsible for the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC) (Zhao et al. 2014). The conversion of 5mC to 5hmC impairs the binding of the repressive methyl-binding protein MeCP2, supporting the hypothesis that 5hmC may regulate gene expression, just as methylation (Valinluck et al. 2004). Whilst 5mC is commonly associated with gene repression, 5hmC is associated with an increase in gene expression (Kholi and Zhang 2013; Coppieters et al. 2014). In addition, it has been found that TET can also covert 5mC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al. 2011). Whilst the DNMT family of enzymes establish and maintain DNA methylation and are associated with a transcriptionally repressed chromatin state, TET enzymes are implicated in DNA demethylation and epigenetic control of gene expression, favouring gene transcription (Williams et al. 2011; Kholi and Zhang 2013).

2.1.2.3. Readers of DNA methylation – methyl-binding domain proteins

Although DNA methylation may decrease gene expression on its own, by inhibiting the binding of transcriptional activators, a second class of proteins with a large affinity for 5mC inhibits transcription factor binding. There are three distinct protein families that recognize DNA methylation: the methyl-CpG-binding domain (MBD) proteins; the ubiquitin-like PHD and RING finger domain-containing proteins (UHRF); and the zinc-finger proteins. The first of these families to be identified was the MBD protein family, which is also the best studied. The MBD proteins contain a conserved methyl-CpG-binding domain that confers a higher affinity for single methylated CpG sites, allowing them to bind to methylated-CpG sequences (Nan et al. 1993; Hendrich and Tweedie 2003). Included in this family are, the first identified methyl-Cap binding protein, MeCP2, along with MBD1, MBD2, MBD3, and MBD4 (Meehan et al. 1989; Lewis et al. 1992; Hendrich and Bird 1998). MeCP2 seems to play a distinctive role in sustaining DNA methylation and in repressing transcription.

The UHRF proteins, including UHRF1 and UHRF2, are multidomain proteins that bind methylated cytosines via a SET- and RING-associated DNA-binding domain (Hashimoto et al. 2008, 2009). UHRF protein's function is to bind to DNMT1 and then target it to hemi methylated DNA to maintain DNA methylation, particularly during DNA replication (Sharif et al. 2007; Achour et al. 2008). Unlike most methyl binding proteins, the primary function of UHRF proteins is not to bind to DNA and repress transcription.

The last family of methyl-binding proteins are the zinc-finger proteins that bind to methylated DNA by a zinc-finger domain. They incorporate Kaiso, a structurally unrelated protein, which is critical regulator of DNA damage responses in multiple cell types and has also been shown to bind methylated CGs through its three Krüppel-like C_2H_2 zinc fingers. Two of this zin-finger proteins include ZBTB4, and ZBTB38, that contain Kaiso-like zinc fingers, although unlike Kaiso they can bind single methylated CpGs (Filion et al. 2006). Zinc-finger domain proteins repress transcription in a DNA methylation-dependent manner, alike the MBD family regardless of their differences (Filion et al. 2006; Lopes et al. 2008).

Gene repression depends on the frequency of methylated-CpG sequences within the gene and on the site of the methylation in relation to the promoter region of the gene (Hsieh 1994; Bian et al. 2013).

2.2. Histone modifications

Histone modification refers to the methylation and acetylation of histone proteins by the action enzymes such as histone methyltransferases, histone acetyltransferases and histone deacetylases (Li et al. 2020; Zhang et al. 2020). Histones are a family of basic proteins that associate with DNA in the nucleus and are crucial for compacting and remodelling chromatin (Neary et al. 2015). Histones are key elements of chromatin and integral parts of the

nucleosome. The nucleosome, which is large part of chromatin, is composed of four different histones that form an octamer of two sets of four histone cores (Histone H2A, Histone H2B, Histone H3 and Histone H4) (Kouzarides 2007; Tessarz and Kouzarides 2014). This octamer binds to DNA and act as "anchors" around which the DNA strands are tightly wrapped (Maeshima et al. 2019). Additionally, there is a histone H1 which regulates the higher order chromatin structure (Fyodorov et al. 2018). Histones have a globular shape core but also contain an N-terminal tail (Mariño-Ramírez et al. 2005) that is subjected to a wide variety of post-translational modifications (Jenuwein and Allis 2001; Peterson and Laniel 2004). These histone post-translational modifications influence the regulation of gene expression, by changes in their interaction with DNA, and are linked with the function of DNMTs in many genes. The list of histone modifications is extensive and include methylation, acetylation, phosphorylation (Banerjee and Chakravarti 2011), ubiquitination, citrullination, sumoylation and ADP-ribosylation (Strahl and Allis 2000; Wang et al. 2004; Bartke et al. 2010; O'Reilly 2017). These histone modifications are mediated by histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (KDMs), which can regulate the chromatin, into active (loosen) or repressed (compacted) state. However, methylation (mono-, di-, or tri-) and acetylation are the most well studied modifications. Both are chemical process but while methylation adds a methyl group to the amino acid lysine that is situated in the histone, acetylation adds an acetyl group. Histone methylation, specially, mono-, di-, and trimethylation of lysines are associated with positive or negative chromatin methylation landscapes (Dhayalan et al. 2010; Loaeza-Loaeza et al. 2020) and may, therefore be a marker for both active and inactive regions of chromatin. For example, methylation of a particular lysine (K9) on a specific histone (H3) may silence gene transcription while methylation of a different lysine (K4) on the same histone (H3) may activate gene transcription (Egger et al. 2004). These modifications frequently compete for the same histone lysine residues, but they may also mobilize antagonistic regulatory complexes (Strahl and Allis 2000) besides being transmissible across generations (Cavalli and Paro 1998; Strahl and Allis 2000). On the other hand, acetylation is usually associated with active chromatin, while deacetylation is generally associated with heterochromatin. Furthermore, acetylation is often a required signal to other modifications, such as phosphorylation, methylation and ubiquitylation (Yang and Grégoire 2007; Yang and Seto 2008).

It has been proved that DNA methylation and histone modifications are closely linked in their regulation and promotion of gene silencing, that also dependents on the type of the cell and physiologic environment (Coward et al. 2014). Furthermore, this interaction between DNA methylation and histone modification appears to be a two-way interaction as both mechanisms can influence each other. Methyl-binding proteins such as MeCPs and MBDs may promote this interaction by recruiting histone deacetylases to methylated areas (Mann et al. 2010). DNA

methylation also plays a key role in the synergy between the epigenetic mechanisms of histone modifications and microRNA expression regulation (Neary et al. 2015). Moreover, besides controlling chromatin architecture and many aspects of the development and differentiation of organisms, posttranslational histone modifications may in some cases regulate the onset of diseases (Pedersen and Helin 2010).

Histone mark writers, such as methyltransferases or acetyltransferases, are generally counterbalanced by a plenitude of histone mark erasers, such as demethylases or deacetylases. Modifications in histone marks writers or erasers, either through environmental factors or abnormalities might induce the onset of serious diseases (Jirtle and Skinner 2007; Gillette and Hill 2015; Zhang et al. 2015; He et al. 2018).

2.2.1. Histone acetyltransferases (HATs)

Histone acetyltransferases (HATs) catalyse the addition of an acetyl group to lysine residues of histones, using acetyl coenzyme A (acetyl-CoA) as the acetyl donor (Hodawadekar and Marmorstein 2007; Berndsen and Denu 2008). Acetylation by HATS removes the positive charge of lysine which reduces its electrostatic attraction to DNA and extends nearby chromatin structure and thus allowing the access to transcription factors that activate gene expression (Kouzarides 2007; O'Reilly 2017; Duong and Hagwood 2018; Jezek and Green 2019). Histone acetylases regulate the acetylation in histone tails and P300 is one of the most important transcriptional regulators as it can also control several transcription factors (Vo and Goodman 2001). The expression of P300 is increased in systemic sclerosis (SSc) and is upregulated by TGF-β1 (Ghosh et al. 2013). Also, the hyperacetylation of H4 on the collagen promoter increases the transcription of the Smads, including Smad2 (Tu and Luo, 2007; Ghosh et al. 2013).

2.2.2. Histone deacetylases (HDACs)

Histone deacetylases (HDACs) remove the acetyl groups from the histone tails which result in the condensing of the chromatin structure and therefore repression of gene expression (Ruijter et al. 2003). Depending on their function, DNA sequence and domain organization, the HDACs can be divided in four different classes (Gray and Ekström 2001). Class I HDACs are widely expressed and are mainly found in the nucleus. They include HDAC1, 2, 3 and 8 (Gray and Ekström 2001). Class II HDACs exhibit a more limited pattern of expression and are mostly located in the cell cytoplasm. Nevertheless, they can transfer to the nucleus in response to relevant signals and feasible alter nonhistone proteins too (Glozak et al. 2005; Kouzarides 2000). Class IIa and IIb HDACs are subdivided based on the number of catalytic domains they possess. HDACs from class IIa comprises HDAC4, 5, 7 and 9 and from class IIb comprises

HDAC6 and 10 (Martin et al. 2007). Class III includes the sirtuins, nicotinamide adenosine-dependent (NAD) enzymes (SIRT family) and class IV contains only one member HDAC11, which shares sequence domains with class I and class III HDACs. Class I HDACs are often found together in large multimeric complexes called Sin3. Class I and Class II HDACs require Zn2þ for their catalysis. HDAC inhibitors are a class of compounds that inhibit the activities of HDACs.

The dynamic interplay between HATs and histone deacetylases determines the final state of acetylation (Berndsen and Denu 2008).

2.2.3. Histone methyltransferases (HMTs and HKMTs)

Histone methyltransferases (HMTs) can selectively methylate lysines and arginines in the tail of histones and induce transcription (Jelinic and Shaw 2007; Zhang et al. 2020). Histone methylation mainly occurs on lysine and arginines residues of histones H3 and H4 (Strahl et al. 1999; Yi et al. 2015). Histone methylation by specific histone methyltransferases (HMTs) or histone lysine methyltransferases (HKMTs) can cause activation or repression of gene transcription (Yi et al. 2015; Yu and Zhuang 2019; Yang et al. 2020). Whereas DNA cytosine methylation and histone deacetylation have a repressive effect (Lee et al. 2020), lysine methylation can either activate or silence gene transcription depending on the specific lysine residue involved (Meyers 2012; Morera et al. 2016). Histone lysine methyltransferases (HKMTs) are the enzymes that post-translationally add one to three methyl groups to lysine residues in proteins and the most well-defined are histone H3 lysine 4 trimethylation (H3K4me3), an activator, and histone H3 lysine 9 di- and trimethylation (H3K9me2/3) and histone H3 lysine 27 trimethylation (H3K27me3) which are transcription repressors (Duong and Hagwood 2018; Naik et al. 2021).

2.2.4. Lysine Histone demethylases (KDMs)

Lysine demethylase enzymes (KDMs) converse lysine methylation and, can be sub-grouped in two families depending on their catalytic mechanism (Anand and Marmorstein 2007; Hauser et al. 2018). The first family, the larger Jumonji C (JmjC), are iron-dependent enzymes, which can demethylate side chains of lysine in all three methylation states and are classified as KDM2-7. The second family, KDM1, are flavin-dependent histone demethylases and include the two main demethylases, LSD1 and LSD2, also known as KDM1A and KDM1B (Karytinos et al. 2009). These enzymes are unable to act on trimethylated lysine residues and can only demethylate mono and dimethyl lysine residues (Forneris et al. 2008).

2.3. Crosstalk between DNA methylation and histone modifications

In eukaryotes, DNA is linked with histone proteins which aids in bundling the extended threads of DNA into the small nucleus (Deal and Henikoff 2011; Moore et al. 2013; Lee et al. 2020). The modifications of three specific amino acids on the N-terminal histone tails, influence not only how DNA strands are packaged but also their transcriptional activity, so that when DNA is extended the transcription is active whereas when DNA and histones are densely packed, the gene expression is inhibited (Deal and Henikoff 2011; Miller and Grant 2013). The DNMTs interact directly with the enzymes that modulate histone modifications and are commonly associated with gene repression (Miller and Grant 2013; Moore et al. 2013; Hervouet et al. 2018). Inversely, histone modifications may also control DNA methylation. In fact, DNA methylation may be induced by increased acetylation of histones (Cervoni and Szyf 2001; D'Alessio et al. 2007). For instance, DNMT1 and DNMT3A bind to a histone methyltransferase (SUV39H1) that methylates histone H3 Lysine9 (H3K9) resulting in transcription repression (Fuks et al. 2003). Moreover, the direct attachment of DNMT3A to the tail of H3 stimulates its methyltransferase activity (Dhayalan et al. 2010; Li et al. 2011a). However, the trimethylation of H3K4 (H3K9me3) hinder DNMT3A, DNMT3B and DNMT3L from binding to H3 histone tails and thus preventing methylation (Ooi et al. 2007; Zhang et al. 2010). One of regions of the genome that are extremely rich in H3K4me3 are the CpG islands (Mikkelsen et al. 2007). Furthermore, both DNMT1 and DNMT3B can attach to histone deacetylases which promote condensation of the DNA and therefore restricting transcription (Fuks et al. 2000; Geiman et al. 2004).

Nevertheless, the most effective linkage between DNA methylation and histone modification is sustained by methyl-binding proteins (Fuks et al. 2003). Gene repression can be magnified by the interaction between the MBDs and the UHRF proteins with the methylated DNA and histones (Nan et al. 1998; Karagianni et al. 2008). Indeed, MeCP2 can repress gene transcription by inducing histone deacetylases to remove active histone modifications (Nan et al. 1998; Fuks et al. 2003). Furthermore, MeCP2 may also increase gene transcription inhibition by recruiting histone methyltransferases that add repressive H3K9 methylation (Fuks et al. 2003). Overall, DNA methylation and histone modifications work closely together to regulate gene expression (Miller and Grant 2013; Moore et al. 2013; Zhang et al. 2015; Lee et al. 2020).

2.4. Long and small non-coding RNAs

Long ncRNAs (lncRNAs) are RNAs with approximately 200 nucleotides which can interact with DNMTs and recruit them to target genes, although they do not encode proteins (Wilusz et al. 2009; Li and Cheng 2018; Loaeza-Loaeza et al. 2020). The lncRNAs represent part of the noncoding regions that account for 98% of the genome (Lander et al. 2001; Djebali et al. 2012; Ma et al. 2013). According to its structure, the lncRNAs have different functional mechanisms. They may act as frameworks, guides, or interfere with the binding of RNAs and

proteins such as DNMTs, and thus regulate DNA methylation (Morris and Mattick 2014; Zhao et al. 2016; Simmons 2018; Zhang et al. 2019). Although non-coding RNAs may modify the function of other epigenetic modifiers they mainly have post-transcriptional effects (Beerman et al. 2016; Duong and Hagood 2018).

Epigenetic modifiers, such as long noncoding RNAs along with histone modifications shape the DNA methylation landscape (Dobosy and Selker 2001; Freitag and Selker 2005). Moreover, a bi-directional relationship has also been described between non-coding RNAs (ncRNA) and DNA methylation.

Long non-coding RNA play a cis-regulatory role at the gene and even the entire chromosome level, and small non-coding RNA regulates gene expression at the genomic level (Kornienko et al. 2013; Statelo et al. 2021). Small non-coding RNA (sncRNA) can mediate the degradation of mRNA, induce changes of chromatin structure and, determine how cells differentiate. They can also degrade foreign nucleic acid sequences to protect the cell's genome (Mattick and Makunin 2006; Li and Liu 2019). The number of sncRNAs increases with the complexity of species and they are potent regulators of molecular pathways, as one sncRNA usually has hundreds of mRNA targets. For instance, according to the latest releases from the Ensembl website (https://ensembl.org) (Flicek et al. 2014), humans possess 25,134 ncRNAs, whilst there are 9,014 known ncRNAs annotated to the equine genome. Besides Ensembl, many recent databases also provide extensive annotations of non-coding genes, such as, the NONCODE database (Zhao et al. 2016) which is specifically committed to the annotation and bioinformatic characterization of long non-coding RNAs in animals and plants.

Evidence has shown that the dysregulation of lncRNAs seems to be related with tumours and modulates the overexpression or the recruitment of DNMTs to specific sites of the genome (Merry et al. 2015; Schmitz et al. 2016).

2.4.1. MicroRNAs

MicroRNAs (miRNA) are possibly the most renowned of the regulatory ncRNA classes. They are transcribed by non-coding genes (Ling et al. 2013; Denham et al. 2021) and, are associated with heterochromatin formation, DNA methylation targeting and gene silencing (Isoda et al. 2017; Tao et al. 2017; Duong and Hagood 2018; Mumbach et al. 2019; Statello et al. 2021). They were first described in 1993 (Lee et al. 1993). MicroRNAs (miRNAs) are crucial epigenetic regulators of post-transcriptional events and, are short non-coding RNAs of approximately 17–27 nucleotides in length (Bartel 2009; Ha and Kim 2014; Makarova et al. 2016). Their roles include the modulation of mRNAs splitting, disruption of mRNAs or translation repression, resulting in the regulation of gene expression (Bartel 2009; Beerman et al. 2016; Dowson and O'Reilly 2016). As miRNAs regulate gene expression they are also regulated by methylation in a two-way relationship (Lehmann et al. 2008; Li et al. 2015; Dowson and O'Reilly

2016; Aure et al. 2021). Furthermore, DNA methylation together with histone modifications can also regulate the expression of miRNAs and this relationship is also bi-directional (Han et al. 2007; Sinkkonen et al. 2008).

It has been described that the expression of miRNAs in a cell can regulate functions up to 200 messenger RNAs as their targets (Lomvardas et al. 2006; Catalanotto et al. 2016). In addition, each mRNA may be regulated by more than one miRNA (Chuang and Jones 2007; Chi et al. 2009, Friedman et al. 2009; Neary et al. 2015). In humans, the miRNAs may also regulate around 60% of protein-coding genes (Holliday and Grigg 1993; Friedman et al. 2009; Catalanotto et al. 2016). Moreover, several miRNAs are epigenetically modulated by methylation in CpG islands or histone modifications or by both (Ramsahoye et al. 2000; Xi et al. 2007).

The whole set of miRNAs expressed in a tissue is referred to as the miRNome (Hollis and Starkey 2018). At present, 2654 human (*Homo sapiens*) and 690 equine (*Equus caballus*) mature miRNA are annotated in the principal miRNA repository, miRbase (www.miRBase.org.). Approximately half of the mature equine miRNAs annotated in miRbase were verified experimentally (Platt et al. 2014), while the remaining part were predicted based on *in silico* analyses (Zhou et al. 2009). MiRNA nomenclature is quite complex, while mature sequences are designated by 'miR', their stem-loop pre miRNA molecules are designated by 'mir' within miRNA names. The miRbase uses three- or four-letter prefixes to designate the species, and in this way human miRNAs are designated hsa-miR (*Homo sapiens*) whereas equine miRNAs are designated eca-miR (*Equus caballus*) (van der Kolk et al. 2015; Hollis and Starkey 2018). RNA editing can crucially alter protein-coding transcripts and particularly non-coding RNAs, by changing RNA nucleotides without affecting the original DNA sequence (Li et al. 2009; Farajollahi and Maas 2010). The interaction of RNA modifications, epigenetics, and histone modifications further suggests that RNA editing may play an important role in the epigenetic regulatory networks (Chahwan et al. 2011; Zhang et al. 2020; Kan et al. 2022).

2.5. Transcription factors

Transcription factors (TFs) are proteins that recognize and bind cis-regulatory regions (promoters and enhancers) at their binding sites to regulate transcription (Lambert et al. 2018). Most of TFs recruit co-factors to activate or inhibit the transcription of their target genes (Lambert et al. 2018; Wang et al. 2021). They can regulate DNA methylation through specific DNA sequence binding by recruiting DNMTs for methylation or protecting DNA from methylation. Also, the DNMTs may bind to TFs or to elements of repressor complexes to complete DNA methylation (Brenner et al. 2005; Miller and Grant 2013; Lemma et al. 2022). Nonetheless, TFs binding can hinder *de novo* methylation on CpG sites, irrespective of whether the gene is expressed, protecting them from methylation (Straussman et al. 2009; Lienert et al. 2011). However, the modification in TF binding sites or TFs down-regulation may lead to CpG island's

inaptitude to preserve their unmethylated status, as exposed CpG sites can be targeted for DNA methylation (Deaton and Bird 2011; Lienert et al. 2011).

The role of TFs in DNA methylation remains to be unveiled, yet it seems that DNMTs can recruit a transcription factor as a co-repressor. Moreover, in normal cells, the transcriptional activity of DNMTs promoters is modulated by the binding of the specif protein 1 (Sp1) and 3 (Sp3) TFs, among others (Robertson 2000; Margot et al. 2001; Peterson et al. 2003; Hervouet et al. 2009; Loaeza-Loaeza et al. 2020). In fact, the inhibition of Sp1 or Sp3 binding to the promoters of DNMT3A and DNMT3B resulted in its decreased expression (Jinawath et al. 2005; Loaeza-Loaeza et al. 2020).

While most of transcription factors recognize complex motifs of several nucleotides, it is uncertain whether lower complexity sequence features, such as dinucleotides, can promote gene activity on their own (Hartl et al. 2019). The aforementioned data highlights the complexity of the interplay between TF binding and DNA methylation.

3. Epigenetics and disease

The in-depth knowledge of the complex biological mechanisms behind genetics and epigenetics is becoming more and more significant for the prevention, diagnosis, and treatment of diseases. Francis Crick in 1958 was the first to suggest the central dogma of biology, i.e., the process by which the information in DNA is translated into a functional product (Crick 1958), which provided an intellectual foundation for such a revolution. It was a major discovery that allowed us to understand that DNA mutations may have a permanent harmful effect on protein function which in turn, may lead into disease (Lander 2011). The understanding of classical genetic diseases was further improved by the sequencing of the entire human genome, which was the result of an international endeavour (Lander et al. 2001; Pareek et al. 2011; Claussnitzer et al. 2020). Nevertheless, additional approaches are necessary to understand the nature of non-Mendelian and complex diseases, which probably result from a combination of genetic and epigenetic aberrations (Ptak and Petronis 2008). This aim is again sustained by the occurrence of epigenetic differences, expressed in unique traits and susceptibility to disease during the lifetime of monozygotic twins (Fraga et al. 2005; Chahwan et al. 2011). Conrad Waddington in 1942 introduced for the first time the term epigenetics, and since then the definition has gradually evolved over time (Waddington 1942). Emerging studies and projects are underway to rapidly unveil the considerable extent of epigenetic information and its role in disease development (Mehler 2008; Chahwan et al. 2011; Li et al. 2020).

The epigenetic role in cancer is probably the most extensively reported by far, and through which silencing of tumour suppressor genes resulting from hypermethylation of DNA, enables the development of disease (Jones and Baylin 2007; Ng and Yu 2015; Chatterjee et al. 2017). However, in a lesser extent, hypomethylation can also induce aberrant expression of oncogenes (Laird and Jaenisch 1996; Nishigaki et al. 2005; Good et al. 2018). Histone modifications and microRNA alterations also seem to be involved in several disease processes (Ramzan et al. 2021; Park et al. 2022). Like DNA methylation, these histone or microRNA alterations are considerably clear in cancer development. Therefore, the inhibition of epigenetic processes has become noticeable as a prospective therapeutic target (Neary et al. 2015; Bennett and Licht 2018).

The first human disease to be linked to epigenetic changes was cancer. Feinberg and Vogelstein discovered that tissue lesions from patients with colorectal cancer had lower DNA methylation than normal tissue from the same patients (Feinberg and Vogelstein 1983). The loss of DNA methylation may induce an aberrant increase in gene activation, through chromatin arrangement modification because characteristically, methylated genes are silenced. However, hypermethylation can silence the protective tumour suppressor genes (Baylin 2005; Zeisberg and Zeisberg 2013; Pfeifer 2018). The CpG islands become hypermethylated in cancer cells, thus causing genes that should not be silenced to turn off. This aberration is a characteristic

epigenetic modification of tumors and occurs in the initial stages of cancer development (Jones and Baylin 2002; Robertson 2002; Egger et al. 2004; Nishiyama and Nakanishi 2021). In fact, these types of changes may be more common in human cancer than DNA sequence mutations (Shen and Laird 2013; Vogelstein et al. 2013; Guo et al. 2019).

Epigenetic alterations have been associated to several diseases, such as, cardiovascular disease (Ordovás and Smith 2010; Webster et al. 2013; Luo et al. 2022), and pulmonary disease (Robertson et al. 2019; Zhang et al. 2020; Benincasa et al. 2021), colon (Goel and Boland 2012; Jung et al. 2020) and breast cancer (Pasculli et al. 2018; Garcia-Martinez et al. 2021), where it was observed hypomethylation of large regions of the genome and hypermethylation in the promoter of tumour suppressor genes (Hansen et al. 2011; Berman et al. 2012; Dowson and O´Reilly 2016; Zhang et al. 2020). The gene promoter CpG islands develop aberrant hypermethylation in the course of many diseases, resulting in the transcription inhibition (Lee and Dunn 2008; Poplawski et al. 2008; Pfeifer 2018; Schwarzenbach and Gaham 2022). Most literature indicate that inherited and environmental factors are the two major sources for cancer-causing mutations. However, a recent study by scientists from the Johns Hopkins Kimmel Cancer Center featured a third source by providing evidence that random, unpredictable DNA copying "mistakes" are responsible for nearly two-thirds of the mutations that cause cancer (Tomasetti et al. 2017).

More recently, methylation changes have been associated with fibrotic diseases such as hepatic (Mann et al. 2010; Moran-Salvador and Mann 2017), pulmonary (Rabinovich et al. 2012; Bartczak et al. 2020; McErlean et al. 2021), renal (Tampe and Zeisberg 2014; Bontha et al. 2017; Gluck et al. 2019), and cardiac fibrosis (Xu et al. 2015; Shao et al. 2022).

3.1. Epigenetics and fibrosis

Recent findings suggest a role for epigenetic modifications in the development of fibrosis (Helling and Yang 2015; Moran-Salvador and Mann 2017; O'Reilly 2017; Bartczak et al. 2020; McErlean et al. 2021; Shao et al. 2022). It has been proposed that the inability of myofibroblasts to return to their latent state results in the maintenance of their activated state during fibrotic disease (Hinz et al. 2007; Neary et al. 2015; Gibb et al. 2020). Myofibroblasts develop crucial alterations in their gene expression profiles during the process of differentiation (Phan 2008; Fortier et al. 2021). Several environmental factors possibly participate in the induction of fibroblast differentiation, within the injured tissue, and it is also suggested that the maintenance of the myofibroblast phenotype is due to alterations in gene expression caused by chromatin modifications (Hinz et al. 2012; Tao et al. 2013; Peyser et al. 2019). Moreover, other studies suggest that all mesenchymal cells subtypes take part in the excessive ECM production in pulmonary fibrosis in mouse and human (Liu et al. 2021). It should be noted that fibroblasts, *in vivo*, are heterogenous and when exposed to the same stimulus not all differentiate into

myofibroblasts, while *in vitro,* the activated fibroblasts that become myofibroblasts can be identified by α-SMA expression (Kalluri and Zeisberg 2006). Numerous genes promote the differentiation of fibroblasts into myofibroblasts (Mann et al. 2007; Hinz et al. 2012; Tai et al. 2021). The signalling pathways that induce myofibroblast activation and disease progression are regulated by a large and growing number of epigenetic modifications, which can be organ and/or disease specific (Mann et al. 2007; Hinz et al. 2012; Duong and Hagood 2018; Tai et al. 2021; Yu et al. 2022). In fact, epigenetic mechanisms are the major regulators of the phenotype of the cells, and although their role in myofibroblast differentiation is intensively studied, it is still not thoroughly understood (Kouzarides 2007; Hu et al. 2010; Duong and Hagood 2018; Tuong et al. 2022).

3.1.1. DNA methylation and fibrosis

DNA methylation is a crucial mechanism of inhibiting gene expression and is especially important in the regulation of development and cell differentiation (Hu et al. 2010; Moore et al. 2013). Transforming growth factor β1 is closely involved in in fibrosis processes and stimulates fibroblast differentiation into the myofibroblast phenotype. This change in phenotype coincides with numerous changes in gene expression (Ding et al. 2008). In cardiac cells from rats, TGF-β1 induced upregulation of COL1A1 mRNA and protein and was also accompanied by decreased expression of DNMT1 and DNMT3A (Pan et al. 2013). The same was observed in rat lung fibroblasts where TGF-β1 increased COL1 and decreased DNMT1 and DNMT3A expression. Another study also demonstrated that DNMTs expression was decreased by the action of TGF-β1 at the same time with an increase in α-SMA expression (Hu et al. 2010; He et al. 2019). However, another study demonstrated that DMNT1 expression was increased by TGF-β in mouse renal fibroblasts (Bechtel et al. 2010). This was also observed in a study in hepatic stellate cells, where a more methylated genome was associated with liver myofibroblast activation (Page et al. 2016). Nonetheless, a study by Götze and collaborators observed that early activation of stellate cells *in vitro*, was associated with global hypomethylation (Götze et al. 2015). Thus, TGF-β can induce both hypermethylation and hypomethylation, highlighting the intricacy of the mechanisms that regulate the methylation framework. In addition, regulation of α-SMA expression has been extensively studied as a means of understanding the mechanism of fibroblast differentiation. Hence, although the knowledge regarding the regulation of the α-SMA gene expression is substantial (Orimo and Weinberg 2006; Hinz 2007; Hinz et al. 2012), studies concerning the epigenetic regulation of α-SMA gene expression by myofibroblasts are scarce. Nevertheless, some studies are shedding a light into this subject (Hu et al. 2010; Neveu et al. 2015; He et al. 2019; Xiang et al. 2020). The stimulation of α-SMA expression by an inhibitor of DNA methyltransferase, 5-Aza-2'-deoxycytidine (5-aza-dC), demonstrated the significance of DNA methylation (Christman 2002; He et al. 2015). Moreover, when fibroblasts

were treated with TGFβ to promote myofibroblast differentiation, DNMT expression was suppressed by TGFβ, and it was hypothesized that α-SMA expression could be regulated by another pathway to cause myofibroblast differentiation, through the inhibition of DNA methylation (Hu et al. 2010; He et al. 2019). However, the treatment with a DNMT inhibitor attenuated cardiac hypertrophy (Vujic et al. 2015) and cardiac fibrosis by suppressing the dysregulation of pro-fibrotic gene expression, in rats (Stenzig et al. 2018). This agrees with another study where the treatment with DNMT inhibitor, 5-aza-dC, decreased the expression of the pro-fibrotic gene α-SMA in human lung fibroblasts (Xiang et al. 2020). Also, the same effect of 5-aza-dC was observed in hepatic stellate cells, where it inhibited their differentiation into myofibroblasts (Mann et al. 2007) and in human conjunctival fibroblasts where it suppressed the fibrogenic changes in fibroblasts (Yonemura et al. 2019). In another study, 5-aza-dC improved (ameliorated) experimental kidney fibrosis in mice (Bechtel et al. 2010). A decrease in COL1 expression, after the treatment of TGFβ1 induced fibroblasts, from different organs, with 5-aza-dC was also observed (Neveu et al. 2015; Yonemura et al. 2019; Xiang et al. 2020). Nevertheless, contrary reports have found that the treatment with 5-aza-dC of TGFβ1 induced fibroblasts, increased COL1 expression (Hu et al. 2010; Pan et al. 2013).

These conflicting results might derive from the alteration of the methylation pattern in different genes, as fibrosis may be due to both hypomethylation of pro-fibrotic genes and hypermethylation of anti-fibrotic genes (Neary et al. 2015). However, it also plausible that this could be the result of the passage number of the cells that were used in each study, as it has been shown that methylation patterns can change with passage (Shmookler Reis and Goldstein 1982). Another explanation could be due to the diversity of the type of cells used, as the methylation pattern is different between tissues and individuals (Zhang et al. 2013).

3.1.2. Histone modifications and fibrosis

Histone deacetylase inhibition has shown to be effective in fibrosis models (Glenisson et al. 2007; Guo et al. 2009; Conforti et al. 2017; Bombardo et al. 2018). Deacetylation by histone deacetylases (HDACs) usually results in gene silencing, whereas acetylation of histones is generally associated with activation of gene expression (Wade 2001; Marks et al. 2004). The inhibitors of HDAC may be beneficial in fibrosis, regulating fibrotic genes and its pathways through the inhibition of HDACs. They can also target inflammatory cytokines that arise due to inflammation, which occurs in fibrotic processes following tissue damage (Saouaf et al. 2009).

It was described that the histone acetylase and transcriptional coactivator, P300, activated the TGFβ-induced expression of collagen in myofibroblasts, through the hyperacetylation of histone H4 in COL1A2 gene and activated TGFβ-induced expression of collagen in myofibroblasts (Ghosh et al. 2013). Moreover, the lysine acetylation may establish

binding sites for bromodomains, where “reader” proteins related to transcription can bind to form protein complexes that regulate transcription programs (Tang et al. 2013).

Epigenetic histone modifications were reported to be involved in liver fibrosis (Mannaerts et al. 2010; Liu et al. 2021). A role for histone acetylation in fibrosis appeared initially in liver fibrosis (Niki et al. 1999; Rombouts et al. 2002). It was observed that the histone deacetylase inhibitor trichostatin A (TSA), inhibited the synthesis of procollagens type I and type III, and α-SMA filament formation and hepatic stellate cell (HSC) proliferation, and it was preceded by hyperacetylation of histone H4 (Niki et al. 1999; Rombouts et al. 2002). Also, in hepatic fibrosis in mice, HDAC4 promoted the epigenetic repression of MMPs 9 and 13, inhibiting their expression and disturbing the MMP-TIMP balance, and resulting in fibrosis (Qin and Han 2010). Additionally, a histone H3K9 demethylase enzyme was also shown to regulate liver fibrosis (Jiang et al. 2015).

Histone modifications were found accountable for the fibroblast’s phenotype and their persistent activated state, in idiopathic pulmonary fibrosis (Coward et al. 2009; Huang et al. 2013; Sanders et al. 2014; Noguchi et al. 2015; Coward et al. 2018; Yang et al. 2022). In systemic sclerosis (SSc), which is a fibrotic disease in humans, it was also reported a dysregulation in HDAC activity (Hemmatazad et al. 2009; Bergmann et al. 2018; Tsou et al. 2019). Indeed, histone hypoacetylation has been associated with systemic sclerosis (Noda et al. 2014; Wang et al. 2016). The histone deacetylase inhibitor, TSA has also been reported to effectively abolish α-SMA expression and suppress the myofibroblast differentiation induced by TFGβ1, in human skin fibroblasts (Glenisson et al. 2007). Epigenetic alterations in histones were also described in cardiac fibrosis (Rai et al. 2019; Yang et al. 2020; Lim et al. 2021; He et al. 2022) and renal fibrosis (Nie et al. 2020; Shen and Zhuang 2022).

3.1.3. MicroRNAs (miRNAs) and fibrosis

It’s becoming more acknowledged that RNA editing plays a role in disease, particularly with the new high-throughput sequencing technologies (Chan et al. 2016; Destefanis et al. 2021). MiRNAs appear to be involved in almost all cellular processes and seem to be disturbed in several diseases (Lu et al. 2008; Bartel 2009; Mendell and Olson 2012; Hata 2013; Peng and Croce 2016; Eichmüller et al. 2017; Pawlica et al. 2021). Regarding fibrotic diseases, miRNAs can regulate different elements that take part in fibrosis in multiple types of cells (Ciechomska et al. 2014; Meng et al. 2016; Riedel et al. 2018). Over 2500 miRNAs, have been reportedly associated with fibrotic diseases by many studies (Mann et al. 2010; Roderburg et al. 2011; Dakhlallah et al. 2013; Li et al. 2014; Yu et al. 2015; Christmann et al. 2016; Zhao et al. 2022). There are numerous external signals that induce or repress the miRNAs in cells. These specific miRNAs may function as signalling axis, targeting hundreds of mRNA, and therefore influencing protein production (Bartel 2009; O’Brien et al. 2018). Alterations in the miRNA balance may

significantly impact protein production and subsequently fibrotic pathways, as many of these miRNAS modulate pro- or anti-fibrotic genes (O'Reilly 2016; O'Brien et al. 2018; Usman et al. 2021). Several studies have reported that aberrant miRNAs expression is associated with numerous fibrotic diseases, including cardiac fibrosis (van Rooij et al. 2008; Zhao et al. 2020; Schimmel et al. 2021, Zeng et al. 2021;), liver fibrosis (Mann et al. 2010; Yu et al. 2015; Zhang et al. 2019; Wang et al. 2021), lung fibrosis (Pandit et al. 2011; Dakhlallah et al. 2013; Mora et al. 2017; Cadena-Suárez et al. 2022), renal fibrosis (Krupa et al. 2010; Yu et al. 2019; Fan et al. 2020) and systemic sclerosis (Christmann et al. 2016; Zhang et al. 2020; Szabo et al. 2021).

One of the first examples of this association was reported by van Rooij et al. (2008), which described that miR-29 was involved in the regulation of cardiac diseases. Besides being strongly associated with fibrinogenesis, miR-29 seems to play a crucial role in the repression of collagen and other ECM components (van Rooij et al. 2008). It is now recognized that miR-29 is also dysregulated in liver fibrosis (Roderburg and Luedde 2014; Liang et al. 2016; Huang et al. 2019), kidney fibrosis (Wang et al. 2012; Huang et al. 2020; Hu et al. 2021), as well as systemic sclerosis (SSc) (Takemoto et al. 2013; Shimada et al. 2020). MiR-29 may induce changes in methylation by targeting the DNMT family, so a decrease in miR-29 may result in hypermethylation of genes that modulate fibrosis. It was also observed that miR-29a regulates TIMP-1 gene expression altering collagen accumulation (Ciechomska et al. 2014; O´Reilly 2017). In addition, miR-21 was increased in myofibroblasts from IPF lungs, and its inhibition diminished the severity of bleomycin-induced lung fibrosis in mice (Liu et al. 2010). At the same time the levels of miR-29 were decreased in these myofibroblasts, resulting in a concerted increase in the ECM (Yang et al. 2013).

Interestingly, decreased miR-29 expression seems to be regulated by fibrotic ECM, through a positive feedback loop between fibroblasts and aberrant ECM (Parker et al. 2014). Other miRNAs were described to be dysregulated in idiopathic pulmonary fibrosis (IPF) in humans, such as hsa-miR-17-92 which was considerably reduced (Mora et al., 2017).

3.2. Epigenetic treatments and drugs

Considering that so many diseases, such as cancer, involve epigenetic changes, the use of epigenetic treatments to restrain these modifications appears to be a rational and acceptable strategy. Furthermore, as these modifications are reversible, it renders them ideal targets for therapeutic interventions (Kelly et al. 2010; Cheng et al. 2019). The most used epigenetic treatments aim to alter either DNA methylation or histone acetylation (Egger et al. 2004; Cheng et al. 2019). Today, seven agents have been approved by the USA Food and Drug Administration (FDA), from three epigenetic target classes (two DNMT inhibitors, four HDAC inhibitors and one EZH2 inhibitor) for the treatment of different diseases, but many more are

undergoing in clinical trials (Kaminskas et al. 2005; Marks 2007; Barbarotta and Hurley 2015; Laubach et al. 2015; Sanaei and Kavoosi 2020).

DNMT inhibition is reviewed as an efficient approach for the prevention of DNA hypermethylation alterations (Nepali and Liou 2021). The ability of DNMT inhibitors to reverse epimutations is the basis of their use as novel strategies for cancer therapy (Hu et al. 2021). These medications act like the nucleotide cytosine and incorporate themselves into DNA while it is replicating (Constantinides et al. 1978; Santi et al. 1984; Stresemann and Lyko 2008; Moore et al. 2013; Jones et al. 2019; Nunes et al. 2020). Demethylating agents are currently used to treat myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). However, they can cause cytotoxicity at high doses and therefore the rationale is to use lowest dose that can still activate silenced genes (Qin et al. 2007; Ahuja et al. 2016; Sato et al. 2017). Hence, they are being experimentally used in solid tumours in low dose administration, with very promising results (Li et al. 2015; Takeshima et al. 2020). The combination of a demethylating agent (decitabine) with another compound (a ncRNA) was able to block breast cancer growth and metastasis in mice (Fu et al. 2022). Moreover, numerous clinical trials are ongoing to evaluate the efficacy of the treatment in nasopharyngeal carcinoma, prostate cancer, lung cancer, thyroid cancer, ovarian cancer, and many others (Fu et al. 2011; Ramos et al. 2015; Singal et al. 2015; Bohl et al. 2018; Lu et al. 2018).

Histone modifications have been investigated in a wider variety of diseases than DNA methylation, and include solid tumours, hematological malignancies, and even many inflammatory diseases (Cheng et al. 2019). It has been reported that targeting histone modifiers, such as histone deacetylases inhibitors can induce changes on histone methylation (Huang et al. 2011) and alter the binding of chromatin remodelling factors at the same locus. Currently, histone deacetylases inhibitors are being used for the treatment of cutaneous T-cell lymphoma (CTLC), peripheral T-cell lymphoma (PTCL), multiple myeloma and chronic myelogenous leukemia (CML). They are also undergoing several clinical trials for the treatment of advanced thyroid cancer, gliomas, brain tumors, kidney cancer, metastatic prostate cancer, breast cancer, among others. Other epigenetic drug, the EZH2 inhibitor (tazemetostat), which is a histone methyltransferase inhibitor has been in FDA approved in 2020 for the treatment of metastatic or locally advanced epithelioid sarcoma and for relapsed or refractory follicular lymphoma in humans (US FDA, 2020). Also, some trials, in advanced breast cancer, are combining both DNMTs inhibitors and HDCA inhibitors, with promising results (Connolly et al. 2017).

HDAC inhibition may alter the CpG methylation of specific promoters by affecting the activity of DNMTs (Clozel et al. 2013) because epigenetic modifiers work in complexes with other chromatin-related proteins (Weigt et al. 2016). Thus, the interference on one partner can cause unpredicted effects on the other partners of the complex. It should be kept in mind that

effectively targeting a specific epigenetic enzyme in *in vitro* does not imply that the same will happen in the cellular context (Ganesan et al. 2019). Another challenge is, the appropriate dose to use (low doses have better effect for DNMT inhibitors) (Tsai et al. 2012; Li et al. 2015; Takeshima et al. 2020), or duration of the treatment.

To be efficient, epigenetic treatments must target the "diseased" cells, or else, the activation of gene transcription in normal cells could cause undesirable effects (Simmons 2008). Another pitfall of the conventional epigenetic drugs is that they lack local specificity and have global and non-chromatin effects. Therefore, the need to find more adequate and competent epigenetic therapies is imperative, besides improving the combined therapies with already existing drugs (Majchrzak- Celinska et al. 2021).

3.2.1. DNA methyltransferase (DNMT) inhibitors

The DNMT inhibitors can be divided in two classes: nucleoside analogues and non-nucleoside analogues (Nepali and Liou 2021).

3.2.1.1. Nucleoside analogues

The nucleoside analogues, which contain a modified cytosine ring, can be transformed in nucleotides, and incorporated into newly synthesized DNA or RNA (Presant et al. 1981). DNMT nucleoside inhibitors induce the reduction of DNMTs and a decrease in the levels of DNA methylation (Nunes et al. 2020; Majchrzak-Celinska et al. 2021). Among many nucleosides analogues only azacytidine and decitabine are FDA approved as epigenetic drugs while gemcitabine is approved as an anticancer agent (Table 2).

Table 2: Epigenetic nucleoside analogues drugs

Nucleoside analogue	Mechanism of action	Clinical trials	References	FDA approval (USA)
Azacytidine (5-Azacitydine, 5-Aza)	DNMT inhibitor	Recurrent brain tumor, systemic autoimmune diseases, solid tumors, metastatic colorectal cancer.	NCT02940483 NCT02985190 NCT02959437 NCT01193517	MDS, AML (Kaminskas et al. 2005; Ma and Ge 2021)
Decitabine (5-aza-2′-deoxycytidine, 5-aza-dC, Dac)	DNMT1, DNMT3B inhibitor	Pancreatic adenocarcinoma, recurrent ovarian carcinoma, esophageal squamous cell carcinoma, COVID-19	NCT05360264 NCT03017131 NCT05265962 NCT04482621	MDS (Kantarjian et al. 2006)
Guadecitabine (SGI-110)	DNMT inhibitor	AML, MDS, recurrent ovarian carcinoma, melanoma, metastatic colorectal cancer	NCT03603964 NCT02901899 NCT04250246 NCT01966289	
4'-thio-2'-deoxycytidine (TdCyd)	DNMT1 inhibitor	Neoplasms and solid tumors	NCT03366116.	
5-Fluoro-2′-deoxycytidine (FdCyd)	DNMT1 inhibitor	Head, neck, lung, and breast neoplasms	NCT00978250	
Gemcitabine (GCB)	DNMT inhibitor	Solid tumors, metastatic pancreatic cancer	NCT05093322 NCT04498689	Pancreatic, ovarian and metastatic breast cancer (Hidalgo 2010; deVita 2006; Tkaczuk 2009)
Zebularine (4-Deoxyuridine)	DNMT1 inhibitor	*In vitro* assay in colon cancer cell lines and cholangiocarcinoma cell lines	(Sanaei and Kavoosi 2020; Nakamura et al. 2015)	

AML – acute myeloid lymphoma; CMML – chronic myelogenous leukemia; DNMT1, DNMT3B - DNA methyltransferase 1, 3B; FDA – Food and Drug Administration, MDS – myelodysplastic syndromes

The most used nucleoside agents in cancer treatment are azacytidine and decitabine (Liu et al. 2018). Both azacytidine and decitabine are integrated in the DNA after being absorbed by the cells but, only when they are at the S phase of their cycle at the time of exposure (Constantinides et al. 1978; Jones et al. 2019). Then, DNMTs identify the azacytosine–guanine dinucleotides and form a covalent bond with the cytosine ring. The covalently trapped DNMTs are degraded, resulting in the depletion of cellular DNMTs leading to methylation inhibition (Santi et al. 1984; Stresemann and Lyko 2008; Nunes et al. 2020). Both drugs have poor bioavailability and low chemical stability, undergoing hydrolysis in either aqueous acid or base, and require incorporation into DNA to exert their effects as covalent inhibitors (Wong et al. 2019; Majchrzak-Celinska et al. 2021). Azacytidine and decitabine are currently used in the treatment for myelodysplastic syndromes (MDS), chronic myelomonocytic leukaemia (CMML) and acute myeloid leukemia (AML) (Issa et al. 2005; Gore et al. 2006; Prebet et al. 2014; Smith et al. 2014; Dombret et al. 2015; Diesch et al. 2016; Liu et al. 2019).

Azacytidine is an analogue of the cytidine pyrimidine nucleoside, that inhibits the DNMT1. It undergoes phosphorylation to get incorporated into the RNA but can also be in incorporated in DNA (Ferlay ey al., 2015; Nepali and Liou 2021). Decitabine is a unique cytosine analogue that inhibits both DNMT1 and DNMT3B (Ferlay et al. 2015) and can only be

incorporated into DNA (Momparler 1985) and is one of the most powerful known specific DNMT inhibitor (Zhang et al. 2020). It directly inhibits DNA methyltransferase activity reducing DNA methylation, thereby further inhibiting the proliferation of tumour cells, and preventing the occurrence of drug resistance. Furthermore, it was reported that decitabine treatment abrogates the effects of TGF-β induced myofibroblasts in cardiac cells (Watson et al. 2014). The use of azacytidine in patients with solid tumors is limited by its toxicity, myelosuppression; and low response rates (Weiss et al. 1972; Weiss et al. 1977). Recently, it was suggested that CC-486 (an oral formulation of azacytidine) has clinical activity as monotherapy in nasopharyngeal cancer, besides being well tolerated by patients (von Hoff et al. 2018).

3.2.1.2. Nonnucleoside analogues

Novel nucleoside and non-nucleoside DNMT inhibitors are continuously being developed and tested (Zwergel et al. 2019). The latter are usually found in natural sources, like many polyphenols, flavonoids, antraquinones, and others, and are capable to inhibit the activity and/or expression of DNMTs (Zwergel et al. 2016; Akone et al. 2020). Their evaluation in the context of cancer chemotherapy is ongoing, and the results are promising (Zwergel et al. 2016; Akone et al. 2020; Majchrzak-Celińska, et al, 2021). Also, some drugs (including procainamide) have been repurposed after they demonstrated demethylating effects. These agents show affinity for CpG-rich regions of DNA, blocking the activity of DNMTs and reactivating some tumor silencing genes (Castillo-Aguilera et al. 2017) (Table 3).

Table 3: Epigenetic nonnucleoside analogues drugs

Nonnucleoside analogue	Mechanism of action	*In vitro*/clinical studies	References
SGI-1027	Inhibits DNMT1, DNMT3A and DNMT3B	Cervical cancer; hepatocellular carcinoma tumor	(Sun et al. 2008; She et al. 2020)
MC3343	Inhibits DNMT1	Cancer stem cells, leukemic cells, solid cancer cell lines	(Valente et al. 2014; Zwergel et al. 2020)
MC3353	Inhibits DNMT1	Colon cancer cells, AML cells, lymphoma cells, prostate cancer, cells	(Zwergel et al. 2019)
MG98	Inhibits DNMT1	Metastatic renal carcinoma, advanced renal cell carcinoma, advanced solid tumors	(Winquist et al. 2006; Plummer et al. 2009; Amato et al. 2012)
RG108	Inhibits DNMT1 and DNMT3B	Endometrial cancer cells	(Yang et al. 2017)
Nanaomycin A	Inhibits DNMT3B	Human cancer cells	(Kuck et al. 2010; Caulfield and Medina-Franco 2011)
Procainamide	Inhibits DNMT1	Prostate cancer, lung cancer cells	(NCT02103088; Gao et al. 2009)

AML – acute myeloid lymphoma; DNMT1, DNMT3A, DNMT3B - DNA methyltransferase 1, 3A, 3B

3.2.2. Histone deacetylase (HDAC) inhibitors

Histone deacetylase (HDAC) inhibitors block the removal of acetyl groups from DNA, leading to gene expression. Histone deacetylase inhibitors may be useful in fibrosis by directly

inhibiting HDACs that regulate fibrotic genes but also by targeting the inflammatory cytokines released due to tissue damage, during the inflammation component of fibrosis. In fact, HDAC inhibition suppresses inflammation and has proved advantageous in rheumatoid arthritis animal models (Saouaf et al. 2009) and alters the levels of the MMPs degrading enzymes (Young et al. 2005). Vorinostat and romidepsin were the first drugs to be approved that influence epigenetic post-translational modification of histone proteins (Ganesan 2016), but later on other compounds were developed (Table 4).

Table 4: Histone deacetylase (HDAC) inhibitor drugs

HDAC inhibitors	Condition/Trial	FDA approval (U.S.)	References
Romidepsin (FK-228)	CTCL	2009	(Barbarotta and Hurley 2015)
Vorinostat (SAHA; suberoylanilide hydroxamic acid	CTCL	2006	(Marks 2007)
Belinostat (PXD101)	PTCL	2014	(Lee et al. 2015)
Panobinostat (LBH589)	Multiple myeloma	2015	(Laubach et al.2015; Cavenagh and Popat 2018)
Pracinostat (SB939)	Solid tumor, AML lung fibroblasts		(Novotny-Diermayr et al. 2010; Jones et al. 2019)
Valproic Acid	Liver fibrosis		(Mannaerts et al. 2010)
Chidamide * (CS055/HBI-8000)	PTCL	2015*	(Guan et al. 2020)

AML – acute myeloid lymphoma; CTCL – cutaneous T-cell lymphoma; PTCL – peripheral T-cell lymphoma; * approved by the Chinese FDA

While DNMT inhibitors, azacytidine and decitabine, have a significantly recognized role in first-line chemotherapy for the treatment of MDS with additional use in CMML and AML, the HDAC inhibitors vorinostat and romidepsin have a limited application despite their initial promise in a variety of tumour types in cell-based and animal models (Ganesan et al. 2019).

3.2.3. Histone methyltransferase (HMT and KMT) inhibitors

Histone methyltransferase (HMT) inhibitors can be classified according to their specificity for different types of methyltransferases. Both lysine and arginine HMTs use S-adenosylmethionine (SAM) as a co-factor and methyl group donor (Jin et al. 2022). Lysine histone methyltransferases (KMTs) are the enzymes that post-translationally add one to three methyl groups to lysine residues in proteins (Han et al. 2019; Ganesan et al. 2019). The lysine methyltransferase (KMT) enhancer of zeste homolog 2 (EZH2) functions both as a transcriptional suppressor and as a transcriptional co-activator, depending if tri-methylation of H3K27 occurs or not (Gan et al. 2018).

Tazemetostat (EPZ-6438) is a EZH2 inhibitor that received US FDA approval in January of 2020, for the treatment of metastatic or locally advanced epithelioid sarcoma. A study with another EZH2 inhibitor, GSK126, reported the inhibition of atrial enlargement and fibrosis in mice (Song et al. 2019). Also, a study with DZNeP, another EZH2 inhibitor, suppressed hepatic fibrosis, evidenced by the decrease of hepatic fibrosis markers (*α*-SMA and Collagen I), suggesting that DZNep may represent a novel treatment for fibrosis (Ding et al. 2021). Moreover, the treatment with 3-DZNeP significantly improved lung injury and fibroproliferation by blocking EMT through TGF-β1/Smad signaling pathway and regulating shift of macrophage phenotypes, in lipopolysaccharide (LPS)-induced mice (Bao et al. 2021). Also, other studies are being carried out for the use in AML, advanced solid tumours and MDS, among many others (National library of Medicine, NCT03701295; NCT01897571; NCT02936752).

3.2.4. Lysine histone demethylase (KDM) inhibitors

Lysine methylation is reversed by the lysine demethylase enzymes (KDMs) (Hauser et al. 2018). Although there are interesting links between lysine demethylases and human disease, at present, inhibitors of the enzymes are still undergoing clinical trials (Hauser et al. 2018), aiming for the treatment of AML, solid tumours, and relapsed ewing sarcoma (National library of Medicine, NCT02273102; NCT02959437; NCT03514407).

3.2.5. Bromodomains

The bromodomains were the first epigenetic readers that had a great influence on therapeutic research and among them the BET (bromo and extra terminal) family has been the main focus for drug discovery (Cochran et al. 2019) as they are key activators of oncogenic networks in different cancers. Although some BET inhibitors have already been developed, such as JQ1, AZD5153, and I-BET762 (molibresid), many more are still in clinical trials (Majchrzak-Celinska et al. 2021; Shorstova et al. 2021). Moreover, JQ1 and AZD5153, showed prominent antifibrotic effects *in vitro* and *in vivo*, reducing skin fibrosis in bleomycin treated mice with increased dermal thickness (Vichailkul et al. 2022).

3.2.6. miRNAs (miRNAs) therapy

A promising approach is to target disease-related miRNAs using anti-miRNA oligos (miRNA inhibitors) to inhibit overexpressed miRNAs either in their mature or precursor form (Zhang 2008). On the opposite of conventional therapies, miRNA therapy can potentially target many genes of a specific pathway, instead of a single protein (Sayed and Abdellatif 2011; Caroli et al. 2013; Bronze-da-Rocha 2014). MiRNAs can be regulated by oligonucleotides composed of high affinity nucleotide mimics, designated miR-mimics, and single-stranded antisense oligonucleotides, termed antimiRs or antagomirs (Bronze-da-Rocha 2014).

Currently, there are no FDA-approved miRNAs, although many miRNA therapies have achieved significant preclinical efficacy and entered in clinical trials (Wang et al. 2021; Smith et al. 2022; Zogg et al. 2022). The miR-122 inhibitor (miravirsen) has completed phase II clinical trials for the treatment of hepatis C (Janssen et al. 2013; Panigrahi et al. 2022) and MRG-110 (miR-92a inhibitor) has completed phase I clinical trials for the improvement of wound healing (Gallant-Behm et al. 2018; Abplanalp et al. 2020). Many others are still in clinical trials for the treatment of keloid and tennis elbow, but also as potential biomarkers of lung cancer and multiple sclerosis (National Library of Medicine, NCT03601052; NCT04670289; NCT02247453; NCT05290688).

3.3. Epigenetic treatment of fibrosis

No current epigenetic therapies have been approved to treat fibrosis, even though some drugs that inhibit the profibrotic epigenetic modifications seem promising *in vitro* and *in vivo*; (Duong and Hagood 2018). Epigenetic drugs have been used successfully in the treatment of cancer. Since myofibroblasts have a neoplastic-like behaviour, this has triggered investigations in search of epigenetic drugs that may alter myofibroblast phenotype. In some fibrotic diseases the increase in DNA methylation has been attributed to specific upregulation of either the maintenance enzyme DNMT1 or the *de novo* methyltransferases DNMT3A and DNMT3B. Although one might expect an increase in DNMT3A or DNMT3B to be primarily involved in initiating pro-fibrotic DNA methylation patterns, studies in the liver, lung, heart, kidneys and systemic sclerosis have implicated a role for elevated DNMT1 and/or DNMT3A or DNMT3B in fibrosis (Wang et al. 2006; Bechtel et al. 2010; Sanders et al. 2012; Dakhalallah et al. 2013; Tao et al. 2014; Watson et al. 2014). These important observations further highlight the link between dysregulation of DNMTs and fibrosis.

Some studies *in vitro* and a few *in vivo* have reported the successful use of therapeutics in the inhibition of DNA, histone methylation and histone deacetylation, in the repression of myofibroblasts (Niki et al. 1999; Mann et al. 2007; Kaimori et al. 2010; Mann et al. 2010; Mannaerts et al. 2010; Liu et al. 2013; Page et al. 2016; Watson et al. 2016; Zeybel et al. 2017). As fibroblasts and myofibroblasts are the central mediators of fibrosis, epigenetic modifiers that stimulate their apoptosis are a possible therapeutic option, such as HDAC inhibitors which prevent histone modifications responsible for apoptosis resistance (Huang et al. 2013; Sanders et al. 2014). Combined therapies that incorporate more than one epigenetic mechanism to synergistically reverse or inhibit pro-fibrotic myofibroblast behaviour is a promising route. Epigenetic modifiers may also serve as biomarkers of disease severity (Duong and Hagood 2018).

Even though fibrosis has been confirmed to be reversible in preclinical and clinical studies (Hu et al. 2011; Tao et al. 2016), regrettably, clinical treatment options are still limited

(Xu et al, 2021). Unfortunately, few data are reported in epigenetic based drugs for clinical treatment.

Nevertheless, the therapeutic intervention with 5-aza, at a dose 5 times lower than clinically given for oncology treatment, attenuated myocardial hypertrophy and fibrosis, in mice (Russell-Hallinan et al. 2020). Also, the treatment with 5-aza-CdR attenuated lung fibrosis in neonatal rats exposed to hyperoxia by lowering hydroxyproline and TGF-β1 expression through re-expression of P16 in neonatal rats (Zhao et al. 2018). In another *in vivo* study, romidepsin, a HDAC inhibitor, inhibited bleomycin-induced pulmonary fibrosis in mice, in association with suppression of lysil oxidase expression showing its anti-fibrotic effects. Romidepsin also significantly prevented up-regulation of pro-fibrotic genes including *FN1* and *COL3A1* mRNA in the bleomycin-exposed mice, whilst a trend for suppression of *COL1A1* was observed (Conforti et al. 2017). Moreover, compound 38, a selective BET bromodomain inhibitor, has been shown to have a potential role in in the treatment of liver fibrosis as it effectively treated injurious inflammation and fibrogenesis in liver fibrosis in mice, which qualifies it as a strong candidate for clinical therapy (Fu et al. 2022). However, given the difference between human disease and animal models, more research is needed to translate the current results for the clinical treatment of human diseases (Fu et al. 2022). A novel BRD4 inhibitor, C-34 tethering a 4-phenylquinazoline scaffold was also reported to be able to serve as a lead compound in the further development of fibrotic cardiovascular disease treatment (He et al. 2022). One *in vivo* experiment demonstrated that the EZH2 inhibitor, GSK126, significantly inhibited cardiac fibrosis in an angiotensin-II (Ag-II) induced mouse model (Song et al. 2019). In another study, it was demonstrated for the first time that inhibition of EZH2 with 3-DZNeP attenuated lung injury and subsequent fibrosis by repressing the EMT in mice (Bao et al. 2021).

Zhang et al. (2017), reported that miR-30a supplementation suppressed myofibroblast accumulation and reduced the number of lung fibrotic lesions in mice that were exposed to bleomycin. Also, the effect of administration of miR-200b/c on lung fibrosis was evaluated by Cao et al. (2018), in lipopolysaccharide (LPS)-induced lung injury model in mice, where it was reported an improvement in the visual appearance of the tissue, a lowered production of TGF-β1 and attenuated ECM. Later it was demonstrated that the intravenously administration of nuclease-resistant naked miR-542-5p, suppressed the production of *COL1A1* and *FN* as well as the extent of EMT and reversed established silica-induced fibrosis in mice (Yuan et al. 2019). Therefore, that miRNA-based therapeutics have great potential as they repress multiple fibrosis-related genes simultaneously.

Epigenetic drugs that target active myofibroblasts in fibrotic conditions appear to be very auspicious in the treatment of a wide range of diseases, however, a thorough knowledge of how epigenetic mechanisms interplay with myofibroblast behaviour is still to be achieved. This

objective is even more complex due to the discrepancies of the epigenetic networks that occur depending on the type of organ and disease involved.

Epigenetic modifications, such as DNA methylation, are important in normal physiology; thus, inhibiting this process is likely to yield some unwanted side effects. It is also apparent that the half-life of DNA demethylating agents is quite short; hence, the development of 5-aza therapeutic strategies for the treatment of fibrotic pathologies as opposed to blood malignancies would require investigations into drug formulations which could improve efficacy as an anti-fibrotic agent. Such approaches that could be explored include controlled release preparations and pro-drug formulation approaches to optimise bioavailability and increase the therapeutic window for the hypomethylation indication. Unlike in cancer, a sustained low-dose release may be more beneficial for the treatment of fibrosis, avoiding high cytotoxic doses. Furthermore, it would be of value if new formulations could be devised that enable specific organs to be targeted depending on the fibrotic pathology.

3.4. Epigenetics in the horse

Very few epigenetics studies have been pursued in the equine field and as such the knowledge is very limited. In 2013 Dindot and collaborators studied the epigenetic regulation of gene expression and explored the emerging field of epigenetics in the horse (Dindot and Cohen 2013). The applications of epigenetics have been mainly developed in the oncology field, but epigenetics also has applications to many areas of equine health. Therefore, the study of the relationship between epigenetic modifications and a variety of phenotypes, including disease, is desirable. The use of next-generation, whole-genome sequencing coupled with whole-genome epigenetic techniques (e.g., chromatin immunoprecipitation sequencing (ChIP-seq)) revealed the noncoding functional elements and associated genetic variants in the equine genome (Doan et al. 2012).

Usually, interferon-gamma (IFN-γ) expression is reduced in neonates of most species (Vuillermin et al. 2009), including foals (Breathnach et al. 2006), and is associated with an increased risk of intracellular bacterial infections, such as those caused by *Rhodococcus equi* (R. equi). Sun et al. (2013) demonstrated that the DNA methylation in the proximal promotor region of IFN-γ was increased (hypermethylated) in neonatal foals when compared to adult horses and that the hypermethylation in this region contributes to the reduced IFN- γ expression in neonatal foals. The same has been described in human neonates (White et al. 2002). The first genome-wide DNA methylation characteristics data from skeletal muscle, heart, lung, and cerebrum tissues of thoroughbred (TH) and Jeju (JH) horses, an indigenous Korea breed, was described by Lee et al. (2014) by methyl-DNA immunoprecipitation sequencing. The analysis of the DNA methylation patterns indicated that the average methylation density was lower in the promoter region and higher in other genome sequence regions (Lee et al. 2014). Some studies

have suggested that exercise induces methylation changes (Gomez-Pinilla et al. 2011; Barrès et al. 2012), and athletic ability is closely associated with methylation (Brutsaert and Parra 2006; Terruzzi et al. 2011). Like humans and rodents, the skeletal muscle miRNAs of horses, are dynamically regulated soon after a single bout of training (Denham et al. 2021). The transcriptional reprogramming after exercise seems to be in part resultant of epigenetic reprogramming through the DNA methylome. This was particularly evident in the whole blood of two Thoroughbred racehorses, where it was observed a great difference in methylation regions after a single session of exercise (Gim et al. 2015).

As sarcoids are the most common neoplasms occurring in horses (Knottenbelt 2003), Semik-Gurgul et al. (2018), using bisulfite sequencing and clone sequencing, analyzed equine sarcoid tissues, and detected that the DNA methylation in the lesioned samples was considerably higher than the corresponding normal skin tissue. DNA methylation of the fibrinogen locus was also studied in equine conceptus, liver, and endometrium, and across the full gene cluster, as preimplantation equine embryos synthesize and secrete fibrinogen, even though fibrinogen synthesis almost exclusively occurs in the liver (Grant et al. 2020). DNA methylation was found to be significantly higher in the endometrium than in the liver and conceptus, supporting the hypothesis of DNA methylation being a regulator of fibrinogen expression in the conceptus (Grant et al. 2020).

Understanding the role of miRNAs in equine health and disease is still in its infancy. Currently, more than 700 equine miRNAs were identified, with different subgroups of miRNAs being differently expressed according to distinct tissues (Desjardin et al. 2014; Kim et al. 2014; McGivney et al. 2017). Nevertheless, there are only a few studies on small RNA next-generation sequencing of equine tissue (Desjardin et al. 2014; Kim et al. 2014; Lee et al. 2016; Pacholewska et al. 2016; Pawlina, et al. 2017). Interestingly, some miRNAs have been reported to be expressed in a tissue-specific pattern, like in equine plasma (Kim et al. 2014; Lee et al. 2016), highlighting the potential application of miRNAs in diagnostics and therapeutics. In mare ovaries, the miRNA levels in the follicular fluid showed similarities between antral fluid and granulosa cell fractions, demonstrating that antral fluid can be a useful indicator of follicular miRNA status (da Silveira et al. 2012). Moreover, a subset of miRNAs was related with different stages of ovarian follicular development, along with regulation of cell survival and differentiation (Schauer et al. 2013). In addition, differences in miRNA expression have been reported between equine ovulatory and anovulatory follicles, suggesting a physiological association between increases in follicular miRNA and seasonal anovulation in mares (Donadeu and Schauer 2013; Schauer et al. 2013). Serum miRNA was also evaluated in pregnant mares, and one miRNA (miR-374b) was differentially regulated through late gestation and four miRNAs were differentially regulated between the pregnant and non-pregnant mares (Loux et al. 2017). Other studies linking equine biological processes and diseases with miRNA were reported, such as miRNA expression in spermatozoa

from different regions of the epididymis (Twenter et al. 2017), insulin resistance in horses (da Costa Santos et al. 2017), polysaccharide storage myopathy and recurrent exertional rhabdomyolysis (Barrey et al. 2010), osteochondrosis (Desjardin et al. 2014), tendinopathies (Millar et al. 2015), laminitis (Lecchi et al. 2018), sarcoids (Unger et al. 2021) and equine viral arteritis (Carossino et al. 2018). Therefore, miRNAs might be used as novel diagnostic markers for many diseases including myopathies and osteochondrosis (van der Kolk 2015), and for pregnancy-related complications in mares, including fetal growth restriction and placental infection (Loux et al. 2017).

Regarding treatment of equine diseases with epigenetic therapies the reports are very scarce. Vorinostat, a histone deacetylases inhibitor, was found to suppress TGF-β1-mediated *in vitro* differentiation of equine corneal fibroblasts into myofibroblasts (Donnelly et al. 2014). Another study demonstrated a favourable effect of the histone demethylase inhibitor OG-L002 in restraining the load of equine herpesvirus type 1 (EHV-1) and gene expression in equine kidney cells, suggesting that maintaining a repressive epigenetic state on the EHV-1 genome in the host equine cell decreases viral load during infection (Tallmadge et al. 2018). One of the benefits of using epigenetic drugs to alter epigenetic modifications in the host cell, instead of targeting viral proteins, is that is less likely to induce resistant viral strains (Tallmadge et al. 2018). It was also reported a synergistic interaction between decitabine and valganciclovir (an antiviral compound and prodrug of ganciclovir) in an antiviral *in vitro* assay against EHV1 (Thieulent et al. 2020). In the same study it was also demonstrated for the first time that three deocycytidine analogues, i.e., decitabine, gemcitabine and cytarabine, were all effective against EHV-1 infection *in vitro* (Thieulent et al. 2020).

An additional investigation showed that *in vitro* pharmacotherapy of autologous adipose stem cells with a combination of 5-azacytidine and resveratrol (natural polyphenol compound, with antioxidant, anticancer, antimicrobial properties, among others) before their clinical use in the treatment of equine metabolic syndrome, reversed the phenotype of mesenchymal stem cells (MSC) isolated from equine metabolic syndrome diagnosed animals (Marycz et al. 2016; Kornicka et al. 2018). Later, the use of microvesicles isolated from 5-azacytidine and resveratrol treated MSC was also described for the treatment of suspensory ligament injury in the horse (Kornicka-Garbowska et al. 2019).

Interestingly, the first equine cloned foal born in Australia (Gambini et al. 2016) was produced with somatic cells treated with chetomin, a fungal secondary metabolite reported to inhibit the trimethylation on histone 3 lysine 9 (H3K9 me3) (Damasceno Teixeira et al. 2019).

3.5. Future Perspectives

Epigenetics is a promising area of research with clinical impact, due to the developments in epigenetic drug discovery, along with the widen understanding of the importance of epigenetic

mutations in diseases and the consequent biomarker discovery (Dirks et al. 2016; Graça et al. 2016; Jones 2016; Morera et al. 2016; Esteller, 2017; Berdasco and Esteller 2019; Velasco and Francastel 2019). An extremely pertinent challenge is the election of the proper druggable target to achieve a functional effect on a specific disease (Ganesan et al. 2019). Differential reprogrammable methylation in a sequence-specific site is now possible through several genome-editing tools based on DNA recognition domains such as transcription activator-like effectors (TALENs), zinc finger proteins (ZNFs), and the system of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) proteins without genetic editions (Pabo et al. 2001; Boch et al. 2009; Jinek et al. 2012).

Over the past decade, CRISPR/Cas has raised exponential interest as a tool to correct genetic mutations but also offers powerful possibilities to silence any given gene (Geel et al. 2018). Moreover, if loss-of-function is caused by epigenetic silencing of expression, CRISPR/Cas can be reprogrammed to target effectors to that genomic locus to re-express the silenced gene. This latter application of CRISPR/Cas is achieved by mutating (and abolishing) the nuclease activity of the Cas protein (resulting in a dead Cas protein; dCas) (Geel et al. 2018; Brezgin et al. 2019). If the catalytic domain of an epigenetic enzyme is fused to dCas, the outcome will be the rewriting of the epigenetic signature at this specific location, and this process is referred to as epigenetic editing (Geel et al. 2018). In the case of diseases where the final goal is not the death of the cells (opposite to cancer diseases), the endurance of the effect is of great relevance, and it can be attained using epigenetic editing (Ganesan et al. 2019). Several epigenetic writers or erasers can be targeted to a specific locus, at the same time, to rewrite the epigenetic signature, using epigenetic editing, resulting in stable mitotic effects for many cell divisions (Cano-Rodriguez and Rots 2016; Mlambo et al. 2018; Ganesan et al. 2019).

The fast advance in microarrays and the extensive sequencing technologies is allowing genome-wide DNA methylation profiling of different cell types, diseases, and species (Zilberman and Henikoff 2007; Suzuki et al. 2010). Furthermore, the development of new high-throughput technologies, together with several epigenetic databases hold great promise to improve the understanding of epigenetics underlying health and disease. Data bases such as ENCODE (Encyclopedia of DNA Elements; Dunham et al. 2012), ROADMAP Epigenomics (the NIH Roadmap Epigenomics Mapping Consortium; Bernstein et al. 2010), IHEC Data Portal (The International Human Epigenome Consortium; brings forth reference epigenomes relevant to health and disease), WashU Epigenome Browser (a web browser that offers tracks from ENCODE and Roadmap Epigenomics projects), EWAS Atlas (Epigenome-Wide Association Studies), NGSmethDB (Whole-genome bisulfite sequencing (WGBS) database for many different tissues, pathological conditions, and species), MethBase (Hundreds of methylomes from well-studied organisms; Song et al. 2013), miRbase (published miRNA sequences) and NonCode (a database of all kinds of noncoding RNAs for 16 species).

Regarding animals, the Model Organism Encyclopedia of DNA Elements (modENCODE (http://www. genome.gov/26524507) provided similar insight into the functional elements in the widely used Mus musculus, Drosophila melanogaster, and Caenorhabditis elegans model organisms (Gerstein et al. 2010; Roy et al. 2010; Dunham et al. 2012; Stamatoyannopoulos et al. 2012) as well as the FAANG (Functional Annotation of Animal Genomes; Andersson et al. 2015) consortium. The latest progresses in genomic sequencing technology and computational assembly methods enabled a deeper knowledge of reference genome assemblies (Kalbfleisch et al. 2018). Equus caballus (horse) genome assembly EquCab2, a reference genome for the domestic horse, was completed in 2007 (Wade et al. 2009). In 2018, the EquCab3, an improved reference genome was released (Kalbfleisch et al. 2018) and was the result of new technologies for sequencing of longer reads, that aided in the characterization of the genome repetitive regions (Petersen and Coleman 2020). Presently, EquCab3 is the primary reference genome assembly for the horse and ought-to be used for the analysis of future sequence data (Petersen and Coleman 2020). The supplementary accomplishments to annotate both protein-coding regions, noncoding RNA and regulatory features sustain the improvement of EquCab3 (Burns et al. 2018). Besides the identification of genomic variants and transcript expression, next generation sequencing (NGS) may serve to understand DNA modifications, such as methylation, or to identify regions of the genome that interact with proteins (Petersen and Coleman 2020).

Finally, the increasing spectrum of antibodies against post translationally modified proteins as well as the study of post-translational histone modifications by immunoprecipitation with high-throughput sequencing (ChIP-Seq), enables an accurate profiling of target modifications along the genome, allowing a better understanding of the chromatin epigenetic landscape (Park 2009; Hawkins *et al.* 2010; Stepanov et al. 2022). Although DNA methylation in mammals has been studied for several decades, there is still much to understand about the storage of epigenetic information in DNA.

For molecular medicine, the fast development of these new approaches and the reversible nature of the epigenome offer the promise of great advances in the fields of biomarker discovery, drug targeting, and personalized medicine. In the equine domain, although a better knowledge of the alteration of individual genetic variation in response to a particular treatment or performance is building up, the goal of a personalized management for horses is far off to be accomplished (Petersen and Coleman 2020).

1. Collagen type III as a possible blood biomarker of fibrosis in equine endometrium

Alpoim-Moreira J, Fernandes C, Rebordão MR, Costa AL, Bliebernicht M, Nunes T, Szóstek-Mioduchowska A, Skarzynski DJ, Ferreira-Dias G. 2022. Collagen Type III as a Possible Blood Biomarker of Fibrosis in Equine Endometrium. *Animals*; 12(14):1854. doi: 10.3390/ani12141854.

1.1. Abstract

Collagen pathological deposition in equine endometrium (endometrosis) is responsible for infertility. Kenney and Doig´s endometrial biopsy histopathological classification is the gold standard method for endometrosis evaluation, whereby blood biomarkers identification would be less invasive and could provide additional information regarding endometrosis diagnosis and fertility prognosis. This study aimed to identify blood biomarkers for endometrosis diagnosis (42 mares were used in experiment 1), and fertility assessment (50 mares were used in experiment 2). Reproductive examination, endometrial biopsy histopathological classification (Kenney and Doig) and blood collection were performed. Endometrium and serum collagen type I (COL1) and type III (COL3), and hydroxyproline concentrations were measured (ELISA). Serum COL3 cut-off value of 60.9 ng/mL allowed healthy endometria (category I) differentiation from endometria with degenerative/fibrotic lesions (categories IIA, IIB or III) with 100% specificity and 75.9% sensitivity. This cut-off value enabled category I+IIA differentiation from IIB+III (76% specificity, 81% sensitivity), and category III differentiation from others (65% specificity, 92.3% sensitivity). COL1 and hydroxyproline were not valid as blood biomarkers. Serum COL3 cut-off value of 146 ng/mL differentiated fertile from infertile mares (82.4% specificity, 55.6% sensitivity), and was not correlated with mares´ age. Only COL3 may prove useful as a diagnostic aid in mares with endometrial fibrosis and as a fertility indicator.

Keywords: collagen, biomarker, blood, endometrium, fibrosis, mare, endometrosis

1.2. Introduction

Endometrosis, a term first introduced by Kenney in 1992 (Kenney 1993), is one of the causes of infertility in mares. Endometrosis is mainly characterized by periglandular fibrosis of

the endometrium (Schoon et al. 1992; Schoon et al. 1997), which compromises the integrity and function of the endometrial glands required for embryo survival in the preimplantation period and for placental development (Gray et al. 2001). The degree of endometrosis in mares increases with age (Schoon et al. 1997; Schoon and Schoon 2003; Ebert et al. 2014), even though it is thought not to be associated with the number of foalings (Ricketts and Alonso 1991; Hoffmann et al. 2009; Aresu et al. 2012). Atypical morphological and functional differentiation of periglandular endometrial stromal cells is the first sign of endometrosis. The first stage of fibrosis is characterized by large, polygonal periglandular stromal cells, which synthesize collagen fibres, whereas in advanced fibrosis, without signs of collagen synthesis (Raila 2020), predominate metabolic active or inactive stromal cells, as well as myofibroblasts (Hoffmann et al. 2009; Evans et al. 1998; Walter et al. 2001; Hoffmann et al. 2009). Myofibroblasts, which are fibroblast-derived cells, have been recognized as the main source of type I collagen (COL1) and of fibrogenic/inflammatory cytokines in fibrotic lesions (Guyot et al. 2006; Masseno et al. 2012). The type of collagen present in endometrosis characterizes the chronology of this condition. In repair and fibrotic processes, collagen type III (COL 3) is the first to be expressed, followed by its replacement by COL1 in the extracellular matrix (ECM) (Martinez-Fernandez 1999; Bochsler and Slauson 2002). Most degenerative changes typical for endometrosis can be diagnosed only through the histological evaluation of an endometrial biopsy (Ricketts and Alonso 1991; Aresu et al. 2012; Kenney 1978; Kenney and Doig 1986; Flores et al. 1995; Ricketts and Barrelet 1997; Katkiewicz et al. 2007; Zajac et al. 2008; Schlafer 2007; Snider et al. 2011; Hanada et al. 2014). Endometrial biopsy has been the gold standard for evaluation of the health of the mare's uterus for over 50 years. Currently, the fertility prognosis is based on the categorization scale of Kenney and Doig (Kenney and Doig 1986), together with amendments of Schoon (Schoon et al. 1997; Schoon and Schoon 2003). Even though it has been considered as a safe, practical, and especially useful method (Kenney 1972; Ricketts and Barrelet 1997) and as an essential part of the breeding standard examination, it does not provide a 100% accurate information as the biopsy sample taken may not represent the state of the whole uterus. The search for an additional and less invasive technique would be desirable. Therefore, the use of a blood biomarker should be considered. One key consideration in the assessment of any biomarker is the biological likelihood of its relationship to the pathological or physiological condition it measures. The use of collagen fragments for the assessment of fibrotic disorders is now well established in humans (Luo et al. 2018). Blood biomarkers have been studied in horses to assess musculoskeletal conditions like osteochondrosis (Frisbie et al. 1999; Laverty et al. 2000; Billinghurst et al. 2004; Frisbie et al. 2008), and some types of collagens have been used in humans for diagnostic purposes (Luo et al. 2018; Gressner et al. 2007; Karsdal et al. 2020); thus, the rationale of the present study was to find a putative correlation between endometrial fibrosis and blood COL, to be used as a diagnostic blood marker of endometrosis in mares. Due

to the highly restricted distribution of hydroxyproline in collagen and elastin, the hydroxyproline content generally reflects the amount of collagen in samples. Therefore, since quantification of hydroxyproline has been utilized for evaluating tissue fibrosis or collagen deposition (Bruckner 2010; Karsdal et al. 2011; Neto da Silva 2022), it was used in this study. As the degree of endometrosis in mares increases with age (Schoon et al. 1997; Schoon and Schoon 2003; Ebert et al. 2014), the age effect was also studied. Thus, we hypothesized that COL1, COL3 and hydroxyproline could be used as blood biomarkers of endometrial fibrosis in the mare. As such, we aimed to investigate the concentrations of COL1, COL3 and hydroxyproline in blood serum and endometria from mares with different endometrial categories, graded according to the histopathological system of Kenney and Doig (Kenney and Doig 1986), and fertility outcome in experiment 2.

1.3. Materials and Methods

1.3.1. Experimental design

The present work included two different experiments. In experiment 1, venous blood and endometrial biopsies were obtained from the same mares for determination of COL1, COL3 and hydroxyproline concentrations, both in serum and in endometrial tissue graded according to Kenney and Doig´s classification (Kenney and Doig 1986). Experiment 2 was conducted in different mares, after conclusion of experiment 1. Only venous blood samples were retrieved from mares later determined as fertile or infertile. In experiment 2, only COL3 was assessed based on experiment 1 results. All the procedures complied to to welfare mandates, authorized by the Ethic Committee for Research and Teaching (Comissão de Ética para a Investigação e Ensino - CEIE) of the Faculty of Veterinary Medicine, from the University of Lisbon, Lisbon, Portugal. They were performed by a veterinary doctor as part of a breeding exam, requested by the mare owner's, who consented on data use for research purposes.

1.3.2. Animals

1.3.2.1. Experiment 1

This study was carried out in the breeding season (from May to July) on a group of 42 Lusitano mares, ranging from 3 to 23 years old, from different stud farms. All animals were kept outdoors and maintained on pasture and had free access to water. Mares' internal genitalia (ovaries, uterus, and cervix) were examined by transrectal ultrasonography (Sonovet 600; rectal linear probe 7.5 MHz), to assess their reproductive status at the time of endometrial biopsy and blood collection. The age of the mares in category I ranged from 3 to 4 years (mean 3.5 ± 0.18; n=13); in category IIA from 3 to 19 years (5.9 ± 2.01; n=8), in category IIB from 8-17 years (11.6 ± 1.28; n=8) and in category III from 10-24 years (mean 16.3 ± 1.26; n=13). None of the mares

had foaled recently. The endometrial biopsy was withdrawn from the uterine body and in the estrous cycle before the first insemination of the breeding season. Endometrial biopsies were collected with a biopsy alligator jaw forceps (ref. 141965; Kruuse, Langeskov, Denmark). Right after endometrial biopsy procurement, tissue was divided into two portions: one piece was immersed in 4% buffered formaldehyde solution, and the other one in RNAlater® (R0901; Sigma-Aldrich, St Louis, MO, USA) that was further kept at -80ºC.

1.3.2.2. Experiment 2

Experiment 2 was performed, after accomplishment of experiment 1, and on different mares. Therefore, only serum COL3 was assessed, based on the results from experiment 1. In this experiment, 50 Lusitano cyclic mares (27 in follicular phase and 23 in luteal phase) ranging from 3 to 25 years old, from the same stud farm, located in Portugal (Ribatejo region), were used. All animals were kept outdoors and maintained on pasture and had free access to water. None of the mares had recently foaled or had a foal at foot. Mares' internal genitalia (ovaries, uterus, and cervix) were examined by transrectal ultrasonography (Sonovet 600; rectal linear probe 7.5 MHz), to confirm their reproductive status at the time of blood collection. Insemination of those mares was performed afterwards, during estrus, with fresh semen from different stallions with proven fertility. Since uterine biopsy was not performed in most of the mares, it was not possible to obtain data on endometrial category. Mares were classified as fertile (n = 25; n = 12 in luteal phase; n = 13 in follicular phase) when a gestation diagnosis was positive and confirmed by ultrasonography at 60 days post insemination. Mares were classified as infertile (n = 25; n = 11 in luteal phase; n = 14 in follicular phase) when a negative gestation diagnosis was made, after at least 3 attempts (cycles) of insemination. The age of the fertile mares ranged from 3 to 21 years (7 ± 0.95), and from 5 to 25 years (14.7 ± 1.2) in the infertile mares.

1.3.3. Blood sampling and processing

Jugular venous blood was collected from all mares (experiment 1 and experiment 2), at the time of ultrasound examination, into a dry vacutainer, coated with silica as the clot activator (BD Vacutainer® 367896; Becton Dickinson, Winnersh, UK). Blood was centrifuged at 1540× g for 10 min and serum was separated, aliquoted, and stored at −80 ◦C, until COL and hydroxyproline assays were performed.

1.3.4. Endometrium biopsies processing

Formaldehyde-fixed endometrium biopsy (experiment 1) was embedded in paraffin, and 4µm-thick histological sections were stained with hematoxylin (05-06014E; Bio-Optica, Milan,

Italy) and eosin (HT1103128; Sigma-Aldrich) and were observed by light microscopy (Leica DM500) at 400× magnification. Equine endometrial biopsies histopathological features, such as the extent of inflammation and/or fibrosis, were the grounds for Kenney and Doig's classification (Kenney and Doig 1986). Therefore, the endometrium was classified, as follows: category I—when the endometrium presented a normal histology or with very mild, focal inflammation or fibrosis; category IIA—when there was mild to moderate inflammation and/or multifocal fibrosis with 1–3 layers of fibroblasts surrounding glands, or less than 2 fibrotic nests per 5 mm linear field; category IIB—when there was moderate inflammation and/or multifocal–diffuse fibrosis with 4 or more layers of fibroblasts surrounding the endometrial glands or 2–4 fibrotic nests per 5 mm linear field; category III— when there was severe inflammation and/or extensive fibrosis with 5 or more fibrotic nests per 5 mm linear field (Kenney and Doig). The endometrial biopsies were assigned to category I (n = 13; 6 in luteal phase and 7 in follicular phase), to category IIA (n = 8; 4 in luteal and 4 in follicular phase), to category IIB (n = 8; 4 in luteal and 4 in follicular phase) or to category III (n = 13; 6 in luteal phase and 7 in follicular phase).

1.3.5. Collagen determination

Quantification of COL was performed in endometrial biopsy tissue, kept in RNAlater® (experiment 1) and in blood serum (experiments 1 and 2). For quantification of COL in endometrial tissue, the Enzyme-Linked Immunosorbent Assay Kit for Collagen Type 1, COL1 (CEA571Eq; Cloud-Clone Corp., Katy, TX 77494, USA) was used, and for Collagen Type III, COL3 trimer form (CEA176Eq; Cloud-Clone Corp., Katy, TX 77494, USA) was used. Endometrial tissue was macerated and processed according to the manufacturer's instructions. Briefly, 20 mg of endometrium were homogenized with a lysing solution (1 mL solution/20–50 mg of tissue) and macerated using the TissueLyser II (QIAGEN GmbH, 40724 Hilden, Germany) for 4 cycles of 30 s each, at 75 Hz. After that, cell disruption was performed using an ultrasonic homogenizer (Bandelin Sonopuls, Berlin, Germany) until the solution was clear. Then, centrifugation was performed (10,000× g for 5 min). The supernatant was collected, and the assay was performed according to the manufacturer's kit. The reaction was developed using the tetramethyl benzidine reaction (TMB) substrate, and the absorbance was read using a microplate reader (FLUOstar Optima, BMG LabTech; Baden-Württemberg, Germany) at the wavelength of 450 nm. The concentrations of COL1 and COL3 in each sample were calculated using the standard curve. The detection limit of COL1 and COL3 were 7.15 ng/mL and 4.98 ng/mL, respectively. For each COL type, all samples were run in a single assay. For COL1, the standard curve ranged from 18.52 to 1500 ng/mL, and the intra-assay coefficient of variation (CV) was 8.9%. For COL3, the standard curve ranged from 12.35 ng/mL to 1000 ng/mL, and the intra-assay CV was 6.7%. Protein was extracted from the endometrial tissues as described in (37), and quantification of total protein was performed by Bradford (5000006; Bio-Rad,

Hercules, CA, USA) method. Concentrations of COL1 or COL3 were expressed in nanogram (ng) per microgram (µg) of total endometrial protein. Quantification of COL was also assessed in serum of all mares (experiments 1 and 2), using the same ELISAs for COL1 and COL3, as referred to above for the endometrium, and following the manufacturer's protocol. Serum samples were used directly with no need to undergo the same procedures for tissue samples. Samples were run in duplicate in a single assay. In experiment 1, the intra-assay CVs were 8.8% and 6.9% for serum COL1 and COL3, respectively. In experiment 2, the intra-assay CV was 8.7% for serum COL3. Serum concentrations of COL1 or COL3 were expressed as ng per mL.

1.3.6. Hydroxyproline determination

Hydroxyproline content was measured in the endometrial tissue (experiment 1) and in the serum (experiments 1 and 2) using Hydroxyproline Colorimetric Assay Kit (K555-100; Biovision, Milpitas, CA, USA). As hydroxyproline is a major component of collagen and comprises about 13.5% of its amino acid composition (Tanwar et al. 2017; Metschl et al. 2019), it was used in this study to evaluate total COL concentration in mare endometrium and serum. Briefly, 10 mg of endometrium tissue was homogenized with 100 µL dH20 (100 µL dH20/10 mg of tissue) and macerated using the TissueLyser II (QIAGEN GmbH; 40724 Hilden; Germany) for 4 cycles of 30 s, at 75 Hz. After that, 100 µL of HCL (1 µL HCL/1 µL of sample) were added. For serum samples, 250 µL was used and equal volume of HCL was added. The samples were then placed in a heater, at 119 ◦C, for 3 h. Afterwards, 4 mg of activated charcoal/100 mL of sample (102186; Sigma-Aldrich; Merck KGaA; St. Louis, MO, USA) was added to the serum samples, as a purification process, and then vortexed, and centrifuged. For each sample of serum or tissue, 10 µL was used, and the protocol was carried out following the manufacturer's instructions. The hydroxyproline content in tissue and serum samples was quantified colorimetrically by using the chloramine T method, according to the manufacturers protocol, and absorbance was measured at 560 nm, using a microplate reader (FLUOstar Optima, BMG LabTech; Baden-Württemberg, Germany). Hydroxyproline concentration was quantified using a standard curve of high purity hydroxyproline and expressed as ng of hydroxyproline per µg of total endometrial protein or as ng per µL of serum.

The endometrial concentrations of COL1, COL3 or hydroxyproline were expressed as ng/µg of total protein to normalize the parameters assessed in the tissues, as there are no reference values for total protein for the equine endometrium. It is known that in blood, there is a small physiological variation of total protein values. Therefore, serum concentrations of COL1, COL3 or hydroxyproline were expressed in ng/mL, as reference values for equine serum total protein are known.

1.3.7. Statistical Analysis

To establish whether data were normally distributed, variables were tested with Shapiro–Wilk test in both experiment 1 and 2. In experiment 1, to detect differences in COL1 concentration between endometrial categories, one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were performed. Kruskal–Wallis followed by Dunn's multiple comparisons test were used to detect differences between age, endometrial or serum COL3 concentrations, and endometrial categories. Mann–Whitney test was used to assess differences in the following: (i) concentration of serum COL1, endometrial or serum hydroxyproline among endometrial category; (ii) COL1 or COL3 concentration in serum, or endometrial hydroxyproline and age groups (3–9 years vs. over 9 years). Unpaired t-test was performed to evaluate putative differences in COL1 or COL3 concentrations in endometrial tissue and age groups (3–9 years vs. over 9 years) and in hydroxyproline concentration in serum and age groups (3–9 years vs. over 9 years). Spearman correlation was used to assess the following relationships: between endometrial category and (i) age, (ii) endometrial and serum COL1, COL3, and hydroxyproline; among age and (i) endometrial COL1, COL3 and hydroxyproline, or (ii) serum COL1 and COL3. Spearman correlation was also performed to assess the relationship between endometrial and serum concentrations of (i) COL1, (ii) COL3, and (iii) hydroxyproline. Pearson correlation was performed to evaluate the degree of association between age and serum hydroxyproline. In experiment 2, unpaired t-test was performed to evaluate differences in COL 3 serum concentration between (i) fertile and infertile mares, and between (ii) age of the mares. Spearman correlation was performed to assess relationship between serum COL3 concentration and (i) fertility, and (ii) age of the mares. The area under receiver operating characteristic curves was determined for COL3, and sensitivity and specificity were calculated. In experiment 1, category I endometrium (healthy) was compared with endometria with degenerative/fibrotic changes (category IIA, IIB, and III), category I and IIA with IIB and III, and severe endometriosis (category III) with all other categories. In experiment 2, fertile mares were compared with infertile mares. The statistical analyses were performed by GraphPAD PRISM (Version 6.01, GraphPad Software, San Diego, CA, USA). Significance was defined as $p < 0.05$. Results were expressed as mean ± standard error of the mean (SEM).

1.4. Results

1.4.1. Experiment 1:

The severity of fibrosis in mares' endometrial biopsies histopathologically graded (Kenney and Doig 1986), increased with age ($p < 0.0001$; Figure 1). A very strong correlation was found between mares' age and endometrial category ($\rho = 0.8033$; $p < 0.0001$).

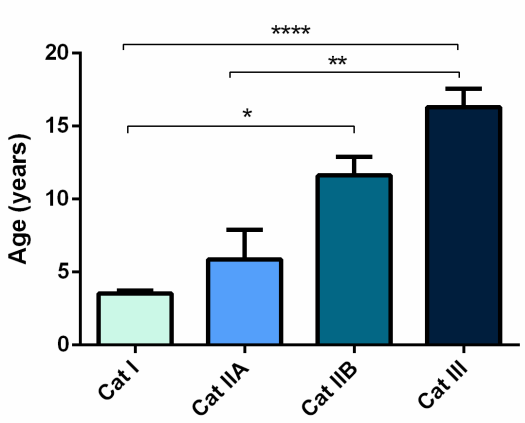

Figure 1. Age of the mares (mean ± SEM) with endometria assigned to different Kenney and Doig's histopathological categories as I, IIA, IIB or III. Asterisks indicate statistical differences (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

The COL1 protein concentration in the endometrial biopsy was higher in category III, when compared to category I (Figure 2a, $p < 0.05$). A moderate correlation was found between endometrial category and endometrial COL1 ($\rho = 0.6024$; $p < 0.001$). However, in serum, COL1 concentration was higher in category I, healthy endometrium, with respect to category IIB (Figure 2b), and a low negative correlation between endometrial category and serum COL1 was observed ($\rho = -0.42$; $p < 0.05$). Regarding COL3 in the tissue (Figure 3c), it was higher in category IIB ($p < 0.001$), and III ($p < 0.01$), when compared to category I, and higher in category IIB, when compared to category IIA ($p < 0.05$). A moderate positive correlation was obtained between endometrial category and COL3 in the endometrial tissue ($\rho = 0.6360$; $p < 0.0001$). The high concentration of COL3 in category IIB, and III endometria was consistent with serum concentration, which was also higher in category IIB ($p < 0.05$), and III ($p < 0.0001$), with respect to category I (Figure 2d). There was a strong correlation between endometrial category and serum COL3 ($\rho = 0.7048$; $p < 0.0001$). A moderate correlation between endometrial COL3 and serum COL3 ($\rho = 0.55$; $p < 0.001$) was also observed. Hydroxyproline tissue concentration was elevated in category IIB and III, when compared to category I ($p < 0.05$) (Figure 2e), while in serum the concentration was higher in category I than in category III ($p < 0.05$) (Figure 2f). A positive correlation was observed between endometrial category and hydroxyproline concentration in endometrial tissue ($\rho = 0.6770$; $p < 0.001$), but no correlation was observed for serum. No correlation was found between endometrial and serum concentrations of COL1 or hydroxyproline. No differences were found between estrous cycle phases (Figure S1).

Mares were also analyzed by age group. When mares were assigned to two different age groups (22 mares, 3–9 years old; 20 mares, over 9 years old), COL1 concentration in endometrium was the highest in the oldest mares, but the lowest in their serum ($p < 0.05$; Figure 3a,b). A positive moderate correlation was found between the age of the mares and endometrial COL1 ($\rho = 0.45$; $p < 0.05$), and a negative low correlation with serum COL1 ($\rho = -0.48$; $p <$

0.01). Regarding COL3, its concentration was higher both in endometrial tissues ($p < 0.01$), and in serum ($p < 0.001$) in the oldest animals (Figure 3c,d). There was a low correlation between mares age, and endometrial COL3 ($\rho = 0.36$; $p < 0.05$), and between mares age, and serum COL3 ($\rho = 0.37$; $p < 0.05$). Hydroxyproline concentration was elevated in endometrial tissues ($p < 0.05$) in the oldest animals (Figure 3e), but not in serum (Figure 3f). A low correlation between age and endometrial hydroxyproline was observed ($\rho = 0.48$; $p < 0.05$), but no correlation was found between age and serum hydroxyproline.

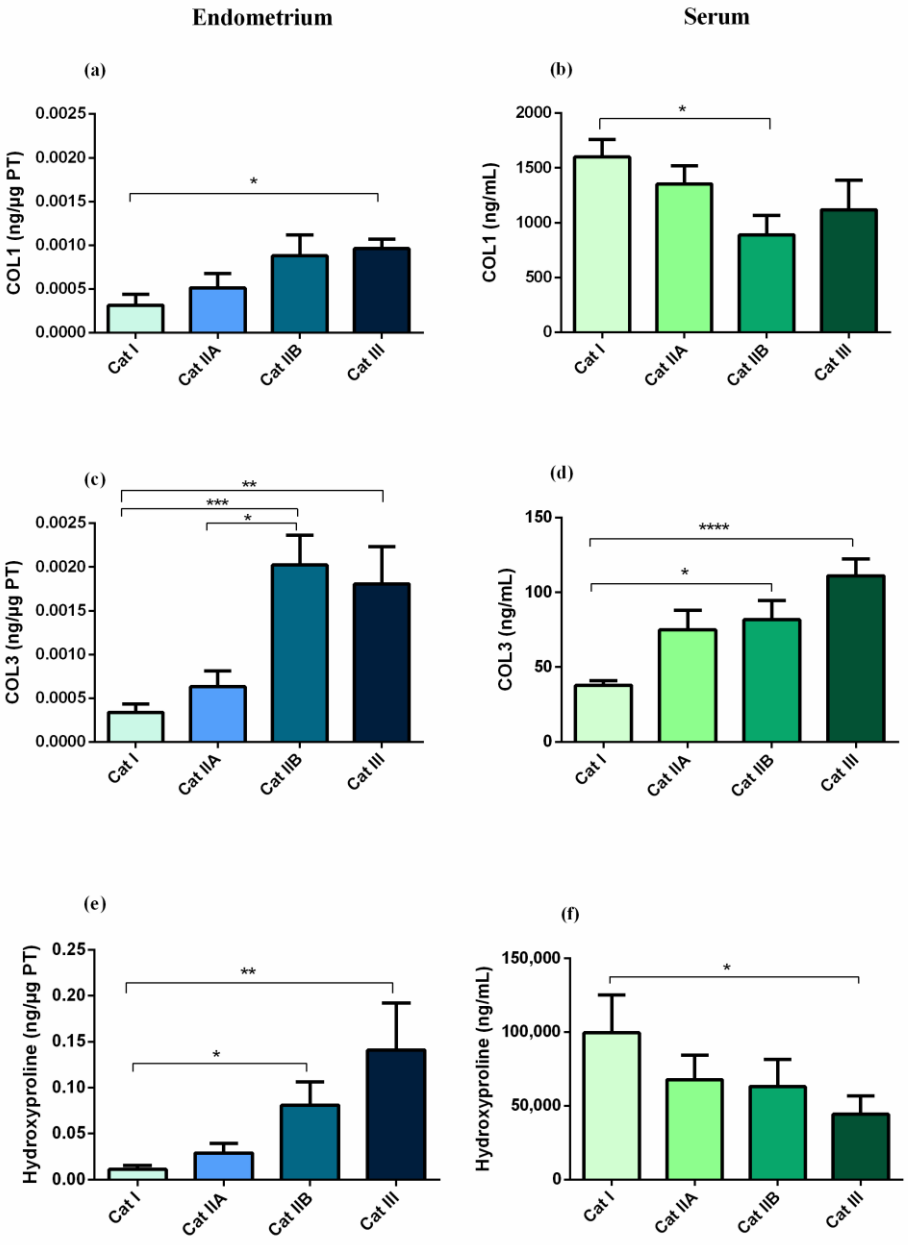

Figure 2. Effect of equine endometrial category (category I, IIA, IIB or III) on the concentration of (a) type I collagen (COL1) in endometrial tissue, (b) COL1 in serum, (c) type III collagen (COL3) in endometrial tissue, (d) COL3 in serum, (e) hydroxyproline in endometrial tissue, and (f) hydroxyproline in serum. Bars represent mean ± SEM. Asterisks indicate significant differences between endometrial categories (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

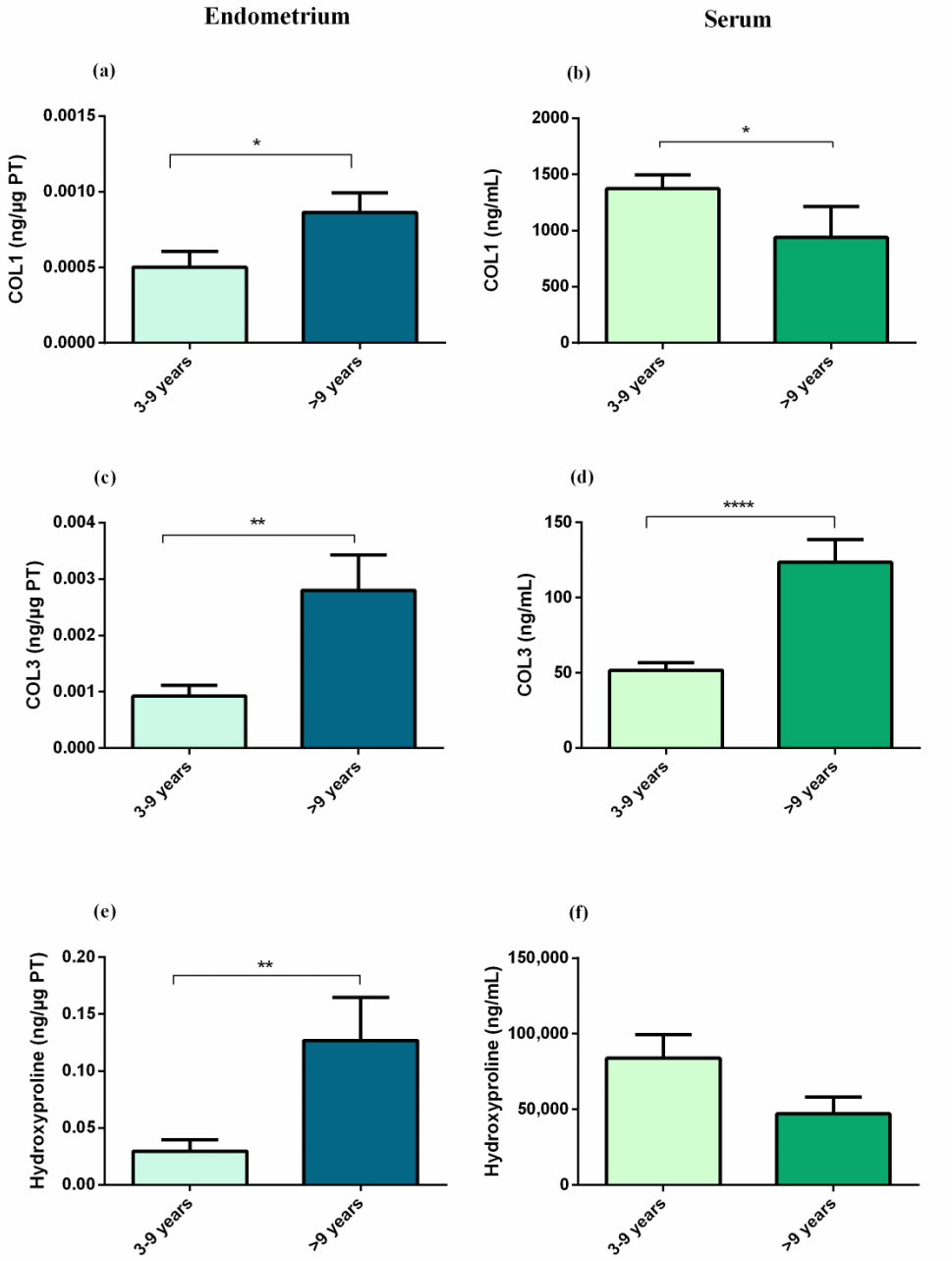

Figure 3. Effect of mares age (3 to 9 years old vs. over 9 years old) on the concentrations of (a) type I collagen (COL1) in endometrial tissue, (b) COL1 in serum, (c) type III collagen (COL3) in endometrial tissue, (d) COL3 in serum, (e) hydroxyproline in endometrial tissue, and (f) hydroxyproline in serum. Bars represent mean ± SEM. Asterisks indicate significant differences between age groups (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

The sensibility and specificity of serum COL3 were also determined (Figure 4). Serum COL3 cut-off value of 60.9 ng/mL allowed the differentiation of healthy mares (category I) from mares with endometrial degenerative/fibrotic lesions (categories IIA, IIB, and III), with a specificity of 100% and a sensitivity of 75.9%, and the area under the curve (AUC) was 0.90 (95% confidence interval (CI), 0.81 to 0.99; $p < 0.0001$). The same cut-off value also allowed

the differentiation between category I + IIA from IIB + III with a specificity of 76%, a sensitivity of 81%, and an AUC of 0.85 (95% CI, 0.73 to 0.96; p = 0.0001), and it allowed the differentiation of category III from all the other endometrial categories with a specificity of 65%, a sensitivity of 92.3%, with an AUC of 0.85 (95% CI, 0.73 to 0.96; p = 0.0003). COL1 and hydroxyproline did not prove valid as blood biomarkers of endometrosis.

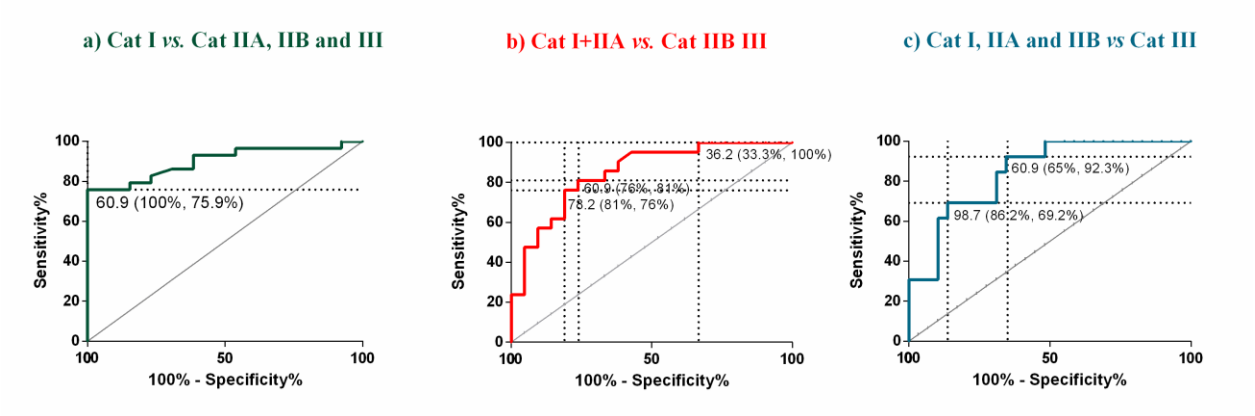

Figure 4. Receiver operating characteristic curves (ROC) of type III collagen for differentiation of the following: (a) healthy mares (category I endometrium—Cat I) from mares with endometrial degenerative/fibrotic lesions (Cat IIA, IIB, and III); (b) Cat I + IIA endometrium from IIB + III endometrium; (c) severe endometriosis (Cat III) from all the other endometrial categories. Cut-off values are expressed in ng/mL (specificity, sensitivity).

1.4.2. Experiment 2:

There were higher COL3 serum concentrations in infertile mares when compared to fertile mares ($p < 0.05$; Figure 5a). Higher COL3 in the older mares' serum (>9 years) was also observed ($p < 0.01$; Figure 5b). There was a low correlation between serum COL3 and fertility of the mares ($\rho = 0.35$, $p < 0.05$), and no correlation was observed between serum COL3 and mare's age. Mare's age was higher in the infertile group (14.68 years ± 1.2), when compared to the fertile group (7 years ± 0.95; $p < 0.0001$) (Figure 5c), and there was a moderate correlation between fertility and the age of the mares ($\rho = 0.68$, $p < 0.0001$). There were no differences with respect to the phase of the estrous cycle (Figure S2).

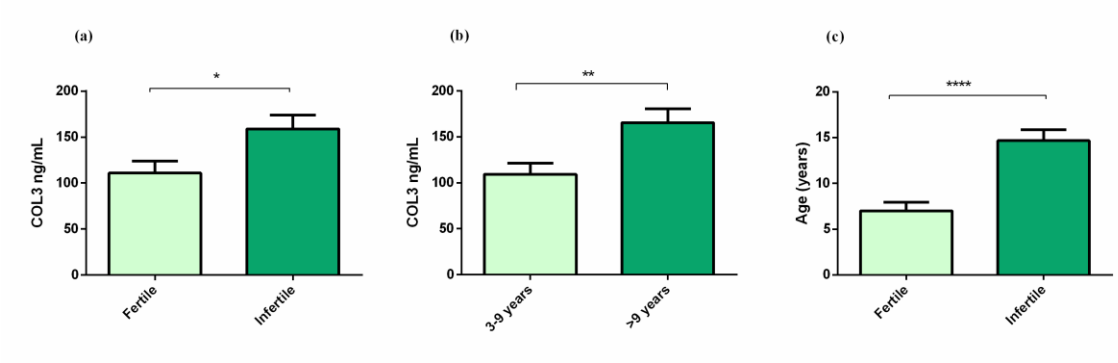

Figure 5. (a) Effect of fertility on serum type III collagen (COL3); (b) effect of age on serum COL3; (c) effect of age on fertility. Bars represent mean ± SEM. Asterisks indicate significant differences between fertility or age groups (* $p < 0.05$; ** $p < 0.01$, **** $p < 0.0001$).

The mares were then analyzed separately in each group (fertile and infertile) to assess the age effect (3 to 9 years old *vs*. over 9 years old) on serum COL3 concentrations. In both fertile and infertile groups, there were no differences between mare's age and serum COL3 (Figure S3). Additionally, no correlation was found between age and serum COL3 in both fertile and infertile mares ($r = 0.128$, $p > 0.05$; $r = - 0.009$, $p > 0.05$, respectively). The sensibility and specificity of serum COL3 were also determined. Serum COL3 cut-off value of 146 ng/mL allowed the differentiation of fertile from infertile mares, with a specificity of 82.4% and a sensitivity of 55.6% and the AUC was 0.72 (95% CI, 0.55 to 0.89; $p = 0.029$) (Figure 6).

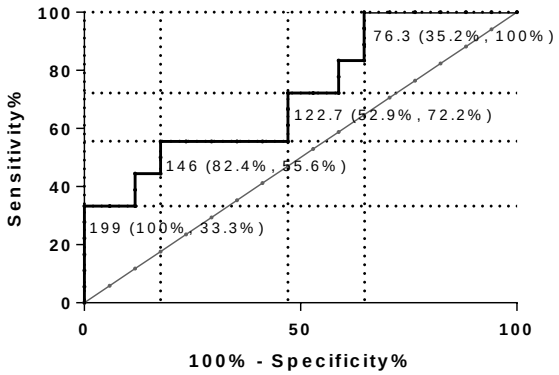

Fig. 6 – Receiver operating characteristic curves (ROC) of serum type III collagen for differentiation of fertile from infertile mares. Cut-off values are expressed in ng/mL (specificity, sensibility).

1.5. Discussion

In humans, many studies are being conducted to find diagnostic and prognostic fibrotic biomarkers for cardiac (Ong et al. 2020; Zannad et al. 2010), renal (Ix et al. 2015), and hepatic fibrosis (Rosenberg et al. 2004; Tanwar et al. 2017; Soylemezoglu et al. 1997), among many others (Gressnet et al. 2007). Biomarkers may be used for early detection of otherwise subclinical disease, diagnostic assessment of an acute or chronic clinical syndrome, risk

stratification of patients with a suspected or confirmed diagnosis, prognosis, and selection of an appropriate therapeutic intervention, and monitoring the response to therapy (Zannad et al. 2010). As an example, a study in abdominal aortic aneurysm in humans, higher amounts of hydroxyproline, COL1, and COL3 were found in samples of patients compared with healthy controls, and a positive correlation was found between tissue and serum concentrations of COL1, and COL3 (Metschl et al. 2019). Therefore, we aimed to identify blood biomarkers for the diagnosis of endometrosis and fertility in mares. Uterine biopsy is an invasive procedure and evaluation of blood biomarkers could facilitate the diagnosis of endometrosis, and/or to predict fertility, and provide additional information.

In the present work, up-regulation of COL1 protein production in mare endometrium, as the severity of histopathological lesions increased, according to Kenney and Doig's classification, was noted, as previously shown (Lunelli et al. 2013). This raise was also seen in COL3 and hydroxyproline concentrations in the endometrium, suggesting that both collagens are being produced when fibrosis increases. Nevertheless, it has been previously described that COL1 fibers are replaced by COL3 fibers with fibrosis, in the equine endometrium, and in several human organs, such as in liver and heart (Martinez-Hernandez 1999; Bochsler and Slauson 2002; Masseno 2012). However, other studies suggest that COL3 appears to regulate COL1 fibril formation in many human organs (Liu et al.,1997; Kuivaniemi and Trump 2019). In humans and animals (Miller and Gay 1987), COL1 exists in higher amounts, accounting for over 90% of bone protein, being also very high in skin and other soft tissues. Therefore, the use of COL1 as a blood biomarker should be interpreted with caution, as it may be related to pathologies of other organs (Karsdal et al. 2020). In contrast to COL1, the expression of COL3 is restricted to soft tissues, and correlates to the number of myofibroblasts in fibrotic tissue (Badid et al. 2000). Consequently, the accuracy for the presence of fibrotic processes in soft tissues is greater for COL3 (and other minor collagens), as compared to COL1 (Badid et al. 2000). In fact, type III procollagen peptides have been regarded as good prognostic biomarkers for liver fibrosis in humans (Nielsen et al. 2015; Leeming et al. 2016). However, it should be emphasized that COL3 is not an endometrial specific biomarker since its plasma elevation can occur due to other organ diseases (Gressner et al. 2007). The increase in COL1 and COL3 production in the endometrium was substantiated by a raise in hydroxyproline concentration (major component of COL) in tissues with severe endometrosis, and advanced fibrosis (categories IIB and III). Due to the highly restricted distribution of hydroxyproline in collagen and elastin, the hydroxyproline content generally reflects the amount of collagen in samples. In the serum, there was a significant decrease in COL1 between category I and IIB, and in hydroxyproline in category III compared to category I. Since COL1 is the most abundant fibrillar collagen, making up about 95% of the total collagen in animal tissue (Miller and Gay 1987), hydroxyproline in the mare also displayed the same patterns of COL1, both in endometrium and in serum, when endometrosis was

present. In addition, no correlation was found between endometrial and serum concentrations of COL1 or hydroxyproline. Thus, neither of them seems viable as a biomarker of endometrial fibrosis, and therefore, they should be ruled out. In our study, only COL3 data suggest it may be related to fibrosis, increasing both in serum and endometrium with fibrosis. Indeed, a moderate correlation between endometrial and serum COL3 concentration was observed, and a strong correlation was noted between endometrial category and serum COL3.

A ROC curve is an established method for evaluating the clinical viability of a biomarker (Luo et al. 2018). The sensibility and specificity of COL3 was most effective to differentiate mares from category I from all other categories, when compared to the ability to differentiate category I + category IIA from category IIB + III, or to identify mares with severe endometrosis (category III) among all the other endometrial categories. Serum COL3 was also higher in infertile mares, when compared to fertile mares, and a low positive correlation was observed between serum COL3 and infertile mares. Sensibility and specificity of COL3 was more accurate in differentiating healthy mares from mares with endometrial fibrosis, than for separating fertile mares from infertile mares. This might be explained by the fact that infertility in mares may be due to several causes other than endometrosis, and because both fertile and infertile groups of mares may include mares with different endometrial categories.

Old age has been associated with increased endometrial inflammation, increased embryo-loss rate, and subsequently reduced pregnancy rate in the mare (Carnevale and Ginther 1992). Although the age factor is not by itself the cause of endometrosis, in this study the endometrial fibrosis assessed by histopathological examination, was higher in older mares (category IIB and III) compared to the youngest ones, and a strong correlation between age and severity of the disease was found. This agrees with previous work (Schoon et al. 1992; Ricketts and Alonso 1991; Hoffmann et al. 2009a; Hoffmann et al. 2009b; Rebordão et al. 2019. Aging of the mares has also been related to increased fibrosis in the oviduct (Pinto-Bravo et al. 2018), which might be an additional cause of infertility/sub-fertility in this species. Furthermore, there was an increase in both types of collagens under study and hydroxyproline in the endometrium with mares aging, suggesting that the age factor plays an important/significant role and should not be dismissed. Regarding serum concentrations, COL1 was higher, and COL3 was lower in young mares, when compared to older mares, and no differences were found in hydroxyproline. Higher serum COL1 concentrations in younger horses, and lower in older horses, have been previously described (Price et al. 1995). Most of those studies refer to equine musculoskeletal pathologies, and the described high serum COL1 concentrations appear to be ascribed to the ongoing bone formation in young horses. Therefore, serum COL1 is more likely to represent bone and cartilage status than endometrosis. Hence, the age factor may interfere with its use as an endometrial fibrosis biomarker. In the present study, the highest serum COL1 concentrations observed in mares with healthy endometrium (category I), and the lowest ones

in mares with endometrial fibrosis, might be explained by the inclusion of younger mares in category I, and of older ones in categories IIB and III. The infertile mares were older than the fertile mares, and a moderate positive correlation was observed between age and infertility. However, no correlation was observed between serum COL3 and mare's age.

To the best of our knowledge no other studies were carried out on serum COL3, to evaluate endometrial fibrosis and fertility in mares. As stated above, it should be emphasized that COL3 is not an endometrial specific biomarker, and alterations in its concentration can occur due to other organ diseases (Gressner et al. 2007). Therefore, further studies are necessary to study the possible involvement of COL3 in other organs. Additionally, many collagen biomarkers detect a specific type of COL, but do not distinguish between newly formed and older matrix-deposited COL, or between COL formation and degradation (Karsdal et al. 2011), as in this study. Hence, the investigation of COL turnover, by determination of its synthesis and degradation products, could give a more precise information regarding the stage of fibrosis, and should be further investigated.

1.6. Conclusion

In conclusion, serum COL3 concentration might be considered as a potential aid for the diagnosis of endometrial fibrosis and fertility prognosis in the mare. In contrast, COL1 and hydroxyproline did not prove to be effective as putative biomarkers of endometrial fibrosis in this species. Although it is very unlikely a single blood biomarker could replace a histopathological evaluation, serum COL3 may have clinical applications. As such, it may be used to evaluate a group of mares as possible recipients in embryo transfer programs, where performing endometrial biopsies of several mares is not feasible.

Supplementary Figures

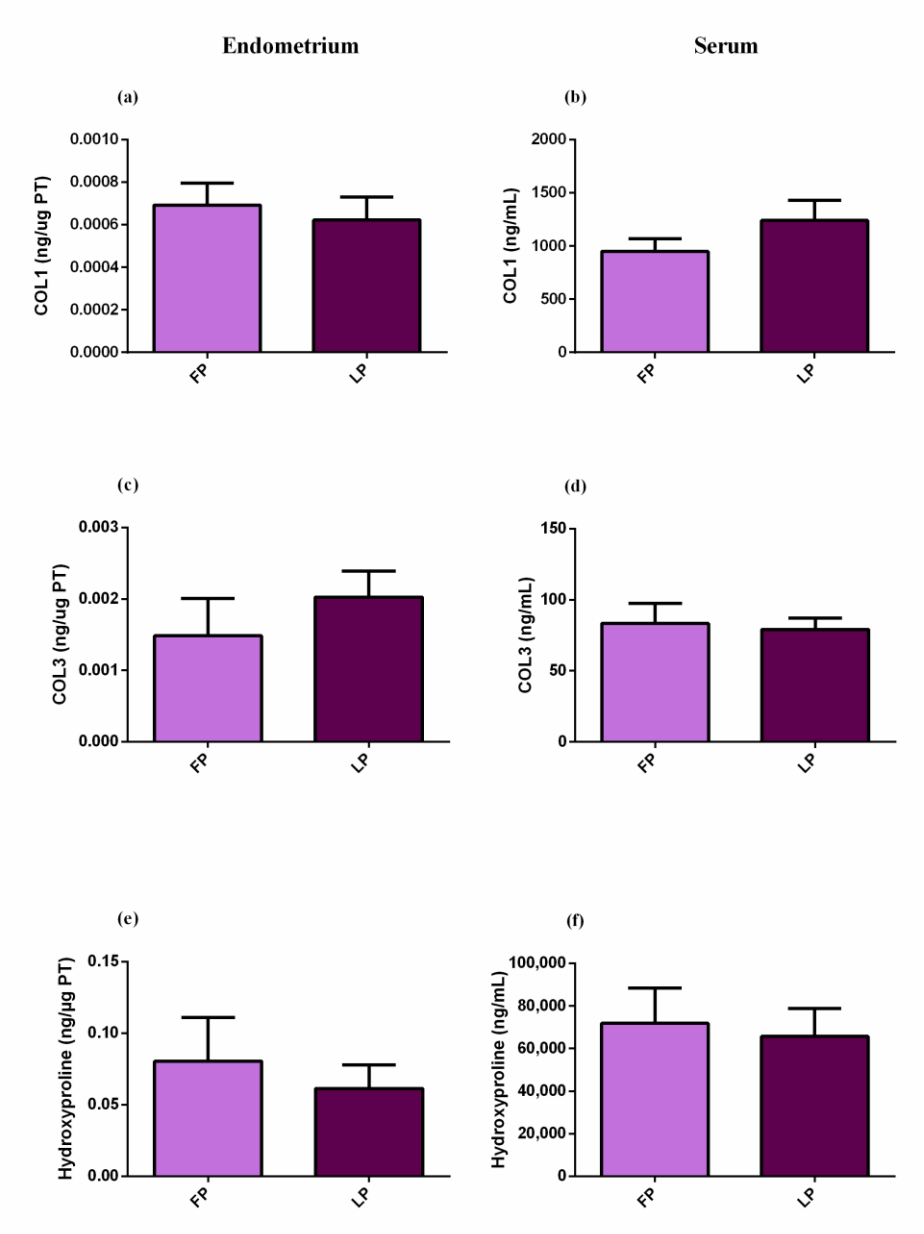

Figure S1. Effect of equine estrous cycle phases (Folicular phase – FP and Luteal phase – LP) on the concentration of (a) type I collagen (COL1) in endometrial tissue; (b) COL1 in serum; (c) type III collagen (COL3) in endometrial tissue; (d) COL3 in serum; (e) hydroxyproline in endometrial tissue; and (f) hydroxyproline in serum. Bars represent mean±SEM.

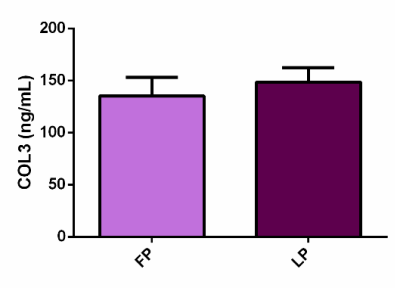

Figure S2. Effect of equine estrous cycle phases (Folicular phase – FP and Luteal phase – LP) on the concentration of serum COL3. Bars represent mean±SEM.

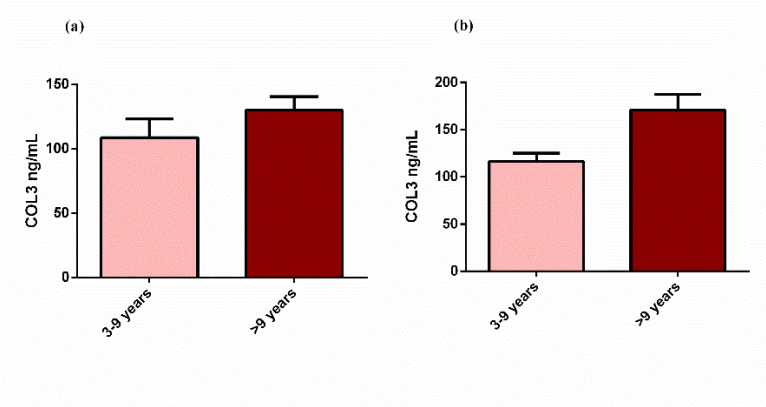

Figure S3. Effect of mares age (3 to 9 years old *vs*. over 9 years old) on the serum concentrations of type III collagen (COL3) in (a) fertile mares and (b) infertile mares. Bars represent mean±SEM.

2 - Collagens and DNA methyltransferases in mare endometrosis

Alpoim-Moreira J, Fernandes C, Rebordão MR, Amaral A, Pinto-Bravo P, Bliebernicht M, Skarzynski DJ; Ferreira-Dias G. 2019. Collagens and DNA methyltransferases in equine endometrosis. Reprod Dom Anim. 54:46–52. doi:10.1111/rda.13515

2.1. Abstract

Inflammation and fibroproliferative diseases may be modulated by epigenetic changes. Therefore, we suggest that epigenetic mechanisms could be involved in equine endometrosis pathogenesis. DNA methylation is one of the methods to evaluate epigenetics, through the transcription of methyltransferases (DNMT1, DNMT3A, DNMT3B). The correlation between DNMTs and collagen (COL) transcripts was assessed for the different Kenney and Doig's (1986) endometrium categories. Endometrial biopsies were randomly collected from cyclic mares. Histological classification (category I, n=13; II A, n=17; II B, n=12; and III, n=7) and evaluation of COL1A2, COL3A1 and DNMTs transcripts by qPCR, were performed. Data were analysed by one-way analysis of variance (ANOVA), Kruskal–Wallis test and Pearson correlation. As mares aged, there was an increase in endometrium fibrosis ($p < 0.01$), and in DNMT1 mRNA ($p < 0.001$). Considering DNMT3B transcripts for each category, there was an increase with fibrosis ($p < 0.05$). No changes were observed for DNMT1 and DNMT3A transcripts. However, DNMT3A mRNA levels were the highest in all categories ($p < 0.01$). In category I endometrium, a positive correlation was observed for transcripts of all DNMTs in both COLs ($p < 0.01$). In category IIA, this correlation was also maintained for all DNMTs transcripts in COL1A2 ($p < 0.05$), but only for DNMT3B in COL3A1 ($p < 0.05$). In category IIB, there was a positive correlation between DNMT3B and COL3A1 ($p < 0.05$). In category III, a positive correlation was only observed between DNMT3B and COL3A1 ($p < 0.05$). Our results suggest that there is a disturbance in COLs and DNMTs correlation during fibrosis.

Keywords: collagen, DNA methylation, endometrium, epigenetic, fibrosis, mare

2.2. Introduction

Epigenetic mechanisms are responsible for heritable changes in genome function that do not alter the nucleotide sequence. The most common epigenetic mechanism is DNA methylation, which regulates gene activity. The predominant mammalian DNA methylating enzyme is DNMT1. This protein is responsible for the restoration of hemi-methylated sites to full methylation (maintenance methylation) (Du et al. 2015), while DNMT3A and DNMT3B are *de novo* enzymes mainly involved in the methylation of new sites (Jeltsch 2006). In the promotor

regions of many genes the most commonly hypermethylation events lead to gene repression (Mohn et al. 2008), whereas hypomethylation results in gene induction (Karouzakis et al. 2009). Epigenetic changes are potentially induced by environmental factors, are potentially reversible, and thus promising targets in therapeutics.

Endometrosis has long been recognized as a major cause of infertility (Kenney 1992). In fibrotic tissues, myofibroblasts are the primary contributors to the excessive production of extracellular matrix proteins, such as collagen. Collagen type I (COL1) and type III (COL3) are the most common collagens in mare endometrium (Lunelli et al. 2013). Alterations in DNA methylation have a substantial impact on fibroblast phenotype and promote the differentiation to pathological myofibroblasts leading to fibrosis (Neary et al. 2015). Recently, epigenetic alterations have been linked to inflammation and chronic fibrosis (Hahn et al. 2008; Dakhlallah et al. 2013).

Overcoming endometrial fibrosis is a major unmet clinical need. Therefore, our aim was to study the existence of a possible correlation between mRNA levels of *DNMTs* and *COL1A2* and *COL3A1* in different Kenney and Doig's endometrial categories.

2.3. Material and Methods

All mares' internal genitalia were systematically examined by transrectal ultrasonography (Sonovet 600; rectal linear probe 7.5 MHz). Endometrial biopsies were randomly collected from cyclic mares (May to July) with a biopsy alligator jaw forceps (ref. 141965; Kruuse), complying to welfare mandates as a clinical procedure, and with owner's consent. The mean age of mares within category I was 3 ± 0 years (year; mean ± SEM); in category IIA, 8.7 ± 1.46 years; in category IIB, 11.08 ± 1.61 years and 16.71 ± 1.67 years in category III.

Right after endometrial biopsy procurement, tissue was immersed in RNA later for qPCR or in 4% buffered formaldehyde solution. Formaldehyde-fixed endometrium was paraffin embedded, and haematoxylin (05-06014E; Bio-Optica) and eosin- (HT1103128; Sigma-Aldrich) stained sections were examined under a light micro- scope (Leica DM500). Endometrial biopsies were graded based on the extent of inflammation and /or fibrosis, following Kenney and Doig′s classification (1986). They were assigned to category I (n = 13) when the endometrium was healthy or with slight or sparse inflammation or fibrosis; to category IIA (n = 17) when there was mild and scattered inflammation and fibrosis; to category IIB (n = 12), when moderate inflammation or fibrosis was present; or to category III (n = 7), characterized by severe irreversible fibrosis and/or inflammation. For qPCR studies, RNA isolation and cDNA synthesis were performed as described (Rebordão et al. 2018). Briefly, total RNA was extracted using TRI Reagent (Ref T9424; Sigma Life Science) according to manufacturer's instructions. Quantification and quality of RNA were carried out with a Nanodrop system (ND; Fisher

Scientific, Spain) and by agarose gel electrophoresis. The cDNA was obtained from total RNA (1 µg), using M-MLV Reverse transcriptase (Ref. M1705; Promega) and oligo (dT) 15 primer (Ref. C101; Promega). Specific primers were designed, as well as the reference gene (Table 5), using the Internet-based program Primer-3 (Untergasser et al. 2012) and Primer Premier software (Premier Biosoft Interpairs). Mitochondrial ribosomal protein L32 (MRPL32) was chosen as the most stable internal control gene (Dheda et al. 2004), among four validated reference genes, as described (Rebordão et al. 2018). Using Power SYBER Green PCR Master Mix (Ref. 4367659; Applied Biosystems) and a StepOne-Plus TM Real-Time PCR System (Applied Biosystems), qPCR studies of target and reference genes were performed simultaneously. Zhao and Fernald (2005) method was used to analyze the relative mRNA data.

Kruskal–Wallis analysis followed by Dunn's multiple comparison test was performed to compare DNMTs transcripts between endometrial categories. One-way analysis of variance (ANOVA) was used to analyze COLs data with endometrial categories and age. Paired t test was used to compare COLs within each category. Pearson correlation test was used to assess a putative correlation between COLs and DNMTs by endometrial category (GraphPAD PRISM, Version 8.1.0, 253, San Diego, CA, USA). Significance was considered when $p < .05$. Data are presented as mean±SEM.

Table 5: Primers sequences used in qPCR study

Gene (Accession number)	Sequence 5'-3'	Amplicon
COL1A2 (XM_001492939.3)	Forward: CAAGGGCATTAGGGGACACA	196
	Reverse: ACCCACACTTCCATCGCTTC	
COL3A1 (AF117954.1)	Forward: CAAAGGAGAGCCAGGAGCAC	98
	Reverse: CTCCAGGCGAACCATCTTTG	
DNMT1 (XM_023645449.1)	Forward: CAAGGCAAACAACCAGGCA	237
	Reverse: CTTCCTCCTCTTCCGTGTGTGT	
DNMT3A (XM_023619394.1)	Forward: GCCTCAATGTCACCCTGGAA	206
	Reverse: AAGAGGTCCACACATTCCACG	
DNMT3B (XM_023626333.1)	Forward: GAGCTGGCAAGACTTTCCCC	198
	Reverse: TTGGGTGGAGGGCAGTAGTC	
MRPL32 (XM_001492042.6)	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

COL1A2-collagen type 1, alpha2; *COL3A1*-collagen, type III, alpha 1; *DNMT1*, *DNMT3A*, *DNMT3B* - DNA methyltransferase 1, 3A; 3B; *MRPL32*-mitochondrial ribosomal protein L32.

2.4 Results

Histological classification of mares' endometrium biopsy showed an increase in endometrium fibrosis ($r = 0.872$; $p < 0.0001$), and in *DNMT1* mRNA ($r = 0.516$; $p < .001$) with ageing (data not shown).

Regarding *DNMT1* and *DNMT3A* transcripts, there were no changes within endometrial categories (Figure 7A,B). However, *DNMT3B* transcripts increased with fibrosis establishment

from category I to III ($p < 0.05$; Figure 7C). For each category, relative *DNMT3A* mRNA levels were the highest compared with the other *DNMT*s ($p < 0.01$; data not shown). Concerning *COL1A2* and *COL3A1* transcripts, no differences were noted within categories (Figure 7D,E).

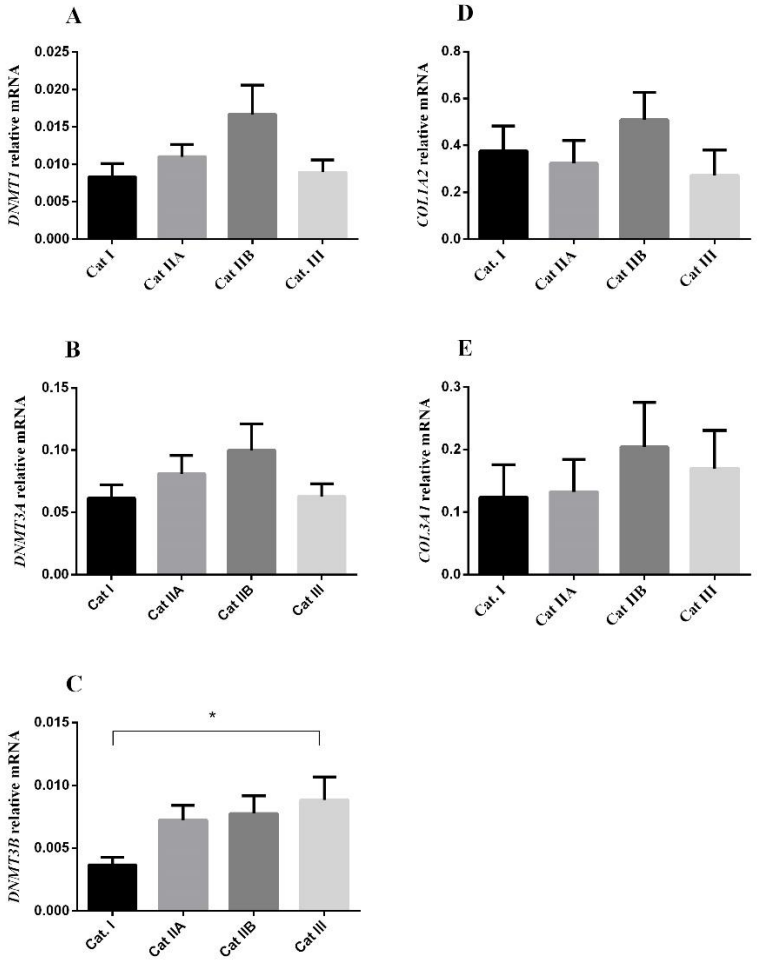

Figure 7. Relative *DNMT1* (a), *DNMT3A* (b), *DNMT3B* (c), *COL1A2* (d) and *COL3A1* (e) transcripts by equine endometrium graded as Kenney and Doig's; categories I (n = 13), IIA (n = 17), IIB (n = 12) and III (n = 7). Bars represent means±SEM. The asterisk indicates significant differences between endometrial categories (*$p < 0.05$).

The correlation between *DNMT*s and *COL*s transcripts was also assessed. In category I endometria, a positive correlation was observed between *DNMT1* ($r = 0.699$; $p < .001$), *DNMT3A* ($r = 0.789$; $p < 0.01$) or *DNMT3B* ($r = 0.827$; $p < 0.001$) and *COL1A2* (Figure 8A,B and C); and between *DNMT1* ($r = 0.703$; $p < 0.01$), *DNMT3A* ($r = 0.744$; $p < 0.01$) or *DNMT3B* ($r = 0.793$; $p < 0.01$) and *COL3A1* (Figure 8D,E and F).

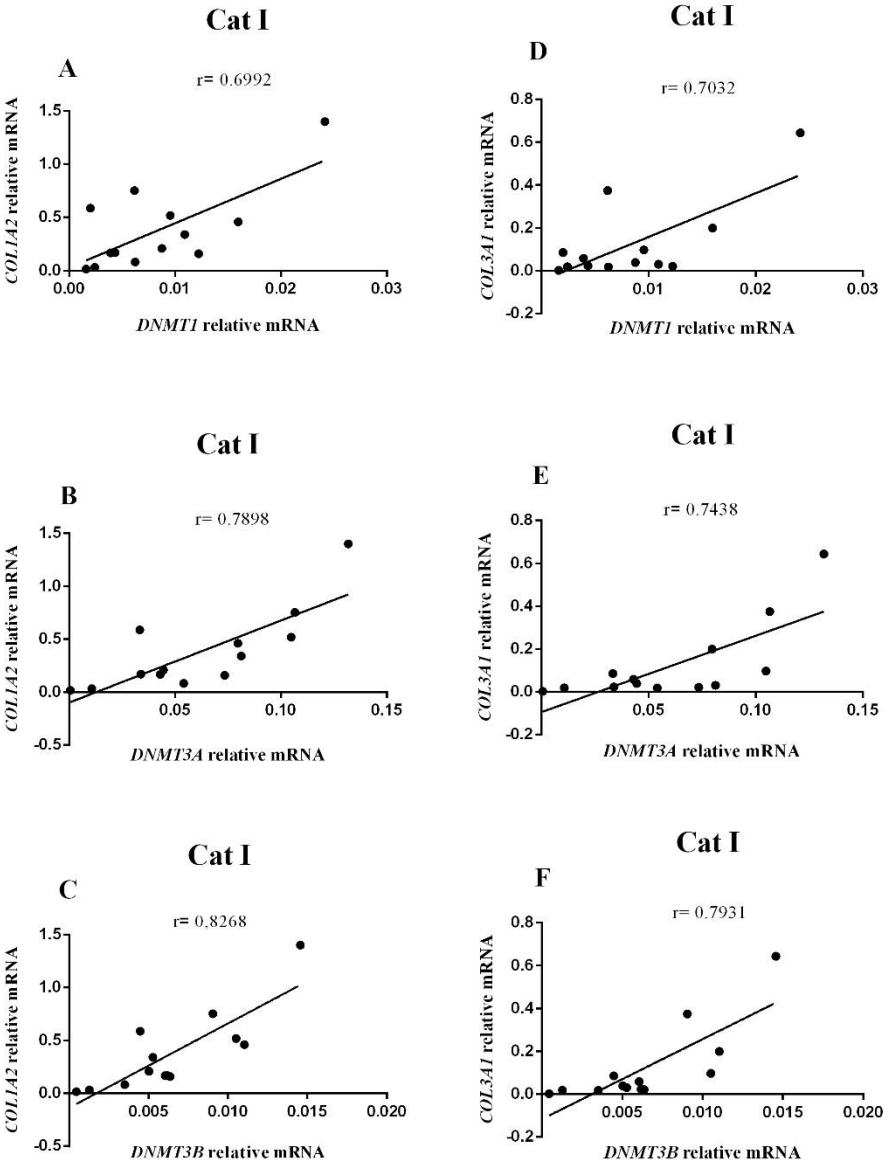

Figure 8. Correlation between transcripts of *DNMT*s and *COL1A2* or *COL3A1* in equine category I endometria. r = Pearson correlation coefficient values. p-values between *DNMT1* (a; $p < .001$), *DNMT3A* (b; $p < 0.01$) or *DNMT3B* (c; $p < 0.001$) and *COL1A2*, and between *DNMT1* (d; $p < 0.01$), *DNMT3A* (e; $p < 0.01$) or *DNMT3B* (f; $p < 0.01$) and *COL3A1*, in category I endometria.

In category IIA endometria, there was a positive correlation between *DNMT1* ($r = 0.594$; $p < 0.05$), *DNMT3A* ($r = 0.676$; $p < 0.05$) or *DNMT3B* ($r = 0.794$; $p < 0.001$) and *COL1A2* (Figure 9A,B and C), and between *DNMT3B* ($r = 0.646$; $p < 0.01$) and *COL3A1* (Figure 9D). However, in category IIB endometria, there was a positive correlation only for *DNMT3B* ($r = 0.585$; $p < 0.05$) and *COL3A1* (Figure 9E). Likewise, in category III, the only positive correlation was between DNMT3B transcripts and those of COL3A1 ($r = 0.780$; $p < 0.05$; Figure 9F).

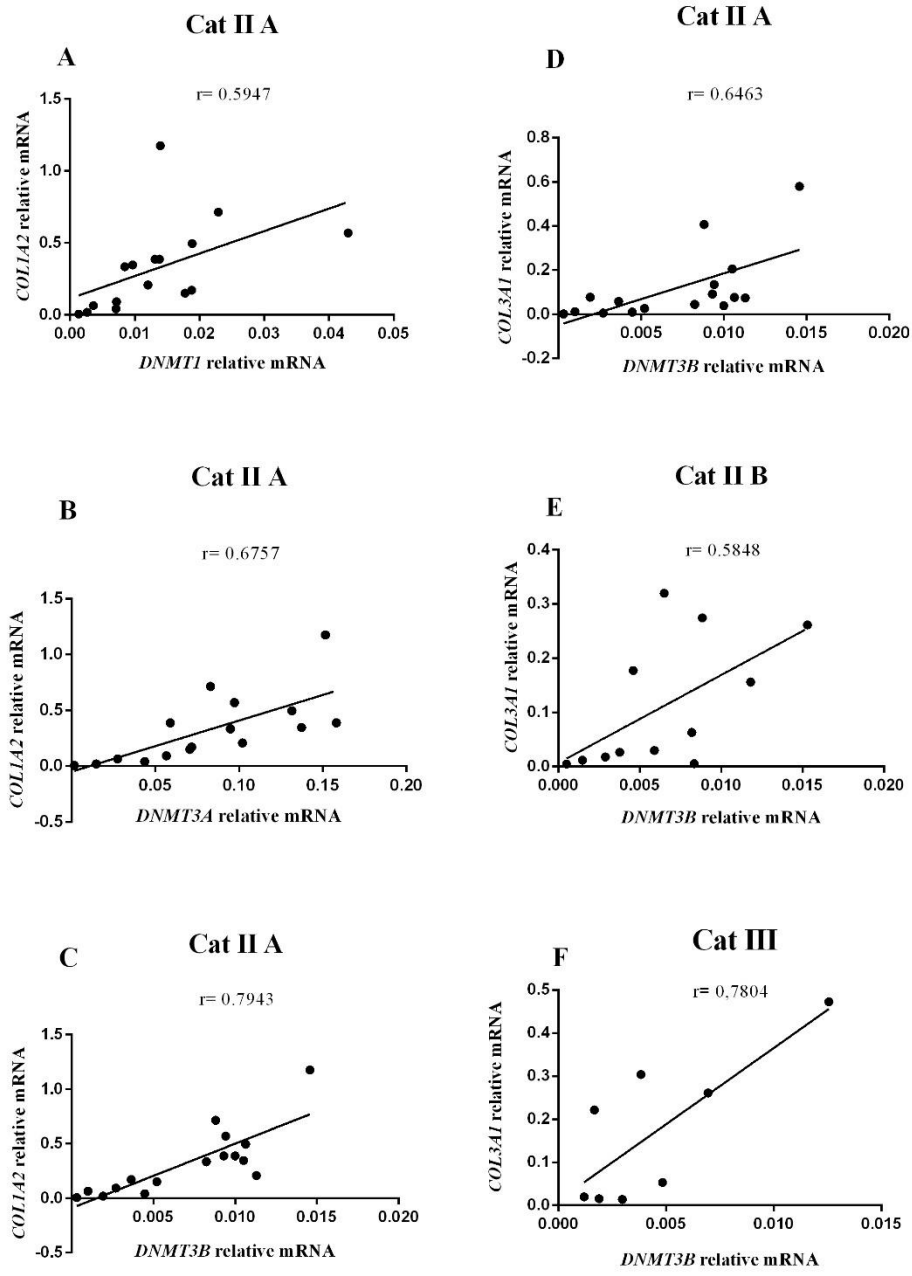

Figure 9. Correlation between transcripts of *DNMT*s and *COL1A2* or *COL3A1* in equine category IIA, IIB and III endometria. r = Pearson correlation coefficient values. p-values between *DNMT1* (a; $p < 0.05$), *DNMT3A* (b; $p < 0.05$) or *DNMT3B* (c; $p < .001$) and *COL1A2*; *DNMT3B* (d; $p < 0.01$) and COL3A1 in category IIA endometria; between *DNMT3B* and *COL3A1* (e; $p < 0.05$) in category IIB endometria, and between *DNMT3B* and *COL3A1* (f; $p < 0.05$) in category III endometria.

2.5 Discussion

In this study, endometrial fibrosis assessed by histopathological examination, raised in older mares (category IIB and III) compared to the young ones. This is in agreement with previous work (Rickets and Alonso 1991). Nevertheless, this increase in fibrosis was not simultaneous with a raise in *COL1A2* or *COL3A1* transcripts in severe endometrosis (category III). Gene expression is regulated by a dynamic balance between transcriptional and post-

transcriptional events, including processing and destruction of mRNAs, translation, alteration and damage of the resulting proteins (Vogel and Marcotte 2012). Thus, a discrepancy between *COL* transcripts and the presence of the collagen fibers (protein) in the mare endometrium might be expected. Pathogenesis of endometrosis might be associated to ageing, but also to a plethora of other factors, such as epigenetics, and neutrophil extracellular traps involvement (Rebordão et al. 2018).

To the best of our knowledge this is the first study to evaluate mRNA levels of DNA methylases and their correlation with *COL* transcripts in the endometrium of mares with different degrees of fibrosis. In the last stage of endometrosis (category III) *DNMT3B* transcripts increased. *DNMT3A* mRNA levels were the highest compared to the other *DNMTs* in all categories, which has also been reported for rat lung fibroblasts and epithelial cells (Hu et al. 2010). Also, in older mares *DNMT1* mRNA levels were elevated. This upregulation could reflect the downregulation of an anti-fibrotic gene, and therefore contributing to further fibrosis establishment, as described for idiopathic pulmonary fibrosis in humans (Sanders et al. 2008). During aging, global DNA methylation may be lost (Richardson 2003), but locus-specific hypermethylation may occur in many genes, such as collagen (Johnson et al. 2012). However, in this study, identification of specific methylation sites is yet to be performed to better understand if methylation occurred at the promoter gene region or elsewhere.

As endometrial fibrosis increased with Kenney and Doig's grade (categories IIB and III), there was no correlation between *COL1A2* and *DNMT1*, *DNMT3A* and *DNMT3B* transcripts. Concerning *COL3A1*, similar data were found except for *DNMT3B*. Our results suggest that there might be an association between *COLs* and *DNMTs* in the healthy endometrium.

Although *DNMT3B* mRNA showed a correlation with *COL3A1* mRNA in categories IIB and III, the same did not occur for *COL1A2* mRNA. This lack of correlation may suggest that with fibrosis progression, DNA methylation patterns no longer relate to *COL* transcription. This study suggests an involvement of epigenetic changes in mare endometrial collagen deposition and fibrosis.

3. Metallopeptidades 2 and 9 genes epigenetically modulate equine endometrial fibrosis

Alpoim-Moreira J, Fernandes C, Pimenta J, Bliebernicht M, Rebordão MR, Castelo-Branco P, Szóstek-Mioduchowska A, Skarzynski DJ, Ferreira-Dias G. 2022. Metallopeptidades 2 and 9 genes epigenetically modulate equine endometrial fibrosis. *Frontiers in veterinary science*, *9*, 970003. https://doi.org/10.3389/fvets.2022.970003

3.1. Abstract

Endometrium type I (COL1) and III (COL3) collagen accumulation, periglandular fibrosis and mare infertility characterize endometrosis. Metalloproteinase-2 (MMP-2), MMP-9 and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) are involved in collagen turnover. Since epigenetic changes may control fibroproliferative diseases, we hypothesized that epigenetic mechanisms could modulate equine endometrosis. Epigenetic changes can be reversed and therefore extremely promising for therapeutic use. Methylation pattern analysis of a particular gene zone is used to detect epigenetic changes. DNA methylation commonly mediates gene repression. Thus, this study aimed to evaluate if the transcription of some genes involved in equine endometrosis was altered with endometrial fibrosis, and if the observed changes were epigenetically modulated, through DNA methylation analysis. Endometrial biopsies collected from cyclic mares were histologically classified (Kenney and Doig category I, $n = 6$; category IIA, $n = 6$; category IIB, $n = 6$ and category III, $n = 6$). Transcription of *COL1A1, COL1A2, COL3A1, MMP2, MMP9, TIMP1*, and *TIMP2* genes and DNA methylation pattern by pyrosequencing of *COL1A1, MMP2, MMP9, TIMP1* genes were evaluated. Both *MMP2* and *MMP9* transcripts decreased with fibrosis, when compared with healthy endometrium (category I) ($P < 0.05$). *TIMP1* transcripts were higher in category III, when compared to category I endometrium ($P < 0.05$). No differences were found for *COL1A1, COL1A2, COL3A1* and *TIMP2* transcripts between endometrial categories. There were higher methylation levels of (i) *COL1A1* in category IIB ($P < 0.05$) and III ($P < 0.01$), when compared to category I; (ii) *MMP2* in category III, when compared to category I ($P < 0.001$) and IIA ($P < 0.05$); and (iii) *MMP9* in category III, when compared to category I and IIA ($P < 0.05$). No differences in *TIMP1* methylation levels were observed between endometrial categories. The hypermethylation of *MMP2* and *MMP9*, but not of *COL1A1* genes, occurred simultaneously with a decrease in their mRNA levels, with endometrial fibrosis, suggesting that this hypermethylation is responsible for repressing their transcription. Our results show that endometrosis is epigenetically modulated by anti-fibrotic genes (*MMP2* and *MMP9*) inhibition, rather than fibrotic genes activation and therefore, might be promising targets for therapeutic use.

Keywords: DNMT; MMP; collagen; endometrial fibrosis; endometrosis; epigenetics; mare.

3.2. Introduction

Equine endometrosis is a multifactorial disease considered to be one of the most important causes of equine infertility, especially in older mares (Buczkowska et al. 2014) and has an economic impact on the horse breeding history (Schöniger and Schoon 2020). Endometrosis is characterized by periglandular fibrosis of the equine endometrium (Kenney 1978) which compromises the integrity and function of the endometrial glands required for embryonic preimplantation and placental development (Gray et al. 2001). However, this condition is still a puzzle regarding its pathogenesis and treatment. Periglandular arrangement of myofibroblasts, associated with the deposition of extracellular matrix (ECM), such as collagen (COL), is a cardinal feature of endometrosis in mares (Walter et al. 2005). Bochsler and Slauson (Bochsler and Slauson 2002) stated that the deposition of collagen types I (COL1) and III (COL3) occurs in fibrotic processes, which promotes the development of cicatricial tissues with major tensile strength. In a healthy endometrium, the first collagen to be synthesized is COL3, which in turn is gradually replaced by COL1 following the development of fibrotic lesions (Masseno 2009; Cost, 2015). Collagen type I is usually predominant in Kenney's category III endometrium (severe fibrosis) (Masseno 2009), and COL3 in category I healthy endometrium (Lunelli et al. 2013). Matrix metalloproteinases (MMPs) are a family of extracellular endopeptidases (Ra and Parks 2007) that are important factors in the process of fibrosis. Data concerning MMP expression in equine endometrial fibrosis are limited but MMP-2 and MMP-9 (now called 72 kDa gelatinase and 92 kDa gelatinase, in the horse), seem to be involved in this process (Aresu et al. 2012; Centeno et al. 2018; Crociati et al. 2019; Szóstek-Mioduchowska et al. 2020). MMP-2 and MMP-9 are gelatinases that denature collagens (gelatins) and other ECM substrates (Vandooren et al. 2013; Djuric and Zivkovic, 2017). The endogenous inhibitors of MMPs are tissue inhibitors of metalloproteinases (TIMPs) and neutralize the activity of MMPs. Among the four types of TIMPs, TIMP-1 is a specific inhibitor for MMP-9 (Vandooren et al. 2013) while TIMP-2 regulates MMP-2 activity (Giannandrea and Parks 2014). A key feature of fibrosis is the imbalance between MMPs and TIMPs resulting the loss of the homeostasis between fibrolysis and fibrogenesis (Hemmann et al. 2007).

Novel findings implicate a role for epigenetic modifications contributing to the progression of fibrosis by alteration of gene expression profiles (Neary et al. 2015; Bergmann and Distler 2017; Avci et al. 2022; Yang et al. 2022). Epigenetic modifications, heritable changes in the genome that do not alter the DNA sequence, influence, or regulate gene expression (Duong and Hagood 2018). Epigenetic changes, unlike genetic alterations, can be reversed (Simmons 2008) as thus extremely promising for therapeutic use. In mammals, the most studied epigenetic

events are DNA methylation and histone modifications, such as methylation, acetylation, ubiquitination, and phosphorylation (Jenuwein and Allis 2001; Das and Singal 2004). DNA methylation constitutes a major epigenetic modification of the genome and is essential for cellular reprogramming, tissue differentiation, and normal development related to many biological processes including gene expression regulation. DNA methylation is known to occur at the 5′ of cytosine in CpG dinucleotides which are found mostly in so-called CpG islands present in promoters (Newell-Price et al. 2000; Curradi et al. 2002; Ehrlich and Lacey 2013) and is catalyzed by DNA methyltransferases (DNMTs) such as DNMT1, DNMT3a, and DNMT3b (Robertson 2002). These CpG islands are enriched in promotor regions close to transcriptional start sites and their methylation might prevent the transcription of the respective gene (Jones 2012; Klein and Gay 2015). Hypermethylation of a promoter has long been well recognized as an efficient means of repressing transcription (Fuks 2005). The majority of CpG sites outside of CpG islands are methylated, suggesting a role in the global maintenance of the genome, while the majority of CpG islands in gene promoters are unmethylated, which allows active gene transcription (Herman and Baylin 2003; Webber et al. 2007). Transcriptional factors bind to the unmethylated promotor region of a gene to allow its transcription. But, if that region becomes hypermethylated this binding does not occur, and this transcription is not activated. However, most recently, it was demonstrated that intragenic DNA methylation could also affect the gene expression (Singer et al. 2015). In fact, differential methylation within the gene body plays a role in several gene regulation processes (Kullis et al. 2013). One way to evaluate epigenetics mechanisms is through DNA methylation by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B). However, this only reflects the level of these enzymes, which in turn may indicate the level of global methylation. Another way to assess the DNA methylation pattern is by bisulfite pyrosequencing. This method is very accurate and is commonly used for quantitative analysis of DNA methylation at single nucleotide level, and in a particular region of the gene (CpG islands), providing more detailed information.

As such, we proposed to evaluate the transcriptomic pattern of some of the most relevant genes involved in mare endometrosis (*COL1A1, COL1A2, COL3A1, MMP2, MMP9, TIMP1*, and *TIMP2*) and secondly, perform epigenetic studies of the genes that have shown alterations (through DNA methylation analysis) to determine whether there is an epigenetic regulation of endometrial fibrosis in mares.

3.3. Materials and Methods

3.3.1. Animals

During the breeding season, Lusitano cyclic mares (*n* = 24; 6 per Kenney and Doig's category; 3 in luteal phase and 3 in follicular phase per category) were used for endometrial

biopsy procurement. Mare's internal genitalia were examined by transrectal ultrasonography (Sonovet 600). Endometrial biopsies were randomly obtained from cyclic mares (May to July), with a biopsy alligator jaw forceps (ref. 141965; Kruuse), complying to welfare mandates as a clinical procedure, and with owner's consent. The age of mares ranged from 3 ± 0 years within category I; from 3 to10 years (5.17 ± 1.38) in category IIA, from 6 to 14 years (9.50 ± 1.26) in category IIB, and 11 to 23 years (18.5 ± 2.72) in category III.

The tissue was divided into small pieces with a scalpel and then immersed in RNA later for qPCR or in 4% formaldehyde solution for histopathological evaluation. Formaldehyde-fixed endometrium was paraffin embedded and hematoxylin (05-06014E; Bio-Optica) and eosin (HT1103128; Sigma-Aldrich) stained sections were examined under a light microscope (Leica DM500; Leica Microsystems, Mannheim, Germany). Endometrial biopsies were graded based on the extent of inflammation and /or fibrosis, following Kenney and Doig's classification (Kenney and Doig 1986). They were assigned to category I (n = 6) when the endometrium was healthy or with slight or sparse inflammation or fibrosis; to category IIA (n = 6) when there was mild and scattered inflammation and fibrosis; to category IIB (n = 6), when moderate inflammation or fibrosis were present; or to category III (n = 6), characterized by severe irreversible fibrosis and/or inflammation.

3.3.2. Real time PCR

Endometrial biopsies, from different Kenney and Doig´s categories, were used for the evaluation of *COL1A1*, *COL1A2*, *COL3A1*, *MMP2*, *MMP9, TIMP1* and *TIMP2* transcripts, after RNA isolation, cDNA synthesis and qPCR studies, performed as described (Rebordão et al. 2018). Briefly, total RNA was extracted using TRI Reagent (Ref T9424; Sigma Life Science), including a DNA-digestion step with an RNase-free DNase (Ref. 79254, RNase-Free DNase Set, Qiagen, Germany), according to manufacturer's instructions. Quantification and quality of RNA was carried out with a Nanodrop system (ND; Fisher Scientific, Spain) and by agarose gel electrophoresis, respectively. The cDNA was obtained from total RNA (1µg), using M-MLV Reverse transcriptase (Ref. M1705; Promega) and oligo (dT) 15 primer (Ref. C101; Promega). Specific primers were designed, as well as the reference gene (supplementary Table 1), using the Internet-based program Primer-3 (Untergasser et al. 2012) and Primer Premier software (Premier Biosoft Interpairs). Mitochondrial ribosomal protein L32 (*MRPL32*) was chosen as the most stable internal control gene (Dheda et al. 2004) among four validated reference genes, as described (Rebordão et al. 2018). Using Power SYBER Green PCR Master Mix (Ref. 4367659; Applied Biosystems) and a StepOne-Plus TM Real-Time PCR System (Applied Biosystems), qPCR studies of target and reference genes were performed simultaneously. Zhao and Fernald (Zhao and Fernald 2005) method was used to analyze the relative mRNA data.

3.3.3. DNA Preparation

DNA extraction was performed using the kit Quick-DNA 96 Plus Kit (Zymo Research®). Briefly the biopsy sample (25 mg) was diluted with 95 µL of DEPC water and 95 µL Buffer Solid Tissue as indicated in the Kit. The samples were then macerated using the TissueLyser (QIAGEN), 5 cycles of 25mHz for 30 s. The protocol was performed as instructed by the Kit. Quantification and quality of DNA was carried out with a Nanodrop system (ND; Fisher Scientific, Spain). 500 µL of each sample (24 in total; 6 per Kenney and Doig's category) were sent to an external lab for DNA bisulfite pyrosequencing methylation analysis.

3.3.4. Promoter annotations

CpG islands and CG percentage were predicted for *COL1A1, MMP2, MMP9* and *TIMP1* equine gene sequences with MethPrimer software (Li and Dahiya 2002). Within these, hotspot regions with the highest percentage of CpGs were identified and used in our analysis (supplementary Table 2). Among the four genes (*COL1A1, MMP2, MMP9* and *TIMP1*) studied, three were annotated in this study to possess CpG islands within the promoter region (*COL1A1, MMP2* and *TIMP1*). Only *MMP9* did not have CpG islands in the first 1,000 bp and therefore the studied region was exon 8, by homology with human studies, which have demonstrated regulation of *MMP9* gene transcription in this region (Falzone et al. 2016).

3.3.5. DNA bisulfite pyrosequencing analysis (CpG islands)

The bisulfite modified DNA sample was then 10-fold diluted and 1 µL of diluted DNA was used in PCR reactions with 3 µL 10xPCR buffer, 200 µL/L of dNTPs, 6 pmol forward primer, 6 pmol reverse primer, and 3 mmol/L $MgCl_2$, 0.75 U Qiagen HotStar Taq polymerase (Qiagen Inc., Valencia, CA., 205203) in 30 µL total volume adjusted using double distilled H_20, as necessary. The PCR cycling condition was as follows: 95ºC 15 minutes; 45 x (95ºC 30s; 51ºC 30s: 72ºC 30s); 72ºC 10 minutes; 4ºC∞. The PSQ96HS system was used according to standard procedures for the Pyrosequencing TM analysis. DNA methylation pattern of *COL1A1, MMP2, MMP9,* and *TIMP1* was analysed by bisulfite pyrosequencing in an external lab (IMIBA, Malaga, Spain). Quantitative sodium bisulfite pyrosequencing was performed, as previously described (Castelo-Branco et al. 2013), for the genes that showed differences in the transcriptomic analysis between endometrial categories, and comprised: *COL1A, MMP2, MMP9* and *TIMP1*. In brief, targeted assays were designed using the PyroMark Assay Design Software 1.0 (Qiagen). Forward, reverse, and sequencing primers were used for PCR and pyrosequencing (supplementary Table 2). The % of methylation was calculated as a mean of the CpG sites that passed quality control. Samples were considered for the study where at least 80% of the CpG sites passed quality control.

3.3.6. *In silico* analysis

The *in-silico* analysis was performed in the genes where methylation was correlated to transcription: *MMP2* and *MMP9* genes. CpG islands sequences were analysed using two different programs: TRANSFAC® database (Matys et al. 2006) and Alibaba 2.1 (Grabe 2002) in search for possible binding transcription factors of the regulatory region of the genes (Figure 15).

3.3.7. Statistical Analysis

Normal distribution of the data was evaluated by Shapiro-Wilk test. Kruskal-Wallis analysis followed by Dunn's multiple comparison test were performed to compare *COL1A1* and *MMP9* transcripts and *COL1A1, TIMP1* and *TIMP2* methylation between endometrial categories. One-way analysis of variance (ANOVA) followed by post-hoc Tukey multiple comparison test were used to analyse *COL1A2*, *COL3A1* and *TIMP2* mRNA and *MMP9* methylation, between endometrial categories. Unpaired t-test was used to compare *MMP2* and *TIMP1* mRNA and *MMP2* methylation, between endometrial categories. Pearson correlation test was performed to analyse transcription and methylation of *MMP2* and *MMP9* genes. GraphPAD PRISM (Version 8.1.0, 253, San Diego, CA, USA) was used. Significance was considered when $P < 0.05$. Data are presented as mean±SEM.

3.4. Results

3.4.1. *MMP2* and *MMP9* expression is downregulated during fibrosis progression

Firstly, we interrogated if expression levels of the COL genes and some of their regulators (MMPs and TIMPs) were altered in the different endometrial categories. Although no statistical differences in mRNA levels were observed in *COL1A1*, *COL1A2* and *COL3A1* between different stages of endometrosis (Figure 10), a striking decrease in mRNA levels was observed for the *MMP2* and *MMP9* metallopeptidase genes ($P < 0.05$) between endometrial categories (Figure 11). As for the metallopeptidase tissue inhibitors studied, *TIMP1* transcripts were higher in category III when compared to category I endometrium ($P < 0.05$) whereas no differences were found for *TIMP2* transcripts (Figure 12). Mares were then grouped by phase of estrous cycle for each category and no differences were found between luteal and follicular phase (data not shown).

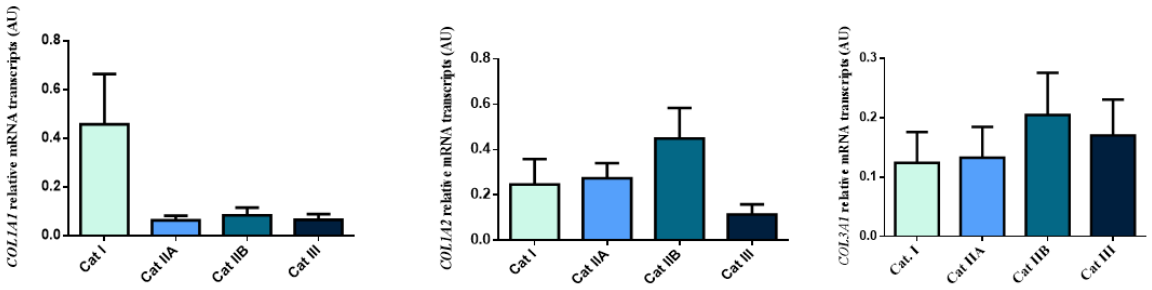

Figure 10. Relative *COL1A1, COL1A2* and *COL3A1* mRNA transcripts in equine endometrium graded as Kenney and Doig´s categories I, IIA, IIB and III. Bars represent mean±SEM.

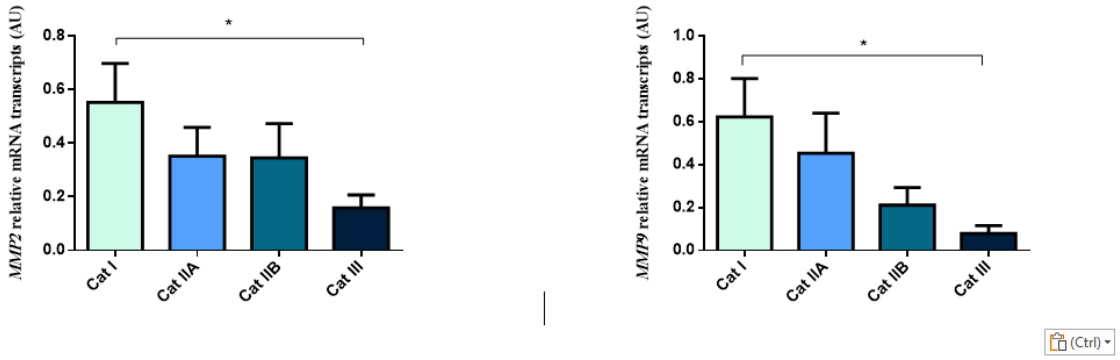

Figure 11. Relative *MMP2* and *MMP9* transcripts in equine endometrium graded as Kenney and Doig´s categories I, IIA, IIB and III. Bars represent mean±SEM. The asterisk indicates significant differences between endometrial categories (* *P*<0.05).

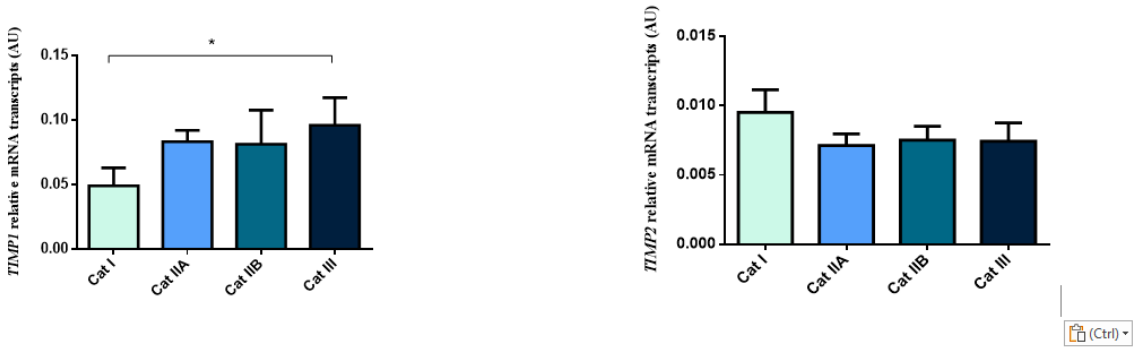

Figure 12. Relative *TIMP1* and *TIMP2* transcripts in equine endometrium graded as Kenney and Doig´s categories I, IIA, IIB and III. Bars represent mean±SEM. The asterisk indicates significant differences between endometrial categories (* *P*<0.05).

3.4.2. DNA methylation plays a role in endometrial fibrosis regulation

Next, we questioned if the observed alterations in mRNA levels of the studied genes were associated with epigenetic mechanisms. As such, we performed DNA methylation analysis and observed higher methylation levels of *COL1A1* in category IIB ($P < 0.05$) and III ($P < 0.01$), when compared to category I endometrium (Figure 13 A). In addition, higher levels of

methylation were observed for *MMP2* in category III when compared to category I ($P < 0.001$) and IIA ($P < 0.05$) (Figure 13 B) and for *MMP9* gene in category III with respect to category I and IIA endometrium ($P < 0.05$) (Figure 13 C). No difference in methylation levels between endometrium categories were observed for *TIMP1* (data not shown). There were no methylation differences between luteal and follicular phase for each endometrial category.

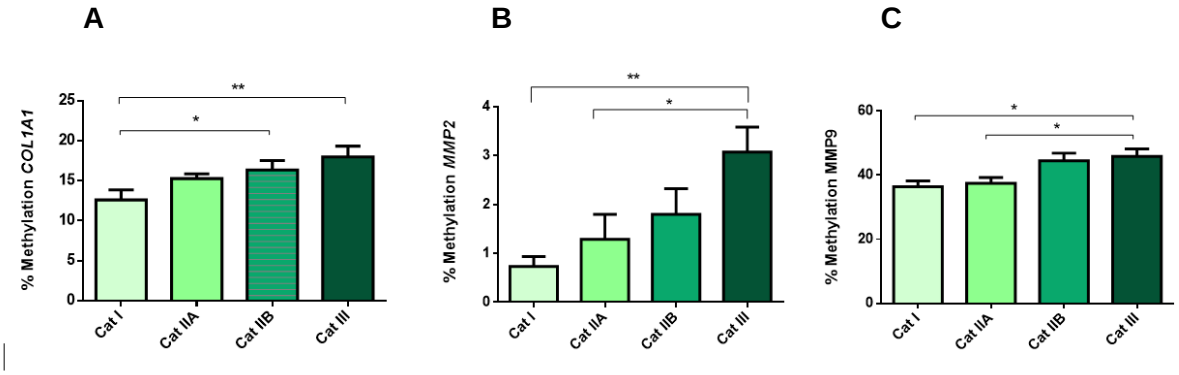

Figure 13. Methylation of DNA (%) of **(A)** *COL1A1*, **(B)** *MMP2* and **(C)** *MMP9* in equine endometrium graded as Kenney and Doig´s categories I, IIA, IIB and III. Bars represent mean±SEM. The asterisks indicate significant differences between endometrial categories (* $P<0.05$, ** $P<0.01$).

3.4.3. Methylation of *MMP2* and *MMP9* is negatively correlated with its transcription

We than analysed the correlation between the observed DNA methylation events and potential alterations in gene expression. The higher methylation levels observed in *MMP2* and *MMP9* were strongly correlated with the decreased transcription levels of both genes (r=-0.967, $P < 0.05$ and r= -0,956, $P < 0.05$ respectively) upon endometrial fibrosis progression (Figure 14).

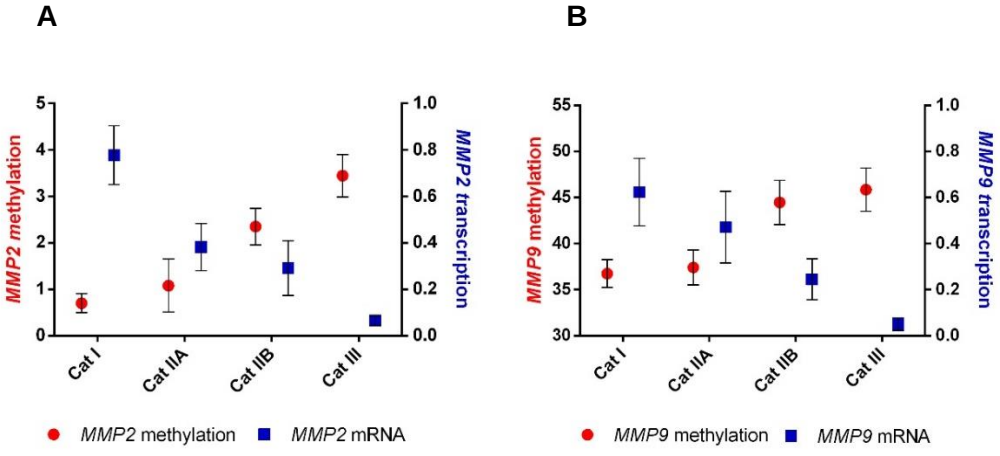

Figure 14. Correlation between methylation and transcription of **(A)** *MMP2* and **(B)** *MMP9* in equine endometrial categories graded as Kenney and Doig's classification.

3.4.4 Sp-1 and Ap-2α transcription factors identified as predicted binding factors in regulatory regions of *MMP2* and *MMP9*

Several transcription factors from each software program were detected and the overlapping factors from the two programs were identified. The predicted transcription factors for *MMP2* and *MMP9* were Sp-1 and Ap-2α (Figure 15).

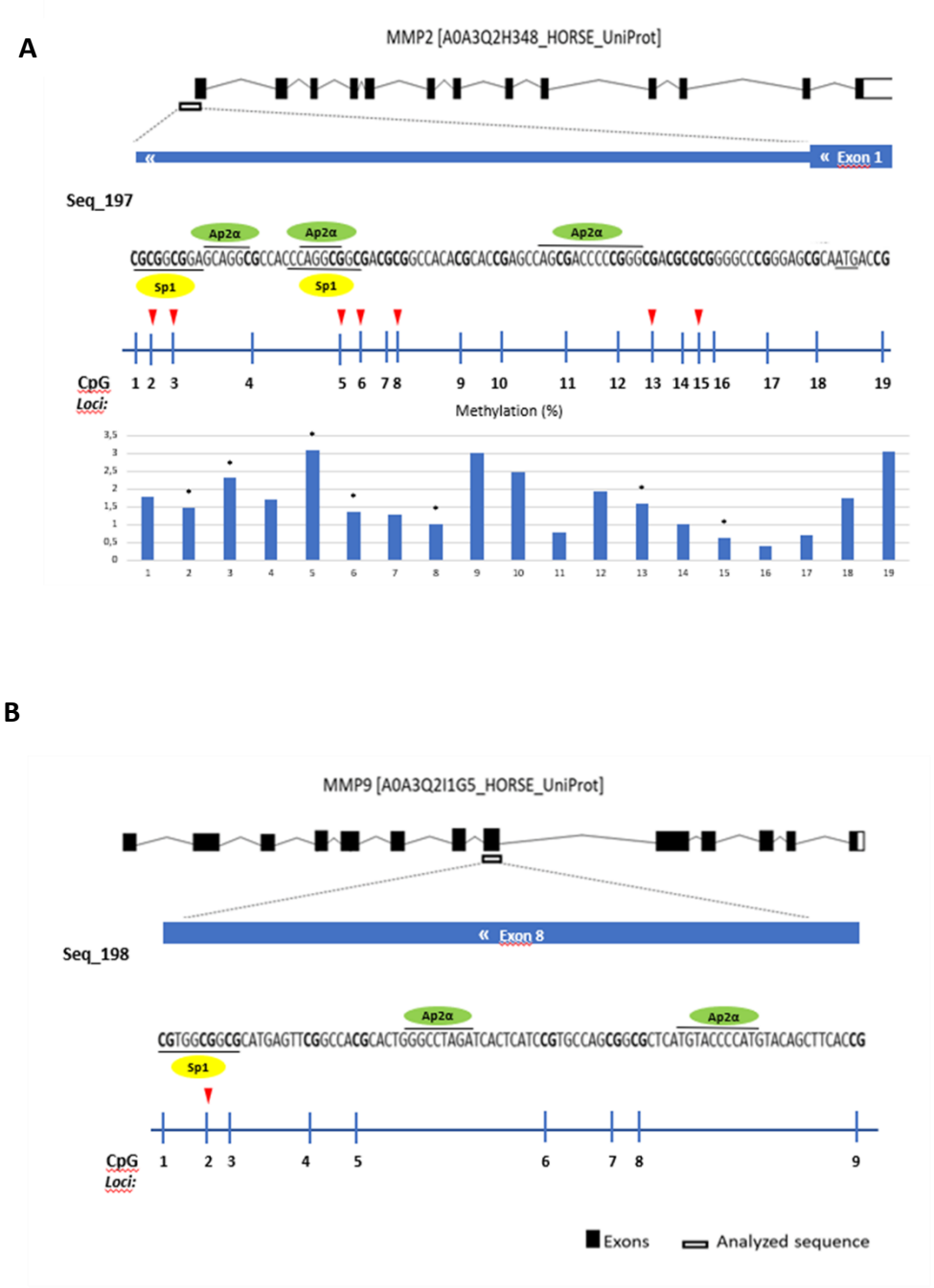

Figure 15. Gene analysed sequence, binding transcription factors and methylation (%) in equine endometrial categories graded as Kenney and Doig's classification per CpG position in (A) *MMP2* and (B) *MMP9.* Sp1 - proximal specificity protein 1, AP2α- Activator protein 2. Red arrows and asterisks indicate significant differences between CpG positions (* *P*<0.05).

Then we analysed which positions within the CpG island were accountable for the observed alterations. For *COL1A1* the CpG positions that showed more alterations in methylation levels between endometrial categories were sites 2, 6 and 8 (Figure 16); for *MMP2* site 2, 3, 5, 6, 8, 13 and 15 (Figure 15A); and *MMP9* only site 2 (Figure 15B).

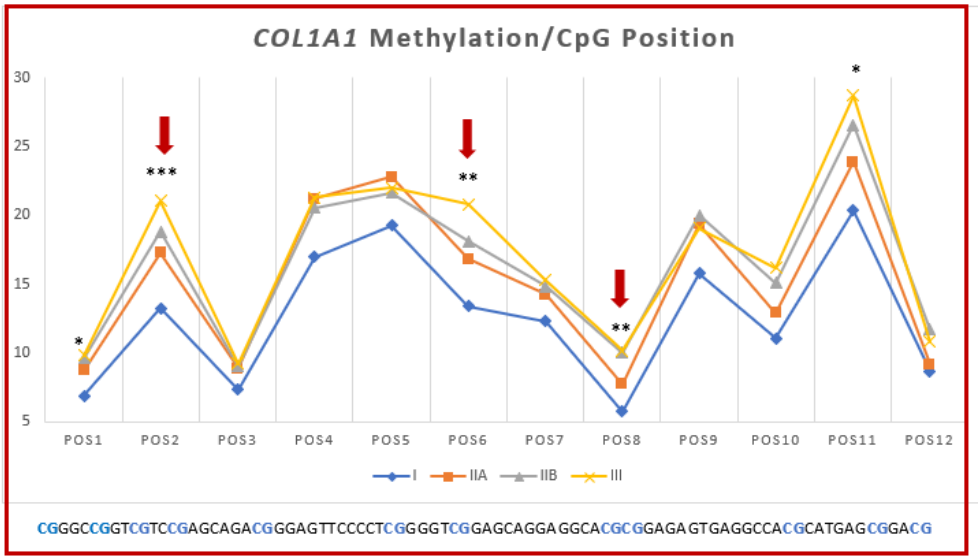

Figure 16. Methylation (%) of *COL1A1* by CpG position in equine endometrial categories graded as Kenney and Doig's classification. Red arrows and asterisks indicate significant differences between CpG positions (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

3.5. Discussion

Novel findings implicate a role for epigenetic modifications contributing to the progression of fibrosis by alteration of gene expression profiles. Furthermore, accumulating evidence suggests that epigenetic alterations are central in maintaining the myofibroblast phenotype (Bergmann and Distler 2017). Several studies demonstrated that the hypermethylation of gene promotors of antifibrotic mediators plays important roles in pathologic fibroblast activation and that inhibition of DNMTs prevents fibrosis in many fibrotic diseases (Wang et al. 2006; Bechtel et al. 2010; Huang et al. 2010; Dees et al. 2014; Noda et al. 2014; Watson et al. 2014; Altorok et al. 2015; Dees et al. 2020). Previously, we reported an increase in *DNMT3B* mRNA levels with equine endometrial fibrosis (category III) when compared with healthy endometrium (category I), indicating there was hypermethylation with the advance stage of endometrosis (Alpoim-Moreira et al. 2019). However, more detailed information is needed to understand which genes were involved and if hypermethylation was occurring in the gene promoter region and therefore could be regulating its transcription.

Our first proposed goal to evaluate transcriptomic of some of the genes involved in mare endometrial fibrosis, showed a decrease in *MMP2* and *MMP9* mRNA transcripts and an

increase in *TIMP1* mRNA transcripts with endometrial fibrosis. Although the *COL1A1* mRNA transcripts did not show statistical differences we decided to include it in the DNA methylation study, along with the genes where an alteration was observed, to understand if they were epigenetically regulated.

The main constituents of fibrotic lesions are interstitial collagens, such as COL1 and COL3, and excessive deposition of these durable fibers can result in disruption of proper tissue structure and function (Giannandrea and Parks 2014). Activated fibroblasts are the central mediators in the pathogenesis of fibrosis and they differentiate into myofibroblasts, which are characterized by the increased secretion of collagen and other components of the extracellular matrix (Abraham et al. 2007; Wynn and Ramalingam 2012). In the present study, no differences were found for *COL1A1, COL1A2* or *COL3A1* transcripts between different endometrial categories. In a study in equine endometrium (Pinto-Bravo 2018) neither the estrous cycle phase, nor the age of mares had any effect on *COL1* mRNA levels. Another study in jennies showed no differences in *COL1A2* and *COL3A1* transcripts between endometrial categories (Miró et al. 2020). However, some *in vitro* studies with mare cultured endometrial fibroblasts showed increased *COL1A1* and *COL3A1* mRNA transcripts after TGF-β1induced fibrosis (Szóstek-Mioduchowska et al. 2018) together with high COL1 and COL3 protein levels. Additionally, a study in equine tendon fibroblasts also demonstrated an increase in *COL1A1* and *COL1A2* gene expression after stimulation with TGF-β (Gumbs 2019). This divergence could be due to differences in signaling and regulatory pathways in tissues examined *ex vivo* or cultured *in vitro*. Nevertheless, data from other study, with the same mares (unpublished data), have shown elevated COL1 and COL3 protein concentrations in endometrial tissue with fibrosis, regardless of unaltered *COL1* and *COL3* gene transcription. This suggests that accumulation of collagen in the endometrial tissue might be due to lack of degradation, rather than increased collagen production. Other explanation could be that the mRNA signal for COL1 and COL3 production might have occurred before the biopsy was performed. It is known that mRNAs are less stable than proteins with a maximum half-life of approximately 7 h, compared to 46 h for proteins (Vogel and Marcotte 2012). Thus, protein abundance mainly depends on a dynamic balance amongst transcription, mRNA processing and damage, translation, modification, and destruction of the resulting proteins (Vogel and Marcotte 2012). DNA methylation of the CpG islands in the promotor zone of the *COL1A1* gene increased with endometrial fibrosis. However, hypermethylation of *COL1A1* gene with fibrosis, did not result in its altered transcription. It is important to consider that collagen synthesis is precisely controlled at multiple levels, including *via* post-transcriptional and post-translational mechanisms that are still being discovered (Roche and Czubryt 2014).

One class of molecules that is thought to be important in the maintenance of the ECM and processes of tissue repair is the class of matrix metalloproteinases (MMPs). The MMPs

have been considered to play an important role in the extracellular matrix turnover (Vandooren et al. 2013) and a balance between activation and inhibition of MMPs is crucial for maintaining tissue homeostasis (Szóstek-Mioduchowska et al. 2020). Dysregulated expression of various MMPs is associated with many pathological processes, such as fibrosis, weakening of ECM or tissue destruction, e.g., in cancer metastasis (Di Nezza et al. 2002; Amălinei et al.,2010). However, data concerning MMP expression in equine endometrium during endometrial fibrosis is limited. In our study both *MMP2* and *MMP9* gene expression decreased with endometrial fibrosis, suggesting that the reduced transcription may result in diminished degradation of collagen and its accumulation in the endometrium. Other studies also demonstrated lower *MMP2* gene transcription in fibrotic endometrium but *MMP9* transcription was higher (Falcomo et al. 2015). In another study with endometrial fibroblasts in mares (Szóstek-Mioduchowska et al. 2020), *MMP9* gene transcription increased after TGF-β1 stimulation. Also, a study by Centeno and collaborators (Centeno et al. 2018) showed that *MMP2* transcription was upregulated in endometrial fibrosis. On the other hand, a study in mice with induced liver fibrosis reported increased *MMP2* mRNA and decreased *MMP9* mRNA (Qin and Han, 2010). Many studies in other animals and humans have shown a decrease in *MMP2* (Sugihara et al. 2009; Kendziorski and Belcher 2015) and *MMP9* gene expression in several fibrotic diseases (Bailey et al. 2012) while others reported the opposite (Kim et al. 2005; Andersen et al. 2007; Dancer et al. 2011). These inconsistent results regarding *MMP2* and *MMP9* expression might be partially explained by the fact that MMPs demonstrate tissue-dependent and disease-specific expression and function (Leong et al. 2021). Moreover, current knowledge about MMP regulation is largely based on cell culture systems, raising a major question as to whether identical mechanisms apply to MMP expression in the whole organism as well (Yan and Boyd 2007).

Our results showed increased methylation in *MMP2* and *MMP9* in category III when compared to category I and IIA. Our study agrees with many others in that hypermethylation of antifibrotic gene promotors is involved in fibrosis development (Wang et al. 2006; Bechtel et al. 2010; Huang et al. 2010; Dees et al. 2014; Noda et al. 2014; Watson et al. 2014; Altorok et al. 2015). Furthermore, we observed that hypermethylation of *MMP2* and *MMP9* genes occurred concomitantly with a decrease in their transcription levels as fibrosis increased, showing an epigenetic regulation as suggested in Figure 17. Other studies also observed that silencing of MMP *genes* is likely mediated by epigenetic alterations (Qin and Han, 2010).

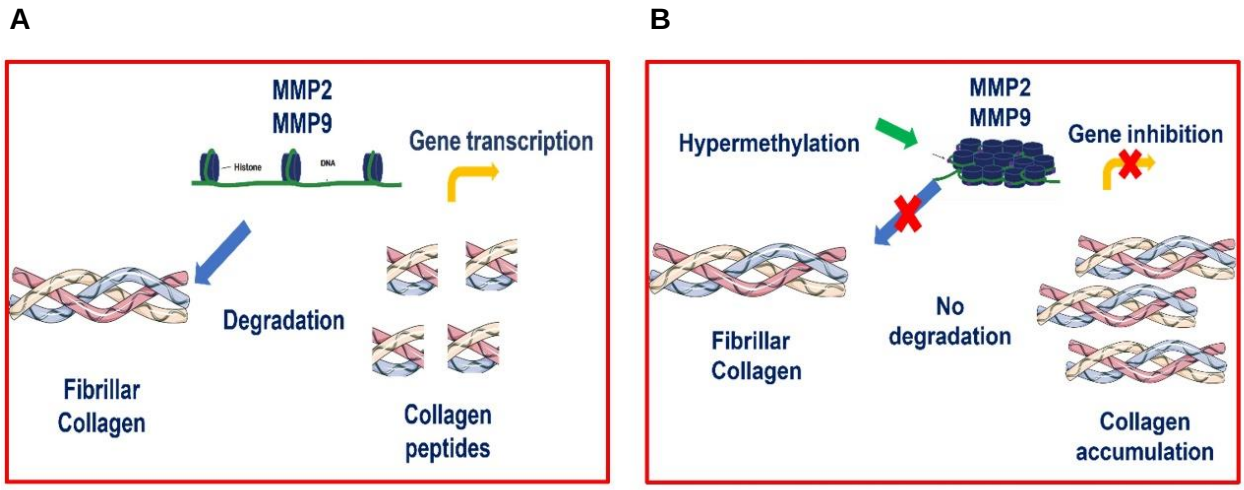

Figure 17. **(A)** Transcription of *MMP2* and *MMP9* genes is possible when there is no hypermethylation of these genes, as chromatin is accessible and thus allows transcriptional factors to bind to the promoter region and activate the transcription process **(B)** Epigenetic modulation of *MMP2* and *MMP9* in equine endometrial fibrosis. When hypermethylation occurs, the chromatin becomes condensed and the transcriptional factors are not able to bind to this site and therefore, transcription is inhibited.

Tissue inhibitors of metalloproteinases (TIMPs) are the major endogenous regulators of MMP activities in the tissue microenvironment and have the capacity to modify cellular activities and to modulate matrix turnover (Madtes et al. 2001). TIMP-1 and TIMP-2 proteins bind to and inhibit activated collagenases, subsequently protecting newly synthesized collagen from immediate degradation by MMPs (Hemmann et al. 2007). In our study *TIMP1* mRNA transcripts raised with fibrosis when compared to healthy endometrium whereas no differences were observed for *TIMP2* mRNA. Our data agrees with a study in human lung fibrosis, where *TIMP1* mRNA was markedly increased in response to lung injury, whereas there was no change in *TIMP2* mRNA levels (Madtes et al. 2001). Also, studies by Heymans in human cardiac disease (Heymans et al. 2005) and Wang in rat lungs (Wang et al. 2011) reported upregulated TIMP1 gene expression during fibrosis. Since in our study, there were no alterations in DNA methylation pattern between endometrial categories for the TIMP1 gene, this might suggest that its inhibition does not occur, continuing to be expressed and increased with fibrosis, thus contributing to collagen accumulation in mare endometrium.

Some of the studied CpG island positions showed more differences in methylation between categories than others. Transcriptional factors (TF) play an important role in gene transcription as they can regulate transcription by binding to the activator or promoter regions of DNA and control gene expression through various mechanisms (Gill 2001). The *in silico* analysis performed (Messeguer et al. 2002; Farré et al. 2003) in the analyzed regions of the *MMP2* and *MMP9* genes, detected Sp-1 and Ap-2α as possible binding transcription factors for *MMP2* and *MMP9*. These putative binding regions encompass CpG sites that showed significant methylation differences across the four studied endometrial categories. Specificity

protein 1 (Sp-1) binding sites are often located close to binding sites for activator proteins (Ap-1/Ap-2) (Sato and Furukawa 2004), as it was observed in our analysis. Transcription factor Ap-2, an important TF for the expression of many genes (Wang et al. 2017) has a role in the transcriptional regulation of *MMP2* in humans (Bergman et al. 2003; Craig et al. 2015). In this study, the binding region for Sp-1 in *MMP9* comprised loci 2, where the differences in methylation between endometrial categories occurred. Therefore, we speculate that these factors may play a role in the complex mechanism of endometrial fibrosis regulation and should be further investigated.

Overall, it seems like a different approach might be needed to address fibrosis treatment, as up to date, no effective therapy exists for equine endometrosis. “Epi-drugs” that target active myofibroblasts in fibrotic disorders are a promising direction in the treatment of a myriad of diseases. Nevertheless, we are far from a comprehensive understanding of how epigenetic modulators influence each other and myofibroblast behavior (Duong and Hagood 2018). If epigenetic mechanisms are involved in mare endometrial fibrosis development, as suggested by our results, then therapeutic agents that can reverse these epigenetic changes may represent a new and promising approach, for a condition that still has no available treatment.

3.6. Conclusion

In this study we have showed that equine endometrial fibrosis seems to be epigenetically modulated. Furthermore, that modulation seems to occur through the inhibition of antifibrotic genes like *MMP2* and *MMP9*, rather than fibrotic genes (*COL1* and *TIMP1*) and therefore might be promising targets for therapeutic use. Nevertheless, further studies are required to understand in depth this mechanism and possible role of other genes involved in mare endometrosis.

Supplementary Tables

Supplementary Table 1 – Primer sequences used in qPCR study

Gene (Acession number)	Sequence 5´- 3		**Amplicon (base pairs)**
	Forward	**Reverse**	
COL1A1 (XM_023652710.1)	TATGGAAACCCGAGCCCTG	ACTCCTGTGGTTTGGTCGTCTG	175
COL1A2 (XM_001492939.3)	CAAGGGCATTAGGGGACACA	ACCCACACTTCCATCGCTTC	196
COL3A1 (AF117954.1)	CAAAGGAGAGCCAGGAGCAC	CTCCAGGCGAACCATCTTTG	98
MMP2 (XM_001493281.2)	TCCCACTTTGATGACGACGA	TTGCCGTTGAAGAGGAAAGG	115
MMP9 (NM_001111302.1)	GCGGTAAGGTGCTGCTGTTC	GAAGCGGTCCTGGGAGAAGT	177
TIMP1 (NM_001082515.1)	CAAGTTCGTGGGGACCTCAG	CTCTCCATAGCGGGGGTGTA	141
TIMP2 (XM_023651899.1)	ATCTACGGCAACCCCATCAA	CTTCTTCCCTCCAACGTCCA	144
RPL32 (XM_001492042.6)	AGCCATCTACTCGGCGTCA	GTCAATGCCTCTGGGTTTCC	144

COL1A1 - collagen type 1, alpha1; *COL1A2* - collagen type 1, alpha2; *COL3A1* - collagen type 3, alpha1; *MMP2* - matrix metalloproteinase 2; *MMP9* - matrix metalloproteinase 9; *TIMP1* - tissue inhibitor of matrix metalloproteinase 1; *TIMP2* - tissue inhibitor of matrix metalloproteinase 2; *RPL32* - ribosomal protein L32

Supplementary Table 2 - Primer sequences used in DNA methylation study.

Gene	**PCR Primers**		**Target region (length)**	**CpG sites (nº)**
	Forward	**Reverse**		
COL1A1	GGGTAGGGTTAGGTAGTTTTGATT	CATATCTAAACCCTAAACATATAAACTCTT	85 bp	12
MMP2	GGGGTTTTAAATATATAAAGGGATTGT	ACATCTCCAAAAAACTTAATAATAAAC	95 bp	19
MMP9	GGTTGGGAGTTTAGTTTAGGG	CCCACAACCTCACCATAAAAATACT	61 bp	8
TIMP1	TTTAGGGGGAGGGAGTGG	CCCCCCCTACCTCTACTAAAATCTCTCTA	103bp	7

COL1A1 - collagen type 1, alpha1; *MMP2* - matrix metalloproteinase 2; *MMP9* - matrix metalloproteinase 9; *TIMP1*- tissue inhibitor of matrix metalloproteinase 1

4 - 5-aza-2'-deoxycytidine (5-aza-dC or decitabine) inhibits collagen type I and III expression in TGF-β1 treated equine endometrial fibroblasts

Alpoim-Moreira J, Szóstek-Mioduchowska A, Słyszewska M, Rebordão MR, Skarzynski DJ, Ferreira-Dias G. 2023. 5-Aza-2'-Deoxycytidine (5-aza-dC, Decitabine) Inhibits Collagen Type I and III Expression in TGF-β1-Treated Equine Endometrial Fibroblasts. *Animals,* 13(7):1212.doi: 10.3390/ani13071212.

4.1. Abstract

Endometrosis is associated with changes in structure and function of the endometrium that has a negative impact in endometrial function and mare's fertility. These pathological changes involve excessive extracellular matrix (ECM) deposition in mare endometrium, such as collagen type I (COL1), collagen type III (COL3) and α smooth actin (α-SMA). Transforming growth factor (TGF-β1) is considered one of the major pro-fibrotic signals for myofibroblast differentiation and promotes collagen production. Alterations in fibroblast phenotype are associated with epigenetic alterations in chromatin structure. In addition, other studies have shown that TGF-β1 induces myofibroblast differentiation and collagen synthesis by epigenetic mechanism in humans. Nevertheless, this has not been described in mare endometrium so far. As such, the aim of our study was to investigate the *in vitro* epigenetic regulation in endometrial fibroblasts challenged with TGF-β1 and the use of 5-aza-2′-deoxycytidine (5-aza-dC; decitabine), an epigenetic modifier, as a putative treatment option for endometrial fibrosis. Methods and Results: The *in vitro* effects of TGF-β1 on DNA methyltransferases (*DNMT1, DNMT3A* and *DNMT3B*) mRNA levels and the effects of DNA methylation inhibition with 5-aza-dC were examined in equine endometrial fibroblasts. The results from this work demonstrate that TGF-β1 upregulates *DNMT3A* mRNA levels and COLs secretion in equine endometrial fibroblasts. Administration of the DNA methylation inhibitor 5-aza-dC significantly decreased collagen type I and III mRNA and secretion, but not *α-SMA* transcripts in response to TGF-β1 in endometrial fibroblasts. Conclusion: These findings suggest a role for epigenetic mechanisms in contributing for the evident profibrotic action of TGF-β1 in equine endometrial fibroblasts, during fibrosis establishment. The *in vitro* effect of 5-aza-dC on fibroblast collagen reduction, highlights its potential use as a therapeutic option for endometrial fibrosis.

Keywords: endometrosis, mare, collagen, epigenetics, DNMTs, fibroblasts, 5-aza-dC, demethylating inhibitor

4.2. Introduction

Endometrosis is responsible for infertility in the mare and is characterized by excessive deposition of collagen (COL) in the endometrium, being collagen type I (COL1) and type III (COL3) the most abundant. Transforming growth factor β1 (TGF-β1) is over expressed in several fibrotic tissues (Yang et al. 2010; Ueha et al. 2012; Zeisberg and Kalluri 2013; Seki and Brenner 2015) and induces COL production in cultured fibroblasts, regardless of their origin (Zeisberg and Kalluri 2013). It not only regulates cell growth, development, and tissue remodelling, but it also participates in the pathogenesis of tissue fibrosis. In the equine endometrium, the activity of TGF-β1 is correlated with endometrosis (Ganjam and Evans 2006; Szóstek-Mioduchowska et al. 2019a). In other tissues, TGF-β1 induces differentiation of many cell types into myofibroblasts. These cells are characterized by α-smooth muscle actin (α-SMA) expression and the ability to deposit excessive amounts of extracellular matrix (ECM) components. Increased expression of α-SMA in fibroblasts is therefore widely interpreted as a marker of fibroblast activation (Smith et al. 2019).

Moreover, aberrant expression of TGF-β1after injury stimulates expression of α-SMA (Rønnov-Jessen and Petersen 1993) and ECM (Hewittson et al. 2017) in fibroblast-like cells. Fibroblasts are key effector cells in tissue remodelling. They remain persistently activated in fibrotic diseases, resulting in progressive deposition of extracellular matrix. Although fibroblast activation may be initiated by external factors, prolonged activation can induce an “autonomous,” self-maintaining profibrotic phenotype in myofibroblasts (Dees et al. 2020).

Accumulating evidence suggests that epigenetic alterations play a central role in establishing this persistently activated pathologic phenotype of fibroblasts (Dees et al. 2020). Epigenetic changes, unlike genetic alterations, can be reversed as thus extremely promising for therapeutic use (Duong and Hagood 2018). DNA methylation, considered a stable epigenetic marker, can be assessed through the action of DNA methyltransferases (DNMTs: DNMT1, DNMT3A and DNMT3B) and commonly mediates gene repression (Hermann and Jeltsch 2004; Du et al. 2015). Recent studies have shown an epigenetic involvement in several human fibrotic disorders (O´Reilly 2017; Felisbino and Kinsey 2018; Weiskirchen et al. 2019; Bartczak et al. 2020; Henderson et al. 2020). Also, epigenetic has been involved in equine endometrial fibrosis, as demonstrated in our previous studies (Alpoim-Moreira et al. 2019; Alpoim-Moreira et al. 2022b). As such we have hypothesized that endometrial fibroblasts might be under epigenetic regulation.

Therefore, our aim was to analyze the effect of TGF-β1 on methylating enzymes and the effect of the licensed demethylating agent 5-aza-2’deoxycytidine (5-aza-dC, 5-aza or decitabine) on TGF-β1 stimulated equine endometrial fibroblasts. For that purpose, we first evaluated DNA methylation through the expression pattern of *DNMT1, DNMT3A* and *DNMT3B* on TGF-β1 stimulated mare fibroblasts to determine if equine endometrial fibroblasts were under epigenetic

regulation. Further on, we aimed to confirm if the TGF-β1 induced alterations on COL1 and COL3 expression by equine endometrial fibroblasts could be reversed by the action of 5-aza-dC. Therefore, we determined the transcription levels of *DNMT* enzymes, ECM components and α-SMA and, COL1 and COL3 protein concentration, before and after fibroblasts were treated with TGF-β1, 5-aza-dC or both.

4.3. Materials and Methods

Uteri (n=5) were obtained *post-mortem* from cyclic mares, at a local abattoir (Rawicz, Poland) from April to June according to the protocols approved by the local institutional committee for animal care and use. The mares were clinically healthy, as declared by official government veterinary inspection and individual veterinary histories of animal health. Immediately before death, peripheral blood samples were collected into heparinized tubes for subsequent progesterone (P_4) analysis. The animals were slaughtered for meat, as part of routine breeding slaughter animals, and in agreement to the European mandates (EFSA, AHAW/04-027). The internal genitalia (uteri and ovaries) were retrieved within 5 min. of animal death. In this study, uteri from mares in the follicular phase of the estrous cycle were used. The follicular phase was identified based on the macroscopic observation of ovaries and progesterone ($P_{4)}$ analysis of blood plasma. This phase was characterized by the absence of an active *corpus luteum* (CL) and the presence of follicles of various sizes, but always >35 mm in diameter, with a concentration of P_4 <1 ng/mL (Roberto da Costa et al. 2007). Samples of endometria were placed in 4% buffered formaldehyde for histological examination and for endometrial categorization, according to Kenney and Doig (1986) (Kenney and Doig 1986).

4.3.1. Isolation and culture of fibroblasts

The fibroblasts isolated from healthy endometria (Kenney and Doig's, category IIA endometria) were isolated according Szóstek-Mioduchowska et al. (Szóstek-Mioduchowska et al. 2019b). In the laboratory, the uterine lumen was washed three times with 10 mL of sterile Hanks' balanced salts (HBSS; H1387; Sigma-Aldrich, Saint Louis, Missouri, USA) containing 0.01% of antibiotic/antimicotic (AA) solution (AA5595; Sigma Aldrich, Saint Louis, Missouri, USA). A uterine horn was slit open with scissors to expose the endometrial surface. Endometrial strips were excised from the myometrium layer with a scalpel, washed once with sterile HBSS containing 0.01% of AA solution and cut into very small pieces (1-3 mm^3) with a scalpel. The minced tissues were digested once by stirring for 45 min in 100 mL of sterile HBSS containing 0.05%, (w/v) collagenase I (C2674, Sigma-Aldrich, Saint Louis, Missouri, USA) and 0.005% (w/v) DNase I (11284932001; Roche-Sigma Aldrich, Saint Louis, Missouri, USA) and 0.01% AA, 0.1% (w/v) Bovine serum albumin (BSA; A9418, Sigma Aldrich, Saint Louis, Missouri, USA). The cell suspension was filtered through 70μm and 40 μm strainers to remove undigested tissue

fragments. The filtrate was mixed gently with 1ml of Red Blood Cell Lysing Buffer Hybri-Max™ (R7757; Sigma-Aldrich, Saint Louis, Missouri, USA) to lyse red blood cells. Afterwards the filtrate was washed three times by centrifugation (4°C, 100×g, 10 min) in HBSS supplemented with antibiotics and 0.1% (w/v) BSA. The final pellet of endometrial cells was resuspended in FBM™ Basal Medium (CC-3131, LONZA, Basel, Switzerland) supplemented with FGM™-2 SingleQuots™ supplements and ascorbic acid (100 ng/ml; A4544; Sigma-Aldrich, Saint Louis, Missouri, USA) and 0.01% of AA solution. The cells were counted using a hemocytometer. The viability of endometrial cells was higher than 95% as assessed by the trypan blue exclusion test. The homogeneity of fibroblast was evaluated using immunofluorescent staining for vimentin based on the protocol described (24) (Figure 1). The dispersed cells were seeded separately at a density of 1×10^5 viable cells/mL and cultured at 38.0°C in a humidified atmosphere of 5% CO_2 in the air. To purify the fibroblast population, the medium was changed 18 h after plating, by which time selective attachment of fibroblasts had occurred and other types of endometrial cells were eliminated (*i.e.*, epithelial, endothelial, and immune cells). The medium was changed every second day until the cells reached confluence. The purity of fibroblast after isolation was around 96%. After reaching 90% of confluency, the cells were cryopreserved as described previously (Szóstek-Mioduchowska et al. 2021).

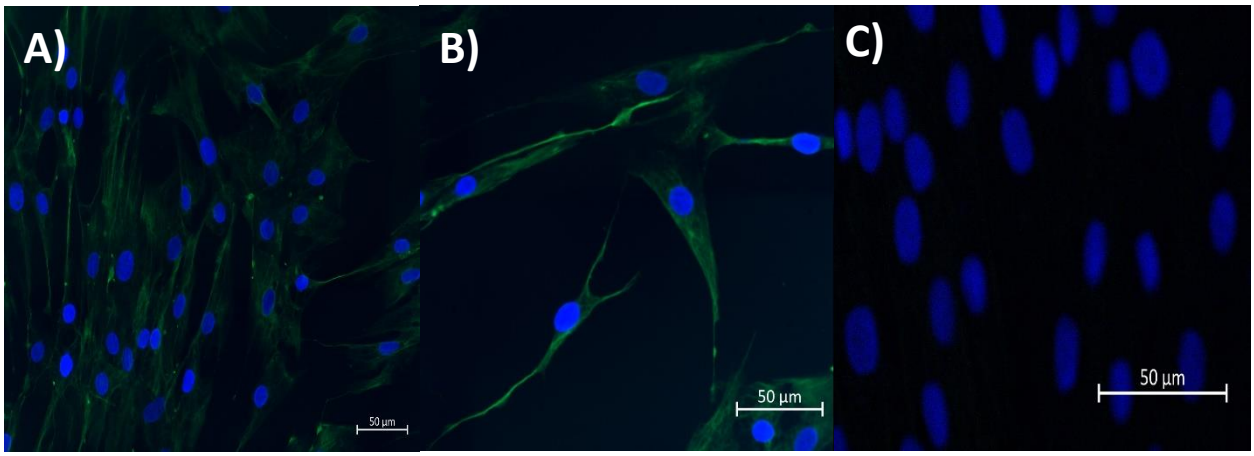

Figure 18 – Representative pictures of immunofluorescence staining of vimentin (A, B) in cultured fibroblasts. C) DAPI staining. The scale bar = 50 µm (magnification: 20, 40).

4.3.2. Preliminary studies

To determine the most adequate protocol to be used in our study, preliminary studies were performed. The dose of 10 ng/mL of TGF-β1 was chosen based in other studies as the treatment has previously shown to maximally activate myofibroblasts (Pan et al. 2013; Smith et al. 2017; Szóstek-Mioduchowska et al. 2019a). Transforming grow factor β1 period treatment of

48 h was also chosen based on other studies, where maximum increase in collagen expression was achieved at 48 h (Pan et al. 2013; Szóstek-Mioduchowska et al. 2019a; Dees et al. 2020).

To establish the appropriate dose of 5-aza-dC a preliminary study was performed with 0 µM, 1 µM or 5 µM. The 1 µM dose achieved the same results in collagen reduction as 5 µM. Therefore, the rationale was to choose the lowest concentrations due to the toxic side effects of 5-az-dc (in clinical use) and based in other studies that achieved the same results (Hu et al. 2010; Yonemura et al. 2019; Dees et al. 2020). To establish the duration and the protocol of treatment with 5-aza-dC a preliminary study was also performed. As such, endometrial fibroblasts were incubated with 1 µM 5-aza-dC and 10 ng/mL TGF-β1 at the same time for 48 h (first protocol) or with 10 ng/mL TGF-β1 for 48 h and then stimulated with 1µM 5-aza-dC for another 48 h (second protocol). With the first protocol no reduction was not observed in COL1, COL3 or α-SMA in fibroblasts. In contrast, in the second protocol, both mRNA and protein concentration of COLs were reduced. Hence, the second protocol was chosen since it also mimics the clinical conditions in which it may be used (treatment after fibrosis development and not its prevention). The duration of 5-aza-dC treatment was chosen as the minimum hours (48 h) at which positive results were achieved based in other studies (Pan et al. 2013; Yonemura et al. 2019).

The effect of TGF-β1 and of 5-aza-dC on cell viability was also analyzed. After reaching confluence in a 96-well plate, fibroblasts were exposed to TGF-β1 (10 ng/mL) or 5-aza-dC (1 µM or 5 µM) for 48 h, and viability was measured using In Vitro Toxicology Assay Kit, MTT based (TOX-1KT, Sigma Aldrich, Madison, USA). None of the doses used showed toxic effects (Supplementary figure 4).

4.3.3. Treatment of cultured fibroblasts

Thawed fibroblasts were seeded at a density of $1x10^5$ viable cells/mL on T75 cm^2 cell culture flasks. After reaching 90% of confluence, fibroblasts were seeded on 24-well plates. When fibroblasts from passage 1 reached the desired 80% confluence for 48 h treatment, the culture medium was replaced with fresh Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/Ham's F-12; D2906; Sigma-Aldrich, Saint Louis, Missouri, USA) supplemented with 0.01% of AA solution, and 0.1% (w/v) BSA, and the cells were incubated at 38.0°C in 5% CO_2. Additionally, the medium used for fibroblast culture was supplemented with ascorbic acid (100 ng/mL). Then, fibroblasts were treated with vehicle (as control), with 10 ng/mL of TGF-β1 or with 1 µM of 5-aza-dC at 37ºC, 5% CO2 for 48 h. Then 1 µM 5-aza-dC was added to TGF-β1 group for another 48 h under the same conditions. Since the half-life of 5-aza-dC is very short the medium was changed every 24 h and fresh 5-aza-dC was added daily for 48 h. Fibroblasts were also incubated alone (control), with 1 µM 5-aza-dC or with 10 ng/mL TGF-β1 for 96 h. The cells and the conditioned media were collected at 48 h and 96 h and stored at -

80ºC. Conditioned media were collected into 1.5 mL tubes for ECM determination using ELISA. After incubation, the cells were disrupted with 1 mL of lysis buffer RTL (1015750; Qiagen GmbH, Hilden, Germany) and stored at -80 ºC for RNA extraction and PCR.

4.3.4. Total RNA isolation, cDNA synthesis and qPCR

Total RNA was extracted using Qiagen RNeasy® mini kit (74104; QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's information, including a DNase digestion step. RNA samples were stored at -80 ºC. Before use, RNA concentration and quality were determined spectrophotometrically and by agarose gel electrophoresis, respectively. The ratio of absorbance at 260 and 280 nm (A260/280) was approximately 2. The RNA (1 mg) was reverse transcribed into cDNA using a QuantiTect Rev. Transcription Kit (no. 205313; QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. The cDNA was stored at -80 ºC until qPCR was carried out. Real-time PCR was performed with an ABI Prism 7900 sequence detection system using SYBR Green PCR master mix (Applied Bio-systems, Foster City, CA) with 384-well plates. The amplified genes used were α-*SMA, COL1A1, COL3A1, DNMT1, DNMT3A* and *DNMT3B.* Specific primers were designed, as well as the reference gene (Table 6), using the Internet-based program Primer-3 (Untergasser et al. 2012) and Primer Premier software (Premier Biosoft Interpairs). *SDHA2* was chosen as the most stable internal control gene, among four validated reference genes, as described (Rebordão et al. 2018). All primers were synthesized by Sigma-Aldrich (Saint Louis, Missouri, USA). The total reaction volume was 10 mL containing: 3 mL cDNA (1 ng), 1 mL each forward and reverse primers (500 nM) and 5 mL SYBR Green PCR master mix. Real-time PCR was carried out as follows: initial denaturation (2 min at 50 ºC; 10 min at 95 ºC), followed by 42 cycles of denaturation (15 s at 95 ºC) and annealing (1 min at 60 ºC). After each PCR reaction, melting curves were obtained by stepwise increases in temperature from 60 ºC to 95 ºC to ensure single-product amplification. The specificity of product was also confirmed by electrophoresis on 2% agarose gel. The data were analysed using the method described by Zhao and Fernald (Zhao and Fernald 2005).

Table 6 – Sequences of primers designed for real time PCR

Gene (Acession number)	Sequence 5´- 3		Amplicon (base pairs)
	Forward	**Reverse**	
a-SMA (XM_001503035.6)	TCAGCTTCCCTGAACACCAC	GCAAAGCCAGCCTTACAAAG	151
COL1A1 (XM_014736922.1)	TAAGGGTGACAGAGGCGATG	GGACCGCTAGGACCAGTTTC	144
COL3A1 (AF117954.1)	CAAAGGAGAGCCAGGAGCAC	CTCCAGGCGAACCATCTTTG	98
DNMT1 (XM_023645449.1)	CAAGGCAAACAACCAGGCA	CTTCCTCCTCTTCCGTGTGTGT	237
DNMT3A (XM_023619394.1)	GCCTCAATGTCACCCTGGAA	AAGAGGTCCACACATTCCACG	206
DNMT3B (XM_023626333.1)	GAGCTGGCAAGACTTTCCCC	TTGGGTGGAGGGCAGTAGTC	198
SDHA2 (DQ402987.1)	GAGGAATGGTCTGGAATACTG	GCCTCTGCTCCATAAATCG	91

a-SMA-alpha smooth actin; *COL1A1*-collagen type 1, alpha2; *COL3A1*-collagen type III, alpha 1; *DNMT1, DNMT3A, DNMT3B*- DNA methyltransferase 1, 3A, 3B; *SDHA2*- succinate dehydrogenase complex flavoprotein subunit A, mitochondrial

4.3.5. Collagen protein quantification

ELISA techniques were performed to quantify COL1 and COL3 concentrations, in conditioned media from cultured cells.

The concentration of COL1 in conditioned medium were determined using the 96 Tests Enzyme-Immunosorbent Assay Kit for Collagen Type I (COL1) (Cloud-clone; SEA571Eq). The standard curve for COL1 ranged from 3.12 to 200 ng/mL. The intra- and inter-assay coefficients of variation (CVs) on average were 11.5% and 9%, respectively. The concentrations of COL3 in conditioned media were determined using 96 Tests Enzyme-Immunosorbent Assay Kit for Collagen Type III (COL3) (Cloud-clone; SEA176Eq). The standard curve for COL3 ranged from 1.56 to 100 ng/mL. The intra- and inter-assay CVs on average were 10% and 9%, respectively.

4.3.6. Statistical Analysis

Data are shown as the mean±SEM. For each analysis, the Gaussian distribution of results was tested using the Shapiro and Wilk normality test (GraphPad Software version 9; GraphPad, San Diego, CA, USA). If the assumptions of normal distribution were not met, nonparametric statistical analyses were done. Significance was considered when $P<0.05$. Unpaired t-test was performed to evaluate the differences in: *COL1A1, COL3A1, a-SMA*, *DNMT1, DNMT3A, DNMT3B* mRNA, COL1 and COL3 protein concentration between 48 h and

96 h of stimulation with a) TGF-β1 or b) 5-aza-dC. One way-anova, followed by Tukey's test was used to analyze the differences in *COL1A1, COL3A1* and *a-SMA* mRNA between control and treatment with 5-aza-dC, TGF-β1 or both. Unpaired t-test was also performed to assess the differences in *DNMT1, DNMT3A, DNMT3B* mRNA, COL1 and COL3 protein concentration between control and treatment with 5-aza-dC, TGF-β1 or both.

4.4. Results

4.4.1. TGF-β1 upregulated collagen type I, III and α-SMA expression in endometrial fibroblasts

Treatment of endometrial fibroblasts with TGF-β1 increased mRNA levels of *COL1A1* and *COL3A1* at 48 h (P<0.001) and 96 h (P<0.01) and *α-SMA* only at 96 h (P<0.01) (Figure 2A). It also increased protein concentration of COL1 and COL3 at both 48 h (P<0.01 and P<0.0001, respectively) and 96 h (P<0.0001 and P<0.05, respectively) (Figure 2B). Although the time we chose for TGF-β1 treatment was 48 h based on previous studies, the maximum increase in *COL1A1* and *COL3A1* mRNA levels and protein concentration were achieved at 96 h. There was an increase in *COL3A1* and *α-SMA* mRNA levels between 48 h and 96 h (P<0.01), but the same was not observed for *COL1A1* (Figure 19A). However, there were no differences between COL1 and COL3 protein concentration between 48 h and 96 h (Figure 19B).

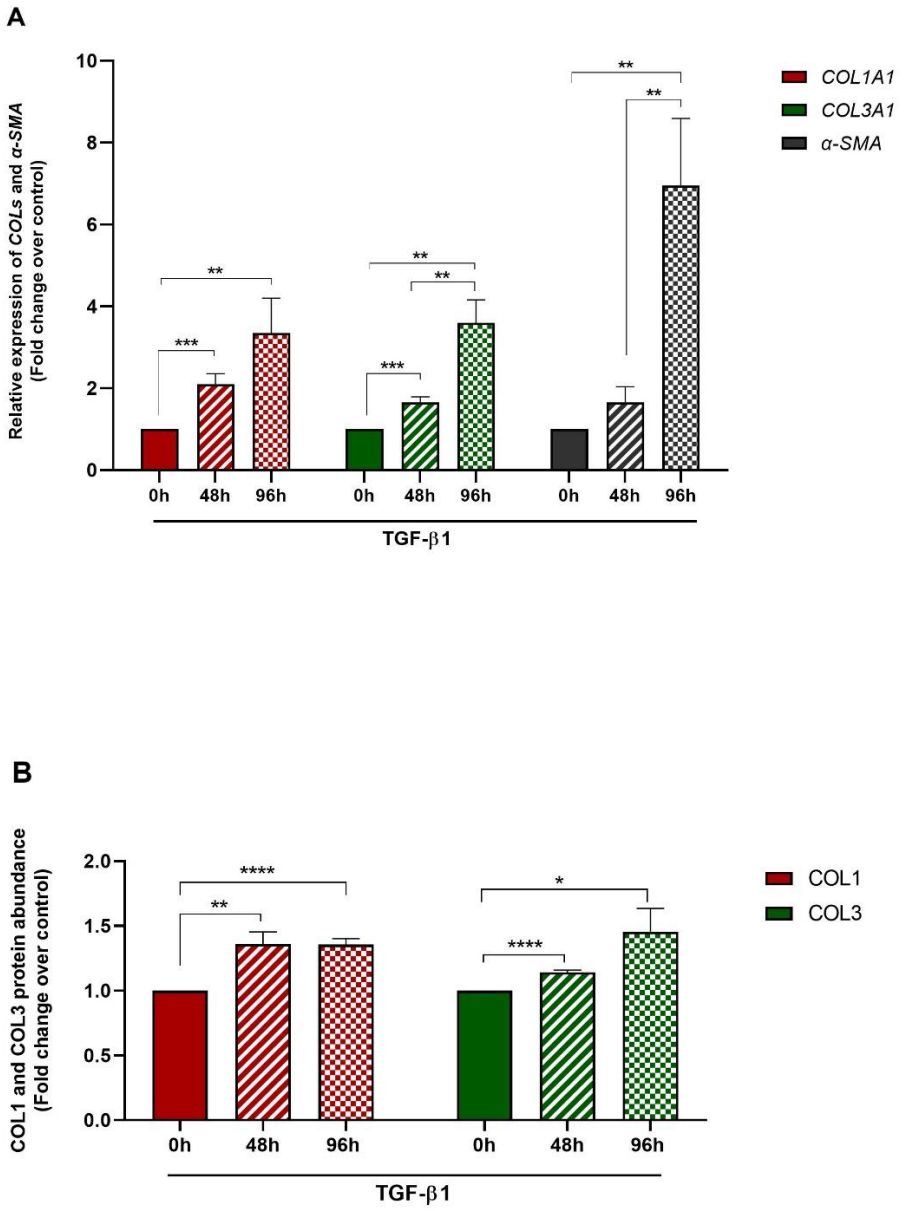

Figure 19. A) Relative collagen type I (*COL1A1*) and type III (*COL3A1*) and α-smooth muscle actin (*α-SMA*) mRNA levels, and **B)** relative COL1 and COL3 protein concentrations after 0 h, 48 h and 96 h of TGF-β1 treatment in endometrial fibroblasts. Bars represent mean±SEM. Asterisks indicate significant differences between hours of treatment (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

4.4.2. TGF-β1 upregulated *DNMT3A* expression in endometrial fibroblasts

We examined *DNMT1, DNMT3A* and *DNMT3B* gene mRNA levels in TGF-β1 treated endometrial fibroblasts to determine whether *DNMTs* regulate the collagen expression through DNA methylation, at 48 h and 96 h. TGF-β1 upregulated *DNMT3A* at 48 h and 96 h (P<0.01), but no differences were observed for *DNMT1* or *DNMT3B* mRNA levels (Figure 20). There were also no differences in *DNMT*s mRNA levels between 48 h and 96 h (P>0.05).

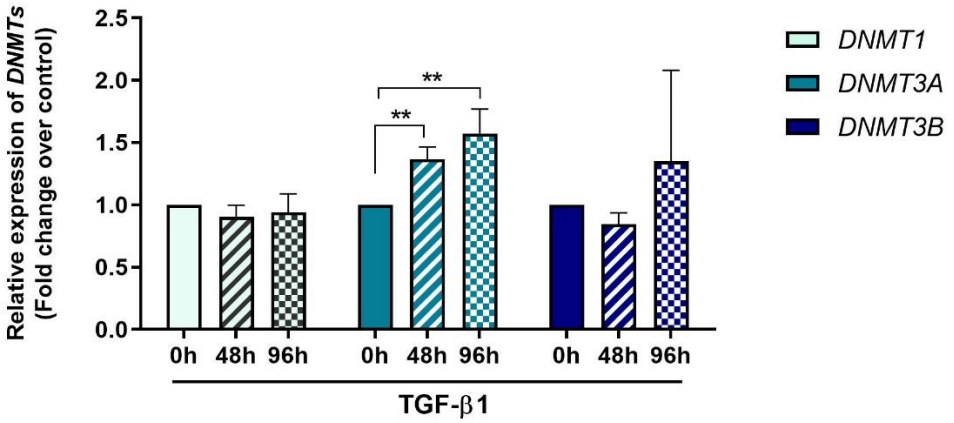

Figure 20. Relative DNA methyltransferases *DNMT1, DNMT3A* and *DNMT3B* mRNA levels in TGF-β1 (10ng/mL) treated endometrial fibroblasts at 0 h, 48 h and 96 h. Bars represent mean±SEM. Asterisks indicate significant differences between hours of treatment (** *P*<0.01).

4.4.3. 5-aza-dC downregulated collagen type I and III expression induced by TGF-β1 in endometrial fibroblasts

Demethylating DNMT inhibitor 5-aza-dC (decitabine) was used to test whether epigenetic regulation is involved in collagen expression. Endometrial fibroblasts were treated with TGF-β1, TGF-β1+5-aza-dC or 5-aza-dC. TGF-β1 upregulated *COL1A1* and *COL3A1* mRNA levels (*P*<0.01 and *P*<0.001, respectively) and the administration of 5-aza-dC to the TGF-β1 treated fibroblasts (TGF-β1+5-aza-dC) significantly downregulated their expression (*P*<0.001 and *P*<0.01, respectively) (Figure 21A). The same pattern was observed for COL1 and COL3 protein concentration (*P*<0.05) (Figure 21B). However, the TGF-β1 induced increase of α-SMA mRNA levels in endometrial fibroblasts was not reduced with the 5-aza-dC treatment (Figure 21A) (*P*>0.05).

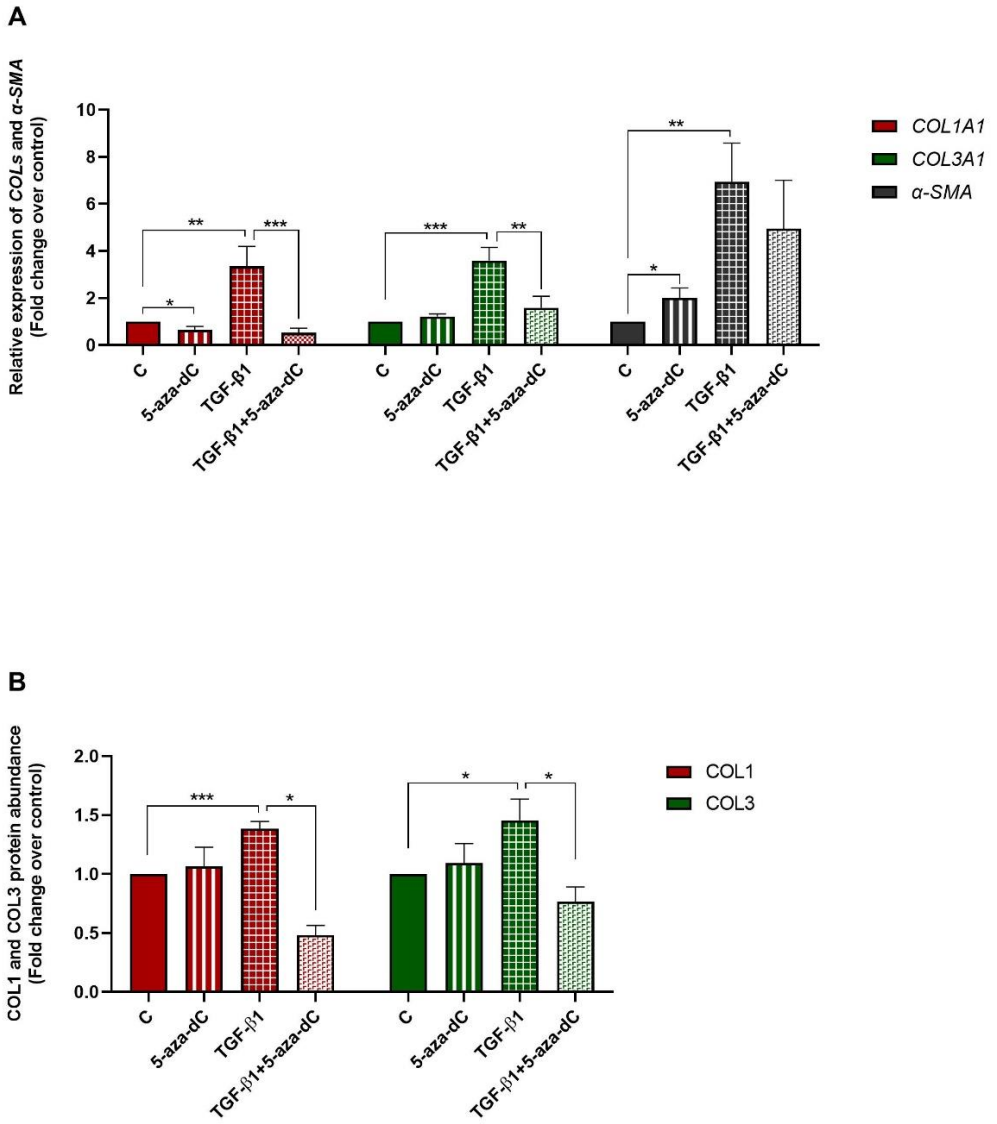

Figure 21. A) Relative collagen type I (*COL1A1*) and type III (*COL3A1*) and α-smooth muscle actin (*α-SMA*) mRNA levels, and **B)** relative COL1 and COL3 protein concentrations in endometrial fibroblasts treated with 5-aza-dC (1μM), TGF-β1 (10ng/mL) or both combined (TGF-β1+5-aza-dC). Bars represent mean±SEM. Asterisks indicate significant differences between treatments (**P*<0.05, ** *P*<0.01, *** *P*<0.001). C – control (with no factors added to culture medium).

4.4.4. TGF-β1 + 5-aza-dC downregulated *DNMT1* and *DNMT3A* expression

After administration of 5-aza-dC to the TGF-β1 treated fibroblasts, a decrease was observed in *DNMT1* and *DNMT3A* mRNA levels (*P*<0.05) (Figure 22). No alterations were found for *DNMT3B* mRNA levels.

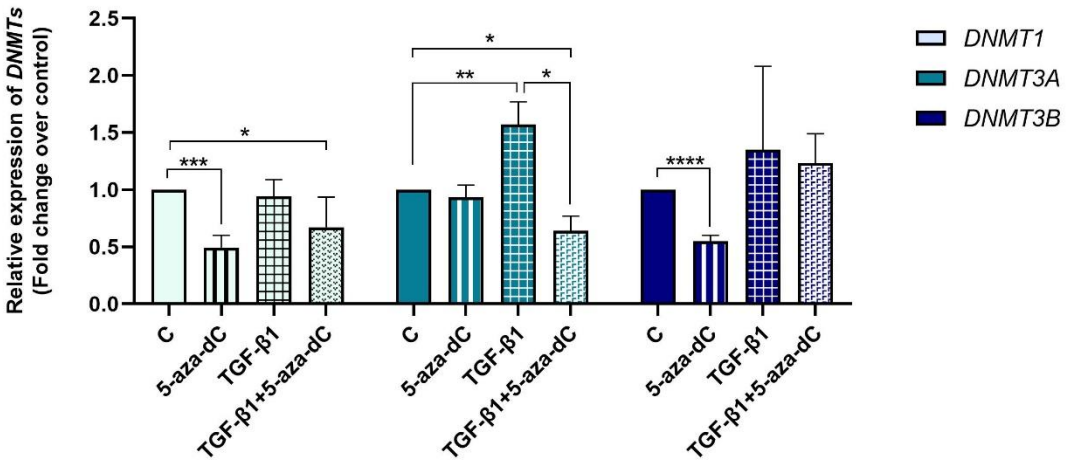

Figure 22. Relative DNA methyltransferases (*DNMT1, DNMT3A* and *DNMT3B*) mRNA levels in endometrial fibroblasts treated with 5-aza-dC (1μM), TGF-β1 (10ng/mL) or both combined (TGF-β1+5-aza-dC). Bars represent mean±SEM. Asterisks indicate significant differences between treatments (* *P*<0.05, ** *P*<0.01, *** *P*<0.001, **** *P*<0.0001). C - control (with no factors added to culture medium).

4.4.5. 5-aza-dC down-regulated *COL1A1* and upregulated α-SMA at 48 h and 96 h

We also aimed to study the effect of demethylating 5-aza-dC in endometrial fibroblasts. When administered alone, 5-aza-dC downregulated *COL1A1* at 96 h (*P*<0.05) and upregulated *α-SMA* mRNA levels at 48 h and 96 h (*P*<0.05) (Figure 23A). Regarding COL1 and COL3 protein concentrations only COL1 increased at 48 h (*P*<0.05), while no other differences were found at 96 h, neither in COL3 (Figure 23B).

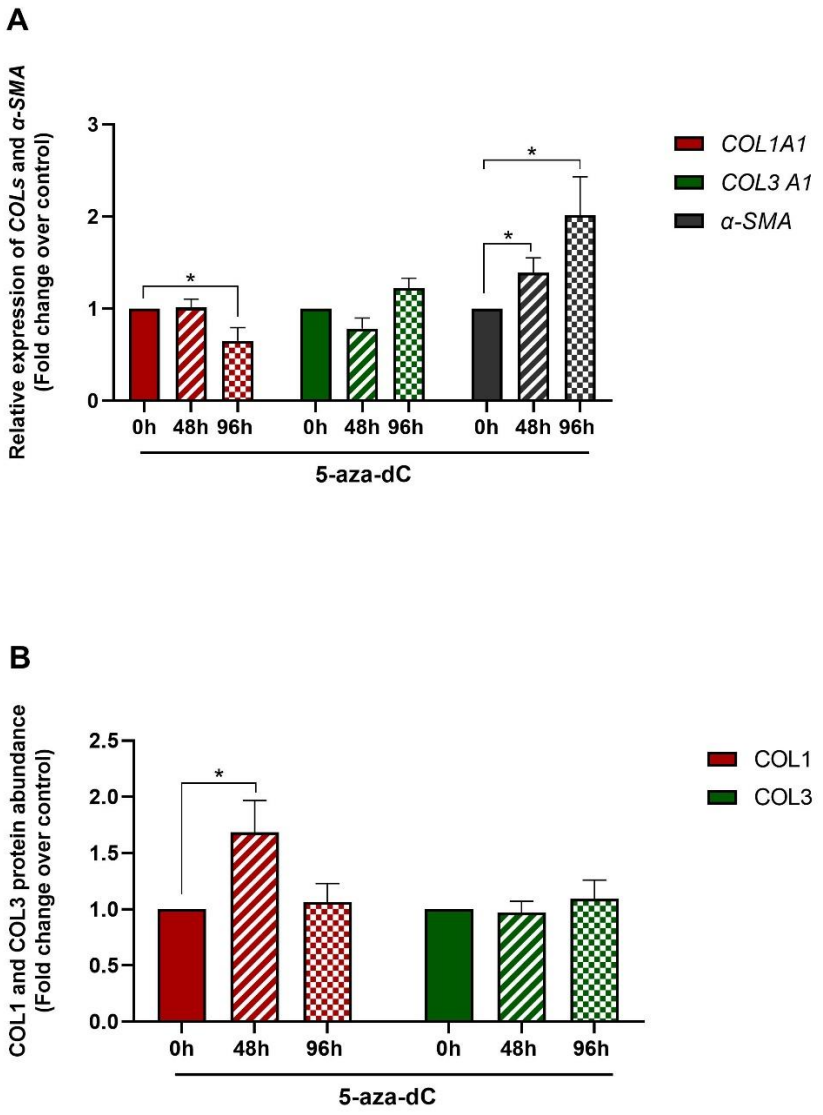

Figure 23. A) Relative collagen type I (*COL1A1*) and type III (*COL3A1*) and α-smooth muscle actin (*α-SMA*) mRNA levels, and **B)** relative COL1 and COL3 protein concentrations in endometrial fibroblasts treated with decitabine (5-aza-dC) for at 0 h, 48 h and 96 h. Bars represent mean±SEM. Asterisks indicate significant differences between hours of treatment (* $P<0.05$).

4.4.6. 5-aza-dC downregulated *DNMT1, DNMT3A* and *DNMT3B* expression

The effect of 5-aza-dC was also studied in the mRNA levels of methylating enzymes *DNMT1, DNMT3B* and *DNMT3A.* There was a decrease in mRNA levels of *DNMT1* and *DNMT3B* at 96 h ($P<0.001$ and $P<0.0001$, respectively) and of *DNMT3A* at 48 h ($P<0.001$) (Figure 24). It was also observed a decrease of *DNMT1* mRNA levels between 48- and 96 h treatment ($P<0.01$) (Figure 24).

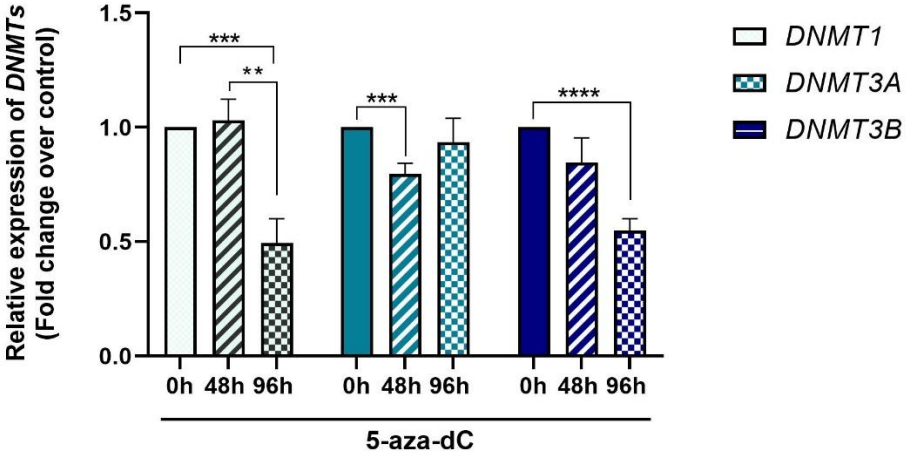

Figure 24. Relative DNA methyltransferases (*DNMT1, DNMT3A* and *DNMT3B*) mRNA levels in decitabine (5-aza-dC) treated endometrial fibroblasts at 0 h, 48 h and 96 h. Bars represent mean±SEM. Asterisks indicate significant differences between hours of treatment (** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

4.4.7. TGF-β1 induced collagen type I and III expression in equine endometrial fibroblasts by DNA methylation and the effects were reversed by 5-aza-dC

The increased mRNA levels of *DNMT3A* in TGF-β1 stimulated endometrial fibroblasts occurred simultaneously with an increase in collagen type I and III expression (both mRNA levels and protein concentration) (Figure 25A and 25B). After treatment of TGF-β1-induced fibroblasts with 5-aza-dC, it was also observed a reduction of *DNMT3A* mRNA ($P<0.05$) simultaneously with a reduction in collagen type I and III mRNA levels ($P<0.001$ and $P<0.01$, respectively) and protein concentration ($P<0.05$) (Figure 25A and 25B).

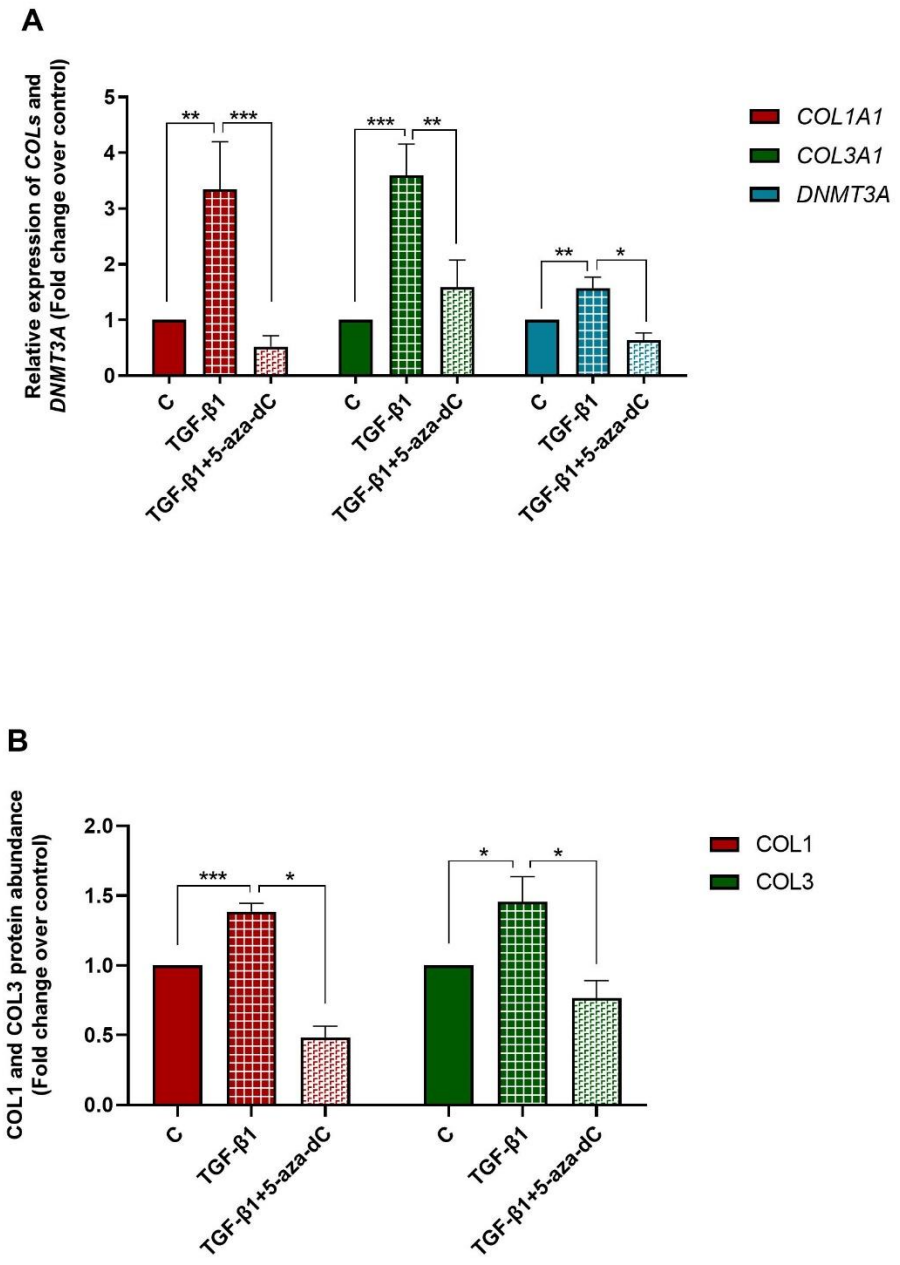

Figure 25. A) Relative collagen type I (*COL1A1*) and type III (*COL3A1*) and DNA methyltransferase 3A (*DNMT3A*) mRNA levels, and **B)** relative COL1 and COL3 protein concentration after treatment of endometrial fibroblasts with TGF-β1 (10ng/mL) and TGF-β1+5-aza-dC (1μM). Bars represent mean±SEM. Asterisks indicate significant differences between treatments (*P<0.05, ** P<0.01, *** P<0.001). C- control (with no factors added to culture medium).

4.5. Discussion

We have previously reported increased *DNMT3B* mRNA levels in equine endometrial fibrosis (Alpoim-Moreira et al. 2019) and increased concentrations of COL1 and COL3 proteins with the degree of fibrosis (Alpoim-Moreira et al. 2022a). We have also demonstrated epigenetic modulation of equine endometrial fibrosis by hypermethylation of the promoter region of the anti-

fibrotic *MMP2* and *MMP9* genes (Alpoim-Moreira et al. 2022b). Thus, to evaluate what was taking place at the cellular level, we aimed to study the epigenetic mechanisms associated to TGF-β1 action in endometrial fibroblasts. Regulation of collagen expression has been extensively studied and a plethora of evidence has indicated that TGF-β1 is an important regulator of ECM metabolism in different organs (Massague 1990).

Our findings indicate that in equine endometrial fibroblasts, TGF-β1 upregulated the expression of collagen type I and III mRNA and their secretion at 48 h and 96 h and *α-SMA* mRNA levels at 96 h. Furthermore, TGF-β1 induced an increase in *DNMT3A* expression at 48 h and 96 h. This agrees with other studies, in humans, which have shown increased DNMTs expression in lung fibrosis (Sanders et al. 2012), cardiac fibroblasts (Xiao et al. 2014) and skin fibroblasts from systemic sclerosis patients (Dees et al. 2020). In addition, in human nasal epithelial cells, *DNMT3A* might be the most affected by TGF-β1 (Park et al. 2022), as it happened in our study. It was suggested in the same study that DNMT inhibitors suppress the progression of chronic rhinosinusitis pathology by regulating DNA methylation. In primary mouse renal fibroblasts, DMNT1 expression was induced by TGF-β1 (Bechtel et al. 2010). Nevertheless, in contrast, TGF-β1 downregulated *DNMT1* and *DNMT3A,* and upregulated *COL1A1* mRNA expression and secreted COL1 in cardiac fibroblasts (Pan et al. 2013). Interestingly, treatment with 5-aza-dC abrogated the effects of TGF-β1-induced myofibroblasts in human cardiac cells (Watson et al. 2014).

These different results may be explained by the passage number of the cells used in each study. Besides, it may be ascribed to the type of cells used (cardiac cells *vs.* renal cells), since it has been established that methylation patterns vary in different tissues and individuals (Pan et al. 2013; Zhang et al. 2013). Thus, TGF-β1 can induce both hypermethylation or hypomethylation in genes, illustrating the complexities of the pathways that control and alter methylation patterns.

To confirm if the increase of methylation, collagen and α-SMA mRNA expression with TGF-β1 could be reverted by epigenetic treatment, a demethylating epigenetic modifier, 5-aza-dC, was used. The administration of 1µg of 5-aza-dC for 48 h to TGF-β1 previously stimulated equine endometrial fibroblasts (for 48 h) was effective in reducing the increased COL1 and COL3 expression (mRNA levels and protein concentration) to normal levels (control), but not for *a-SMA* mRNA. The same results were observed in human cardiac fibroblasts (Watson et al. 2016). Our data also agree with other studies in humans, where it was observed that 5-aza-dC mitigates renal (Bechtel et al. 2010; Pushpakumar et al. 2015), cardiac (Watson et al. 2016; Russell-Hallinan et al. 2020) and pulmonary (Robinson et al. 2012; Neveu et al. 2015; Zhang et al. 2019) fibrosis by reducing hypermethylation of genes associated with fibroblast activation. In another study, it was found that hypermethylation contributes to renal fibrosis and inhibition of DNMTs supresses chronic unilateral ureteral obstruction-induced renal fibrosis (Smith et al. 2019). A similar reduction in the upregulation of *COL1* mRNA and protein expression was reported after administration of 5-aza in TGF-β1 induced human dermal fibroblasts (Dees et al. 2020). Furthermore, in a study in human hepatic stellate cells

it was observed that 5-aza-dC inhibited their differentiation into myofibroblasts (Mann et al. 2007). In addition, 5-aza suppressed fibrogenic changes in human conjunctival fibroblasts (Yonemura et al. 2019). On the contrary, treatment with 5-aza-dC to TGF-β1 treated rat lung fibroblasts, stimulated *a-SMA* gene expression by inhibiting DNMTs (Hu et al. 2010).

In our study, the administration of 5-aza-dC alone to equine endometrial fibroblasts for 48 h decreased *DNMT3A* mRNA levels, but not *DNMT1* or *DNMT3B*. When fibroblasts were exposed to 5-aza-dC for 96 h it reduced *DNMT1* and *DNMT3B* mRNA levels, but not *DNMT3A*. Neveu et al. (Neveu et al. 2015) also reported a decrease in *DNMT1* expression after treatment with 5-aza-dC alone in lung fibroblasts. In our study, it was observed a reduction of *COL1A1* mRNA levels at 96 h and an increase of *a-SMA* mRNA levels at 48 h and 96 h, after treatment of endometrial fibroblasts only with 5-aza-dC. However, COL1 protein concentration was increased at 48 h and no difference was found at 96 h, despite its decreased mRNA levels at 96 h. Other studies have shown opposite results, either with a rise in *COL1A1* mRNA levels after treatment with 5-aza-dC alone (Pan et al. 2013), or no differences were found (Neveu et al. 2015; Watson et al. 2016). Several studies regarding 5-aza-dC effect on ECM components have shown contradictory results. The basis for this difference is unclear, but it may be related to different cell types and/or experimental conditions used.

It the present study, the endometrial fibroblasts were previously challenged with TGF-β1 for 48 h, and then treated with 5-aza-dC for 48h, so the treated group was not exposed to 5-aza-dC for the total duration of the experiment (96 h).

It appears that longer time of exposure to 5-aza-dC might provoke bigger changes in both *DNMT*s, COLs and *a-SMA* expression. However, the 48-h period was enough to reduce the increased collagen expression induced by TGF-β1 in endometrial fibroblasts, although the same did not happen for *a-SMA.* Due to the toxic effects of demethylating agents, the minimal dose that produced the desired effect was used as a rationale for future clinical trials. Some limitations of this study include the lack of information of DNMTs and α-SMA protein expression and DNMTs activity.

The stimulation of endometrial fibroblasts with TGF-β1 increased COLs (mRNA levels and protein concentration), *a-SMA* and *DNMT3A* mRNA levels, and the treatment with 5-aza-dC decreased their expression, except for *a-SMA* gene expression, suggesting an epigenetic regulation through the alteration of DNA methylation.

In summary, although epigenetic alterations have been implicated in the development of many types of cancer (Esteller et al. 2007; Movassagh et al. 2021), the role of epigenetic changes in fibrosis, particularly in equine endometrial fibrosis, needs much further investigation. Based on our findings, along with the evidence of other studies linking DNA methylation and fibrosis, one may suggest that DNA methylation plays a role in the pathogenesis of endometrial fibrosis. Therefore, pharmacological modulation of this process may bring a valuable treatment for endometrosis.

4.6. Conclusion

The increase in *DNMT3A* and COLs (mRNA and protein) after TGF-β1 stimulation of equine endometrial fibroblasts was reduced after the treatment with a demethylating agent (5-aza-dC), suggesting an epigenetic regulation of mare endometrial fibrosis.

Supplementary Figures

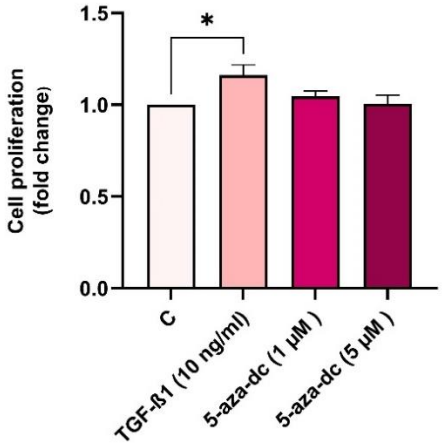

Figure S4 - Effect of TGF-β1 at a dose of 10 ng/mL and 5-aza-dC at doses of 1 µM and 5 µM on cell proliferation. Bars represent mean±SEM. Asterisks indicate significant differences between hours of treatment (*P<0.05). C- control (no factors added to the culture medium).

CHAPTER IV – General discussion and conclusions

1. General discussion

Endometrosis physiopathology understanding is still not fully achieved. Therefore, treatment options are barely non-existent and would benefit from a more in-depth knowledge regarding endometrosis establishment. Endometrosis is one the major causes of infertility in mares (Kenney 1992) and it is characterized by COL1 and COL3 accumulation and periglandular fibrosis (Kenney 1978; Walter et al. 2005). MMPs are involved in collagen turnover and are important factors in fibrosis (Ra and Parks 2007), especially MMP2 and MMP9 in mare endometrial fibrosis (Aresu et al. 2012; Centeno et al. 2018; Crociati et al. 2019; Szóstek-Mioduchowska et al. 2020). TIMP-1 and TIMP-2 inhibit MMP-9 and MMP-2, respectively (Giannandrea and Parks 2014). The imbalance between MMPs and TIMPs is a principal feature of fibrosis and results in the loss of the homeostasis between fibrolysis and fibrinogenesis (Hemmann et al. 2007). Fibroblasts are key effector cells in tissue remodelling. They remain persistently activated in fibrotic diseases, resulting in progressive deposition of extracellular matrix and can induce a self-maintaining profibrotic phenotype in myofibroblasts (Dees et al. 2020). These cells are characterized by α-smooth muscle actin (α-SMA) expression and an ability to deposit excessive amounts of extracellular matrix (ECM) components (Smith et al. 2019).

The diagnosis of endometrosis is based on a uterine biopsy, which remains as the only reliable diagnostic tool for over 50 years. Although it is a safe procedure, nonetheless it is still invasive, not 100% accurate and sometimes not feasible in a large group of mares. Moreover, it does not provide fertility information, but expected foaling rate. As such, a more practical and less invasive method would be advantageous, such as a blood test, particularly when assessing a large group of mares as in an embryo transfer programs, when selecting receptor mares. Since collagen pathological accumulation in the equine endometrium is responsible for infertility, in the first part of the present work, a study was conducted to analyse possible blood biomarkers to aid the diagnosis of endometrial fibrosis and fertility in mares.

Biomarkers may be used for early detection of otherwise subclinical disease, diagnosis of an acute or chronic clinical disease, risk stratification of patients with a suspected or confirmed diagnosis, prognosis, and selection of an appropriate therapeutic intervention, and monitoring the response to therapy (Zannad et al. 2010). As an example, a study in abdominal aortic aneurysm in humans, higher amounts of hydroxyproline, COL1, and COL3 were found in samples (tissue and serum) of patients compared with healthy controls, and a positive

correlation was found between tissue and serum concentrations of COL1, and COL3 (Metschl et al. 2019). In human medicine, the use of collagen fragments as biomarkers for the diagnosis and prognosis of fibrotic disorders, such as for cardiac fibrosis, (Ong et al. 2020; Zannad et al. 2010), renal fibrosis (Ix et al. 2015), and hepatic fibrosis (Rosenberg et al. 2004; Tanwar et al. 2017; Soylemezoglu et al. 1997) is well established (Luo et al. 2018; Gressner et al. 2007; Karsdal et al. 2020).Nevertheless, to the best of our knowledge, in horses, blood biomarkers have been studied only to assess musculoskeletal conditions, such as osteochondrosis (Frisbie et al. 1999; Laverty et al. 2000; Billinghurst et al. 2004; Frisbie et al. 2008).

In the present study, after assessing COL1, COL3 and hydroxyproline in mare endometrium and blood, only COL3 proved to be a possible biomarker of endometrial fibrosis. Serum COL3 concentrations correlated with Kenney and Doig´s mare endometrial category, and higher concentrations corresponded to more advanced categories.

Regarding fertility, higher concentrations of COL3 were observed in the infertile mare's group when compared with the fertile group. It was also noted, an up-regulation of COL1 protein production in mare endometrium, as the severity of histopathological lesions increased (according to Kenney and Doig's classification), as previously shown (Lunelli et al. 2013). This raise was also seen in COL3 and hydroxyproline concentrations in the endometrium, suggesting that both collagens are being produced when fibrosis increases. Nevertheless, it has been previously described that COL3 fibers are replaced by COL1 fibers with fibrosis, in the equine endometrium, and in several human organs, such as in liver and heart (Martinez-Hernandez 1999; Bochsler and Slauson 2002; Masseno 2012). However, other studies suggest that COL3 appears to regulate COL1 fibril formation in many human organs (Liu et al.,1997; Kuivaniemi and Trump 2019). In contrast to COL1, the expression of COL3 is restricted to soft tissues, and correlates to the number of myofibroblasts in fibrotic tissue (Badid et al. 2000). Consequently, the accuracy for the presence of fibrotic processes in soft tissues is greater for COL3 (and other minor collagens), when compared to COL1 (Badid et al. 2000). In fact, type III procollagen peptides have been regarded as good prognostic biomarkers for liver fibrosis in humans (Nielsen et al. 2015; Leeming et al. 2016). Though, it should be emphasized that COL3 is not an endometrial specific biomarker since its plasma elevation can occur due to other organ diseases (Gressner et al. 2007).

The endometrial category increased with age and there was a strong correlation between age of the mares and endometrial category. This agrees with previous work (Schoon et al. 1992; Ricketts and Alonso 1991; Hoffmann et al. 2009a; Hoffmann et al. 2009b; Rebordão et al. 2019). Although the age factor is not by itself the cause of endometrosis, old age has been associated with increased endometrial inflammation, increased embryo-loss rate, and subsequently reduced pregnancy rate in the mare (Carnevale and Ginther 1992). Furthermore, there was an

increase in COL1, COL3 and hydroxyproline in the endometrium as well as serum COL3 with mares aging, whereas in the serum COL1 decreased with age. Higher serum COL1 concentrations in younger horses, and lower in older horses, have been previously described (Price et al. 1995), mostly in equine musculoskeletal pathologies, and appear to be ascribed to the ongoing bone formation in young horses, suggesting that the age factor plays an important role and should not be dismissed. The infertile mares were older than the fertile mares, and a moderate positive correlation was observed between age and infertility. However, no correlation was observed between serum COL3 and mare's age.

A ROC curve is an established method for evaluating the clinical viability of a biomarker (Luo et al. 2018). The sensibility and specificity of COL3 was most effective to differentiate mares from category I from all other categories, when compared to the ability to differentiate category I + category IIA from category IIB + III, or to identify mares with severe endometrosis (category III) among all the other endometrial categories. Serum COL3 concentration was also higher in infertile mares, when compared to fertile mares, and a positive correlation was observed between serum COL3 and infertile mares. The area under the curve for the estimation of endometrial category through COL3 serum concentrations was more accurate than for estimating mare's fertility. This might be explained by the fact that infertility in mares may be due to several causes other than endometrosis, and because both fertile and infertile groups of mares may include mares with different endometrial categories. Although it is very unlikely that a single biomarker can replace a biopsy diagnosis, it could provide additional information to the endometrial biopsy, particularly in category IIB where the percentage of expected foaling rates ranges from 10-50%, with a 40% window of variation.

In the second part of this work, the epigenetic field was explored to understand if endometrosis is under epigenetic regulation. Epigenetic mechanisms are responsible for heritable changes in genome function that do not alter the nucleotide sequence. The most common epigenetic mechanism is DNA methylation, which regulates gene activity. DNA methylation is known to occur at the 5′ of cytosine in CpG dinucleotides which are found mostly in so-called CpG islands present in promoters (Newell-Price et al. 2000; Curradi et al. 2002; Ehrlich and Lacey 2013). It is catalyzed by DNA methyltransferases (DNMTs), such as DNMT1, DNMT3A, and DNMT3B (Robertson 2002). One way to evaluate epigenetics mechanisms is through DNA methylation by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B). However, this only reflects the level of these enzymes, which in turn may indicate the level of global methylation. Another way to assess the DNA methylation pattern is by bisulfite pyrosequencing. This method is very accurate and is commonly used for quantitative analysis of DNA methylation at single nucleotide level, and in a particular region of the gene (CpG islands), providing more detailed information. In the promotor regions of many genes the most commonly hypermethylation events lead to gene repression (Mohn et al. 2008), whereas

hypomethylation results in gene induction (Karouzakis et al. 2009). Epigenetic changes are potentially induced by environmental factors, are potentially reversible, and thus promising targets in therapeutics (Simmons 2008).

In fibrotic tissues, myofibroblasts are the primary contributors to the excessive production of extracellular matrix proteins, such as collagen. Alterations in DNA methylation have a substantial impact on fibroblast phenotype and promote the differentiation to pathological myofibroblasts leading to fibrosis (Neary et al. 2015). Epigenetic alterations have been linked to inflammation and chronic fibrosis (Hahn et al. 2008; Dakhlallah et al. 2013). Furthermore, accumulating evidence suggests that epigenetic alterations are central in maintaining the myofibroblast phenotype (Bergmann and Distler 2017). Several studies demonstrated that the hypermethylation of gene promotors of antifibrotic mediators plays important roles in pathologic fibroblast activation and that inhibition of DNMTs prevents fibrosis in many fibrotic diseases (Wang et al. 2006; Bechtel et al. 2010; Huang et al. 2010; Dees et al. 2014; Noda et al. 2014; Watson et al. 2014; Altorok et al. 2015; Dees et al. 2020).

Therefore, first, the global methylation was assessed, and it was observed a higher transcription level of DNA methyltransferase enzyme *DNMT3B* in mares with category III endometrium when compared to healthy mares (category I). This upregulation could reflect the downregulation of an anti-fibrotic gene, and therefore contribute to further fibrosis establishment, as described for idiopathic pulmonary fibrosis in humans (Sanders et al. 2008).

Then, the specific locus of DNA methylation, was evaluated through bisulfite pyrosequencing. This method allowed to determine if methylation was occurring in a promoter region, important for gene transcription, in some of the genes involved in equine endometrosis. As it was not possible to assess all the genes, *COL1A1, COL1A2, MMP2, MMP9, TIMP1* and *TIMP2* were selected. The transcription of all these genes was evaluated along with DNA methylation in the regulatory regions of the genes, in different endometrial categories. It was found that for the anti-fibrotic *MMP2* and *MMP9* genes, there was a negative correlation between gene transcription and DNA methylation as endometrial fibrosis increased. As such, while methylation levels increased with fibrosis (higher endometrial category) the transcription levels decreased, suggesting an epigenetic modulation through DNA methylation. In category III endometria the concentration of collagen (COL1 and COL3) was increased, although no changes were observed for its transcription. In addition, no differences were found for *COL1A1, COL1A2* or *COL3A1* transcripts between different endometrial categories. A study in jennies showed no differences in *COL1A2* and *COL3A1* transcripts between endometrial categories (Miró et al. 2020). However, some *in vitro* studies with mare endometrial fibroblasts showed increased *COL1A1* and *COL3A1* mRNA transcripts after TGF-β1induced fibrosis (Szóstek-Mioduchowska et al. 2018) together with high COL1 and COL3 protein levels.

Regarding TIMPs, only *TIMP1* had increased mRNA levels in category III, when compared to category I endometrium, and no changes were observed regarding DNA methylation. Our data agrees with a study in human lung fibrosis, where *TIMP1* mRNA was markedly increased in response to lung injury, whereas there was no change in *TIMP2* mRNA levels (Madtes et al. 2001). Also, studies in human cardiac disease (Heymans et al. 2005), and rat lungs (Wang et al. 2011) reported upregulated TIMP1 gene expression during fibrosis. Since in our study, there were no alterations in DNA methylation pattern between endometrial categories for the TIMP1 gene, this might suggest that its inhibition does not occur, continuing to be expressed and increased with fibrosis, thus contributing to collagen accumulation in mare endometrium. Several studies demonstrated that hypermethylation of gene promotors of antifibrotic mediators plays important roles in pathologic fibroblast activation and that inhibition of DNMTs prevents fibrosis in many fibrotic diseases (Wang et al. 2006; Bechtel et al. 2010; Huang et al. 2010; Dees et al. 2014; Noda et al. 2014; Watson et al. 2014; Altorok et al. 2015; Dees et al. 2020). In view of the results obtained in this study it was proposed that the hypermethylation in advanced endometrial fibrosis could be inhibiting the transcription of *MMP2* and *MMP9*, and along with the increased *TIMP1* mRNA levels, could be contributing to the accumulation of collagen in the endometrium, as schematized in Fig. 17. Therefore, it seems that this regulation might be occurring through anti-fibrotic genes silencing rather than activation of fibrotic genes.

Other studies also demonstrated lower *MMP2* gene transcription in fibrotic endometrium, but *MMP9* transcription was higher (Falcomo et al. 2015). In another study with endometrial fibroblasts in mares (Szóstek-Mioduchowska et al. 2020), *MMP9* gene transcription increased after TGF-β1 stimulation. Also, a study by Centeno et al. (2018) showed that *MMP2* transcription was upregulated in endometrial fibrosis. On the other hand, a study in mice with induced liver fibrosis reported increased *MMP2* mRNA and decreased *MMP9* mRNA (Qin and Han, 2010). Many studies in other animals and humans have shown a decrease in *MMP2* (Sugihara et al. 2009; Kendziorski and Belcher 2015) and *MMP9* gene expression in several fibrotic diseases (Bailey et al. 2012), while others reported the opposite (Kim et al. 2005; Andersen et al. 2007; Dancer et al. 2011). These inconsistent results regarding *MMP2* and *MMP9* expression might be partially explained by the fact that MMPs are tissue-dependent and have a disease-specific expression and function (Leong et al. 2021). Moreover, current knowledge on MMP regulation is largely based on cell culture systems, raising a major question as to whether identical mechanisms apply to MMP expression in the whole organism as well (Yan and Boyd 2007).

Once it was established a link between epigenetics (through DNA methylation) and endometrosis the investigation continued *in vitro,* to evaluate if the fibrotic process could be reverted with demethylating drugs. Since TGF-β1 is indicated as an important regulator of extracellular matrix (ECM) metabolism in different organs (Massague 1990), mare endometrial

fibroblasts were cultured and stimulated with TGF-β1, before the treatment with the demethylating agent, decitabine (5-aza-dC) was initiated. TGF-β1 increased the expression of COL1 and COL3 (both mRNA and protein) and upregulated *α-SMA* and *DNMT3A* mRNA expression in mare endometrial fibroblasts. This agrees with other studies, in humans, that have described an increase in DNMTs expression in lung fibrosis (Sanders et al. 2012), and skin fibroblasts from systemic sclerosis patients (Dess et al. 2020). Furthermore, another study also demonstrated that *DNMT3A* was the most affected by TGF-β1, in human nasal epithelial cells, and that DNMT inhibitors suppressed the progression of chronic rhinosinusitis pathology by regulating DNA methylation (Park et al. 2022). Nevertheless, other studies have demonstrated that TGF-β1 downregulated *DNMT1* and *DNMT3A* and upregulated COL1 (mRNA and protein) in cardiac fibroblasts (Pan et al. 2013; Watson et al. 2014). This inconsistency in the results might be due to the type of cells used, as it has been established that methylation patterns vary in different tissues and individuals (Pan et al. 2013; Zhang et al. 2013). Besides, it may also be explained by the passage number of cells used in each study.

The upregulation of collagens (COL1 and COL3 mRNA levels and protein concentration) and *DNMT3A* mRNA transcripts, in TGF-β1 treated fibroblasts, was reduced after the treatment with decitabine, to normal levels (control) but not for *α-SMA* mRNA. Similar results were observed in human cardiac fibroblasts (Watson et al. 2016). Moreover, other studies in humans have reported that 5-aza-dC mitigates renal (Bechtel et al. 2010; Pushpakumar et al. 2015), cardiac (Watson et al. 2016; Russell-Hallinan et al. 2020) and pulmonary (Robinson et al. 2012; Neveu et al. 2015; Zhang et al. 2019) fibrosis, by reducing hypermethylation of genes associated with fibroblast activation. In another study it was found that hypermethylation contributes to renal fibrosis and inhibition of DNMTs supresses chronic unilateral ureteral obstruction-induced renal fibrosis (Smith et al. 2019). The same reduction of the upregulation of *COL1* mRNA and protein expression was reported after administration of 5-aza in TGF-β1 induced human dermal fibroblasts (Dees et al. 2020). Furthermore, a study in human conjunctival fibroblasts also demonstrated that 5-aza-dC suppressed their fibrogenic changes (Yonemura et al. 2019). On the opposite, treatment with 5-aza-dC to TGF-β1 treated rat lung fibroblasts, stimulated *α-SMA* gene expression by inhibiting DNMTS (Hu et al. 2010).

The mechanism by which TGF-β1 induced the upregulation of *DNMT3A* is still to be unveiled, but it was noticeable that the treatment with decitabine reduced this upregulation, besides reducing the collagen expression, further evidencing an epigenetic modulation. Whereas in the study with endometrial biopsies there was an increase in *DNMT3B* transcripts with fibrosis, in the study with endometrial fibroblasts the increase occurred in *DNMT3A* mRNA levels. Many of the studies mentioned above reported alterations in different DNMTs, although the reason for that variation is yet to be elucidated. However, both DNMT3A or DMMT3B are *de novo* methylating enzymes (Okano et al. 1999; Hervouet et al. 2018) and have similar functions.

Although further studies are required to understand these epigenetic mechanisms thoroughly, the use of demethylating agents may present a possible approach to address mare endometrial fibrosis therapy. Nevertheless, the use of demethylating agents should be used with caution due to its side effects, as observed in humans (Howell et al. 2010; He et al. 2017). Also, extrapolating data from *in vitro* studies should be done with caution, as cells do not behave in the same manner *in vitro* as *in vivo*. An example are myofibroblasts, that *in vitro* its confirmation is possible by the expression of *α-SMA*, whereas *in vivo* not all fibroblasts turn into myofibroblasts after an insult or under fibrotic stimulation (D'Urso and Kurniavan 2020). Another major issue is that demethylating agents have not been assayed *in vivo* in horses and as such, a prudent approach should be ensued.

Overall, it seems that a different approach might be needed to address fibrosis treatment, as up to date, no effective therapy exists for equine endometrosis. Epigenetic drugs that target active myofibroblasts in fibrotic disorders are a promising direction for the treatment of multiple diseases. Nevertheless, we are far from a comprehensive understanding of how epigenetic modulators influence each other and myofibroblast behaviour (Duong and Hagood 2018). If epigenetic mechanisms are involved in mare endometrial fibrosis development, as suggested by our results, then therapeutic agents that can reverse these epigenetic changes may represent a new and promising approach, for a condition that still has no available treatment.

2. Conclusions

The present work pinpoints COL3 as a possible blood biomarker to aid in the diagnosis of endometrial fibrosis and fertility in mares, even though it should be further investigated and validated. Also, to the best of our knowledge, no study was performed to evaluate the epigenetic role in mare endometrosis. The results from this work demonstrated a link between epigenetics and endometrosis and demonstrated that *in vitro* treatment with demethylating agent, decitabine, was able to revert the increased *in vitro* collagen production in TGF-β1 treated equine endometrial fibroblasts.
Summarizing, the main conclusions of this work are, as follows:

1) The concentration of COL3 in endometrium and serum was higher with fibrosis. Besides, a serum COL3 cut-off value of 60.9 ng/mL allowed the differentiation of healthy mares from mares with endometrial degenerative/fibrotic lesions, with a specificity of 100% and a sensitivity of 75.9%. The endometrial category increased with the age of the mares, but the correlation between age and endometrial or serum COL3 was low.

2) The concentration of serum COL3 was higher in infertile mares, and a serum COL3 cut-off value of 146 ng/mL allowed the differentiation of fertile from infertile mares, with a specificity of 82.4% and a sensitivity of 55.6%. Although infertile mares were older than fertile mares, no correlation was found between age and serum COL3 concentration in both fertile and infertile mares. In this experiment the endometrial category was not assessed.

3) The phase of the estrous cycle did not interfere with the endometrial and serum concentrations of COL1, COL3 or hydroxyproline, neither with serum COL3 concentrations in fertile and infertile mares.

4) Transcription of DNA methyltransferase *DNMT3B,* in mare endometrium, was upregulated with fibrosis showing that global methylation is increased in Kenney and Doig's higher endometrial categories.

5) Transcription of *MMP2* and *MMP9* in mare endometrium was lower with fibrosis, while-*TIMP1* transcription was higher. Transcription of *COL1A1*, *COL1A2* and *COL3A1* did not differ between endometrial categories. However, COL1 and COL3 protein expression was elevated in category III endometrium, compared with healthy mares.

6) Methylation of CpG islands in regulatory regions of *MMP2* and *MMP9* was elevated in category III endometrium, suggesting that epigenetic modulation seems to occur through the inhibition of anti-fibrotic genes.

7) The transcription and methylation of COLs, MMPs and TIMPs did not depend on estrous cycle phase.

8) Treatment of endometrial fibroblasts with TGF-β1 increased *DNMT3A, COL1A1, COL3A1* and *α-SMA* transcription, as well as COL1 and COL3 production. However, their increase was reverted with treatment with decitabine, except for *α-SMA* transcription.

CHAPTER V – References

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