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BIOMEDICINA

Role of Metformin in the Control of Oxidative Stress and Infertility in Endometriosis

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Role of Metformin in the Control of Oxidative Stress and Infertility in Endometriosis

O papel da Metformina no Controlo do Stress Oxidativo e da Infertilidade na Endometriose

ANA CATARINA SOTOMAIOR NETO CERQUEIRA

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*TESE DE DOUTORAMENTO APRESENTADA
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*Orientação da Professora Doutora Delminda Rosa Gamelas Neves Lopes de
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Co-orientação do Professor Doutor Henrique Manuel Nunes de Almeida

À Professora Doutora Delminda Neves

Ao Professor Doutor Henrique Almeida

Aos Meus Pais

Às minhas irmãs e cunhados
Ao Francisco e ao Diogo

Ao Daniel

“Recomeça...

Se puderes

Sem angústia

E sem pressa.

E os passos que deres,

Nesse caminho duro

Do futuro

Dá-os em Liberdade.

Enquanto não alcances

Não descanses.

De nenhum fruto queiras só metade.”

Miguel Torga

PREFÁCIO

Escrever estas palavras traz-me uma enorme satisfação, sensação de dever cumprido, mas também a memória dos muitos momentos que construíram este percurso. Nem sempre foi um caminho fácil, linear ou vivido com o mesmo entusiasmo. Houve dias de dúvida, de cansaço e até de querer desistir, mas também houve descobertas, conquistas e apoio genuíno. Foi um percurso marcado por desafios, crescimento, resiliência e partilha. E é a todas as pessoas que me acompanharam, apoiaram e acreditaram em mim que quero dedicar algumas palavras.

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Em obediência ao disposto no Decreto-lei nº 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planejamento e execução das experiências, observação do material e análise dos resultados e participei ativamente na redação de todas as publicações que fazem parte integrante desta tese:

- I. Neto AC, Botelho M, Rodrigues AR, Lamas S, Araújo B, Guimarães JT, Gouveia AM, Almeida H, Neves D. Metformin reverses infertility in a mouse model of endometriosis: unveiling disease pathways and implications for future clinical approaches. *Reprod Biomed Online*. 2025 Mar;50(3):104474.
- II. Martins AF, Neto AC, Rodrigues AR, Oliveira SM, Sousa-Mendes C, Leite-Moreira A, Gouveia AM, Almeida H, Neves D. Metformin Prevents Endothelial Dysfunction in Endometriosis through Downregulation of ET-1 and Upregulation of eNOS. *Biomedicines*. 2022 Nov 1;10(11):2782.
- III. Felgueiras R, Neto AC, Rodrigues AR, Gouveia AM, Almeida H, Neves D. Anti-oxidant effect of metformin through AMPK/SIRT1/PGC-1 α /SIRT3- independent GPx1 expression in the heart of mice with endometriosis. *Horm Mol Biol Clin Investig*. 2022 Jul 7;43(4):405-414
- IV. Neto AC, Freitas C, Ribeiro Â, Rodrigues AR, Silva-Carvalho JL, Almeida H, Neves D. Identification of Proteins and MicroRNAs with Prognostic Value for Assisted Reproduction Technology Outcomes in Follicular Fluid of Women with Endometriosis: A Pilot Study. *Int J Mol Sci*. 2025 Oct 7;26(19):9752.
- V. Freitas C, Neto AC, Matos L, Silva E, Ribeiro Â, Silva-Carvalho JL, Almeida H. Follicular Fluid redox involvement for ovarian follicle growth. *J Ovarian Res*. 2017 Jul 12;10(1):44.
- VI. Neto AC, Santos-Pereira M, Abreu-Mendes P, Neves D, Almeida H, Cruz F, Charrua A. The Unmet Needs for Studying Chronic Pelvic/Visceral Pain Using Animal Models. *Biomedicines*. 2023 Feb 24;11(3):696.

A endometriose é uma doença crónica, inflamatória, dependente de estrogénio, que afeta aproximadamente 10% das mulheres em idade reprodutiva. Caracteriza-se pela presença de tecido semelhante ao endométrio fora da cavidade uterina e está frequentemente associada a dor pélvica crónica e infertilidade, manifestando-se esta em cerca de 40% das mulheres com diagnóstico da doença. Embora tradicionalmente considerada uma patologia estritamente ginecológica, a endometriose é atualmente reconhecida como uma doença sistémica, com impacto metabólico, cardiovascular, imunitário e neurológico. Assim, na perspetiva mais vasta da doença reconhece-se a necessidade de estudar, não apenas os mecanismos pélvicos, mas também as alterações sistémicas que não só contribuem para a progressão da endometriose, como também, para o comprometimento da função reprodutiva.

Os tratamentos hormonais recomendados na endometriose, embora eficazes no controlo dos sintomas, não são compatíveis com a conceção ou gravidez, nem apresentam benefícios no controlo das manifestações sistémicas da doença. Como tal, reconhece-se a necessidade de novas terapêuticas não hormonais, em particular de fármacos que possam ser utilizados com segurança antes da conceção e ao longo da gravidez. A avaliação de novas intervenções farmacológicas para a endometriose, inclusivamente recorrendo a reposicionamento de fármacos, implica a utilização de modelos animais. Nestes, é possível estudar a fisiopatologia da doença e avaliar o efeito das intervenções sem as limitações éticas e biológicas inerentes aos estudos estritamente clínicos. No entanto, os estudos clínicos são essenciais para validar os resultados e também para identificar biomarcadores com relevância diagnóstica e prognóstica, especialmente no contexto da Procriação Medicamente Assistida (PMA). A PMA é um recurso fundamental para mulheres inférteis com endometriose, apesar de estas apresentarem taxas de sucesso inferiores a 30%, percentagem observada globalmente na população que recorre a estes tratamentos. Assim, a identificação de biomarcadores que possam melhorar o diagnóstico, prever resultados reprodutivos ou orientar estratégias terapêuticas é de elevado interesse clínico.

Esta tese teve como principal objetivo avaliar o potencial terapêutico de uma dose subterapêutica de metformina – um fármaco não hormonal com propriedades anti-inflamatórias, metabólicas e antioxidantes – num modelo de murganho com endometriose, com foco na progressão das lesões, resultados de fertilidade e alterações no tecido cardíaco, enquanto manifestação sistémica da doença. Adicionalmente, foi conduzida a pesquisa de biomarcadores de diagnóstico e prognóstico, no líquido folicular humano, com o objetivo de auxiliar, quer a identificação da doença, quer a previsão do resultado da PMA nas mulheres.

Apesar da baixa dose, a metformina mostrou regular a expressão do marcador de proliferação, antígeno nuclear de proliferação celular (PCNA) e reduzir o crescimento das lesões e a fibrose nos tecidos ectópicos. Em relação à resposta inflamatória, o tratamento com metformina aumentou a presença de macrófagos (F4/80) no endométrio, concomitantemente com o aumento da expressão de galectina-3, do fator nuclear kappa B (NFkB) e das enzimas antioxidantes heme-oxigenase 1 (HO-1) e glutationa peroxidase 1 (GPX-1), revelando ações anti-inflamatórias, anti-fibróticas e de modulação redox coordenadas.

No que ao tecido cardíaco diz respeito, a metformina preveniu a disfunção endotelial associada à endometriose através do aumento da expressão da síntase do óxido nítrico endotelial (eNOS) e da diminuição da endotelina-1 (ET-1), indicando proteção contra a perda de função vascular associada à doença. Induziu também o aumento da expressão da GPX-1 de forma independente do eixo clássico proteína quinase ativada (AMPK) –sirtuina 1 (SIRT1)– Coativador 1-alfa do receptor gama ativado por proliferador do peroxissoma (PGC-1 α)–sirtuina 3 (SIRT3).

Um achado muito relevante deste trabalho foi o aumento da fertilidade observado nos murganhos com endometriose tratados com metformina, uma vez que apresentaram um número superior de fetos por fêmea gestante em comparação com os animais não tratados, atingindo valores semelhantes aos dos controles.

Nas amostras humanas de líquido folicular, recolhidas de mulheres com endometriose e com infertilidade de causa masculina (controle) em tratamento de PMA, analisamos proteínas secretadas pelo ovócito, pertencentes à família do fator de crescimento transformador beta (TGF- β), tais como a hormona anti-Mülleriana (AMH), a proteína morfogenética óssea 15 (BMP-15) e o fator de diferenciação do crescimento 9 (GDF-9)), bem como um painel de microRNAs (miR-20a_1, miR-145_1, miR-320a_1, miR-125b-5p, miR-212-3p e miR-199a), com função na progressão da endometriose e na qualidade embrionária. Quando comparadas com os controles, as mulheres com endometriose apresentaram alterações marcadas nos perfis proteico e de microRNAs, incluindo aumento de BMP-15 e GDF-9, níveis reduzidos de AMH e desregulação de múltiplos microRNAs. Em particular, microRNAs específicos (miR-125b-5p, miR-212-3p e miR-145_1) demonstraram potencial para uso como marcadores de diagnóstico de endometriose, enquanto outros (miR-145_1 e miR-320a_1) foram associados a gravidez bioquímica nas mulheres controle.

No seu conjunto, os resultados desta tese reforçam o conceito de que a endometriose é uma doença sistêmica que se estende para além da cavidade pélvica. Identifica-se a metformina como uma intervenção terapêutica não hormonal capaz de mitigar a progressão da doença, as suas manifestações sistêmicas e aumentar a fertilidade. Paralelamente, os biomarcadores presentes no líquido folicular emergem como ferramentas promissoras para diagnóstico, prognóstico e, potencialmente, para guiar estratégias personalizadas de PMA.

Ao incorporar investigação experimental e clínica, este estudo contribui para uma compreensão mais abrangente da natureza sistêmica da endometriose, apoiando o desenvolvimento de abordagens terapêuticas direcionadas e capazes de preservar a fertilidade e de uma medicina personalizada apoiada por biomarcadores.

Endometriosis is a chronic inflammatory and estrogen-dependent disease that affects approximately 10% of women in reproductive age. It is characterised by the presence of endometrium-like tissue outside the uterine cavity and is frequently associated with chronic pelvic pain and infertility, affecting up to 40% of women diagnosed with the disease. Although traditionally considered a strictly gynaecological condition, increasing evidence recognises endometriosis as a systemic disease with metabolic, cardiovascular, immune, and neurological systems involvement. This expanded perspective underscores the need to study not only local pelvic mechanisms but also systemic alterations that may contribute to disease progression and impaired reproductive function.

Conventional hormonal therapies, while effective in controlling symptoms, are not compatible with conception or pregnancy and do not address the broader systemic manifestations of the disease. As a result, new non-hormonal therapeutic strategies are needed, particularly those that can be safely used before conception and throughout pregnancy. Given the ethical and biological limitations of studying early lesion development and therapeutic responses in humans, animal models are indispensable for exploring the pathophysiology of endometriosis and evaluating novel pharmacological interventions. In parallel, clinical studies are essential to validate findings and identify biomarkers with diagnostic and prognostic relevance, especially in the context of Assisted Reproductive Technologies (ART), that represent a key treatment pathway for infertile women with endometriosis and where global success rates remain around 30% and outcomes are often poorer in those patients. Thus, identifying biomarkers that could improve diagnosis, predict reproductive outcomes, or guide treatment strategies is of major clinical interest.

This thesis primarily aimed to assess the therapeutic potential of a sub-therapeutic dose of metformin – a non-hormonal drug with anti-inflammatory, metabolic, and anti-oxidative properties – in a mouse model of endometriosis, focusing on its effects on lesion progression, fertility outcomes, and cardiovascular alterations as a systemic feature of the disease. In addition, the investigation of diagnostic and prognostic biomarkers in human follicular fluid that could support disease detection and predict ART outcomes in women, was conducted.

Despite the low dose, metformin regulated expression of proliferating cell nuclear antigen (PCNA), a proliferative marker, and reduced lesion growth and fibrosis of ectopic tissues. In the inflammatory setting, metformin increased macrophage presence (F4/80) in eutopic endometrium and upregulated galectin-3 and nuclear factor kappa B (NF- κ B) in ectopic implants. In addition, metformin upregulated the antioxidant enzymes heme-oxygenase (HO-1) and glutathione peroxidase 1 (GPX-1), indicating coordinated anti-inflammatory, anti-fibrotic, and redox-modulating actions.

Regarding cardiac tissue, metformin prevented endometriosis-associated endothelial dysfunction through increase in endothelial nitric oxide synthase (eNOS) and decrease of endothelin-1 (ET-1) expression, indicating protection against vascular impairment linked to the disease. Notably, it enhanced cardiac GPX-1 expression independently of the classical protein kinase (AMPK) – sirtuin 1 (SIRT1)- Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha (PGC-1 α) – sirtuin 3 (SIRT3) axis activation.

Importantly, fertility outcomes were improved, as treated animals exhibited an increased number of fetuses per pregnancy relatively to untreated animals, reaching numbers similar to controls.

In the human samples of follicular fluid collected from women with endometriosis and controls undergoing ART, we analysed key oocyte-related proteins from the transforming growth factor beta (TGF- β) family, such as anti-Müllerian hormone (AMH), Bone Morphogenetic Protein 15 (BMP-15), and Growth Differentiation Factor 9 (GDF-9), and a panel of microRNAs (miR-20a_1, miR-145_1, miR-320a_1, miR-125b-5p, miR-212-3p, and miR-199a) with demonstrated roles in endometriosis progression and embryo quality. When compared with controls, women with endometriosis exhibited marked alterations in both protein and microRNA profiles, including increased levels of BMP-15 and GDF-9, decreased levels of AMH, and dysregulation of multiple microRNAs. Notably, specific microRNAs (miR-125b-5p, miR-212-3p, and miR-145_1) demonstrated potential as diagnostic markers for endometriosis, while others (miR-145_1 and miR-320a_1) were associated with biochemical pregnancy outcomes in controls.

Collectively, the results of this thesis reinforce the concept of endometriosis as a systemic condition that extends beyond pelvic cavity. Metformin demonstrated therapeutic potential as a non-hormonal intervention capable of improving disease progression, systemic features, and fertility outcomes. In parallel, follicular fluid biomarkers emerged as promising tools for diagnosis, prognosis, and potentially for guiding personalised ART strategies.

By integrating experimental and clinical research, this study contributes to a broader understanding of the systemic nature of endometriosis, while supporting the development of targeted, fertility-preserving therapeutic approaches and biomarker-guided personalised medicine.

I	INTRODUCTION.....	27
1.	ENDOMETRIOSIS OVERVIEW	29
2.	ENDOMETRIOSIS: STAGING AND CLASSIFICATION	32
3.	ENDOMETRIOSIS: A SYSTEMIC DISEASE	33
4.	RELEVANCE OF MICRORNAs AS BIOMARKERS OF ENDOMETRIOSIS.....	46
5.	ENDOMETRIOSIS AND INFERTILITY LINK	49
6.	MEDICAL AND SURGICAL TREATMENTS FOR ENDOMETRIOSIS.....	52
7.	ANIMAL MODELS OF ENDOMETRIOSIS	60
8.	METFORMIN AS A POTENTIAL NON-HORMONAL THERAPY FOR PRESERVING REPRODUCTIVE FUNCTION IN ENDOMETRIOSIS.....	65
9.	OBJECTIVES AND STUDY OUTLINE.....	69
10.	BIBLIOGRAPHY.....	73
II	PUBLICATIONS	95
	PUBLICATION I.....	97
	<i>“Metformin reverses infertility in a mouse model of endometriosis: unveiling disease pathways and implications for future clinical approaches”.....</i>	97
	PUBLICATION II	119
	<i>“Metformin Prevents Endothelial Dysfunction in Endometriosis through Downregulation of ET-1 and Upregulation of eNOS”.....</i>	119
	PUBLICATION III.....	137
	<i>“Anti-oxidant effect of metformin through AMPK/SIRT1/PGC-1α/SIRT3- independent GPx1 expression in the heart of mice with endometriosis”</i>	137
	PUBLICATION IV	149
	<i>“Identification of Proteins and MicroRNAs with Prognostic Value for Assisted Reproduction Technology Outcomes in Follicular Fluid of Women with Endometriosis: A Pilot Study”</i>	149
	PUBLICATION V	169
	<i>“Follicular Fluid redox involvement for ovarian follicle growth”</i>	169

PUBLICATION VI.....	181
<i>“The Unmet Needs for Studying Chronic Pelvic/Visceral Pain Using Animal Models”</i>	181
III DISCUSSION	199
1. METFORMIN ATTENUATES LESION GROWTH IN ENDOMETRIOSIS.....	201
2. METFORMIN IS INVOLVED IN IMMUNE AND ANTI-OXIDATIVE RESPONSES AND FIBROSIS IN ENDOMETRIOSIS	203
3. METFORMIN RESTORES FERTILITY IN ENDOMETRIOSIS	205
4. BIOMARKERS OF FOLLICULAR FLUID ARE PREDICTORS OF ENDOMETRIOSIS AND ART OUTCOMES.....	207
5. TAKE-HOME MESSAGE.....	213
6. BIBLIOGRAPHY	216

I INTRODUCTION

1. Endometriosis Overview

Endometriosis is a chronic gynaecological condition that globally affects approximately 10% of women in reproductive age. It is characterized by the presence of endometrial-like tissue outside the uterus; the ectopic tissue mimics the endometrium, the tissue lining the inside of the uterus (Saunders & Horne, 2021). The growth of ectopic endometrial-like tissue can be found in several locations, such as the ovaries, fallopian tubes, peritoneum, and, less commonly, distant sites like the lungs, diaphragm or brain (L. Ye et al., 2022; Zondervan et al., 2018). Similarly to eutopic tissue, ectopic counterparts respond to hormonal changes during the menstrual cycle, leading to local inflammation, bleeding and pain. Over time, abdominal inflammatory processes contribute to the formation of adhesions, scarring, and cysts filled with endometrial-like tissue that when located in the ovaries are also known as endometriomas (S. E. Bulun et al., 2019; García-Gómez et al., 2019; Sanchez et al., 2014; Wang et al., 2020). Continued exposure to inflammation leads to fibrosis of surrounding tissues and ultimately, contributes to infertility (Saunders & Horne, 2021).

In the first half of the past century three theories had been developed to explain the etiology of endometriosis: Meyer's theory of coelomic metaplasia (1919), which considers that the origin of endometriosis lies in the metaplasia of the coelomic epithelium; Halban's metastatic theory (1925), which explains the appearance of endometrial lesions distant from the uterus by the hematogenous or lymphogenic propagation of viable endometrial cells; and Sampson's (1927) implantation theory, which argues that endometrial debris after retrograde menstruation survives and grows in the peritoneal cavity forming endometrial lesions. Among these theories, the most accepted and

evidence-supported is the implantation Sampson's theory. However, considering that retrograde menstruation is a phenomenon that occurs in the majority of childbearing age women and that only some of them develop endometriosis, it seems very probable that other factors contribute to the development of the disease (Cousins et al., 2023; Kobayashi et al., 2024). These factors include altered cellular immunity, genetic susceptibility, environmental influence and multifactorial modulation of specific gene expression by the microenvironment (Smolarz et al., 2021). In addition, it has been suggested that adult stem cells, which ensure endometrial self-renewal, could also contribute to the pathogenesis of ectopic endometrial growth (Gargett & Masuda, 2010; Maruyama, 2022; Pittatore et al., 2014).

Considering that no single theory is able to completely explain the etiology of endometriosis, the pathophysiology of the disease still remains unclear. Thus, it is largely accepted that endometriosis is a multifactorial disease caused by both endogenous and exogenous contributions (Kobayashi et al., 2024; Smolarz et al., 2021). In what concerns endogenous factors, genetic predisposition seems to play a significant role in endometriosis, because first-degree relatives of affected women have a sevenfold increased risk of developing the disease (Koninckx et al., 2019; Pais & Almeida-Santos, 2023; Rahmioglu et al., 2014). Hormonal influence is also critical, on account that estrogen is central to the pathogenesis of endometriosis by promoting the growth and survival of ectopic endometrial tissue (Mori et al., 2019; Reis et al., 2013; Zhang et al., 2023). Additionally, immune dysfunction is commonly observed in women with the condition, as they often exhibit impaired immune surveillance that allows ectopic implants to evade immune clearance, while chronic inflammation remains a hallmark of the disease (Jaeger-Lansky et al., 2018; Suszczyk et al., 2024; Vallvé-Juanico et al., 2019).

Environmental factors may further contribute to the risk of developing endometriosis; in particular, exposure to endocrine-disrupting chemicals, such as dioxins, is highly associated with an increased likelihood (García-Peñarrubia et al., 2020; Interdonato et al., 2023; Stephens et al., 2021). In addition, nutritional factors can also influence the course of the disease, with certain types of diets potentially offering protection against its development or progression whereas others do the opposite. In this context, some studies suggest that a Mediterranean or anti-inflammatory diet—rich in vegetables, fruits, whole grains, polyphenols, and healthy fats—may help alleviate endometriosis-related pain, by managing estrogen levels and mitigating inflammation and oxidative stress. In contrast, a Western-style diet high in processed foods and red meat is likely to increase the risk and worsen the symptoms (Abramiuk et al., 2024; Barnard et al., 2023; Hipólito-Reis et al., 2022; Markowska et al., 2023; Nirgianakis et al., 2022; Türkoğlu et al., 2024). In line, it was demonstrated that energy restriction initiated after endometriosis onset in a murine model stalled lesion growth (Yin et al., 2018), while another study found that a high-fat diet increased lesion number (Heard et al., 2016).

Endometriosis presents with a wide range of symptoms that vary in severity and do not always correlate with the extent of the disease (Coxon et al., 2018; Vercellini et al., 2006). Chronic pelvic pain is experienced by more than 60% of the diagnosed women, which can be persistent or cyclical, as dysmenorrhea, when accompanying the menstruation (Fauconnier & Chapron, 2005; Maddern et al., 2020; Masciullo et al., 2021). Dyspareunia, or pain during or after sexual intercourse, is a very common complaint with prevalence rates reaching as high as 79% of adolescents and adult woman with endometriosis, compared with 40% of their counterparts without endometriosis (Facchin et al., 2022; Pluchino et al., 2016; Schneider et al., 2020). Bladder symptoms, such as urinary frequency,

or pain during urination, particularly during menstruation, are also frequently reported (de Resende Júnior et al., 2018; Habib et al., 2020; Maddern et al., 2020; Wolthuis et al., 2014). As well, gastrointestinal involvement frequently causes painful bowel movements, diarrhea, constipation, and bloating due to inflammatory and adhesive processes. In addition to these common symptoms, some women experience atypical manifestations like fatigue, depression, lower back or sciatica pain. In rare instances, the respiratory system is impacted, with thoracic endometriosis causing chest pain, difficulty breathing, or hemoptysis (coughing blood). Besides these symptoms, 30-50% of women with endometriosis are diagnosed with infertility, in part due to distorted pelvic anatomy, adhesions, or altered implantation (Bonavina & Taylor, 2022; Tanbo & Fedorcsak, 2017).

The impact of these debilitating conditions on quality of life is profound by causing significant physical, emotional and social well-being impairment (Bourdel et al., 2019; Ruszala et al., 2022; Szyplowska et al., 2023). Delays in diagnosis and treatment, further aggravate the challenges, leading to prolonged suffering and frustration of the affected women (Agarwal et al., 2019; Cromeens et al., 2021; Davenport et al., 2023).

2. Endometriosis: Staging and Classification

The staging and classification of endometriosis have traditionally relied on the American Society for Reproductive Medicine (ASRM) system, initially developed in 1979 and subsequently revised in 1985 and 1997 (American Society for Reproductive, 1997; "Classification of endometriosis. The American Fertility Society," 1979). However, this system has proven insufficient, as it poorly correlates with pain and infertility and fails to account for extra-pelvic lesions (S. E. Bulun et al., 2019; Haas et al., 2011). To address this, the Enzyan system was introduced in 2005 and revised in 2011 (Lee et al., 2021; Tuttles et

al., 2005). This system complements the ASRM classification by considering existence and extent of deep and unusual localization of endometriosis lesions on a setting of deep-infiltrated endometriosis (DIE) (Haas et al., 2011; Lee et al., 2021). Traditionally, endometriosis has been viewed as a localized pelvic disorder primarily affecting the reproductive organs and their surrounding tissues; indeed, it is classified according to the location of lesions as peritoneal, ovarian and deep infiltrated; according to its severity, it is classified into four stages (I: minimal, II: mild, III: moderate, and IV: severe). Beyond the classification based on the location, extent and depth of endometriotic structural lesions, other assessments evolved to consider the prediction of woman fertility, when this point is relevant as it frequently is (for example the Endometriosis Fertility Index (Adamson & Pasta, 2010)). The complexity of the subject resulted in other classifications aiming to refine lesion evaluation, consensual treatment options and condition prognosis (Johnson et al., 2017).

Current evidence indicates that endometriosis manifests systemically, impacting various organ systems and leading to widespread physiological and immunological changes. These findings emphasize the need to reconsider endometriosis as a systemic disease rather than one confined to the pelvis (Alderman et al., 2017; Taylor et al., 2021; Tulandi & Vercellini, 2024). As summarized in Figure 1, endometriosis affects multiple organs and physiological systems, highlighting its multifactorial and systemic nature. In the future, this view will likely lead to a reappraisal of the current classification systems and criteria.

3. Endometriosis: A Systemic Disease

a. Inflammation and immune dysfunction in Endometriosis

A key feature of endometriosis is chronic systemic inflammation, which plays a pivotal role in disease development and progression (Taylor et al., 2021). In fact, inflammation associated with the disease is not limited to the local environment of ectopic lesions. Women with endometriosis frequently exhibit elevated levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and interleukin-1 β (IL-1 β), in both peritoneal fluid and blood (Khorram et al., 1993; Othman Eel et al., 2008). These cytokines are crucial mediators of chronic inflammatory response observed in endometriosis (Taylor et al., 2021) and their elevation in blood indicates that the inflammatory process extends beyond the lesions, rather manifesting as systemic and affecting multiple organs and tissues (Bao et al., 2022; Monsanto et al., 2016; Taylor et al., 2021).

Systemic inflammation is in part due to immune dysfunction, another critical aspect of endometriosis. Immune cells, which typically recognize and eliminate abnormal cells on tissues, often display impaired function in women with endometriosis (Chapron et al., 2019; Vallvé-Juanico et al., 2019). In fact, altered immune cell activity, particularly in vias involving natural killer (NK) cells, was demonstrated in patients (Maeda et al., 2002; Reis et al., 2023; Thiruchelvam et al., 2015; Wu et al., 2000). NK cells are essential for detecting and eliminating abnormal cells, including those from ectopic endometrial lesions. In women with endometriosis, NK cell activity is frequently impaired, allowing ectopic tissue cells to evade immune clearance and to persist in lesions (Reis et al., 2023; Thiruchelvam et al., 2015). The impairment in immune surveillance contributes to the chronic nature of endometriosis, promoting lesions growth and perpetuation of inflammation. In addition to NK cell dysfunction, alterations in T-cell responses, as an increase in T-helper 17 (Th17) cells, are also identified in endometriosis (Gogacz et al.,

2016; Khan et al., 2019). Th17 cells secrete cytokines that exacerbate inflammatory responses, contributing to the endometriotic lesions growth and their associated symptoms (Chang et al., 2017; Kang et al., 2023). Conversely, a decrease in regulatory T-cells (Tregs), which suppress excessive immune responses and maintain immune tolerance, further impairs the immune system's ability to regulate inflammation, aggravating the chronic inflammatory nature of the disease (Khan et al., 2019; Knez et al., 2024; Tanaka et al., 2017).

An additional feature of immune dysfunction in endometriosis is the secretion of autoantibodies, which target self-tissues and trigger autoimmune-like mechanisms (Eisenberg et al., 2012; Greenbaum et al., 2021; Shigesu et al., 2019). Actually, anti-endometrial antibodies have been detected in the blood of women with endometriosis, indicating a potential overlap between the condition and autoimmune diseases (Fernández-Shaw et al., 1993; Greenbaum et al., 2021); some of these anti-endometrial antibodies, such as anti-stomatin-like protein 2 (SLP2), anti-tropomodulin 3 (TMOD3), anti-tropomyosin 3 (TPM3), and anti-PDLIM1-interacting kinase 1 like (PDIK1L) can be used in the diagnosis of the disease (Gajbhiye et al., 2017; Greenbaum et al., 2021; Mathur et al., 1998; Pillai et al., 1996). Supporting immune dysfunction in endometriosis, an increased prevalence of autoimmune diseases, including systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, autoimmune thyroid disorders, celiac disease, multiple sclerosis, inflammatory bowel disease, and Addison's disease (Shigesu et al., 2019) has been demonstrated in patients with endometriosis, comparatively with women not diagnosed with the disease (Porpora et al., 2020; Shigesu et al., 2019; Vanni et al., 2021). In addition, a recent study carried out by Vanni *et al.* suggests that endometriosis is more severe in those who are also affected by autoimmune disturbances (Vanni et al., 2021). Impairment in

immune response mechanisms contribute to systemic manifestations of endometriosis causing widespread symptoms, such as debility and fatigue (Ramin-Wright et al., 2018; Sherwani et al., 2024). Moreover, chronic inflammation and altered immune response increase susceptibility to other chronic conditions, including cardiovascular disease, diabetes and cancer. In fact, their incidence is higher in post-menopause endometriosis patients who discontinued treatments, relatively to disease-free women (L.-H. Chen et al., 2023; Gemmell et al., 2017; Symons et al., 2018).

b. Cardiovascular impact of Endometriosis

Evidence supports that women with endometriosis have an increased risk of cardiovascular disease (CVD), which could be attributed to chronic inflammation and oxidative stress - an imbalance between the production of reactive oxygen species (ROS) and the anti-oxidative defenses— central features of the condition (Parsa et al., 2025; Poeta do Couto et al., 2023). In fact, both inflammation and oxidative stress are known to significantly contribute to atherogenesis, the process that leads to the development of atherosclerotic plaques the major structural change that heightens the risk of cardiovascular events (Batty et al., 2022; Steven et al., 2019). In addition, women with endometriosis often exhibit dyslipidemia, characterized by elevated low-density lipoprotein (LDL) and reduced high-density lipoprotein (HDL) levels that also associate to atheroma plaque and cardiovascular risk establishment (Melo et al., 2010; Mu et al., 2017). Furthermore, hormonal therapies used to manage endometriosis, particularly those involving high-dose estrogen, may exacerbate cardiovascular risk by increasing the likelihood of thrombosis in susceptible individuals (Maeda et al., 2020; Wu et al., 2015).

Our research in a murine model of endometriosis, developed by myself (**publication I** - (A. Catarina Neto et al., 2025)), apart from showing the establishment and

growth of the endometrial cyst, explored the impact of metformin, a common therapeutic agent, of endometriosis-related cardiovascular dysfunction (**publications II** (Martins et al., 2022) and **III** (Felgueiras et al., 2022)). The study demonstrated that mice with surgically-induced endometriosis presented increased heart fibrosis and cardiomyocyte cross-sectional area when compared to controls. Also, in the heart of those animals, a reduced eNOS (endothelial nitric oxide synthase) expression in parallel with an upregulated ET-1 (endothelin-1) expression was found, which points to endothelial dysfunction. In addition, we observed an upregulation of inflammatory pathways, involving increased expression of NFkB (nuclear factor kappa B) expression, an important mediator of metabolic and age-induced myocardial disease and a contributor to myocardial inflammation and to the onset of cardiovascular disease (**publication II**).

Metformin, discussed below, was found to mitigate endothelial dysfunction, which is essential for maintaining proper vascular health. However, treatment did not reduce NF-kB-associated inflammation or prevent cardiac fibrosis, (**publication II**) and led to cardiomyocyte hypertrophy, an enlargement of heart muscle cells with unknown impact on heart function (**publication II**). Regarding the molecular mechanisms that respond to oxidative conditions in the heart, we verified that AMP-activated protein kinase (AMPK – activated protein kinase)/SIRT1 – sirtuin-1 /PGC-1 α - Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha /SIRT3 – sirtuin-3 pathway did not notably alter in the heart of mice with endometriosis comparatively with controls. However, metformin enhanced the expression of the antioxidant enzyme glutathione peroxidase 1 (GPX-1) in mice with endometriosis (**publication III**). These findings do not provide evidence in favor of cardiovascular amelioration in endometriosis; however, in the setting of oxidative

stress, metformin appears to promote a compensatory antioxidant response. To tackle this complex condition, further investigation is necessary.

c. Neurological impact of Endometriosis

Beyond localized pelvic pain, endometriosis has other profound neurological implications including manifestations such as hypersensitization to pain and ensuing anxiety, depression and fatigue (Bajaj et al., 2003; Chen et al., 2016; Lemaire, 2004; Ramin-Wright et al., 2018; Taylor et al., 2021). A key mechanism contributing to the chronic pain experienced by many women with endometriosis is central sensitization (Bajaj et al., 2003). This phenomenon occurs when persistent nociceptive (pain-related) input from endometriotic lesions leads to heightened excitability of neurons in the central nervous system (CNS) (Fan & Li, 2022; Maddern et al., 2020; Neves et al., 2024). Over time, this condition results in an increased sensitivity to stimuli in areas not directly affected by the disease and in widespread chronic pain (Maddern et al., 2020; Vuontisjärvi et al., 2018). Research utilizing functional Magnetic Resonance Imaging (fMRI) has provided valuable insights into how endometriosis impacts brain activity, demonstrating that women with endometriosis exhibit altered activity in brain regions responsible for processing pain, including the insula and the anterior cingulate cortex (As-Sanie et al., 2016; Szabo et al., 2022). These changes in brain activity suggest that the disease not only intensifies the perception of pelvic pain but also alters the way brain processes and responds to pain signals. This evidence provides a neurological basis for the heightened pain experienced by these patients, even in the absence of new tissue damage (Li et al., 2018; Maddern et al., 2020). Furthermore, endometriosis can directly affect the peripheral nervous system, when lesions from endometriosis attach to or infiltrate peripheral nerves, such as sciatic nerve

(Gharaei & Gholampoor, 2023; McKinnon et al., 2015). In this particular case, sciatic endometriosis, women not only feel sciatica pain, but also tingling and legs weakness (Gharaei & Gholampoor, 2023; Possover & Chiantera, 2007). Hence, endometriosis can have a direct and disruptive impact on both central and peripheral nervous systems, further complicating the management of pain and symptoms.

d. Endocrine and metabolic impact of Endometriosis

Endometriosis is closely associated with endocrine and metabolic disturbances and is largely hormonally driven (S. E. Bulun et al., 2019; Patel et al., 2018). The primary contributor to these disruptions is estrogen dominance, which occurs due to the overexpression of aromatase in endometriotic tissue (Bulun et al., 1999). Aromatase catalyzes the conversion of androgens to estrogens, leading to excessive local production and increased circulating estrogen level. Such an imbalance not only promotes disease progression but also impacts various estrogen-sensitive systems throughout the body (Bulun et al., 2002; Da Costa et al., 2022; Ferrero et al., 2014; Zeitoun & Bulun, 1999). Locally, estrogen plays a key role in promoting the survival and proliferation while inhibiting apoptosis of endometrial cells and corresponding growth of ectopic tissue (Mori et al., 2019). Additionally, estrogen produced by endometriotic lesions creates a self-sustaining cycle of disease progression (S. E. Bulun et al., 2019; McCallion et al., 2022). Estrogen also interacts with immune cells, contributing to chronic inflammation and pain (Cutolo et al., 2010; García-Gómez et al., 2019; Harding & Heaton, 2022; Villa et al., 2015). As well, estrogen stimulates angiogenesis (Chung & Han, 2022). Moreover, expression of estrogen receptors ($ER\alpha$ and $ER\beta$) is dysregulated in endometriosis, with an increased $ER\beta/ER\alpha$ ratio in ectopic lesions, which enhances estrogen signaling, effect

causing inflammation and lesion increase (Chantalat et al., 2020; Simmen & Kelley, 2016). Concomitantly with estrogen upregulation, patients evidence progesterone resistance, which is in part due to downregulation of progesterone receptor (PR) expression and function. This condition not only causes privation of anti-inflammatory and antiproliferative effects of progesterone but also leads to inadequate response to progestin-based therapies. These factors, when combined, make endometriosis a highly estrogen-dependent and progesterone-resistant condition, complicating treatment strategies and contributing to the persistence of symptoms (Attia et al., 2000; Bedaiwy et al., 2015; Patel et al., 2017; Tang et al., 2024; Zhang & Wang, 2023). Moreover, endometriosis presence signals a higher risk profile for estrogen-dependent malignancies such as several ovarian cancer subtypes, endometrial and breast cancer (Serdar E Bulun et al., 2019; Mogensen et al., 2016; Wilbur et al., 2017; J. Ye et al., 2022).

Recent research supports that endometriosis is closely linked to metabolic dysregulation (Li et al., 2023; Neves et al., 2024; Sapkota et al., 2017; Taylor et al., 2021), considering that women with endometriosis exhibit disrupted glucose homeostasis and impaired insulin sensitivity and a heightened risk of developing insulin resistance (IR) (Marianna et al., 2017). Moreover, elevated fasting insulin levels in blood and reduced insulin receptor activity in endometriotic tissues were identified, suggesting a localized insulin-resistant state (J.-P. Chen et al., 2023; Noemi Salmeri et al., 2023). Dysregulated glucose uptake and mitochondrial dysfunction in endometriotic cells, contributing to metabolic alterations that may exacerbate disease progression and systemic insulin resistance were also demonstrated (Liu et al., 2024; Xin et al., 2024).

Interestingly, patients with endometriosis often present with a lower body mass index (BMI) and reduced adiposity compared to the general population (Ferrero et al.,

2005; Hong & Yi, 2022). Recent research from our group evidenced that endometriosis impacts both browning (the process by which white adipose tissue acquires characteristics of brown adipose tissue (Bartelt & Heeren, 2014), such as increase of mitochondrial content and thermogenic capability) and inflammatory pathways in visceral adipose tissue. We also found that women with endometriosis exhibit reduced adipocyte size and altered serum catecholamine levels, suggesting dysfunction of adipose tissue with impact in lipid metabolism (Abobeleira et al., 2024).

Furthermore, evidence points that endometriosis modulates the function of other endocrine glands, such as adrenals, pituitary, and thyroid. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis in endometriosis results in glucocorticoid receptor expression changes and reduction of morning cortisol levels, which suggests an impaired stress response (Cuevas et al., 2012; Ortiz et al., 2020; Petrelluzzi et al., 2008; Quiñones et al., 2015). Endometriosis is also linked to dysregulation of thyroid-secreted hormone metabolism. Interestingly, increased thyroxine (T4) and decreased triiodothyronine (T3) secretion was found in endometriotic cells comparatively to control endometrial cells. A higher prevalence of thyroid autoimmune diseases, such as Hashimoto's thyroiditis is observed in endometriosis patients comparatively with women without the disease (Peyneau et al., 2019; Zhang et al., 2023). In those patients, hormonal imbalances involving the pituitary gland, including altered thyroid-stimulating hormone (TSH) levels, may exacerbate disease progression and symptoms (Peyneau et al., 2019; Vannuccini et al., 2022).

e. Impact of Endometriosis in ovarium function

Despite the systemic nature of endometriosis, which impairs multiple organ systems including endocrine regulation, the disease most profoundly affects ovarian

function through mechanisms that compromise fertility (Kitajima et al., 2021; Sanchez et al., 2017). The formation of ovarian endometriomas can mechanically damage ovarian tissue, disrupt blood flow, and impair follicular development (Bulun, 2014; Kitajima et al., 2011). These cysts are often involved in the production of high levels of ROS and inflammatory molecules, leading to oxidative stress and further harm to oocytes and follicular cells (Sanchez et al., 2014). Consequently, women with endometriosis frequently exhibit a reduced ovarian reserve, as indicated by lower anti-Müllerian hormone (AMH) levels, and decreased antral follicle counts (Lemos et al., 2008; Yoo et al., 2011), which aligns with the diminished levels of AMH in the follicular fluid (FF) of women with endometriosis when compared with controls (**publication IV- (Ana Catarina Neto et al., 2025))**).

Owing to both systemic and local manifestations of endometriosis, the composition of FF also shows modifications. FF, which surrounds the developing oocyte, is a critical medium for its development and maturation as it contains proteins, steroid hormones, ROS and antioxidants, all of which modulate oocyte maturation (**publication V - (Freitas et al., 2017))** (Pocate-Cheriet et al., 2020; Yland et al., 2020). Some of these compounds are locally secreted by oocytes and granulosa cells, but other components of FF depend on levels found in blood, considering that an equilibrium is reached between both fluids. FF in endometriosis patients is characterized by elevated levels of inflammatory mediators, such as, IL-6, TNF- α , and TGF- β , which may further compromise oocyte quality and fertility outcomes (Kacem-Berjeb et al., 2023; Y. Li et al., 2019; Oală et al., 2024).

The transforming growth factor-beta (TGF- β) family is a group of multifunctional cytokines involved in cellular processes such as growth, differentiation, immune regulation,

and tissue remodeling (Derynck & Budi, 2019; Y. Zhang et al., 2017). Members of the TGF- β family, including TGF- β isoforms, bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs), play indeed a significant role in the progression of endometriosis and in its link to infertility, as well (De Conto et al., 2021; Santoso et al., 2021; Sikora et al., 2018).

TGF- β 1, TGF- β 2, and TGF- β 3 contribute to fibrosis, angiogenesis, and immune modulation in endometriosis. In particular, elevated TGF- β 1 levels in ectopic lesions drive extracellular matrix deposition and promote adhesion formation, impairing pelvic organ function. Moreover, TGF- β 1 enhances blood vessel growth to support ectopic lesion survival through increment in vascular endothelial growth factor (VEGF - Vascular Endothelial Growth Factor) production (Sikora et al., 2018; Young et al., 2017; Yu et al., 2017).

Activins and BMPs modulate follicular development, granulosa cell function, and reproductive tissue remodeling. In women with endometriosis, altered expression of activins and BMPs contributes to disrupted ovarian dynamics and compromised endometrial receptivity (Knight & Glister, 2006; Wijayarathna & de Kretser, 2016). In fact, in those patients, altered levels of oocyte-secreted factors bone morphogenetic protein-15 (BMP-15) and growth differentiator factor-9 (GDF-9) that have emerged as critical regulators of ovarian function, particularly in folliculogenesis (Fountas et al., 2024; Freitas et al., 2017; Riepsamen et al., 2019), are associated with granulosa-oocyte signaling disruption and reduction of oocyte quality and developmental potential (Gilchrist et al., 2008; Sanchez et al., 2014). BMP-15 and GDF-9 regulate together the interaction between oocytes and granulosa cells ensuring optimal oocyte maturation and competence (Sanfins et al., 2018) and in women with endometriosis, in part owing to pro-inflammatory

cytokines and aberrant estrogen signaling but also to increased oxidative stress, a negative impact on BMP-15 and GDF-9-mediated pathways is observed (Gupta et al., 2006).

Hence, alterations of BMP-15 and GDF-9 levels in FF may serve as biomarkers for assessing oocyte quality and predicting assisted reproductive technologies (ART) outcomes in endometriosis patients (Bulun, 2009; S. E. Bulun et al., 2019; Paulini & Melo, 2011; Riepsamen et al., 2019). However, data is conflicting because while most of the studies evidence a decrease of both proteins in FF from patients with endometriosis, we found them elevated in FF of patients comparatively to controls. Our hypothesis is that the increase in both proteins in FF may reflect an intrinsic compensatory mechanism aimed at improving oocyte quality (**publication IV**). Regularizing BMP-15 and GDF-9 activity through pharmacological agents or supplementation could improve granulosa-oocyte interactions and enhance follicular function. In addition, strategies to mitigate oxidative stress and inflammation in the ovarian microenvironment may indirectly preserve BMP-15 and GDF-9 activity, improving fertility outcomes (Donnez & Dolmans, 2021; Knight & Glister, 2006). Further research is needed to elucidate the precise mechanisms and develop targeted therapies for restoring normal ovarian function.

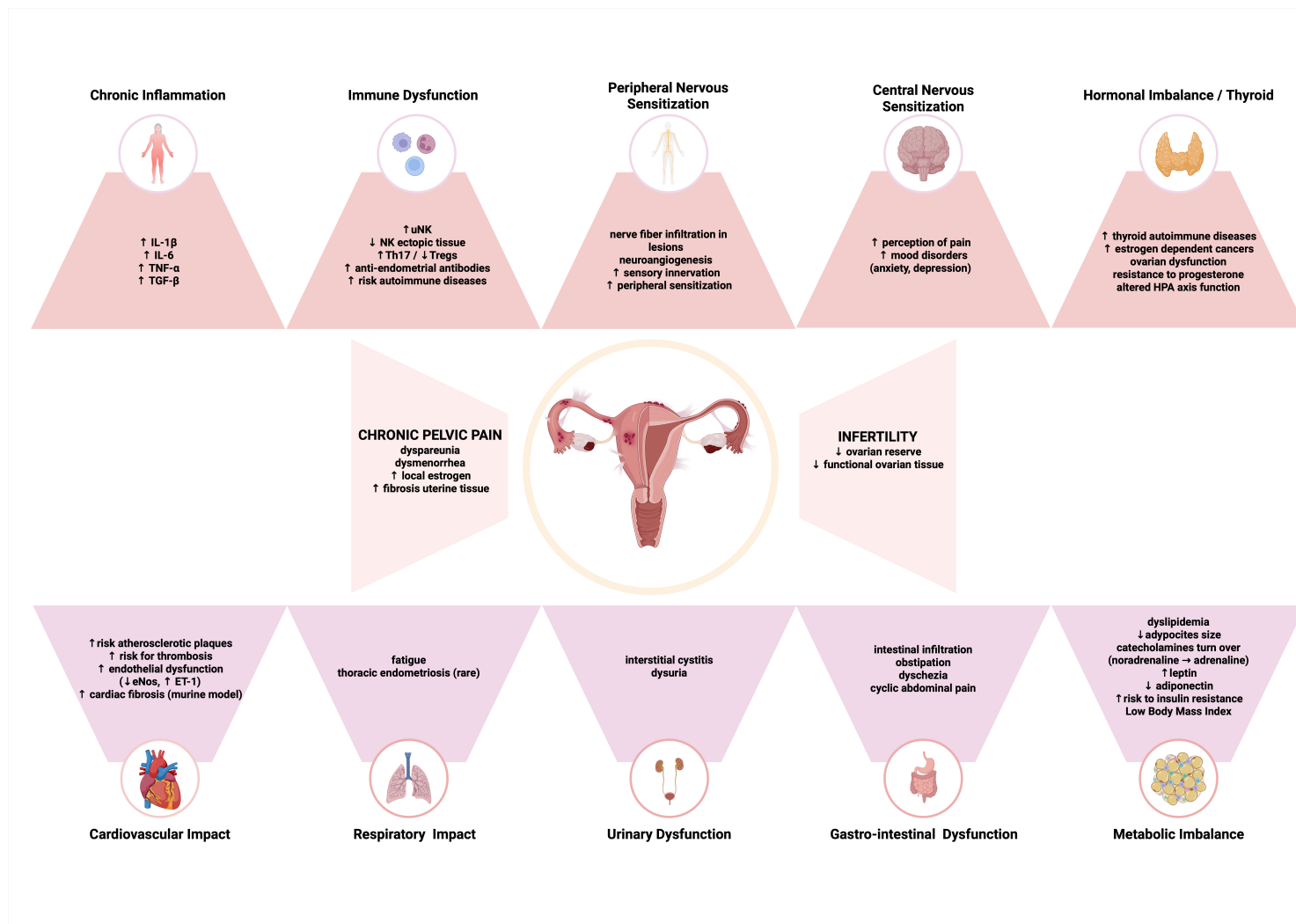


Figure 1 – Endometriosis, a systemic disease

4. Relevance of microRNAs as biomarkers of endometriosis

MicroRNAs are among the biological compounds present in FF. They are small (~22 nucleotides) non-coding RNA molecules, that regulate gene expression by targeting messenger RNAs (mRNAs) for degradation or translational repression (Yates et al., 2013). Their stability in body fluids such as serum and FF, coupled with their tissue-specific expression patterns, makes them attractive candidates for biomarker discovery in endometriosis (Moustafa et al., 2020; Qasemi & Amidi, 2020). In fact, several studies have identified panels of circulating and exosomal miRNAs that are consistently dysregulated in women with endometriosis compared to controls, with some panels achieving high diagnostic accuracy (Area Under Curve (AUC) > 0.9) and robust sensitivity and specificity in distinguishing endometriosis from other gynecologic conditions (Bjorkman & Taylor, 2019; Cho et al., 2015; Gu et al., 2020; Moustafa et al., 2020; Vanhie et al., 2024; Zafari et al., 2022). These miRNAs are implicated in pathways related to inflammation, angiogenesis, cell proliferation, and immune evasion—key processes in endometriosis pathophysiology (Bjorkman & Taylor, 2019; Ghasemi et al., 2022; Santos et al., 2025). For instance, Rekker et al. identified a panel of circulating miRNAs (miR-200a and miR-141) with high diagnostic accuracy, suggesting their utility as non-invasive biomarkers (Rekker et al., 2015). Similarly, Wang et al. performed a genome-wide profiling of serum miRNAs and reported distinct expression signatures in women with endometriosis, where miR-199a and miR-122 were found to be up-regulated and miR-145, miR-141, miR-542-3p, and miR-9 were down-regulated in endometriosis patients (Wang et al., 2013). Additionally, a recent study from Frisendahl et al. identified 14 dysregulated miRNAs in the eutopic endometrium of women with endometriosis (Frisendahl et al., 2024). Moreover, various miRNAs have been found to be differentially expressed in the FF of women with endometriosis (Qasemi & Amidi,

2020). In fact, the study by Li et al. revealed that miR-451, among other miRNAs, was significantly and consistently downregulated in the FF of women with endometriosis. Through both human and murine models, the authors demonstrated that decreased levels of miR-451 negatively impacted embryonic developmental potential, apparently by interference with the expression of genes involved in the WNT signalling pathway, a crucial regulator of early embryogenesis. These findings support the hypothesis that alterations in FF miRNA profile—particularly the suppression of miR-451—may impair oocyte quality and compromise pre-implantation embryo development in the context of endometriosis (X. Li et al., 2019). These findings suggest that FF miRNAs may contribute to the subfertility phenotype observed in endometriosis by altering the follicular milieu and impairing oocyte competence.

Furthermore, miRNAs have emerged as promising biomarkers for predicting reproductive outcomes in ART. In patients without endometriosis, a growing body of evidence supports their utility in evaluating both oocyte competence and endometrial receptivity, two critical determinants of ART success (Charalampos Voros et al., 2025; C. Voros et al., 2025). Vörös et al. highlighted how specific endometrial miRNA signatures—such as miR-30b, miR-30d, and members of the let-7 and miR-200 families—are closely associated with endometrial receptivity and implantation outcomes, modulating key pathways such as WNT, TGF- β , and PI3K/AKT (Charalampos Voros et al., 2025). Other studies have characterized extracellular and exosomal miRNAs within FF, revealing their potential to reflect oocyte quality and embryo development potential. For instance, Scalici et al. and Qasemi & Amidi demonstrated that FF-derived miRNAs could discriminate between successful and unsuccessful IVF outcomes, suggesting their value in predicting fertilization rates and embryo viability (Qasemi & Amidi, 2020; Scalici et al., 2016).

Similarly, Machtinger et al. identified miRNAs (including miR-320 and let-7b) in FF whose altered expression correlated with both oocyte fertilization potential and early embryo morphology (Machtinger et al., 2017). A study by Muraoka et al. further advanced this field by showing that small extracellular vesicles (sEVs) in FF, enriched in specific miRNAs, could serve as robust, non-invasive predictors of clinical pregnancy (Muraoka et al., 2024). Supporting this, another study by Zhang et al. demonstrated that exosomal miRNAs in FF were significantly associated with oocyte quality, reinforcing the hypothesis that these molecules actively participate in intercellular signalling within the ovarian follicle (Zhang et al., 2021).

In line with these findings, our study (**publication IV**) showed that miR-125b-5p and miR-212-3p levels were significantly decreased, while miR-20a_1, miR-145_1, and miR-320a_1 were increased in the FF of women with endometriosis. Furthermore, ROC analysis revealed that FF levels of miR-125b-5p, miR-212-3p, and miR-145_1 may serve as potential biomarkers for the presence of endometriosis. Notably, miR-145_1 and miR-320a_1 levels were also associated with biochemical pregnancy outcomes in women without endometriosis, suggesting their potential utility in predicting reproductive success in ART settings.

Collectively, these findings underscore the diagnostic and prognostic potential of miRNAs—both circulating, vesicle-associated and present in FF—as non-invasive biomarkers for assessing oocyte quality, endometrial receptivity, and overall ART outcomes (Charalampos Voros et al., 2025). Their differential expression in FF and endometrial tissue highlights their relevance in embryo selection and the personalization of fertility treatments (Fang et al., 2021; Liang et al., 2017). Nevertheless, several challenges must be addressed before miRNA-based tools can be translated into routine clinical

practice. These include biological variability linked to the menstrual cycle phase, inconsistencies in sample collection and processing protocols, and the lack of methodological standardization across studies (Diener et al., 2022; Ho et al., 2022; Lamon et al., 2023; Vanhie et al., 2024). Overcoming these limitations through large-scale, well-controlled studies and harmonized analytical approaches will be essential to validate miRNA signatures and fully harness their clinical utility in reproductive medicine.

5. Endometriosis and Infertility Link

Endometriosis is one of the leading causes of infertility, affecting up to 50% of women struggling to conceive (Bonavina & Taylor, 2022; Leone Roberti Maggiore et al., 2024). The mechanisms linking endometriosis and infertility are complex, involving a combination of anatomical, hormonal, inflammatory, and molecular factors (Bonavina & Taylor, 2022; Macer & Taylor, 2012). Understanding the causative factors of infertility in endometriosis is essential for optimizing fertility treatments and developing targeted therapeutic interventions for affected women (Bonavina & Taylor, 2022; Corachán et al., 2021).

Severe endometriosis, particularly on stages III and IV, is characterized by significant anatomical changes that can directly impair fertility. The formation of adhesions in the pelvis causes distortion of normal anatomy, affecting the mobility of the fallopian tubes and their ability to capture the oocyte during ovulation, further reducing the chances of natural conception (Bonavina & Taylor, 2022; D'Hooghe et al., 2003; Rathod et al., 2024). Adhesions or fibrosis may directly block the fallopian tubes, preventing sperm from reaching the oocyte, while inflammation can disrupt the ciliary action of the tubal

epithelium, which is critical for transporting the oocyte and embryo (Holoch & Lessey, 2010; Macer & Taylor, 2012).

Endometriosis also affects the uterine environment creating conditions that may hinder successful implantation and pregnancy (Arici et al., 1996; Boucher et al., 2022; Tsikouras et al., 2024). Altered expression of implantation-related genes, such as HOXA10 (Homeobox A10) and LIF (leukemia inhibitory factor) in the endometrium of women with endometriosis reduces the likelihood of successful embryo implantation (Boucher et al., 2022; Lessey & Kim, 2017). In addition, the peritoneal cavity in women with endometriosis is characterized by heightened inflammation and immune system dysfunction that may lead to increased uterine natural killer (uNK) cell activity and abnormal cytokine secretion, creating a hostile environment for implantation in uterus (Cuadrado-Torroglosa et al., 2024; Thiruchelvam et al., 2015). Activated peritoneal macrophages release ROS that associated with elevated levels of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, impair sperm motility, oocyte quality and embryos development (Cuadrado-Torroglosa et al., 2024; Simopoulou et al., 2021; Yoshida et al., 2004). The inflammatory environment also negatively affects sperm capacitation and interaction with the oocyte (Gou et al., 2019; Máté et al., 2018; Ngô et al., 2009; Ramírez-Pavez et al., 2021; Yamada et al., 2019). In fact, the mechanisms linking endometriosis to infertility are multifactorial and involve synergistic crosstalk between inflammation, oxidative stress, hormonal imbalances, and immune system dysfunction that contribute to an unprepared endometrium and inhospitable environment for gametes and embryos (Bonavina & Taylor, 2022; Gupta et al., 2008; Tanbo & Fedorcsak, 2017). Considering that a unique cause is not identified for endometriosis-related infertility, tailored management strategies are necessary to every patient (Tomassetti & D'Hooghe, 2018). Hormonal therapies to

suppress endometriosis-associated inflammation may help improve ovarian and uterine conditions, though these are often contraindicated or impeditive when attempting conception (Vercellini et al., 2009). Laparoscopic excision of endometriosis lesions has shown to improve fertility outcomes in selected patients, particularly in cases of tubal or pelvic adhesions, though the risks of ovarian damage must be weighed against potential benefits (Bafort et al., 2020; Gasimli et al., 2024). Due to these reproductive challenges, many women with endometriosis require ART to achieve pregnancy (de Ziegler et al., 2019; Moss et al., 2021).

Not infrequently, women with endometriosis face difficulties with ART, including IVF. They typically have fewer oocytes retrieved during IVF cycles, likely due to reduced ovarian reserve (Senapati et al., 2016). In addition, embryos from women with endometriosis may show lower developmental potential, possibly linked to altered follicular and peritoneal environments (Ding et al., 2010; Garrido et al., 2000; Han et al., 2023; Yland et al., 2020). Even when high-quality embryos are transferred, implantation rates are lower in such women, reflecting compromised endometrial receptivity (Boucher et al., 2022; Lessey & Kim, 2017; N. Salmeri et al., 2023). Despite these challenges, ART can still offer hope for many patients with endometriosis. Protocols that optimize ovarian stimulation, manage inflammation, and improve embryo quality have shown promising results, including pre-treatment with anti-inflammatory agents or hormone suppression that may enhance outcomes (Barra et al., 2020; Corachán et al., 2021)

Supporting evidence in women, in the endometriosis-induced mouse model I have developed, we verified that animals in the endometriosis-induced group had a significant decrease of fertility rate (**publication I**). Continued research is essential to unravel the

complex interactions driving infertility in endometriosis, with the ultimate goal of developing more effective and personalized treatment options for affected women.

6. Medical and Surgical Treatments for Endometriosis

Being a chronic inflammatory disease, endometriosis requires a comprehensive treatment approach combining medical and surgical strategies tailored to symptom severity, fertility goals, and patient preferences (Chapron et al., 2019; Olive & Pritts, 2002). Medical treatments primarily aim to suppress excess estrogen-driven proliferation of ectopic endometrial tissue, alleviate pain, and prevent disease progression (Vannuccini et al., 2022; Vercellini et al., 2016). Hormonal therapies, including combined oral contraceptives, progestin-only preparations, gonadotropin-releasing hormone (GnRH) agonists and antagonists, and aromatase inhibitors, play a crucial role in symptom management. In particular, GnRH antagonists like elagolix and relugolix offer advantages over agonists by allowing a more flexible dosage regimen with fewer side effects (Ferrero et al., 2018; Vannuccini et al., 2022). Non-hormonal approaches such as NSAIDs (nonsteroidal anti-inflammatory drugs) for pain relief, neuromodulators for central desensitization, and emerging therapies targeting immune and inflammatory pathways are also under investigation (Clemenza et al., 2018; Dolmans & Donnez, 2022; Ferrero et al., 2018) (**Table 1**). Preoperative medical therapy, particularly GnRH analogs, may help reduce lesion size and inflammation, which facilitates surgery. In addition, postoperative therapy with this class of drugs is often recommended to prevent recurrence in patients who are not planning pregnancy (Chen et al., 2020; Zakhari et al., 2020).

Surgical intervention for removing endometriotic lesions is invasive and indicated for cases of severe pain unresponsive to medical therapy, infertility related to such lesions,

large ovarian endometriomas, or DIE affecting the bowel or urinary tract (Meuleman et al., 2011; Singh & Suen, 2017). Laparoscopic surgery remains the gold standard for both excising or ablating lesions, with excision preferred due to lower recurrence rates and better long-term pain control (Bafort et al., 2020; Byrne et al., 2018). Cystectomy is recommended over simple drainage for ovarian endometriomas as it preserves ovarian reserve and reduces recurrence (Alammari et al., 2017; Daniilidis et al., 2023). In cases of DIE, specialized surgical techniques may be required, sometimes involving segmental bowel resection or ureterolysis. For refractory cases, hysterectomy with or without bilateral salpingo-oophorectomy (BSO) may be considered, eventually followed by hormone replacement therapy (HRT), often necessary to manage hypoestrogenic symptoms (Abrão et al., 2015; Acien et al., 2013; Matorras et al., 2002; Meuleman et al., 2011).

The choice between surgical and medical treatment depends on individual factors of each patient, as surgery provides significant pain relief and may have fertility benefits, particularly in cases of DIE or ovarian endometriomas, whereas medical therapy serves as a first-line approach and is essential in preventing recurrence after surgery. However, a multimodal approach that combines both strategies is often the most effective for long-term management (Bedaiwy et al., 2015; Horne & Missmer, 2022; Saunders & Horne, 2021). Advances in minimally invasive techniques, such as robotic-assisted surgery, are improving surgical outcomes, while research into personalized medicine, molecular profiling, and immune-modulating therapies holds promise for more targeted and effective treatment options in the future (Burnett et al., 2020; Saunders & Horne, 2021; Volpini et al., 2023).

Recent studies have explored the use of non-hormonal pharmacologic treatments for endometriosis (**Table 1**). Notably, dopamine agonists such as cabergoline, quinagolide,

as well as the immunomodulatory JNK inhibitor bentamapimod, have shown promising results in randomized controlled trials or animal models (Chen et al., 2019; Delgado-Rosas et al., 2011; DiVasta et al., 2021; Mendoza-Torreblanca et al., 2023; Shi et al., 2023). However, other agents like statins and TNF- α inhibitors, such as atorvastatin, simvastatin, etanercept or infliximab have not demonstrated efficacy neither in clinical trials nor in animal models (Chen et al., 2019; Soares et al., 2012).

Cannabinoids, the active compounds found in *Cannabis sativa*, have garnered attention for their potential therapeutic applications in endometriosis management, primarily due to their anti-inflammatory, analgesic, and antiproliferative properties (Anvari Aliabad et al., 2024; Whitaker et al., 2024). This group of compounds interact with endogenous cannabinoid receptors CB1 and CB2 that play a pivotal role in modulating pain and inflammation, both central to endometriosis pathology (Bouaziz et al., 2017; Lingegowda et al., 2024; Woodhams et al., 2015). In addition, preclinical studies have demonstrated that cannabinoid receptor agonists can inhibit the proliferation of endometriotic stromal cells while reducing oxidative stress, suggesting a potential therapeutic avenue for managing the disease (Fonseca et al., 2018; Leconte et al., 2010; Lingegowda et al., 2024; Sanchez et al., 2012). Notably, in January 2025, the state of Illinois in USA approved medical cannabis for endometriosis and related conditions, acknowledging studies that suggest cannabis can effectively alleviate pelvic pain and associated symptoms (Illinois.gov, 2025). However, while these findings are promising, caution is needed relatively to cannabinoid-based therapies, particularly because they are not advised to women who desire to conceive, nor before and mostly not during pregnancy, once deleterious effects to the embryo are known (Ezechukwu et al., 2020; Lo et al., 2022).

Interestingly, polyphenols naturally found in foods and plants have been investigated for their potential benefits in managing endometriosis. These bioactive compounds exhibit anti-inflammatory, antioxidant, anti-angiogenic, and immunomodulatory properties, which may contribute to reduce disease progression and alleviate symptoms. Emerging research suggests that polyphenols could play a role in modulating key pathways involved in endometriosis, offering a promising natural and safe non-hormonal therapeutic approach (Dull et al., 2019; Hipólito-Reis et al., 2022; Meresman et al., 2020; Tassinari et al., 2023).

Table 1 – Non-hormonal pharmacologic treatments for endometriosis

Compound	Model and dose	Main effects	Pregnancy security woman)	Reference
Niclosamide	Mouse model; 100-200 mg/kg/day In vitro; 1-10µm	↓growth of end implants; ↓ cell proliferation; ↓ local inflammation; ↓inflammatory mechanisms	Secure for short periods	(Prather et al., 2016; Sekulovski et al., 2018; Sekulovski et al., 2020; Shi et al., 2021; Zhao et al., 2022)
Simvastatin	Mouse model; Baboon 5 – 25 mg/kg/day; 20mg/day In vitro; 0,05-50µm;	↓size of endometrial implants; ↓ lesions volume; ↓ proliferation and estrogen receptor β and ↑ estrogen receptor α in ectopic endometrium and adhesions ↓ proliferation endometriotic stromal cells;	Not established	Bruner-Tran et al., 2009; Cosar et al., 2019; Nasu et al., 2009; Taylor et al., 2017)
Interferon alpha2b	Rat model; injection of 100000u ip or 100000u sc every 48h In vitro; 1-2000 iu/ml Human; 5x106 iu in douglas cul-de-sac	↓ implant size ↓cell migration and endometrioma cell growth ↑ recurrence rate, ↓ t cells populations	Secure	(Acién et al., 2002; Badawy et al., 2001; Dicitore et al., 2018; Ingelmo et al., 2013; Ingelmo et al., 1999)
Interferon beta 1a	In vitro; 1-1000 iu/ml	↓ cell proliferation and migration, ↑ apoptosis	Secure	(Dicitore et al., 2018)
Interferon gama	In vitro; 0,1-100ng/ml	↑ apoptosis and ↓ cell proliferation	Not established	(Nishida et al., 2005)
Amifostin	Rat model; 200mg/day followed by 20mg/kg/day	↓ the size of endometrial implants and inflammatory markers	Not established	(Onalan et al., 2014)

Everolimus	Rat model; 1,5mg/kg/day	↓ endometriosis progression and ↑ ovarian follicles number	Secure	(Kacan et al., 2017)
Bentamapimod	Rat model; 10, 30 and 60 mg/kg twice a day Mouse model; 10mg/kg/day and 30 mg/kg/day Baboon; 20mg/kg/day In vitro; 5 or 15µm	↓ growth of lesions; ↓ inflammation in lesions; ↓ growth of lesions ↓ growth of lesions ↓ MMP-3 secretion	Not established	(Hussein et al., 2016; Palmer et al., 2016)
Amigdaline	Rat model; 5mg/kg once a week; Mouse model; 5-20mg/kg/day In vitro; 50-200 µg/ml	↓ implant volumes and endometriosis recurrence; Restrain growth of lesions; ↓ proliferation, migration and invasion of human endometrial stromal cells	Not established	(Dogru et al., 2017; Yu et al., 2024)
Metformin	Rat model; 100-200 mg/kg/day – 6 weeks; 200 mg/kg/day – 30 days Mouse model; 50mg/kg/day – 3 months In vitro; 1-20 mm – 24 – 72h; 100µm – 24h Human; 1g/day 2 months	↓ lesions growth, VEGF and MMP-9 in lesions; ↓ 17β- estradiol, TNF-α in serum; ↑ LIF and HOXA10 in endometrium; ↓ lesions growth, fibrosis in lesions, endothelial dysfunction; ↑ fertility, antioxidant defenses, autophagy markers ↓ cell proliferation, migration, VEGF, HIF, MIF, MMP-2, MMP-9 and aromatase; ↑ TIMP ↑ endometrial receptivity proteins	Secure	(Cheng et al., 2022; Felgueiras et al., 2022; Jamali et al., 2021; Martins et al., 2022; A. Catarina Neto et al., 2025; Yari et al., 2021; Zhou et al., 2015)
Cannabinoids	Rat model; 5mg/kg/day – 7 days Mouse model; 2mg/kg/day – 32 days; 3mg/kg/day 2 weeks; 5mg/kg/day – 2 weeks In vitro; 0,3-40µm	↓ lesion size and oxidative stress, IL-6 and TNF-α in serum and peritoneal fluid; ↑ total antioxidant status in serum and peritoneal fluid; ↓pain and lesion growth, ↑cognitive function; ↓ lesions size; ↑lesion volume and N-cadherin, integrin β1 and IL-6 genes ↓ cell proliferation and ROS production;	Not secure	(Cobellis et al., 2011; Escudero-Lara et al., 2020; Leconte et al., 2010; Okten et al., 2023; Sanchez, Quattrone, et al., 2017)

INTRODUCTION

	Human; 400mg twice a day – 3 months	↓ dysmenorrhoea, dyspareunia and pelvic pain		
Rapamycin	Rat model; 0,5mg/kg/week	↑ apoptosis and ↓ angiogenesis in the lesions;	Not established	(Laschke et al., 2006; Ren et al., 2016; Siracusa et al., 2021)
	Mouse model; 250 µg/mouse lesion injection once a week	↓ lesions volume, microvessel density and vegf levels;		
	Hamster model; 1,5mg/kg/day	↓ of lesions, microvessel density and angiogenesis		
Quercetin	Rat model; 15mg/kg/day	↓ size of implants;	Not established	(Delenko et al., 2024; Jamali et al., 2021; S. Park et al., 2019; L. Zhang et al., 2023)
	Mouse model; 35-100mg/kg/day	Suppressed the growth of endometriotic lesions;		
	In vitro; 0-100µm	↑ decidualization and apoptosis of endometrial stromal cells; ↓ growth of human endometriotic cells and its fibrosis; ↓ cell proliferation;		
Resveratrol	Rat model; 10-45 mg/kg/day 28 days; 30 mg/kg/day – 14 days; 10 mg/kg/day - 14 days	↓ lesions size and vascularization, ↓ adhesions, GLUT-1, GLUT-3, MCT-1 and MCT-4; ↓ IL-6, IL-8, TNF-α, VEGF and MCP-1 in plasma and/or peritoneal fluid; ↓ VEGF in endometriotic tissue	Not established	(Arablou et al., 2021; Bahrami et al., 2021; Bayoglu Tekin et al., 2015; Bruner-Tran et al., 2011; ErgenoĖylu et al., 2013; Khazaei et al., 2020; Khodarahmian et al., 2021; Kodarahmian et al., 2019; Kolahdouz-Mohammadi et al., 2021; Madanes et al., 2022; Rudzitis-Auth et al., 2013; Wang et al., 2021; Zou et al., 2024)
	Mouse model; 25mg/kg/day – 14 days; 40mg/kg/day – 28 days; 6mg/mouse/day 10 – 20 days	↓ lesions size and its cell density; ↑ ROS in lesions; ↓ cell proliferation and angiogenesis in lesions; ↑ apoptosis in lesions		
	In vitro; 10-200 µm;	↓ proliferation, migration and invasiveness of stromal cells; ↓ cell viability and migration and ↑ apoptosis in normal endometrial and endometriotic cells; ↓ endometrial growth and angiogenesis; ↓VEGF, TGF-β and MMP-9, MCP-1, IL-6 and IL-8 in eutopic and ectopic cells;		
	Human; 400mg/twice a day – 12-14 weeks;	↓VEGF and TNF-α in endometrial tissue; ↓ MMP-2, MMP-9 in endometrium and serum		

Curcumin	Rat model; 48mg/kg/day - 28 days	↑ leptin and ↓ TAC in serum;		
	Mouse model; 12-48 mg/kg/day; 300mg/kg/day -21 days	↓ glandular tissue and ↑ mitochondrial size in lesions; ↓ MMP-3; ↑ apoptosis in stroma and glandular regions; ↓ number of lesions and volume and degree of adhesions; ↓ HIF-1 α and VEGFA in lesions; ↓ IL-6, IL-1 β and VEGFA in peritoneal fluid		
	In vitro; 1-40 μ g/ml 24-72h; 1-50 μ m – 24h; 7,5 μ m	↓ pro-inflammatory and pro-angiogenic molecules; ↓ cell proliferation and mitochondrial function, ↑ apoptosis and oxidative damage in endometriotic cells	Not established	(Chowdhury et al., 2019; Ding et al., 2022; Gudarzi et al., 2024; Jana et al., 2012; Jelodar & Azimifar, 2019; Kapur et al., 2023; Kim et al., 2012)
	Human; 500mg/twice a day – 8 weeks	No effect on pain		

List of Abbreviations: **GLUT-1 / GLUT-3** – Glucose Transporter 1 / 3; **HIF/ HIF-1 α** – Hypoxia-Inducible Factor / 1 alpha; **HOXA10** – Homeobox A10; **IL-6 / IL-8 / IL-1 β** – Interleukin-6 / -8 / -1 beta; **IP** – Intraperitoneal; **LIF** – Leukemia Inhibitory Factor; **MCP-1** – Monocyte Chemoattractant Protein-1; **MCT-1 / MCT-4** – Monocarboxylate Transporter 1 / 4; **MIF** – Macrophage Migration Inhibitory Factor; **MMP-3 / MMP-2 / MMP-9** – Matrix Metalloproteinase-3 / -2 / -9; **ROS** – Reactive Oxygen Species; **SC** – Subcutaneous; **TAC** – Total Antioxidant Capacity; **TGF- β** – Transforming Growth Factor beta; **TIMP** – Tissue Inhibitor of Metalloproteinases; **TNF- α** – Tumor Necrosis Factor alpha; **VEGF/ VEGFA** – Vascular Endothelial Growth Factor /A

7. Animal Models of Endometriosis

Managing endometriosis requires a multidisciplinary strategy that balances medical and surgical interventions. Given the complexity of the disease, research into new treatment options is crucial, and although endometriosis only spontaneously develops in woman and other menstruating primates, rodent models play a fundamental role in advancing understanding of endometriosis pathophysiology, in evaluating novel medical therapies and even refining of surgical techniques in a controlled setting. By using animal models, researchers can explore effects of hormonal or non-hormonal treatments, assess impacts of dietary and environmental factors, and can test innovative pharmacological agents before they are approved for clinical use, ultimately leading to more effective and personalized treatment strategies for women with endometriosis (Grümmer, 2006; Neto et al., 2023; Simitsidellis et al., 2018).

Several rodent models of endometriosis have been developed to mimic key aspects of the human disease (Burns et al., 2022; Zeng et al., 2024; Zhou et al., 2025). These are instrumental in studying features such as pain (**publication VI**) (Neto et al., 2023), fertility (Cohen et al., 2015; A. Catarina Neto et al., 2025), the progression of the disease (Greaves et al., 2014; Wilson et al., 2020), and also in evaluating the efficacy of new therapeutic agents (Zeng et al., 2024). Different approaches have been used to develop endometriosis models, tailored to the specific features under investigation, as summarized in **Table 2**.

The murine model of endometriosis I have developed (**publication I**) was adapted from a previously established model that had already demonstrated reduced fertility comparatively with controls (Cohen et al., 2015). Briefly, endometriosis was surgically induced in recipient mice by transplanting endometrial tissue fragments

(~3 mm³) from B6CBAF1 donor mice into their peritoneal wall. Fragments were sutured to the right and left peritoneal walls of anaesthetised recipient mice. Sham-operated mice, undergoing the same procedure without tissue implantation, served as controls. The study design is depicted in **figure 2**.

Our study further confirmed that these animals exhibited enhanced localized inflammation in both eutopic and ectopic endometrium, concomitantly with increased expression of proliferating cell nuclear antigen (PCNA) in the lesions. The inflammatory and proliferative environment in those animals was associated with a significant decline in fertility rates, as indicated by a lower mean number of pups per pregnant mouse (**publication I**). Additionally, our murine model addressed the systemic nature of endometriosis, including its association with cardiovascular disease (**publications II and III**).

In the search of treatments that preserve fertility, we explored the potential therapeutic effects of metformin, an energy restriction mimetic, in managing endometriosis features, including its impact in fertility. Supporting the choice of metformin, several studies had demonstrated that energy restriction itself or agents mimicking its effects, can positively impact disease control (Jamali et al., 2021; Yin et al., 2018), but none had evidenced beneficial effect in endometriosis-associated infertility.

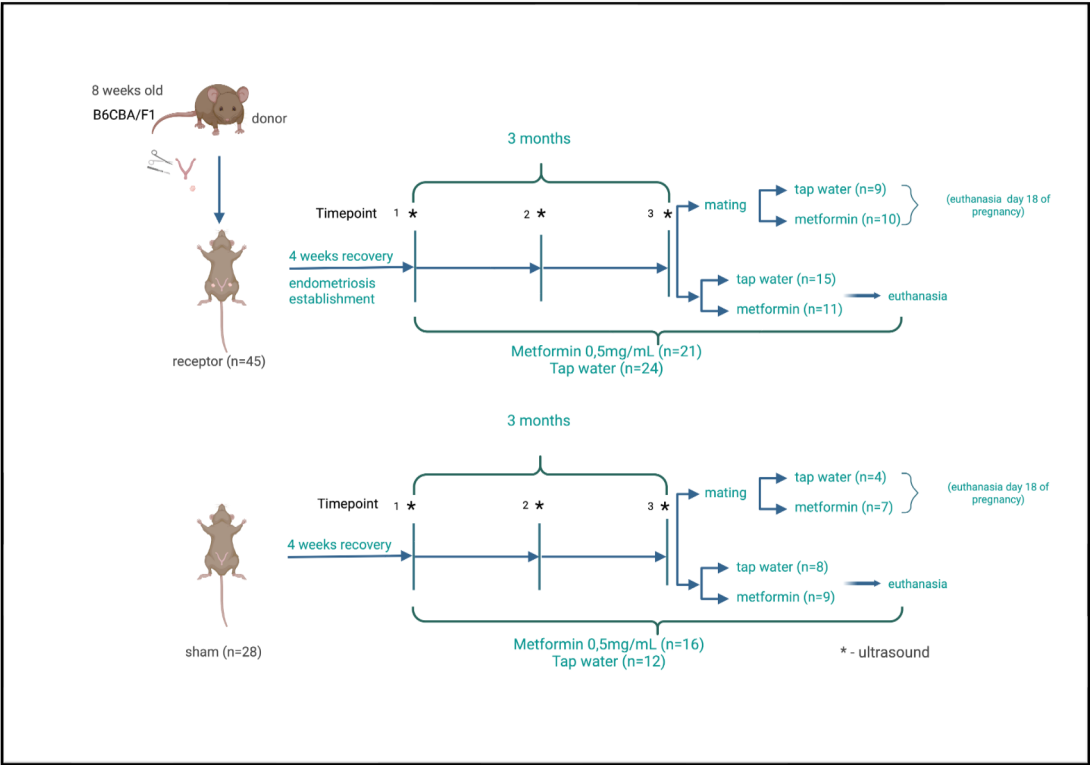


Figure 2 - Study design of mouse model of endometriosis

Table 2 – Rodent models of endometriosis

Model	Animal	Pain	Proliferation, migration, invasion	Inflammation	Angiogenesis	Fertility features	Reference
Retrograde Menstruation	Rat	+					(Persoons et al., 2020; Tal et al., 2019)
	Mouse		+	+	+		
Autologous transplantation	Rat	+	+	+		+	(Berkley et al., 2004; McAllister et al., 2012; McAllister et al., 2016; Pelch et al., 2012; Uchiide et al., 2002; Vernon & Wilson, 1985)
	Mouse		+	+			
Allogeneic transplantation	Rat	+					(van Aken et al., 2020; Yuan et al., 2017)
	Mouse			+			
Syngeneic transplantation	Mouse		+	+	+	+	(Bilotas et al., 2015; Greaves et al., 2014; Henze et al., 2017; Körbel et al., 2010; Richards et al., 2020)
Xenograft transplantation	Mouse		+	+	+		(Abdolmaleki et al., 2021; Abudula et al., 2022; Arosh et al., 2022; Zhang et al., 2022)
Intraperitoneal injection of endometrial dissociated cells	Mouse	+	+	+			(Fattori et al., 2020; Yan et al., 2018)
Heterologous transplantation	Rat		+	+	+		(Burns et al., 2012; Chauhan et al., 2024; Cohen et al., 2015; A. Catarina Neto et al., 2025; Siracusa et al., 2021; Tejada et al., 2022)
	Mouse	+	+	+		+	

INTRODUCTION

Transgenic model (K-Ras, ARID1A & PIK3CA)	Mouse		+				(Dinulescu et al., 2005; Wilson et al., 2020)
Fluorescence labelling of endometrial cells	Mouse			+	+	+	(Li et al., 2024; Marquardt et al., 2022)
Iron overload	Mouse					+	(Ni et al., 2022)
Endometrioma (uterine fragments transplant into ovarian bursa)	Mouse		+	+		+	(Hayashi et al., 2020; Tan et al., 2024)

List of Abbreviations: **ARID1A** - AT-rich interactive domain-containing protein 1A; **PIK3CA** - phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alfa.

8. Metformin as a Potential Non-Hormonal Therapy for Preserving Reproductive Function in Endometriosis

Metformin, a well-established insulin-sensitizing agent, has been recently proposed as a potential non-hormonal therapy for women with endometriosis (Kimber-Trojnar et al., 2022; Triggle & Ding, 2017).

While traditionally used for managing type 2 diabetes, its pleiotropic effects extend far beyond glucose metabolism (Dutta et al., 2023; LaMoia & Shulman, 2020).

Its broad metabolic benefits make it a valuable treatment option for individuals with metabolic syndrome, a cluster of conditions that increases the risk of cardiovascular disease, type 2 diabetes and obesity (Adeshirlarijane et al., 2019; Després, 2003; Jahn et al., 2022; Lv & Guo, 2020). In fact, a variety of reports indicate that metformin reduces the risk of atherosclerosis, hypertension and other cardiovascular complications (Bu et al., 2022; Nesti & Natali, 2017; Zilov et al., 2019). By improving insulin sensitivity and diminishing systemic inflammation, metformin helps regulate hormonal imbalances and supports normal ovarian function (Genazzani et al., 2004; Shpakov, 2021; Velazquez et al., 2024).

Additionally, emerging research suggests that metformin possesses anti-cancer properties, particularly in hormone-sensitive malignancies such as breast and gynecological cancers, where it is thought to inhibit tumor growth by modulating insulin and AMPK-related pathways (Gadducci et al., 2016; Lengyel et al., 2015; Ma et al., 2020; Sorokin et al., 2020; Thakur et al., 2019). In fact, activation of AMPK signaling is a key mechanism by which metformin intervenes, this pathway not only regulates energy balance but also reduces aberrant cell proliferation (Chen et al., 2022; Hasanvand, 2022; Park et al., 2019).

These protective effects are of high relevance given the recognition of endometriosis as a systemic disease with cardiovascular implications that also increase the risk of hormone-dependent cancers (Alderman et al., 2017; Kvaskoff et al., 2021; Poeta do Couto et al., 2023; Taylor et al., 2021; Wilbur et al., 2017).

As well, metformin anti-inflammatory, anti-proliferative, and anti-fibrotic properties are beneficial in mitigating endometriotic lesion progression and the infertility associated with the disease. Metformin lowers local estrogen production by inhibiting aromatase activity, thereby decreasing estrogen-driven endometrial proliferation (J. Cheng et al., 2022; Kimber-Trojnar et al., 2022; J. Zhang et al., 2017; Zhou et al., 2015). By modulating inflammatory cytokines and oxidative stress, metformin creates a more favorable environment for implantation and pregnancy, ultimately improving reproductive outcomes in women with endometriosis through improvement of ovarian function, endometrial receptivity and consequent reduction of miscarriage rates (Ainuddin et al., 2015; Piomboni et al., 2014; Zhai et al., 2019). Also, in the field of reproductive endocrinology, metformin is extensively used to restore ovulatory function in women with polycystic ovary syndrome (PCOS), a condition characterized by insulin resistance, hyperandrogenism, and anovulation (Attia et al., 2023; Johnson, 2014; Sharpe et al., 2019).

A crucial advantage of metformin over traditional hormonal therapies is its well-documented safety profile during pregnancy (**Table 1**). Many hormonal treatments for endometriosis, such as gonadotropin-releasing hormone (GnRH) agonists, progestins, and combined oral contraceptives, need to be discontinued when a patient is trying to conceive due to their contraceptive and potential teratogenic effects (Bedaiwy et al., 2017; Lee et al., 2020). In contrast, metformin is widely used in pregnant women with PCOS and gestational diabetes mellitus (GDM), with numerous studies demonstrating its safety for

both mother and fetus. Those studies showed that no increase in congenital anomalies or adverse fetal outcomes associated with metformin usage were found (Paschou et al., 2024; Tosti et al., 2023), reinforcing its suitability for women with endometriosis who are planning pregnancy. Supporting that, unpublished results from the murine model I have developed evidenced metformin positive effect in the mitigation of placental fibrosis. After treatment, fibrosis levels in mice with endometriosis were comparable to those in healthy controls. In the absence of treatment, levels of fibrosis were higher in placentas of mice with surgically-induced endometriosis, relatively to controls.

In the experimental model, we demonstrated for the first time that metformin improved fertility outcomes in endometriosis-induced mice, besides confirming its known beneficial effects on inflammation and oxidative stress associated with the disease. In addition, metformin demonstrated a positive impact on cardiovascular health, further supporting its role as a potential systemic therapy for the disease (**publications II and III**). Taken together, these findings evidence broader therapeutic implications of metformin in endometriosis, beyond fertility preservation.

Overall, metformin's multifaceted mechanisms of action, favourable safety profile, and potential benefits in improving reproductive function, reducing inflammation, and mitigating cardiovascular risks make it a promising non-hormonal treatment for endometriosis. Its established role in enhancing fertility in women with metabolic disorders, combined with its anti-inflammatory and estrogen-lowering effects, positions metformin as a viable option for women seeking to conceive while managing endometriosis-related symptoms. However, to date, the use of metformin in women has not been reported for the treatment of endometriosis or for improving fertility. Future

clinical trials are needed to further elucidate its efficacy in this context and establish standardized treatment protocols that optimize its benefits in endometriosis care.

9. Objectives and Study Outline

Endometriosis is increasingly recognised as a complex systemic disease that transcends the pelvic cavity, affecting multiple physiological functions including metabolic, cardiovascular, immune, and neurological (Taylor et al., 2021; Tulandi & Vercellini, 2024). This broader perspective highlights the need to investigate endometriosis not only as a localised gynaecological disorder, but as a chronic multisystem condition with far-reaching consequences for women's health, particularly reproductive function and fertility.

To better understand these systemic aspects, animal models provide an essential experimental platform to elucidate the pathophysiology of the disease and explore potential therapeutic interventions (Zhou et al., 2025). In this context, metformin has emerged as a particularly promising non-hormonal agent due to its anti-inflammatory, metabolic, and anti-oxidant properties (Kimber-Trojnar et al., 2022). Therefore, one objective of this work was to investigate, in an experimental mouse model, whether metformin could modulate endometriosis lesion progression and key fertility-related features, while also assessing its impact on cardiovascular alterations as a systemic manifestation of the disease.

Complementing investigation on the experimental model, the clinical impact of endometriosis on fertility was further explored through analysis of human FF samples from women with and without endometriosis undergoing ART. Here, the objectives were to characterise alterations in the follicular microenvironment, identify molecular biomarkers that could improve disease diagnosis, and explore their potential as prognostic indicators of pregnancy outcomes.

Together, these experimental and translational approaches aimed to advance the understanding of endometriosis as a systemic disease, clarify its impact on reproductive

competence, and contribute to the development of more targeted, personalised diagnostic and therapeutic strategies for affected women.

The results obtained within the scope of this work were published in the four original and two review papers that integrate this thesis:

In the first publication, we developed and established a murine model of endometriosis, and we demonstrated that three months of oral metformin treatment boosted fertility, as evidenced by an increased number of fetuses per pregnant mouse, alongside a marked reduction in ectopic implant growth and vascularisation. Metformin normalised PCNA levels and reduced fibrosis in eutopic and ectopic endometrial tissues. While promoting macrophage presence (F4/80) in eutopic tissue, it also downregulated cytochrome P450 (CYP)17A1 in ectopic implants, when compared with non-treated eutopic tissue. Importantly, metformin raised the expression of galectin-3, NF- κ B and the antioxidant enzymes heme-oxygenase 1 (HO-1) and GPX-1 in ectopic tissue, suggesting that its therapeutic effects derive from a combination of anti-inflammatory, anti-fibrotic and redox-modulating actions (**Publication I**).

In the second and third publications, we further characterised systemic actions of metformin, particularly in the cardiovascular system, in the same murine model of endometriosis. Metformin prevented endometriosis-associated endothelial dysfunction as demonstrated in the second publication (**Publication II**). In the third publication, we observed that metformin enhanced cardiac GPX-1 expression, independently of the classical AMPK–SIRT1–PGC-1 α –SIRT3 antioxidant axis (**Publication III**). Together, these studies support metformin's dual protective role, preserving vascular function and antioxidant defences in endometriosis, positioning it as a promising non-hormonal therapy.

In the fourth publication, we analysed FF samples from women with endometriosis undergoing ART. A significant increase in GDF-9 and BMP-15 protein levels was demonstrated, together with a decrease in mature AMH in the FF of women with endometriosis compared to controls. Moreover, miRNAs including miR-20a_1, miR-145_1 and miR-320a_1 were upregulated, while miR-125b-5p and miR-212-3p were downregulated in endometriosis. Our analysis indicated that miR-125b-5p, miR-212-3p and miR-145_1 may act as predictors of disease presence, whereas miR-145_1 and miR-320a_1 correlate with biochemical pregnancy, in control samples. These results suggest the FF milieu in endometriosis harbours molecular biomarkers with potential diagnostic value and this type of analysis may also hold prognostic relevance in ART (**Publication IV**).

In the fifth publication, we reviewed the redox dynamics of human FF and their implications for ovarian follicle development. The article summarises how ROS generated during metabolism are buffered by enzymatic antioxidants, maintaining a tightly regulated redox balance. We emphasised that disruption of this equilibrium could impair oocyte maturation and compromise reproductive competence (**Publication V**).

In the sixth publication, we reviewed and highlighted the limitations of organ-focused animal models in studying chronic pelvic pain, namely in endometriosis. We underscored that pain in the context of such diseases is not solely peripheral but involves systemic and neurobiological mechanisms, including central sensitisation, stress axis dysregulation, and behavioural comorbidities. The article advocates for more comprehensive and translational models that integrate these multisystem interactions, using refined behavioural assessments and advanced tools such as neuroimaging and electrophysiology, to better reflect the human condition (**Publication VI**).

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II PUBLICATIONS

PUBLICATION I

“Metformin reverses infertility in a mouse model of endometriosis: unveiling disease pathways
and implications for future clinical approaches”

RBMO, 2025



ARTICLE



Metformin reverses infertility in a mouse model of endometriosis: unveiling disease pathways and implications for future clinical approaches



BIOGRAPHY

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KEY MESSAGE

Treatment with metformin in a subtherapeutic dose enhances fertility rates in a mouse model of endometriosis by protecting against oxidative stress during pregnancy. Additionally, metformin reduces fibrosis, a process associated with the disease, particularly within endometriosis lesions.

ABSTRACT

Research question: Does metformin reverse endometriosis-associated infertility?

Design: Endometriosis was induced by transplanting uterus fragments from B6CBAF1 mice into recipients of the same strain. The mice were divided into groups: endometriosis (End, $n = 24$), sham-operated (Sham, $n = 12$), endometriosis with metformin (0.5mg/ml) orally administered for 3 months (EndMet, $n = 21$) and sham-operated metformin-treated (ShamMet, $n = 16$). Implant growth was monitored using ultrasonography. Fibrosis was computer-assisted quantified in Masson's trichrome-stained sections of eutopic (EuEnd) and ectopic (EcEnd) endometrium. PCNA, CYP17a1, F4/80 and galectin-3 were analysed by immunofluorescence and western blotting, and NFkB, GPX-1 and HO-1 only by western blotting. Statistical significance was set at $P < 0.05$.

Results: The endometriosis model was successfully established. The End groups showed lower fertility rates than sham-operated mice ($P = 0.0034$), whereas metformin treatment increased the number of fetuses per pregnant mouse ($P = 0.0295$), restoring fertility to control levels; it also slowed implant growth and vascularization. Metformin also restored PCNA expression and fibrosis levels to those of non-treated EuSham mice. PCNA expression decreased in pregnant mice ($P < 0.0178$). Metformin diminished CYP17a1 expression in EcEnd versus EuEnd non-treated tissues and conversely up-regulated F4/80 in EuEnd tissue ($P < 0.0170$), and galectin-3, NFkB and the antioxidant enzymes HO-1 and GPX-1 in EcEnd tissue ($P < 0.0293$), in non-mated mice.

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KEYWORDS

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Oxidative Stress
Reproductive health

Conclusions: These results indicate that application of metformin can alleviate oxidative stress and mitigate fibrosis in endometriosis lesions in a murine model of endometriosis, which highlights metformin's potential as a pharmacological intervention for improving infertility in endometriosis.

INTRODUCTION

Infertility, defined as the absence of conception after 1 year of unprotected and regular intercourse, is a public health concern, driving a rising demand for assisted reproductive technology (ART) (Inhorn and Patrizio, 2015). One of the most prevalent worldwide causes of infertility in the female partner is endometriosis, affecting up to 40% of women facing infertility (Cohen et al., 2015; Evans and Decherney, 2017). Endometriosis is a gynaecological condition with systemic impact, marked by an oestrogen-dependent aberrant growth of vascularized endometrial-like tissue outside the uterus, predominantly within the pelvic region (Alderman et al., 2017; Taylor et al., 2021). This disorder results primarily in pelvic pain, the most common symptom in endometriosis patients, and, in the advanced stages, pelvic organ distortion and infertility.

The pathophysiology of endometriosis, in particular the associated infertility, is far from being completely elucidated, but it is known that disease progression encompasses an inflammatory reaction, cellular proliferation, migration, invasion and angiogenesis (Samimi et al., 2019). Substantial evidence indicates that oxidative stress incites inflammation, through either an escalated production of reactive oxygen species or a decrease in antioxidant defences (Clower et al., 2022). The continuing inflammatory stimulus underlies the loss of function and the fibrosis of endometrial tissue that is closely associated with endometriosis (Viganò et al., 2020).

Endometriosis patients often require ART treatments, primarily due to the inadequacy of the hormone-based treatments currently used to control the disease (Coccia et al., 2022). In addition, these treatments must be interrupted before and during pregnancy, which frequently leads to symptom aggravation (Skorupskaitė and Bhandari, 2021). In this context, there is an unmet need for new pharmacological strategies to control endometriosis both before conception and during pregnancy.

In rodents, endometriosis is not a natural disorder. However, a surgically induced

model of endometriosis in rats is associated with anomalies of the hypothalamic–pituitary–ovarian axis, ovarian follicle development, oocyte quality and embryo development and implantation, as well as ovulatory and hormone/receptor dysfunction and an inflammatory milieu in the peritoneal cavity (Sharpe-Timms et al., 2020). In addition, familial aspects of endometriosis and transgenerational subfertility in both female and male offspring over three generations have been demonstrated in a rat model of endometriosis (Sharpe-Timms et al., 2020; Stille et al., 2009).

In line with this, induced peritoneal endometriosis in a mouse model has been shown to be associated with a decrease in oocyte quality and embryo number. In these animals, fertility is impacted by the volume of tissue implanted as endometriotic lesions, and thus the induced disease can be modulated by various therapeutic approaches (Cohen et al., 2015; Greaves et al., 2014; Park et al., 2022; Pelch et al., 2012; Rosa-E-Silva et al., 2019). Conversely, an earlier study reported that although the pregnancy rate was decreased in a mouse model of endometriosis, no significant differences in resorption rate, litter size and pup weight were noted between groups (Bilotas et al., 2015). These differences may be attributed to differences in the models or murine strains used. In sum, despite the differences observed between experimental rodent models of endometriosis, they provide valuable insights into the pathophysiology of endometriosis-associated infertility and enable the assessment of novel pharmacological interventions.

One of the putative drugs for controlling endometriosis is metformin, a widely employed medication for type 2 diabetes. Interestingly, owing to its antioxidant properties, it instigates the regression of endometrial implants in a rat model of endometriosis (Viollet et al., 2012; Yilmaz et al., 2010). In addition, metformin curtails cell proliferation, suppresses the secretion of pro-inflammatory cytokines and lowers oestrogen production in cultured human endometrioma-derived stromal cells, underscoring its anti-inflammatory attributes (Takemura et al., 2007).

Moreover, metformin has been safely and effectively used in the management of gestational diabetes, demonstrating no adverse effects on women or embryos (Ainuddin et al., 2015).

Despite these encouraging effects on controlling the progression of endometriosis, the particular impact of metformin on fertility in endometriosis has not, to the best of the current authors' knowledge, been elucidated. Thus, the goal of this study was to assess whether metformin would be effective in stopping the progression of endometriosis features and improve the associated infertility. In addition, it aimed to explore the likely mechanisms underlying this benefit.

MATERIAL AND METHODS

Mouse model of endometriosis

All experiments were approved by the Institute for Research and Innovation in Health (i3S) Animal Ethical Committee and the Portuguese Competent Authority (Direcção-Geral de Alimentação e Veterinária, internal reference 2018/27). All the procedures employed in this study adhere to European Community Council directive 2010/63/EU and to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) 2.0 guidelines.

Seventy-three 8-week-old B6CBAF1 female mice descended from female CBA/J mice mated with male C57BL/6J mice (Charles River Laboratories, France) were employed in the experiments, which were carried out at i3S (Porto, Portugal). The mice had free access to food (2014S, Envigo, UK) and water. They were housed five per cage in a room with controlled temperature and humidity (21°C and 50%, respectively), and under a 12/12 h light/dark cycle. All the cages had paper for nesting and cardboard rolls. The oestrous cycle stage of the mice was analysed in each animal before the experiments by vaginal inspection and cytological smear, confirming that all the animals were at a similar stage of the oestrous cycle.

Endometriosis was surgically induced in 45 randomly selected recipient mice by heterologous transplantation of endometrial tissue fragments harvested

from B6CBAF1 mice donors, according to the method described by Cohen and colleagues (Cohen *et al.*, 2015). Briefly, 8-week-old donor mice were subjected to general anaesthesia with isoflurane (B. Braun, Germany) and euthanized by cervical dislocation. Both uterine horns were removed and placed in warm Ringer's lactate solution in a Petri dish. The uterine horns were then longitudinally opened with micro-scissors under a stereomicroscope (Olympus SZX10, Olympus Europa, Germany) and divided under micro-ruler guidance into tissue fragments measuring approximately 3 mm³.

Subsequently, the recipient mice were anaesthetized using isoflurane, and buprenorphine (Bupaq, 0.08 mg/kg subcutaneously) was given as analgesia. After surgical preparation, a small incision was made into the ventral abdominal cavity, followed by a second incision in the peritoneum. One tissue fragment was attached with a 7-0 non-absorbable suture to the right peritoneal wall and an additional fragment to the left peritoneal wall of each recipient mouse, a previous study having demonstrated that the impact on fertility was equivalent whether two or four fragments were implanted in the uterine wall of recipient mice (Rosa-E-Silva *et al.*, 2019). Finally, the peritoneum and the skin were closed with a 6-0 absorbable suture. Mice were treated with buprenorphine 0.1 mg per 100 g body weight administered subcutaneously every 12 h for 72 h. In addition, suture sites and pain signals of pain were checked daily. Sham-operated mice were used as control group (*n* = 28). In these animals the same procedures were performed but, instead of attaching a tissue fragment, a 7-0 non-absorbable suture was placed in the right and in the left abdominal wall.

For each mouse, an endometriosis-like phenotype was confirmed by ultrasound visualization of the implanted endometrial tissue (Micro Ultrasound Vevo 2100, VisualSonics, Canada). The evolution of the implant growth was monitored for 3 months at three timepoints by measuring the area of the implant (pixel²) using AxioVision 4.8 software (Carl Zeiss, Germany) after determining the height and width of the area, including its brim on a two-dimensional image of the implant. The first timepoint was 1 month after the surgery to induce endometriosis, and timepoints 2 and 3 were at 45 and 90 days after timepoint 1. The first image was considered to indicate timepoint 1 and the

growth at the remaining timepoints was evaluated by calculating the ratio of the implant area at each timepoint relative to the area at timepoint 1.

The animals were divided into four groups: mice with surgically induced endometriosis (End, *n* = 24); sham-operated mice (Sham, *n* = 12); mice with endometriosis treated with metformin (EndMet, *n* = 21); and Sham-operated mice treated with metformin (ShamMet, *n* = 16). Metformin (Alfa Aesar Chemicals, USA) was administered orally (0.5 mg/ml diluted in drinking water) for 3 months starting at timepoint 1, and the average dose per mouse was further determined based on the volume of water ingested per day. Each bottle was weighed when it was placed in the animal cage and again at every 2-day change. Bottles were excluded from the calculations if there had been sporadic liquid spillage. The initial weight of the animals was registered on the day of surgery and the final weight just before euthanasia.

Twenty-four hours after the treatment had been completed, the mice in each group were randomly divided into two subgroups. One group was used to assess the fertility rate. The other was anaesthetized for blood collection, abdominal examination and collection of tissue samples; the volatile anaesthetic was maintained until euthanasia was confirmed. The vaginas of the mice were inspected and no differences were identified between the mice, either within the same group or between the different groups, confirming that they were at a similar stage of the oestrous cycle stage at euthanasia. During the abdominal examination, the anatomy of the reproductive system organs was verified by visual macroscopic inspection in every animal at the end-point (FIGURE 1A depicts the study design and exact number of animals per group).

The weight, width, height and length of the implants in each mouse were measured with callipers. One implant was immediately frozen on liquid nitrogen, and the other was placed in 10% buffered formaldehyde. In addition, both uterine horns and the site of the peritoneal wall where the implants/sutures had been placed were collected and divided into two portions. The frozen tissue fragments were stored at -80°C for posterior analysis, and the fixed fragments were embedded in paraffin.

Serum analysis

After blood had been collected from the left ventricle, the serum was separated by blood centrifugation at 300g for 15 min and preserved at -20°C for further biochemical analysis. Serum glucose concentrations were determined at the experimental end-point using a glucose analyser (OneTouch Ultra, LifeScan, USA). Serum total cholesterol and high-density lipoprotein cholesterol (HDL-C) concentrations were determined by enzymatic colorimetry using an Olympus AU5800 autoanalyser (Beckman Coulter, USA) using commercial kits (OSR6616 and OSR6687, respectively; Beckman Coulter) as per the manufacturer's instructions. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using Friedewald's formula ($LDL-C = \text{total cholesterol} - [HDL-C + 1/5 \text{ triglyceride}]$ if the serum triglyceride concentration is 400 mg/dl or less).

Oestradiol concentrations were determined using electrochemiluminescence technology for immunoassay analysis in a Cobas E411 autoanalyser (Roche Diagnostics, USA) utilizing the commercial kit (03000070190, Roche Diagnostics).

Fertility rate assessment

The female mice used for fertility assessment were mated with healthy B6CBAF1 males aged 5 months – one male and two females per cage over 3 days. Mice were checked daily and mating was confirmed by the presence of a vaginal plug. On the 18th day after mating the animals were euthanized as described above. Animals that did not present a vaginal plug (*n* = 4) were excluded from the study, and animals showing a plug were confirmed to be pregnant. Fertility rates were assessed by evaluating the mean number of fetuses per pregnant mouse in each group. Reabsorptions were also quantified in every pregnant mouse.

Morphological analysis of the uterus and implants

Sections of paraffin-embedded uterine horn and ectopically implanted endometrial tissue (5 µm) were assembled on poly-L-lysine-coated slides. The sections were stained with haematoxylin and eosin (H&E) for morphological examination. Briefly, sections were dewaxed by incubating them twice (for 10 and 5 min) in xylol (Merck, Germany) and then hydrated by a series of 5 min incubations in aqueous ethanol solutions of decreasing

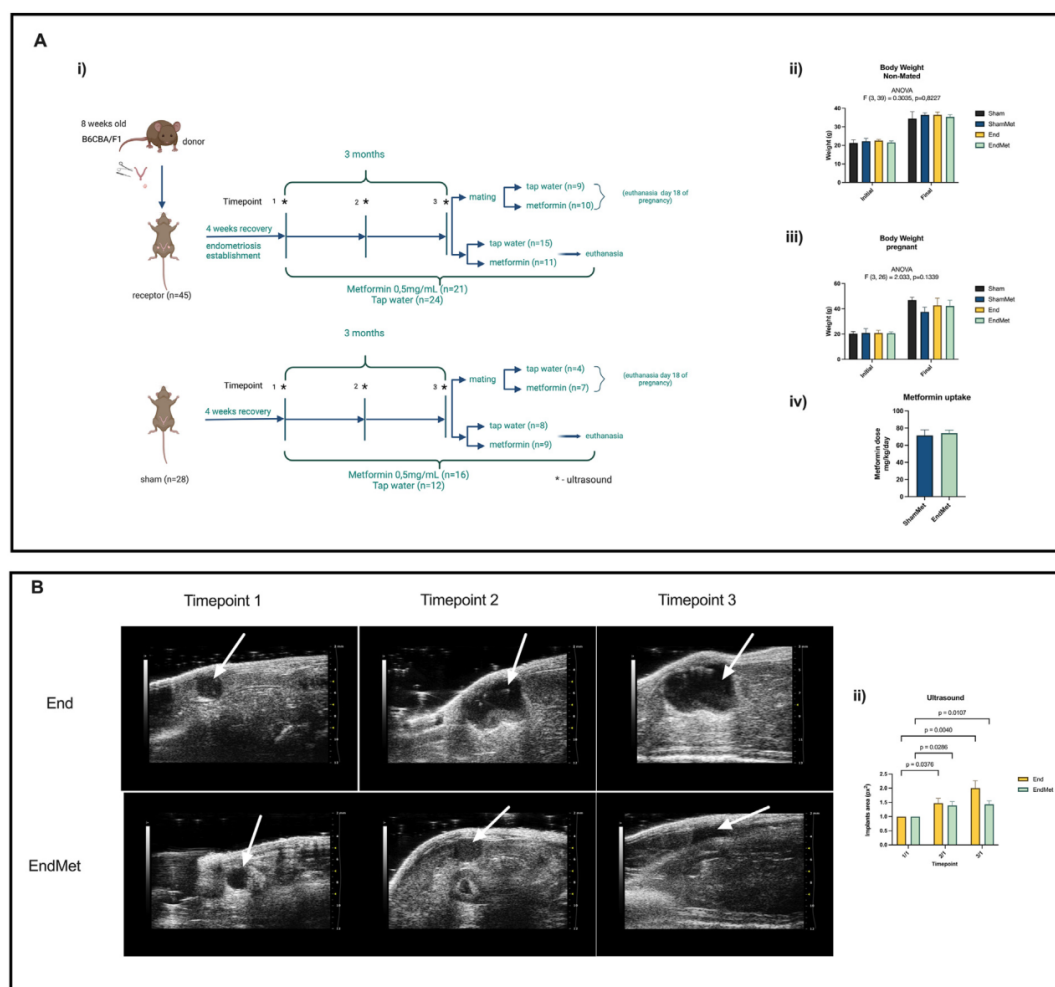


FIGURE 1 Metformin reduces the rate of endometriosis progression. (A) (i) Study design for the mouse model indicating the number used at each stage of the experiment: non-mated Sham (8); non-mated ShamMet (9); non-mated End (15); non-mated EndMet (11); pregnant Sham (4); pregnant ShamMet (7); pregnant End (9); and pregnant EndMet (10). Image created in BioRender. (ii, iii) Graphical depiction of the change in weight of the mice between the day of the surgery and the day of euthanasia in (ii) non-mated and (iii) pregnant mice. (iv) Assessment of the effective dose of metformin imbibed by the mice in the ShamMet and EndMet groups. (B) (i) Representative ultrasound images of implants in mice in the End and EndMet groups at the three timepoints; white arrows indicate the implants. (ii) Graphical analysis of implant growth rate versus time. Values are normalized to timepoint 1 (1 unit in μm^2 was considered for relativization of the area). (C) (i) Representative images showing the non-absorbable sutures (black arrows) in Sham mice. (ii, iii) Endometriosis implants (yellow arrows) in End mice. The magnified inset in (iii) shows the vascularization of an implant (yellow arrow). (iv) Endometriosis implant in EndMet mice. The implants in the mice treated with metformin (yellow arrow) were of a clearer colour than those observed in the non-treated animals, which appeared red. (D) Serum analysis of (i) oestradiol, (ii) total cholesterol, (iii) high-density lipoprotein (HDL) cholesterol, (iv) low-density lipoprotein (LDL) cholesterol and (v) glucose in the experimental groups of non-mated and pregnant mice. Two-way analysis of variance (ANOVA) followed by post-hoc analyses using Tukey's multiple comparisons tests was employed for the statistical analysis, except for metformin uptake, which was analysed using a Student's t-test. Values are presented as mean $\pm$ SEM. Significant *P*-values shown in the figure, and all the *P*-values are shown in [Supplementary Table 1](#). Sham, sham-operated mice; ShamMet, sham-operated mice treated with metformin; End, mice with endometriosis; EndMet, mice with endometriosis treated with metformin.

concentrations (100–50% v/v) and water. Afterwards, the sections were stained with Papanicolaou's solution 1a Harris' haematoxylin solution (Merck) for 2 min followed by washing for 15 min in tap water, and then stained with an alcoholic solution

of eosin 0.5% (w/v) (Sigma-Aldrich, USA; when not specified, the reagents were acquired from Sigma-Aldrich) for 5 min. Finally, the tissue sections were dehydrated with solutions of increasing concentrations of ethanol in water

followed by two passages in xylol. Sections were assembled with a coverslip and Entellan and air dried.

Masson's trichrome stain was applied to differentiate connective tissue, including

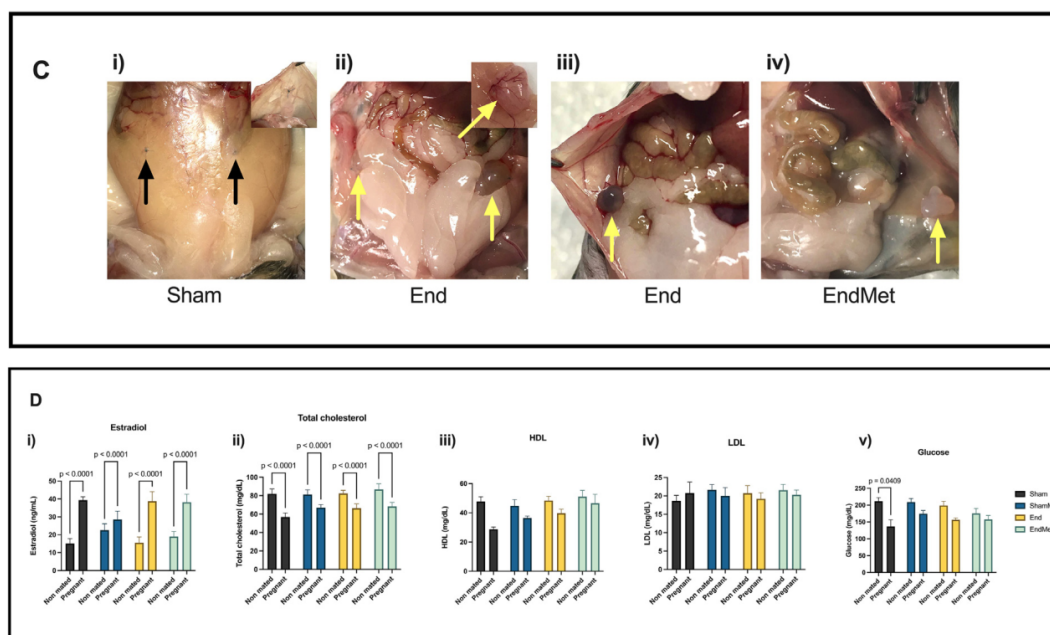


Figure 1 Continued.

collagen fibres and fibrin, from muscle and erythrocytes. The sections were dewaxed and hydrated as previously described and stained with Weigert's haematoxylin (0.5% w/v celestrial, 5% w/v iron ammonium (III) dodecahydrate sulfate, VWR, USA) for 5 min and, immediately afterwards, 0.4% w/v Gill's haematoxylin solution for 5 min. Next, the sections were immersed in 1.3% w/v picric acid aqueous solution for 5 min and then washed in water, followed by staining with Biebrich scarlet–acid fuchsin solution (0.5% w/v Ponceau xylinine, 1% w/v acid fuchsin, Fisher Scientific, USA) for 15 min and incubated in 1% w/v molybdenophosphoric acid hydrate aqueous solution (VWR) for 5 min. After a washing step, the final staining was performed with 0.5% w/v light green (Bio-Optica, Italy) for 10 min. The sections were covered with 1% v/v acetic acid aqueous solution for 2 min and ultimately immersed in water, briefly passed through ethanol and dehydrated for 15 min in xylol before assembly with Entellan.

The sections were observed with 5 × (H&E) and 10 × (Masson's trichrome) magnification objectives lenses under a light Zeiss Axioskop 40 (Carl Zeiss) microscope, equipped with Zeiss Axiocam 208 Color camera (Carl Zeiss), and representative images were captured using Zen software (Carl Zeiss).

The area of fibrosis was quantified on images from Masson's trichrome-stained sections from the uterus and implants by computer-assisted morphometry. Eight fields on each tissue section ($n = 6$ mice per group) were analysed using Image-Pro Plus 6 software (Media Cybernetics, USA); the ratio of the green-coloured fibrotic area to the total tissue area, corresponding to the percentage of interstitial fibrosis, was calculated.

Immunofluorescence

Fluorescent immunolabelling was performed to identify proliferating cell nuclear antigen (PCNA) and cytochrome P450 (CYP)17a1, markers of cell proliferation and steroidogenesis, respectively. Double immunolabelling was performed to identify macrophage marker F4/80 and inflammation mediator and inflammatory cell marker galectin-3.

The uterine and ectopic tissue sections were dewaxed and hydrated as previously described. Sections were washed with phosphate-buffered saline (PBS) solution and permeabilized with 0.5% v/v Triton X-100 in PBS for 5 min, followed by two 5 min washes in PBS. After that, antigen recovery was undertaken with 1 mol/l HCl for 30 min at room temperature followed by neutralization with 2% w/v borax aqueous solution for 5 min. The sections were

blocked with 2% w/v bovine serum albumin in PBS with 0.1% v/v Tween 20 (PBS-T) for 1 h at room temperature in a humid chamber, before overnight incubation at 4°C with rabbit anti-PCNA (0.27 μ g/mL, AB_2636979, Cell Signaling, USA) or rabbit anti-CYP17a1 (0.3 μ g/mL, AB_2800219, Cell Signaling) diluted in blocking solution.

The following day, the sections were washed thoroughly with PBS-T and incubated with anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (2 μ g/mL, AB_2535792, Thermo Fisher, USA) diluted in PBS-T for 1 h at room temperature. Then, after washing, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and the sections assembled with 50% v/v glycerol/PBS. The double immunolabelling was performed as described above with slight differences in the method: the incubation with primary antibodies was undertaken using a mixture of rabbit anti-F4/80 (0.87 μ g/mL, AB_2799771, Cell Signaling) and rat anti-galectin-3 (0.07 μ g/mL, AB_627658, Santa Cruz Technology, USA) primary antibodies diluted in blocking solution, overnight at 4°C.

The next day the sections were incubated a mixture of secondary antibodies Alexa Fluor 568-conjugated anti-rabbit IgG

antibody (2 µg/ml; AB_2534017, Thermo Fisher) and biotinylated anti-rat IgG (0.14 µg/ml, AB_2315779, Jackson ImmunoResearch, USA) diluted in PBS-T for 1 h at room temperature; a fluorescent label was added to the biotinylated antibody through incubation with streptavidin Alexa Fluor 488 (2 µg/ml; Invitrogen, USA) diluted in PBS-T for 1 h. Sections were viewed with a 20 × magnification objective lens and images were recorded on an Apotome fluorescence microscope (AxioImager Z1, Carl Zeiss) equipped with a digital camera (AxioCam MRm, Carl Zeiss) and AxioVision 4.8 software (Carl Zeiss). Negative control experiments were performed with non-immune serum in place of the primary antibody and, additionally, with omission of the secondary antibodies (to exclude auto-fluorescence).

Western blotting

Total protein in samples of eutopic and ectopic endometrial tissue was quantified by the Bradford colorimetric method, after homogenization of tissues in lysis buffer at pH 7.6 (50 mmol/l Tris, 10 mmol/l NaCl, 5 mmol/l EDTA, 2 mmol/l β-glycerophosphate, 0.25% v/v Triton X-100) supplemented with inhibitors of proteases (0.5% v/v) and phosphatases (0.4% v/v) (Sigma-Aldrich). Then, 15 µg of total protein/sample was loaded on a discontinuous buffer electrophoresis system (Bio-Rad Laboratories, USA) using 12% sodium dodecylsulfate-polyacrylamide resolution gel electrophoresis to carry out electrophoretic separation. A constant electric current of 25 mA per gel was applied for approximately 1 h at room temperature.

After electrophoretic separation, the proteins were transferred to a 0.45 µm pore nitrocellulose membrane (Bio-Rad Laboratories), applying a potential difference of 30 V for 90 min. After staining with Ponceau S for 2 min the image of the separated protein bands on the membranes was captured and saved in a Chemidoc XRS (Bio-Rad Laboratories) for further normalization of the studied proteins. The membranes were then incubated with warm blocking solution (5% w/v non-fat powdered milk [Molico, Nestlé, Portugal] diluted in Tris-buffered saline with 0.1% v/v Tween-20 [TBS-T]) for 1 h at room temperature. The membranes were incubated with antibodies for at least two overnight sessions at 4°C with stirring; the antibodies were primary rabbit antibodies

(Cell Signaling when the manufacturer is not indicated) diluted in blocking solution, anti-PCNA (0.134 µg/ml), anti-CYP17a1 (0.06 µg/ml), anti-F4/80 (0.435 µg/ml), anti-nuclear factor kappa B (NFκB; 0.42 µg/ml, AB_10859369), anti-heme oxygenase (HO-1; 0.13 µg/ml, AB_2118685, ProteinTech, USA), anti-glutathione peroxidase (GPX-1; 0.8 µg/ml, AB_2112120, Abcam) and rat antibody anti-galectin-3 (0.02 µg/ml).

The membranes were then washed with TBS-T solution and incubated with the appropriate secondary anti-rabbit antibody conjugated with horseradish peroxidase (AB_10015282, Jackson ImmunoResearch) for 1 h at room temperature with stirring; secondary anti-rat antibody conjugated with horseradish peroxidase (AB_1125219 Santa-Cruz Biotechnology) was used to detect galectin-3. Immunoreaction of each membrane was performed by applying a chemiluminescent substrate for peroxidase (Clarity Western ECL Substrates, Bio-Rad Laboratories) for 5 min, followed by signal detection using the Chemidoc XRS equipment.

To semi-quantify the immunolabelled bands, the intensity of the band, assessed by densitometry and with Image Lab® software (Bio-Rad Laboratories), was divided by the total staining of the proteins in the corresponding lane, stained with Ponceau S. To minimize the variations in exposure times between the membranes, the average intensity value of the uterine samples from the sham group on each membrane served as an internal control. In the experiments that compared only ectopic endometrial tissue samples harvested from non-mated and pregnant mice, the internal control in the membranes was the average value obtained for the samples from the non-mated End group. Each analysis involved a minimum of six distinct samples (mice) per group, and for each protein analysis every sample was quantified in at least two different gels, with the results averaged per sample.

Statistical analysis

Statistical analysis, including an assessment of data distribution normality, was undertaken using Prism version 9.5 (GraphPad Software, USA).

All analyses were performed after verifying normal distribution using the Shapiro–Wilk test. The Student's t-test was performed to analyse metformin

uptake. One-way analysis of variance (ANOVA) was applied to evaluate pregnancy rates. Regular two-way ANOVA was applied to evaluate the effect of the combined factors: endometriosis and metformin, or pregnancy and metformin. In addition, post-hoc analyses were performed using Tukey's multiple comparisons tests.

The results are presented as mean ± standard error of the mean (SEM), and a P-value <0.05 was considered statistically significant.

RESULTS

Metformin reduces the rate of endometriosis progression

The establishment of the endometriosis mouse model was carried out as depicted in [FIGURE 1AI](#) and proved to be successful because a growth of ectopic uterus implants was noted in all the mouse than had been operated on. A high rate of mouse survival was verified as only one death occurred during surgery and none in the follow-up. As metformin could be considered a mimetic of energy restriction, the body weight of the animals was assessed at the final end-point, but no differences were found between groups within the same pregnancy condition (non-mated or pregnant mice) ([FIGURE 1AII, III](#)). Based on the volume of liquid ingested, the effective dose of administered metformin was clinically similar for the ShamMet (71.3 mg/kg per day) and EndMet (74.15 mg/kg per day) groups ([FIGURE 1AIV](#)).

The implant growth in the End and EndMet groups was visualized using ultrasound ([FIGURE 1BI](#)). The End group exhibited a statistically significant growth of the implant area (pixel²) at timepoints 2 and 3 relative to timepoint 1. However, the growth from timepoint 2 (1.47 times the area measured at timepoint 1) to timepoint 3 (2.00 times the area measured at timepoint 1) did not reach statistical significance ($P = 0.1123$). The EndMet group exhibited implant growth at timepoints 2 and 3 relative to timepoint 1, but between timepoints 2 and 3 the area only changed from 1.39 to 1.43 times the initially measured area (timepoint 1) ($P = 0.8396$; [FIGURE 1BII](#)).

At the final end-point, reddish vascularized implants resembling cysts that adhered to several abdominal organs were identified in the mice from the End

group, providing evidence for the macroscopic similarities between the endometriotic implants observed in this animal model and those found in endometriosis patients. The implants in the EndMet group were visually a clearer colour than those in the End group (FIGURE 1CII–IV). The stitches were clearly observed in the Sham group, but inflammatory signs were absent (FIGURE 1CI).

No significant differences in the concentrations of oestradiol, total cholesterol, HDL-C, LDL-C and glucose in mouse serum were observed between the four treatment groups for either the non-mated or the pregnant mice (FIGURE 1D, Supplementary Table 1). However, when the non-mated and pregnant states were compared within the same group, a significant increase in oestradiol concentration and a concomitant decrease in total cholesterol

concentrations were observed in the pregnant mice for all treatment conditions (all $P < 0.0001$; FIGURE 1DI, II). In addition, a decrease in blood glucose concentrations was observed in the pregnant Sham mice compared with their non-mated Sham counterparts ($P = 0.0409$); however, this variation was not evident in either the End groups or the mice treated with metformin. It should also be noted that the animals were not fasting at the time of euthanasia, which augmented the variability of blood glucose concentrations.

Endometriosis increases fibrosis of endometrial tissue

Morphological similarities between eutopic and ectopic endometrial tissue were confirmed by H&E staining. It was possible to identify epithelial and stromal cells, as well as glands, in both tissues and in all the experimental groups. Microscopic analysis confirmed the ability of implants to graft to the peritoneum (FIGURE 2).

Observation of sections stained with Masson's trichrome indicated a noticeable increase in stromal fibrosis in the non-mated, non-treated animals with endometriosis compared with both Sham groups, in both eutopic tissues (non-treated EuEnd versus non-treated EuSham, $P = 0.0186$; and versus EuShamMet, $P = 0.0008$) and ectopic tissues (non-treated EcEnd versus non-treated EuSham, $P = 0.0221$; versus EushamMet $P = 0.0010$); this was confirmed through computer-assisted analysis of the fibrosis to total area ratio (FIGURE 2II). Metformin treatment restored the levels of fibrosis in the tissues to those found in the Sham groups in non-mated animals (FIGURE 2II, Supplementary Table 2). When the ectopic tissue from non-mated mice was compared with that from pregnant mice, a significant decrease in fibrosis in pregnant EcEndMet mice was only observed relative to non-mated EcEnd mice ($P = 0.0187$; FIGURE 2III), again

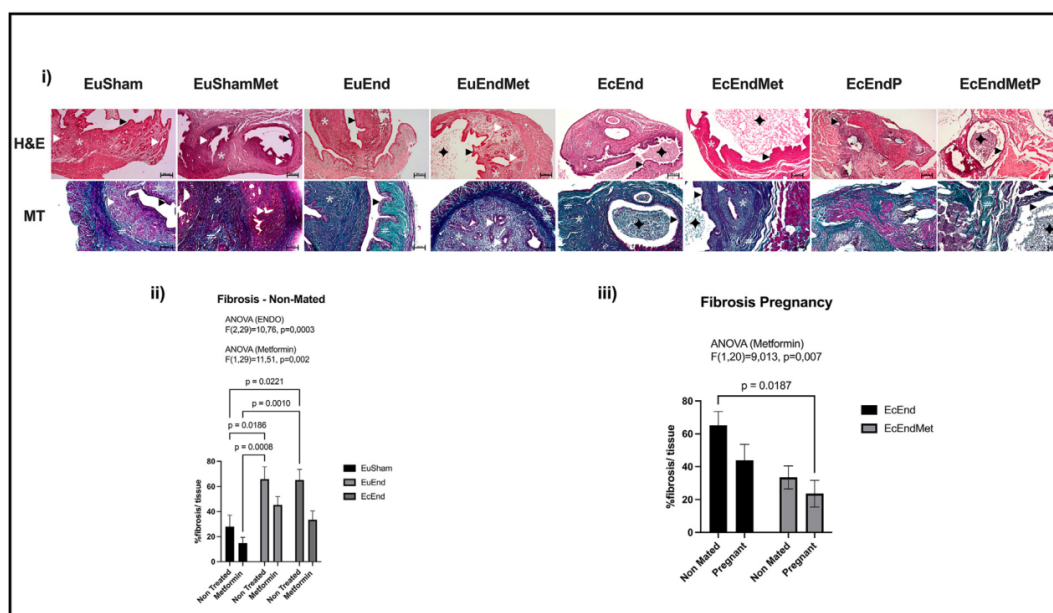


FIGURE 2 Endometriosis (ENDO) increases the fibrosis of endometrial tissue. (i) Representative images of haematoxylin and eosin (H&E; scale bar = 200 μm) and Masson trichrome (MT; scale bar = 100 μm) stained sections of eutopic and ectopic tissues, from the endometriosis experimental groups, either non-mated or pregnant. (ii, iii) Corresponding graphical depictions of the computer-assisted analysis of fibrosis in MT-stained sections. The statistical analysis employed two-way analysis of variance (ANOVA) followed by post-hoc analyses using Tukey's multiple comparisons tests. Values are presented as mean $\pm$ SEM. Significant P -values are shown in the figure, and all the P -values are shown in Supplementary Tables 2 and 3. White arrow heads indicate glands; black arrow heads indicated endometrial tissue epithelium; light grey asterisks denote stroma; white cardinal symbols show fibrosis; black stars indicate flaking cells. EuSham, eutopic tissue from sham-operated mice; EuShamMet, eutopic tissue from sham-operated mice treated with metformin; EuEnd, eutopic tissue from mice with endometriosis; EuEndMet, eutopic tissue from mice with endometriosis mice treated with metformin; EcEnd, ectopic tissue from mice with endometriosis; EcEndMet, ectopic tissue from mice with endometriosis mice treated with metformin; EcEndP, ectopic tissue from pregnant mice with endometriosis mice; EcEndMetP, ectopic tissue from pregnant mice with endometriosis mice treated with metformin.

with metformin not significantly decreasing fibrosis within the group of pregnant mice ([Supplementary Table 3](#)).

Ectopic endometrial tissue shows higher cell proliferation in non-mated mice but lower proliferation in pregnant mice

Specific markers of the cellular mechanisms involved in endometriosis progression, such as abnormal cell proliferation, oestrogen secretion, increased inflammation and oxidative stress, were analysed in the murine model of disease treated with metformin.

To assess the proliferative features of ectopic tissue, the cell proliferation marker PCNA was analysed through immunofluorescence in eutopic and ectopic samples of endometrial tissue. Labelling was noted in the nuclei of the cells, particularly in epithelial cells, from all the analysed tissues ([FIGURE 3AI](#)).

In addition, a band of approximately 36 kDa corresponding to PCNA was identified by western blotting ([FIGURE 3AIV, VII](#)). Two-way ANOVA analysis showed an endometriosis effect in tissues from non-mated mice, as well as a pregnancy effect in the ectopic tissues analysed. Semi-quantification of PCNA ([FIGURE 3AV](#)) revealed an increase in the EcEnd group compared with the EuSham, EuShamMet and EuEndMet mice (all $P \leq 0,0231$). No differences were found, however, when comparing EcEnd with EuEnd ($P = 0,0893$), or EcEndMet with EuSham ($P = 0,0952$), EuShamMet ($P = 0,0704$) or EuEndMet ($P = 0,0901$) ([FIGURE 3AV, Supplementary Table 4](#)). On the other hand, a significant decrease in PCNA expression was found in both the pregnant EcEnd and pregnant EcEndMet mice compared with EcEnd mice (both $P \leq 0,0178$; [FIGURE 3A–VIII](#)). These findings suggested that the extensive proliferative and growth effect in ectopic tissue is hindered by pregnancy, independently of treatment with metformin.

Expression of CYP17a1 is increased by endometriosis but decreased by metformin treatment in ectopic endometrial tissue

CYP17a1, a marker of steroidogenesis, was detected in the analysed tissues by immunofluorescence ([FIGURE 3AII](#)) and by western blotting with a band of approximately 56 kDa ([FIGURE 3AIV, VII](#)). Two-way ANOVA showed that

endometriosis had an effect on CYP17a1 expression in tissues from non-mated animals, and that pregnancy also had an effect on this protein. On the other hand, the effect of metformin in these tissues did not reach significance. The levels of CYP17a1 increased in EuEnd compared with EuSham mice ($P = 0,0228$) but decreased with metformin treatment in EcEndMet mice ($P = 0,0170$; [FIGURE 3AVI](#)). There was a decrease in CYP17a1 levels in ectopic tissue from pregnant mice treated with metformin compared with both treated and non-treated ectopic tissues from non-mated mice (both $P \leq 0,0203$; [FIGURE 3AIX](#)).

Inflammatory cell markers and NFκB were increased by metformin treatment in non-mated but not pregnant mice

Dual immunolabelling of F4/80 (a macrophage marker) and galectin-3 (a molecule involved in the inflammatory response that is expressed by immune cells) was carried out to elucidate the inflammatory features in endometriosis. Labelling for both proteins was noticed in the stroma cells of all the tissues studied but was absent from the epithelia. Both labels had a diffuse, scattered pattern but showed clusters of positive cells in some sections ([FIGURE 3BI](#)). In addition, F4/80 was identified as several bands ranging from 75 to 200 kDa on western blotting ([FIGURE 3BII, VI](#)).

Two-way ANOVA analysis revealed that both endometriosis and metformin had an effect in tissues from non-mated mice, and that both metformin and pregnancy had an effect in ectopic tissues. F4/80 increased in EuEndMet mice compared with the EuEnd ($P = 0,0002$) and EuSham ($P = 0,0146$) groups and also with non-treated EcEnd mice ($P < 0,0001$). In ectopic tissue from EcEndMet animals there was a strong tendency towards an increased expression of F4/80 relative to EcEnd mice ($P = 0,0725$; [FIGURE 3BIII](#)). The variation between these two groups reached significance when the F4/80 expression levels were normalized to those of the ectopic tissues from the End group in the comparison between ectopic tissues from non-mated and pregnant animals. In this case, not only did metformin increase F4/80 levels in non-mated mice ($P = 0,0037$), but an equivalent trend was also observed in pregnant mice ($P = 0,1611$). A significant decrease was observed when comparing pregnant

EcEndMet with pregnant EcEnd mice ($P = 0,0011$; [FIGURE 3BVII](#)).

Galectin-3 was identified by western blotting as a band of approximately 30 kDa ([FIGURE 3BII, VI](#)). Two-way ANOVA revealed that metformin had an effect on the tissues of non-mated animals, and in the comparison between ectopic tissues, pregnancy had an effect while the effect of metformin almost reached significance. In line with the data relative to F4/80 and NFκB ([FIGURE 3BV](#)), metformin increased galectin-3 levels in ectopic tissue (EcEndMet) compared with all the other tissues studied: EuSham ($P < 0,0001$), EuShamMet ($P < 0,0001$), EuEnd ($P = 0,0002$), EuEndMet ($P = 0,0031$) and EcEnd ($P = 0,0039$) ([FIGURE 3BIV](#)). In ectopic tissues from non-mated and pregnant mice, decreased levels of galectin-3 were observed when comparing non-mated EcEndMet mice with their non-treated counterparts ($P = 0,0291$) and with both ectopic tissues in pregnant EcEnd ($P = 0,0099$) and pregnant EcEndMet ($P = 0,0099$) mice ([FIGURE 3BVII](#)).

Regarding NFκB, a transcription factor involved in the inflammatory response, a band of approximately 65 kDa was identified ([FIGURE 3BII, VI](#)). Two-way ANOVA showed that metformin had an effect on the tissues of non-mated mice and that both metformin and pregnancy showed an effect when comparing ectopic tissues. The quantification of NFκB concentrations showed an increase in the ectopic tissue of animals treated with metformin (EcEndMet) compared with all the tissues from the non-treated groups: EuSham ($P = 0,0062$), EuEnd ($P = 0,0070$) and EcEnd ($P = 0,0293$) ([FIGURE 3BV](#)). Within the ectopic tissues, non-mated animals treated with metformin presented increased NFκB concentrations relative to their non-treated counterparts ($P = 0,0038$) and to both groups of pregnant mice: pregnant EcEnd ($P = 0,0025$) and pregnant EcEndMet ($P = 0,0177$) mice ([FIGURE 3BIX](#)).

Metformin increased the expression of antioxidant defence enzymes in the ectopic endometrial tissue of non-mated mice

The oxidative response was evaluated by western blotting quantification of HO-1 and GPX-1, the enzymes with anti-oxidative properties. HO-1 was identified as a band of approximately 28 kDa ([FIGURE 3CI, IV](#)).

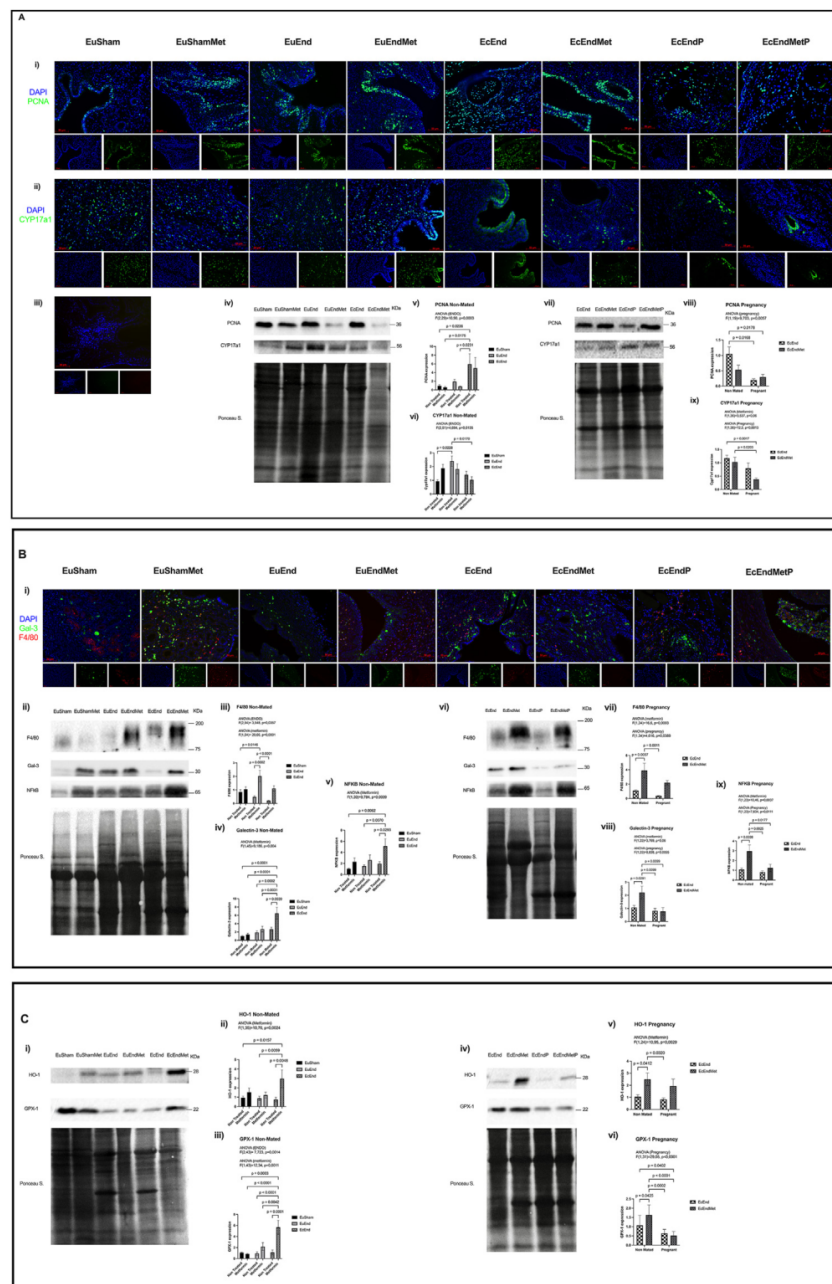


FIGURE 3 Metformin increases inflammatory markers and antioxidative defences in ectopic tissue from non-mated mice, while endometriosis (ENDO) impacts cell proliferation and oestrogen production in the tissues. (A) Representative immunofluorescence images: (i) green labelling for proliferating cell nuclear antigen (PCNA); (ii) green labelling for cytochrome P450 17a1 (CYP17a1); and (iii) negative control. Nuclei are labelled in blue (4',6-diamidino-2-phenyl-indole [DAPI]), and the corresponding single-channels are shown below the merged images. Scale bars = 50 μ m. (iv, vii) Representative images of PCNA and CYP17a1 bands detected by western blotting and representative membrane stained with Ponceau S. Analysis of tissues from non-mated mice compared with pregnant mice was carried out in separate blots. (v, vi, viii, ix) Quantitative analysis of PCNA (v, viii) and CYP17a1 (vi, ix) between treatment groups for non-mated mice and ectopic tissues from pregnant mice compared with their non-mated counterparts. (B) (i) Representative images of immunofluorescence dual labelling for F4/80 (red) and galectin-3 (green), with the corresponding individual channels below each image. Nuclei were labelled blue with DAPI. Scale bars = 50 μ m. (ii, vi) Representative images of F4/80, galectin-3 and nuclear factor kappa B (NFκB) bands detected by western blotting and the corresponding Ponceau S-stained membrane used as a control of loading. (iii, v, vii, ix) Quantitative analysis of F4/80 (iii, vii), galectin-3 (iv, viii) and NFκB (v, ix) between groups of non-mated mice and ectopic tissues from pregnant mice compared with

Two-way ANOVA showed that metformin had an effect on both the tissues of non-mated mice and in the comparison of ectopic tissues. In line with the data observed for the previously analysed proteins, an increase in HO-1 expression in the EcEndMet group was found compared with all the tissues from non-treated animals: EuSham ($P = 0,0157$), EuEnd ($P = 0,0059$) and EcEnd ($P = 0,0048$) (FIGURE 3CII). When looking at the ectopic tissues to analyse the effect of pregnancy, this increase was also observed in non-mated mice treated with metformin relative to non-treated animals ($P = 0,0412$). In addition, pregnant EcEnd mice presented lower levels of HO-1 than EcEndMet mice ($P = 0,0320$). Metformin treatment in pregnant mice, compared with their non-treated counterparts, did not increase HO-1 expression ($P = 0,2522$; FIGURE 3CV).

GPX-1, identified as a band of approximately 22 kDa (FIGURE 3CI, IV), presented a similar variation between the groups to that observed for HO-1. Two-way ANOVA indicated that metformin had an effect on the tissues of non-mated mice, and pregnancy was shown to have effect when comparing ectopic tissues. EcEndMet tissue showed higher GPX-1 expression than EuSham, EuShamMet, EuEnd, EuEndMet and EcEnd tissue (all $P \leq 0,0042$; FIGURE 3CIII). When comparing the ectopic tissues, increased GPX-1 expression was observed in metformin-treated non-mated animals relative to non-treated mice and to both groups of pregnant mice (all $P \leq 0,0425$). In addition, a decrease in GPX-1 expression was found in tissues from pregnant EcEndMet mice compared with non-mated EcEnd mice ($P = 0,0425$; FIGURE 3CVI).

Metformin restores the reduction of fertility in endometriosis

As expected, recipient animals, including those with surgically induced endometriosis, maintained the functional integrity of the reproductive system, which was crucial for the further evaluation of fertility rates. In all groups of pregnant mice the uteruses with fetuses were visible

at the 18th day end-point (FIGURE 4I–IV; the image relative to the ShamMet group is not shown). Adhesions and vascularized implants were still visible in End mice (FIGURE 4II, III) and, as observed before pregnancy, implants in EndMet mice were observed clearer than those in End animals (FIGURE 4IV).

Endometriosis is associated with infertility in women, and the present mouse model of disease successfully replicated this aspect, as a reduction in the average number of fetuses per pregnant mouse was observed in the End group compared with the Sham group ($P = 0,0034$; FIGURE 4V). It was also demonstrated that treatment with metformin played an effective role in restoring the average number of fetuses to levels similar to those found in the Sham group (Supplementary Table 18). Notably, there was a statistically significant increase in the average number of fetuses per pregnant mouse in the EndMet group compared with the End group ($P = 0,0295$; FIGURE 4V). The number of reabsorptions (FIGURE 4I–IV) showed no differences between the groups (FIGURE 4VI–; Supplementary Table 19).

DISCUSSION

Endometriosis is a leading cause of infertility in women (Guo, 2009; Lin et al., 2018) and a painful recurrent condition requiring frequent analgesic and long-term hormone-based treatments. These have the advantage of mitigating much of the pain but at the expense of unwanted side effects, suppression of the menstrual cycle and decisions to postpone pregnancy. Thus, innovative long-term therapeutic approaches that mitigate the symptoms and progression of endometriosis, preserve women's reproductive function and can be used in pregnancy are a still unmet need (Rafique and Decherney, 2017; Taylor et al., 2021).

Despite endometriosis being a natural disease of women and other primates, this has not precluded the use of other mammals including rodents as

experimental models of endometriosis. In general, these animal models aim to recapitulate the natural history of the human disorder, its pathophysiology and its related consequences, notably the reduced fertility (Bilotas et al., 2015; Cohen et al., 2015; Flores et al., 2007; Greaves et al., 2014; Hayashi et al., 2020; Park et al., 2022; Rosa-E-Silva et al., 2019; Sharpe-Timms et al., 2020; Stille et al., 2009). The experimental models also provide a means to study novel pharmacological interventions, notably their functional and structural consequences, aiming for use in humans.

In the current model, endometriosis was established by the peritoneal implantation of allogenic endometrial grafts; the pharmacological intervention was the antioxidant and antidiabetic drug metformin. The use of a model in an animal that does not naturally develop endometriosis is a limitation of the study; similarly, the dose of metformin per animal (70 mg/kg per day) was calculated based on the average ingestion of water by the group. This is lower than the standard dose of metformin used in human treatment (500 mg twice a day), and after adjustment to rodents corresponds to 200 mg/kg per day (Nair and Jacob, 2016). Considering that the animal model of endometriosis does not present insulin resistance and that excessive doses of medication should be avoided in pregnancy, a lower dose of metformin was selected. The use of metformin in the control of gestational diabetes has been demonstrated to be safe for both women and fetuses in doses of 500 mg to 2.5 g per day (Ainuddin et al., 2015; Beyuo et al., 2015).

The concentrations of total cholesterol, HLD-C, LDL-C and glucose in the blood did not differ significantly when the groups were compared, indicating that the subtherapeutic dose of metformin employed did not change glucose and lipid metabolism.

Endometrial implants successfully engrafted in the peritoneum and thrived, as verified by ultrasonography and upon

their non-mated counterparts. (C) (i, iv) Representative images of HO-1 and GPX-1 bands detected by western blotting and corresponding Ponceau S-stained membranes used as a control of loading. (ii, iii, v, vi) Quantitative analysis of heme oxygenase (HO-1; ii, v) and glutathione peroxidase (GPX-1; iii, vi) between groups of non-mated mice and ectopic tissues from pregnant mice compared with their non-mated counterparts. The statistical analysis employed two-way analysis of variance (ANOVA) followed by post-hoc analyses using Tukey's multiple comparisons tests. Values are presented as mean $\pm$ SEM. Significant P-values are shown in the figure, and all the P-values are shown in Supplementary Tables 4–17. EuSham, ectopic tissue from sham-operated mice; EuShamMet, ectopic tissue from sham-operated mice treated with metformin; EuEnd, ectopic tissue from mice with endometriosis; EuEndMet, ectopic tissue from mice with endometriosis mice treated with metformin; EcEnd, ectopic tissue from mice with endometriosis; EcEndMet, ectopic tissue from mice with endometriosis mice treated with metformin; EcEndP, ectopic tissue from pregnant mice with endometriosis mice; EcEndMetP, ectopic tissue from pregnant mice with endometriosis mice treated with metformin.

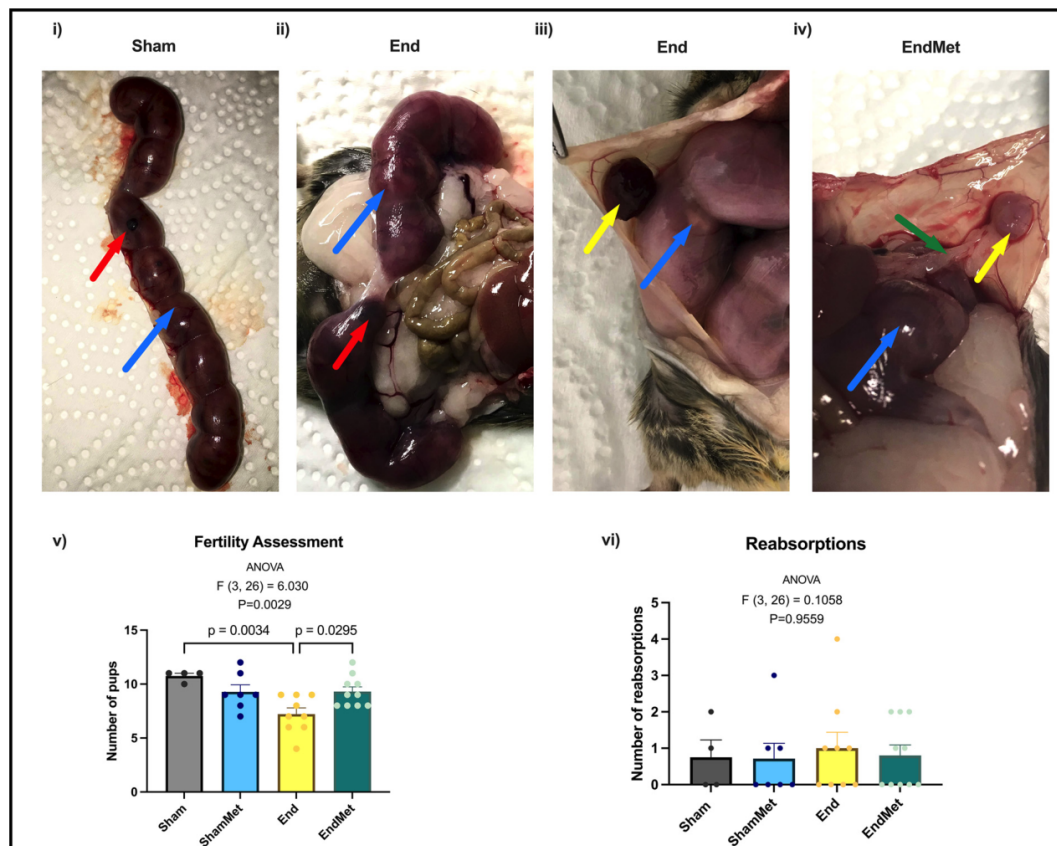


FIGURE 4 Metformin reduces endometriosis (ENDO) and restores fertility in the mouse model of disease. Representative uteri from pregnant mice at the 18th day after the mating end-point: (i) Sham group; (ii, iii) End group; and (iv) EndMet group. Blue arrows indicate a visible fetus; red arrows denote reabsorption sites; the green arrow indicates adhesions; yellow arrows show vascularized implants. (v) Graphical depiction of the number of reabsorptions per pregnant mouse between the groups. (vi) Graphical depiction of the number of reabsorptions per pregnant mouse between the groups. The statistical analysis employed one-way analysis of variance analysis of variance (ANOVA) followed by post-hoc analyses using Tukey's multiple comparisons tests. Values are presented as mean $\pm$ SEM, with the points showing individual mice. Significant *P*-values are shown in the figure, and all the *P*-values are shown in [Supplementary Tables 18 and 19](#). Sham, sham-operated mice; ShamMet, sham-operated mice treated with metformin; End, mice with endometriosis; EndMet, mice with endometriosis mice treated with metformin.

visual inspection after laparotomy. Tissue PCNA evaluation by the current group and others ([Wang et al., 2023](#)) provided additional support for implant growth. A major finding in the ultrasound assessment was that metformin treatment restrained the growth of the implants, in agreement with previous reports in rodents ([Cheng et al., 2022](#); [Oner et al., 2010](#); [Sapmaz et al., 2022](#); [Yilmaz et al., 2010](#)). Moreover, on inspection, metformin also improved the appearance of the implants, as they were clearer in colour, in contrast to lesions in untreated animals that exhibited a darker, brown bloody appearance, resembling 'chocolate cysts' similar to those frequently observed in the ovaries of women with endometriosis.

It is well established that the progression of endometriosis depends on oestrogen ([Rizner, 2009](#)), and that metformin is a known regulator of its synthesis and secretion ([Cheng et al., 2022](#); [Zhang et al., 2017](#)). Oestradiol and other sex hormones predominantly in the ovary require the activity of the steroidogenic CYP17a1 enzyme ([Burris-Hiday and Scott, 2021](#)). The variation in CYP17a1 expression in the uterine tissue suggests that it may be a modulator of local (endometrial) response in endometriosis. In fact, an enhanced expression of CYP17a1 was observed in the eutopic endometrial tissue of non-treated endometriotic mice compared with controls, but not in the ectopic endometrial tissue, emphasizing the

relevance of specific cellular environments. In contrast, CYP17a1 expression evidenced a downward response to metformin in both eutopic and ectopic endometrial tissue. This change suggests that a decrement in oestradiol is the likely mechanism for the growth restriction of endometriotic implants and indicates that metformin has a preventive effect on disease progression.

In mice with endometriosis there was an increase in fibrosis in both eutopic and ectopic tissues. The finding is a frequent occurrence following prolonged inflammation, itself a hallmark of the human disease ([Itoga et al., 2003](#); [Viganò et al., 2020](#)), and is involved in the

establishment and progression of endometriotic lesions (Maybin *et al.*, 2011). Interestingly, following metformin administration, there was a trend for the fibrosis to decrease, probably reflecting a particular ability of the drug (Wu *et al.*, 2021), which may not be similar for every tissue. In fact, a decrease in fibrosis has been reported in ovarian and uterine tissue in a rat model of metformin-treated polycystic ovary syndrome (Zhang *et al.*, 2013), but not in the heart in an endometriosis mouse model (Martins *et al.*, 2022), suggesting a relationship to the turnover capacity of cells in the different tissues, which is very marked when one compares the dynamic endometrial epithelium and stroma, and the cellularly stable myocardium.

Inflammatory pathways are extensive and complex, and recruit a variety of cells and molecules that converge to the site of inflammation, where they attract more cells and molecules. Macrophage secretory products and galectin-3 are major players in inflammation (Capobianco and Rovere Querini, 2013; Hogg *et al.*, 2020; Lousse *et al.*, 2008; Yamashita *et al.*, 2022). Galectin-3, an inflammation-associated protein and marker of surveillance cells, works also as chemoattractant for immune cells, including macrophages, and has been related to the formation of tissue fibrosis (Hisrich *et al.*, 2020). However, in implants and sham-operated uteri at timepoint 3 in the current study, galectin-3 and NFκB expression were similar; in addition, F4/80, a major macrophage marker, had a particularly low level of expression in implants. This low expression profile suggests that, despite the important role of macrophages in inflammation, the tissue may have entered a condition of poorer response, similar to that found in kidney, where F4/80 exhibits a dynamic change from pro-inflammatory to anti-inflammatory action (Kim *et al.*, 2010), while contributing to cell migration (McKnight and Gordon, 1996; Stacey *et al.*, 2000) or adhesion of macrophages to the extracellular matrix or other cell types.

In this study, the low-response condition appears to have changed when metformin was added. In fact, galectin-3, NFκB and F4/80 exhibited all an upward trend, suggesting that the tissues had returned to an inflammatory-like state, which is important to drive the tissues back to a resolution. Nevertheless, tissue-specific properties may again underlie the variety

of responses observed. For example, metformin treatment led to an increase in F4/80 in eutopic tissue, a finding that aligns with a previous demonstration where an increase in F4/80+ cell content in the mouse liver was observed after metformin treatment (Woo *et al.*, 2014), while galectin-3 and NFκB are augmented in ectopic tissue. In contrast, systemic levels of galectin-3 decreased (Weigert *et al.*, 2010), further supporting the view that galectin-3 often plays a dual role, as a pro-inflammatory but also an anti-inflammatory molecule (Hisrich *et al.*, 2020). This versatility is further exemplified by the ability of galectin-3 to scavenge advanced glycosylated end-products, which are irreversibly glycosylated under pro-oxidative conditions (Hogg *et al.*, 2020; Prizzi *et al.*, 2000; Vlassara *et al.*, 1995). By altering gene expression in favour of the expression of anti-inflammatory molecules and excessive cell apoptosis (Lawrence, 2009; Pereira and Oakley, 2008), NFκB promotes inflammation resolution (Lawrence *et al.*, 2001).

The findings discussed so far indicate that, in this model, endometrial implants become established and grow in an oestradiol-dependent environment and cause local inflammation, mimicking the natural disease. More importantly, metformin is able to favour the reversal of these lesions, leading to a beneficial reduction in fibrosis and implant size.

Endometriosis is recognized as a pro-oxidative disease (Donnez *et al.*, 2016), which stems from an increment in the production of pro-oxidative molecules and/or a down-regulation of antioxidative defences. In this setting, GPX-1 serves as safeguard for the cells against oxidative damage. In fact, apart from modulating processes such as metabolism, mitochondrial function, growth and apoptosis, GPX-1 has the capacity to adjust intracellular concentrations of hydrogen peroxide and maintain the overall intracellular redox balance (Lubos *et al.*, 2011). Additionally, HO-1, traditionally seen as an adaptive cellular response to oxidative stress toxicity, has gained recognition for its significant immunomodulatory and anti-inflammatory roles (Campbell *et al.*, 2021).

In this study, both HO-1 and GPX-1 exhibited a similar level of expression at baseline in the control animals and even in eutopic and ectopic tissues in mice with endometriosis. However, upon metformin

administration, the ectopic samples from the endometriosis group showed a significant increase in HO-1 and GPX-1 expression, paralleling the changes in the inflammation-associated molecules NFκB and galectin-3. In line with the current data, metformin has been recognized to activate the antioxidative response in a mouse model of diabetes (Zhang *et al.*, 2022). In addition, a plant-derived flavonoid, naringenin, was shown to improve endometriosis in rats through increasing HO-1 expression in the lesions (Kapoor *et al.*, 2019). In respect to the GPX-1 results, consistency with previous studies was found in terms of the its expression in the cardiac tissue of in a mouse model of endometriosis and a rat model of diabetes (Felgueiras *et al.*, 2022), and also in the brain of rats (Obafemi *et al.*, 2020). Thus, in addition to reversing some of the consequences associated with endometriosis, such as implant growth and local fibrosis, metformin led to an up-regulation of antioxidative enzymatic defences, further limiting the progression and effects of the endometriosis.

An important result was noticed in pregnancy as this affected oestradiol and cholesterol concentrations in every study group. The increase in oestradiol is a recognized change in human pregnancy, here verified in pregnant mice. In contrast, the reduction in cholesterol concentration in pregnant mice (Sweeney *et al.*, 2006), while conflicting with the findings in humans (Green, 1966; Ogura *et al.*, 2002), may reflect a distinct means of cholesterol processing. Indeed, the main supplier of cholesterol to the tissues in humans is LDL-C, whereas in rodents it is HDL-C (Grummer and Carroll, 1988).

Pregnancy is an important issue in the setting of endometriosis. The assessment of ectopic endometriotic tissue from pregnant mice receiving metformin showed a decrease in expression of PCNA, CYP17a1 and GPX1 relative to non-mated EcEnd animals, and CYP17a1, galectin-3, NFκB and GPX-1 relative to non-mated EcEndMet animals. This similarity in the response of these molecules emphasizes the importance of pregnancy and indicates that it overrode the endometriotic condition and buffered its effects.

As a corollary of the beneficial consequences of metformin administration, the assessment of fertilization revealed a most important finding. After the significant reduction in

the number of fetuses per pregnant mouse in the group with induced endometriosis compared with their sham-operated counterparts, also observed by Cohen and collaborators (Cohen *et al.*, 2015), a reversal of infertility was found in the group of mice with endometriosis that were treated with metformin. This evidence marks the first documented reversal of endometriosis-associated infertility with metformin in a mouse model despite a previous report of an equivalent effect with the natural compound oleuropein (Park *et al.*, 2022).

In conclusion, the current study establishes a mouse model illustrating the reversal of infertility associated with endometriosis through metformin treatment, probably through its anti-inflammatory properties manifested in pregnancy. The subtherapeutic dose employed, tailored to this animal model, demonstrated promising outcomes. Although metformin showed consistency of action in the oxidation response pathways, its impact on inflammatory pathways necessitates further exploration. These findings constitute valuable insights into the potential benefits and complexities of metformin in the context of endometriosis, paving the way for future research and clinical considerations.

DATA AVAILABILITY

Data will be made available on request.

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BioRender was used in the construction of **FIGURE 1** and the graphical abstract (Neto, A., 2024, <https://BioRender.com/d99y595>).

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.rbmo.2024.104474](https://doi.org/10.1016/j.rbmo.2024.104474).

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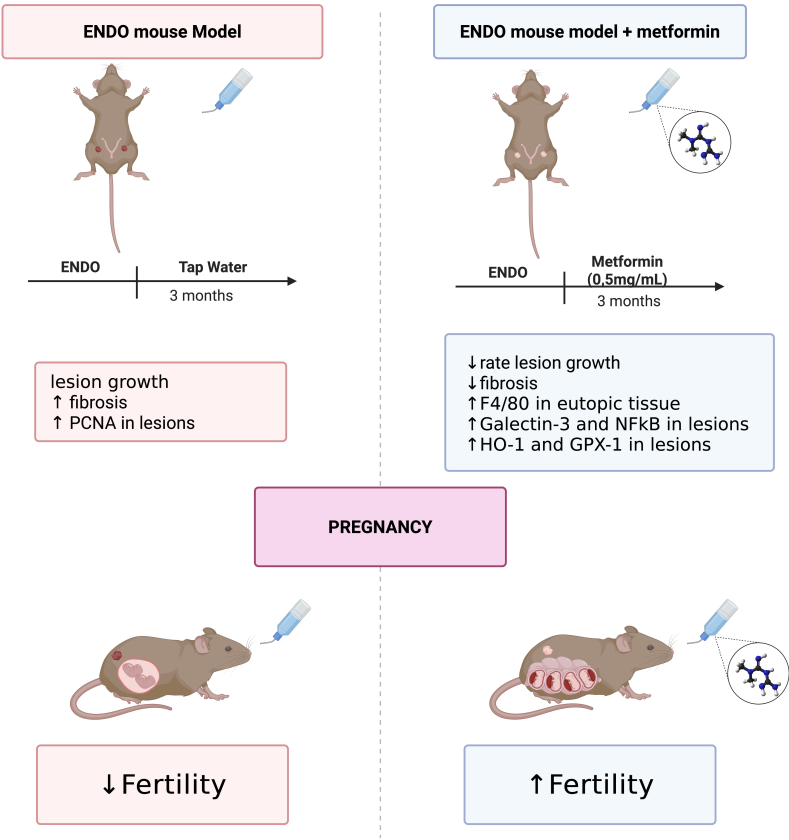
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Graphical Abstract:



Supplementary Materials:

Supplementary tables: All pair-wise p-values by Tukey's post-hoc testing unless otherwise specified

Supplementary table 1: Figure 1 D All p-values

Comparison groups	Estradiol	Total cholesterol	HDL	LDL	Glucose
ANOVA p-value	ANOVA p-value				
Non-mated: Sham vs ShamMet vs End vs EndMet	F (3, 56) = 0,1938 p=0,9002	F (3, 61) = 0,6904 p=0,5614	F (3, 59) = 2,151 p=0,1034	F (3, 59) = 0,1757 p=0,9125	F (3, 59) = 1,387 p=0,2557
Pregnant: Sham vs ShamMet vs End vs EndMet	F (1, 56) = 36,09 p<0,0001	F (1, 61) = 27,92 p<0,0001	F (1, 59) = 10,64 p=0,0018	F (1, 59) = 0,1553 p=0,6949	F (1, 59) = 20,41 p<0,0001
	Pairwise (Tukey's multiple comparison)				
Sham: non-mated vs pregnant	<0.0001	<0.0001	0,2350	>0,9999	0.0409
Sham Met: non-mated vs pregnant	<0.0001	<0.0001	0,8766	>0,9999	0,5884
End: non-mated vs pregnant	<0.0001	<0.0001	0,6844	>0,9999	0,1281
EndMet: non-mated vs pregnant	<0.0001	<0.0001	0,9912	>0,9999	0,9656

Supplementary table 2: Figure 2ii

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,8366	0.0186	0,6618	0.0221	0,9956
EuShamMet	-	0.0008	0,1182	0.0010	0,5442
EuEnd	-	-	0,4812	>0,9999	0,0602
EuEndMet	-	-	-	0,5236	0,9049
EcEnd	-	-	-	-	0.0705

Supplementary table 3: Figure 2iii

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,0734	0,2933	0.0187
EcEndMet	-	0,8121	0,8641
EcEndP	-	-	0,3776

Supplementary table 4: Figure 3A v

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,09998	0,9670	>0,9999	0.0236	0,0952
EuShamMet	-	0,9067	>0,9999	0.0175	0,0704
EuEnd	-	-	0,9473	0,0893	0,2919
EuEndMet	-	-	-	0.0231	0,0901
EcEnd	-	-	-	-	0,9935

Supplementary table 5: Figure 3A vi

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,4022	0.0228	0,4963	0,8937	>0,9999
EuShamMet	-	0,8229	>0,9999	0,8781	0,4349
EuEnd	-	-	0,7945	0,1076	0.0170
EuEndMet	-	-	-	0,9311	0,5419
EcEnd	-	-	-	-	0,9381

Supplementary table 6: Figure 3A viii

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,1351	0,0168	0,0178
EcEndMet	-	0,5965	0,7641
EcEndP	-	-	0,9794

Supplementary table 7: Figure 3A ix

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,8746	0,2639	0,0017
EcEndMet	-	0,6965	0,0203
EcEndP	-	-	0,2740

Supplementary table 8: Figure 3B iii

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,9944	0,9090	0,0146	0,4561	0,9719
EuShamMet	-	0,5797	0,0502	0,1559	>0,9999
EuEnd	-	-	0,0002	0,9446	0,3854
EuEndMet	-	-	-	<0,0001	0,0589
EcEnd	-	-	-	-	0,0725

Supplementary table 9: Figure 3B iv

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,9991	0,9425	0,5082	0,5433	<0,0001
EuShamMet	-	0,9937	0,7133	0,7435	<0,0001
EuEnd	-	-	0,9376	0,9486	0,0002
EuEndMet	-	-	-	>0,9999	0,0031
EcEnd	-	-	-	-	0,0039

Supplementary table 10: Figure 3B v

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,8514	0,9961	0,7068	0,9396	0,0062
EuShamMet	-	0,9658	0,9998	0,9993	0,1098
EuEnd	-	-	0,8775	0,9960	0,0070
EuEndMet	-	-	-	0,9875	0,1908
EcEnd	-	-	-	-	0,0293

Supplementary table 11: Figure 3B vii

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,0037	0,8205	0,4770
EcEndMet	-	0,0011	0,1372
EcEndP	-	-	0,1611

Supplementary table 12: Figure 3B viii

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,0291	0,9063	0,8746
EcEndMet	-	0,0099	0,0099
EcEndP	-	-	0,9997

Supplementary table 13: Figure 3B ix

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,0038	0,9460	0,9849
EcEndMet	-	0,0025	0,0177
EcEndP	-	-	0,8444

Supplementary table 14: Figure 3C ii

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,9117	>0,9999	0,9962	0,9988	0,0157
EuShamMet	-	0,8317	0,9947	0,7344	0,2256

EuEnd	-	-	0,9851	0,9998	0,0059
EuEndMet	-	-	-	0,9502	0,0625
EcEnd	-	-	-	-	0,0048

Supplementary table 15: Figure 3C iii

Pairwise p-value	EuShamMet	EuEnd	EuEndMet	EcEnd	EcEndMet
EuSham	0,9999	>0,9999	0,8422	>0,9999	0,0003
EuShamMet	-	>0,9999	0,6740	0,9996	<0,0001
EuEnd	-	-	0,7296	>0,9999	<0,0001
EuEndMet	-	-	-	0,8254	0,0042
EcEnd	-	-	-	-	0,0001

Supplementary table 16: Figure 3C v

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,0412	0,9771	0,3541
EcEndMet	-	0,0320	0,7585
EcEndP	-	-	0,2522

Supplementary table 17: Figure 3C vi

Pairwise p-value	EcEndMet	EcEndP	EcEndMetP
EcEnd	0,0425	0,2119	0,0425
EcEndMet	-	0,0002	<0,0001
EcEndP	-	-	0,9279

Supplementary table 18: Figure 4 v

Pairwise p-value	ShamMet	End	EndMet
Sham	0,4292	0,0034	0,3877
ShamMet	-	0,0550	>0,9999
End	-	-	0,0295

Supplementary table 19: Figure 4 vi

Pairwise p-value	ShamMet	End	EndMet
Sham	>0,9999	0,9814	0,9998
ShamMet	-	0,9553	0,9986
End	-	-	0,9789

Sham – Sham-operated mice; ShamMet – Sham-operated mice treated with metformin; End – Endometriosis mice; EndMet – Endometriosis mice treated with metformin. EuSham – Eutopic tissue from Sham mice; EuShamMet – Eutopic tissue from Sham mice treated with metformin; EuEnd – Eutopic tissue from endometriosis mice; EuEndMet – Eutopic tissue from endometriosis mice treated with metformin; EcEnd – Ectopic tissue from endometriosis mice; EcEndMet – Ectopic tissue from endometriosis mice treated with metformin; EcEndP – Ectopic tissue from pregnant endometriosis mice; EcEndMetP – Ectopic tissue from pregnant endometriosis mice treated with metformin. HDL- high-density lipoproteins; LDL - low-density lipoproteins

PUBLICATION II

“Metformin Prevents Endothelial Dysfunction in Endometriosis through Downregulation of ET-1 and Upregulation of eNOS”

Biomedicines 2022



Article

Metformin Prevents Endothelial Dysfunction in Endometriosis through Downregulation of ET-1 and Upregulation of eNOS

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Abstract: This study aimed to evaluate if the treatment with metformin affects the morphologic structure, endothelial function, angiogenesis, inflammation and oxidation-responsive pathways in the heart of mice with surgically induced endometriosis. B6CBA/F1 mice (n = 37) were divided into four groups; Sham (S), Metformin (M), Endometriosis (E) and Metformin/Endometriosis (ME). The cross-sectional area of cardiomyocytes was assessed after Hematoxylin–Eosin staining and fibrosis after Picrosirius-Red staining. ET-1, nitric oxide synthases-iNOS and eNOS, and VEGF and VEGFR-2 were detected by immunofluorescence. Semi-quantification of ET-1, eNOS, VEGF, NF-κB, Iκβα and KEAP-1 was performed by Western blotting. MIR199a, MIR16-1, MIR18a, MIR20a, MIR155, MIR200a, MIR342, MIR24-1 and MIR320a were quantified by Real-Time qPCR. The interaction of endometriosis and metformin effects was assessed by a two-way ANOVA test. Compared with the other groups, M-treated mice presented a higher cross-sectional area of cardiomyocytes. Heart fibrosis increased with endometriosis. Treatment of endometriosis with metformin in the ME group downregulates ET-1 and upregulates eNOS expression comparatively with the E group. However, metformin failed to mitigate NF-κB expression significantly incremented by endometriosis. The expression of MIR199a, MIR16-1 and MIR18a decreased with endometriosis, whereas MIR20a showed an equivalent trend, altogether reducing cardioprotection. In summary, metformin diminished endometriosis-associated endothelial dysfunction but did not mitigate the increase in NF-κB expression and cardiac fibrosis in mice with endometriosis.

Keywords: cardiac fibrosis; endometriosis; endothelial dysfunction; inflammation; metformin

1. Introduction

Endometriosis, characterized by the presence of endometrial tissue outside of the uterus, is a gynecological disease estimated to affect up to 10% of women of reproductive age [1,2].

In recent years, evidence that links endometriosis to additional health issues emerged [1], which supports the notion that endometriosis is a chronic systemic disease [3]. Moreover, epidemiological data indicate that endometriosis patients present an increased risk for cardiovascular disease (CVD) [4], including major cardiovascular events [5]. This is partly explained by the hormonal treatments or hysterectomy/oophorectomy [5], but endometriosis-associated conditions such as chronic systemic inflammation [6] and imbalance between oxidative species and anti-oxidative defenses (oxidative stress) [7] also independently contribute to CVD onset and progression [8]. Independently of the hormonal treatments, endometriosis patients present a hypercoagulable status that, whilst supporting the healing of the endometriotic lesions, contributes to the occurrence of thrombo-embolic

events and tissue fibrosis [9]. Regarding what concerns the influence of hormonal therapies on the CVD risk in patients with endometriosis, it was recently demonstrated that dienogest therapy or oral contraception for less than 3 years does not increase the atherosclerosis risk. On the other hand, a positive correlation was found between the duration of oral contraception and the risk for CVD in women with endometriosis [10]. No evidence of deleterious effects on heart after gonadotropin-releasing hormone analogues or antagonists treatment was reported [3].

Molecular pathways dysregulated in CVD are also affected in endometriosis; vascular dysfunction is associated with upregulation of endothelin-1 (ET-1) production [11] that intervenes in endometriosis-altered pain response [12]. On the other hand, the protective role of endothelial nitric oxide synthase (eNOS) is impaired in endometriosis by increased expression of the eNOS inhibitor asymmetric dimethylarginine (ADMA) [13]. NF- κ B activation leads not only to the onset of CVD [14], including atherosclerosis [15], but also to endometriosis-associated inflammation [16]. Angiogenesis, fundamental for the growth of endometriotic tissue [17], also plays a role in atherosclerosis plaque formation [18], with vascular endothelial growth factor (VEGF) appointed, in both processes, as an essential contributor.

MicroRNAs (MIRs) are small non-coding RNAs that post-transcriptionally regulate gene expression by targeting mRNAs [19]. MIRs dysregulation plays an important role in the control of the molecular pathways that underlie endometriosis-associated CVD. Levels of MIR18a, MIR199, MIR342 and MIR310a that regulate cell survival and cardiac regeneration processes increase in the blood of patients with endometriosis [20–26]. On the other hand, MIR16-1, MIR155 and MIR24-1 [27] have been reported to target elements of the angiogenic pathways. Other MIRs have been identified in the regulation of inflammatory and oxidative stress responses, such as MIR20a [28] and MIR200a [29], respectively.

Current treatments for endometriosis consider hormone-based medication, analgesics and, ultimately, surgical excision of endometriotic lesions. Unfortunately, those interventions do not alter the course of the disease and the discontinuation of conceiving comes with the recurrence of symptoms [30]. In addition, a hysterectomy is far from being infallible, with up to 15% of patients remaining with persisting and 3–5% with worsened pain [31].

The failure of the currently used treatments encourages the identification of alternative pharmacological strategies for the control of endometriosis. Metformin, a biguanide used in the treatment of diabetes, including gestational diabetes, with recognized effects in the control of CVD, emerged as a potential medication for the treatment of endometriosis. Hepatic glucose production by 5' adenosine monophosphate-activated protein kinase (AMPK)-dependent and independent mechanism is the main target of metformin. AMPK activation promotes autophagy and inhibits the mammalian target of rapamycin (mTOR), a major regulator of cell growth and proliferation [32,33]. Metformin also presents pleiotropic effects, which supports a potential benefit for the treatment of endometriosis [34]. Metformin, through molecular pathways not yet fully established, presents anti-inflammatory, antioxidant, anti-steroidogenic and anti-angiogenic effects that together mitigate the progression of the disease [35–39]. In fact, metformin has been shown to cause the regression of lesions in a rat model of endometriosis [36] and to mitigate inflammation and expression of angiogenesis-related genes in human ectopic endometrial cells [40]. Studies in women with endometriosis treated with metformin are scarce, but it was demonstrated that treatment incremented endometrial receptivity of minimal/mild endometriosis in a study that only enrolled five patients [41]. Moreover, a reduction in inflammatory cytokines in the blood, dysmenorrhea, pelvic pain, dyspareunia and an increment in the percentage of pregnancy after 6 months of metformin therapy was demonstrated [42].

In this study, we aimed to evaluate the role of metformin in the mitigation of CVD risk associated with endometriosis. Pathways related to endothelial function, angiogenesis, inflammation and oxidative stress were characterized in the heart of a mice model of endometriosis. Morphologic changes and expression of the main miRNAs involved in endometriosis and cardiac damage were also identified.

2. Materials and Methods

2.1. Animal Model

Thirty-seven female B6CBA/F1 mice, first-generation from C57BL/6J males and CBA/J females mating, chosen for their tolerance to tissue-transplantation, were used in the experiments and kept at i3S (Instituto de Investigação e Inovação em Saúde, Porto, Portugal) animal facility. All procedures were performed by blinded trained staff and approved by the Ethical Committee of i3S and the Portuguese competent authority DGAV (Direção-Geral de Alimentação e Veterinária) for animal experimentation, with the internal reference 2018/27. All animal procedures followed the 2010/63/EU directive of the European Parliament and adhered to the ARRIVE 2.0 guidelines. During experiments, mice were housed 4/cage with freely available water and standard rodent chow in 12 h light/dark cycles in a temperature and humidity-controlled room (21 °C and 50%, respectively).

At the age of eight weeks, mice were randomly divided into four groups according to the intervention to be performed: S—sham-operated mice (n = 6), M—metformin-treated sham-operated mice (n = 8), E—endometriosis-induced mice (n = 14) and ME—metformin-treated endometriosis-induced mice (n = 9). Sample size calculation was based on the resource equation method [43] that indicates an n of 4–6 animals per group as appropriate. In order to guarantee an n = 6 in groups E and ME, endometriosis was induced in a higher number of mice. Since the process was successful in all of them, a higher n was obtained in those groups. The surgically induced endometriosis model was achieved by implanting a ~2 mm³ uterus fragment in either side of an incision in the peritoneum, collected from a longitudinally opened uterus from a donor of the same mouse strain. The endometrium was sited to the peritoneum, according to the method described by Cohen et al. [44] adapted by Neto et al. (data not published). Animals were anesthetized with volatile isoflurane (5% for induction and 2–3% for maintenance), monitored by cardiac and respiratory frequency and treated with buprenorphine (1 mg/kg subcutaneous twice daily) on the surgery day and in the 72 h after. The implants were visualized by ultrasound (Micro Ultrasound Vevo 2100) 4 weeks after surgery. Mice from groups S and M were subjected to a sham operation consisting of a suture on both sides of the peritoneum. Four weeks after the intervention, animals in groups M and ME started the treatment with metformin (Alpha Aesar Chemicals, Haverhill, MA, USA) diluted in the drinking water at a dose of 50 mg/kg/day that was maintained for 3 months. Water consumption was monitored during experiments to ensure no differences in dosage between cages. Animals were euthanized by cardiac puncture under deep isoflurane anesthesia, and the hearts were collected and divided into two equal parts. One half was frozen in liquid nitrogen and stored at −80 °C for molecular analysis, and the remaining half was immediately fixed in 10%-buffered formaldehyde and embedded in paraffin for histological analysis and MIRs quantification.

2.2. Computer-Assisted Histologic Study of the Heart

Serial sections (3 µm thick), deparaffinized through incubation in a stove at 60 °C (30 min) and then in xylol (10 min), were hydrated in a series of incubations (5 min) in a decreasing series of ethanol aqueous solutions (100%, 90% and 70% v/v) and water. For each animal, 4 sections were stained with Hematoxylin–Eosin (HE) (Hematoxylin H and Eosin Y 1% v/v alcoholic, Biognost, Zagreb, Croatia) to assess cardiomyocyte area, and another 4 with 0.1% w/v Picrosirius Red (Direct Red 80, Sigma Aldrich, St Louis, MO, USA), to assess myocardial fibrosis.

Sections were visualized by an experienced and blinded individual in an optical microscope (Leitz Wetzlar—Dialux 20, Wetzlar, Germany) equipped with a camera (Olympus XC30, Tokyo, Japan). A cross-sectional area of 50 orbicular cardiomyocytes was measured per animal using Cell B software (Olympus), and eight fields were analyzed with Image-Pro Plus 6 software (Media Cybernetics, Rockville, MD, USA); the ratio of fibrotic area to total tissue area, corresponding to the percentage of interstitial fibrosis, was calculated.

2.3. Dual Immunolabelling in Heart Sections

Deparaffinized and hydrated 5 µm-thick heart sections were incubated in 1% w/v Sudan Black B in 70% v/v ethanol aqueous solution (20 min) to reduce autofluorescence, followed by two washes with phosphate-saline buffer (PBS), two with 0.02% v/v Tween-20 in PBS (PBS.T) and water.

The sections were then incubated in 1 M HCl aqueous solution (30 min) for antigen retrieval, neutralized in 2% w/v sodium borate (5 min), washed with water and PBS, blocked with 1% w/v bovine serum albumin (BSA) and 10% v/v normal swine serum diluted in PBS (1 h) and incubated with a mixture of primary antibodies diluted in blocking solution overnight in a wet chamber at 4 °C (Table 1). Negative controls were prepared with the omission of primary antibodies.

Table 1. List of antibodies used in immunofluorescence experiments.

Primary Antibodies	Concentration (v/v)
ET-1—Goat IgG Polyclonal—Santa Cruz Biotech, Dallas, Texas, USA. ref. sc-21625	1:100
VEGF—Goat IgG Polyclonal—R&D, Minneapolis, Minnesota, USA. ref. AF564	1:50
VEGFR-2—Rabbit IgG Polyclonal—Santa Cruz Biotech. ref. sc-504	1:100
iNOS—Rabbit IgG Polyclonal—Abcam Cambridge, UK. ref. ab178945	1:200
eNOS—Rabbit IgG Monoclonal—Santa Cruz Biotech. ref. sc-7271	1:200
α-SMA—Mouse IgG Monoclonal—Millipore, Burlington, Massachusetts, USA. ref. CBL171	1:300
Secondary Antibodies	Concentration (v/v)
Anti-Goat Alexa Fluor 568 Molecular Probes Engene, Oregon, USA. ref. A11057	1:2000
Anti-Rabbit Alexa Fluor 488 Molecular Probes ref. A21206	1:2000
Anti-Mouse Alexa Fluor 568 Molecular Probes ref. A10037	1:2000

The next day, sections were incubated with the appropriate fluorescence-labeled secondary antibodies diluted in 0.1% v/v Triton X-100 PBS (PBS.Tx) (Table 1) in a wet and dark chamber (1 h) at room temperature after washing with PBS/PBS.T. Sections were then washed with PBS.Tx and PBS and nuclei stained with 4',6-diamidino-2-phenyl-indole (DAPI) (5 min) before mounting in 50% v/v glycerol/PBS and visualization in an Apotome fluorescence microscope (Carl Zeiss AxioImager Z1—Carl Zeiss, Oberkochen, Germany) equipped with a digital camera (AxioCam MRm—Carl Zeiss).

2.4. Western Blotting

Heart fragments were homogenized in lysis buffer pH 7.6 (50 mM Tris-HCl, 10 mM NaCl, 5 mM EDTA, 2 mM β-glycerophosphate, 0.25% v/v Triton X-100) supplemented with proteases inhibitors (1:200 v/v) and phosphatases inhibitors (1:250 v/v) (Sigma-Aldrich). Protein concentration was determined using the Bradford Reagent colorimetric assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Hercules, CA, USA); 25 µg of total protein/sample diluted in 4x gel loading buffer (50 mM Tris-HCl pH 8.8, 10% v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), 2 mM EDTA and 0.0125% w/v bromophenol blue) with 0.1 M v/v dithiothreitol were loaded in the gels. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous buffer system (Bio-Rad Laboratories) under a constant current of 15 mA/gel until stacking was completed, followed by 25 mA/gel. The concentration of acrylamide in the resolving gel was either 10%, 12% or 14% (Table 2), according to the molecular weight of the studied

proteins. The proteins were then transferred to a nitrocellulose membrane with a pore size of 0.45 μm (Bio-Rad Laboratories) in a transfer system (Bio-Rad Laboratories) at a voltage of 30 V (90 min). Blots were stained with Ponceau S, and the images were acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories) to use as the loading control for the proteins analyzed in the corresponding lanes. Then, membranes were washed in a 0.1% v/v Tween-20 in tris-buffered saline solution (TBS.T) and blocked for 1 h in 5% w/v dried skimmed milk (Molico®). After washing in TBS.T, membranes were incubated for 2 overnights at 4 °C with the primary antibodies (Table 2) diluted in TBS.T with 5% w/v BSA and 0.1% v/v NaN_3 . Membranes were washed in TBS.T and incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibody diluted in 5% w/v dried skimmed milk in TBS.T (1 h) followed by additional washes in TBS.T. Finally, membranes were reacted with Clarity Western ECL substrate (Bio-Rad Laboratories), and protein bands images were acquired in the ChemiDoc.

Table 2. List of antibodies used in Western Blotting.

Primary Antibodies	Concentration (v/v)	% Acrylamide Resolving Gel
ET-1—Rabbit IgG Polyclonal-R&D ref. sc-21625-R	1:1000	12
eNOS—Rabbit IgG Monoclonal-Cell Signaling Technology, Danvers, Massachusetts, USA. ref. D9A5L	1:1000	10
VEGF—Mouse IgG Monoclonal R&D ref. MAB564	1:100	14
NF- κB —Rabbit IgG Monoclonal-Cell Signaling Tech. ref. D14E12	1:1000	10
I $\kappa\text{B}\alpha$ —Mouse IgG Monoclonal-Cell Signaling Tech. ref. L35A5	1:1000	10
keap-1—Rabbit IgG Monoclonal-Cell Signaling Tech. ref. D6B12	1:1000	12
Secondary Antibodies		Concentration (v/v)
Goat Anti-Mouse IgG-HRP-Santa Cruz Biotech. ref. sc-2005		1:10,000
Donkey Anti-Rabbit IgG-HRP-Jackson ImmunoResearch, West Grove, Pennsylvania, USA. ref. 711-035-152		1:5000

The intensity of bands was quantified by densitometry using the Image Lab® software (Bio-Rad Laboratories), and semi-quantification of each protein band was calculated through the ratio of the intensity of the band relative to the total stain of the proteins in the respective lane stained with Ponceau S. In order to correct differences due to variations in the exposure time between membranes, the average value of the sham group in each membrane was used as an internal control. Each experiment was repeated four times and averaged per sample; at least 6 different samples/group were used in each analysis.

2.5. Real-Time qPCR

Five 15 μm sections were cut with a fresh microtome blade from each formalin-fixed paraffin-embedded block ($n = 6\text{--}7/\text{group}$) and placed in RNase-free microtubes. Paraffin was dissolved with xylene at 50 °C (Fisher Scientific, Merelbeke, Belgium), ethanol (Millipore, Darmstadt, Germany) and airdrying of the remaining pellet before incubation with a protease at 50 °C (2 h) and 70 °C (15 min) to remove protein contamination.

RNAs were extracted using the RecoverAll™ kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. Purified RNA was converted to cDNA using MystiCq™ MicroRNA cDNA synthesis mix (Sigma-Aldrich). Two negative controls per experiment were performed with the omission of Poly(A) Polymerase and of reverse transcriptase.

MIRs were amplified in 96 well plates after mixing 1 µL of cDNA with 1.2 µL of miRNA-specific primer (miScript Primer Assay, Qiagen, Hilden, Germany) (Table 3), 0.25 µL of Universal Primer (Sigma-Aldrich) and 6 µL of SYBR Green (ThermoFisher Scientific). Real-time polymerase chain reaction (RT-PCR) was conducted on the StepOne-Plus Real-Time PCR System (Applied Biosystems, Bedford, MA, USA) and started with denaturation (10 min) at 95 °C, followed by 45 cycles of 15 s denaturation at 95 °C, 30 s annealing at 55 °C and 30 s extension at 60 °C. Each PCR was normalized with a control, RNU6-1, using the formula $2^{-(CT_{\text{target}} - CT_{\text{control}})}$ to quantify MIR expression levels.

Table 3. List of primers used in MIRs quantification by Real-Time qPCR.

MiRNA	Reference
RNU	Hs_RNU6-2_1 miScript Primer Assay (ref. MS00033740)
MIR199a	Hs_miR-199a_1 miScript Primer Assay (ref. MS00006741)
MIR16-1	has_miR-16-5p miRCURY LNA™ miRNA PCR Assay (ref. YP00205702)
MIR18a	has_miR-18a-5p miRCURY LNA™ miRNA PCR Assay (ref. YP00204207)
MIR20a	Hs_miR-20a_1 miScript Primer Assay (ref. MS00003199)
MIR155	Mm_miR-155_1 miScript Primer Assay (ref. MS00001701)
MIR342	has_miR-342-3p miRCURY LNA™ miRNA PCR Assay (ref. YP00205625)
MIR200a	Hs_miR-200a_1 miScript Primer Assay (ref. MS00003738)
MIR24-1	has_miR-24-3p miRCURY LNA™ miRNA PCR Assay (ref. YP00204260)
MIR320a	Hs_miR-320a_1 miScript Primer Assay (ref. MS00014707)

2.6. Statistical Analysis

Statistical analysis was made using GraphPad Prism version 8.0.2 (GraphPad Software Inc. San Diego, CA, USA).

A regular two-way ANOVA was applied to evaluate the effect of the combined factors, endometriosis and metformin. In addition, *post-hoc* analyses using Tukey's test were performed. Following ANOVA, to elucidate the role of metformin treatment, multiple *t*-tests by row were conducted by assuming consistent scatter and correction by the Holm–Sidak method.

Results are presented as mean ± SEM, and a *p*-value < 0.05 was considered statistically significant. All measurements were relativized to the expression of the sham group by dividing their value by the mean of the sham group.

3. Results

3.1. Endometriosis Increases Cardiac Fibrosis and Metformin Increases Cardiomyocyte Cross-Sectional Area

Cardiomyocyte cross-sectional area was increased in group M comparatively with the other experimental groups (Figure 1a–d,i); such increment was of 24% in comparison with group S (*p* < 0.001) and of 14% relative to both groups E (*p* < 0.01) and ME (*p* = 0.02). Groups E and ME showed a trend towards increased cardiomyocyte area when compared with group S (*p* = 0.13 and *p* = 0.19, respectively). The two-way ANOVA revealed a significant interaction between the effects of metformin and endometriosis on cardiomyocytes size (*F* (1.33) = 15.00, *p* < 0.001).

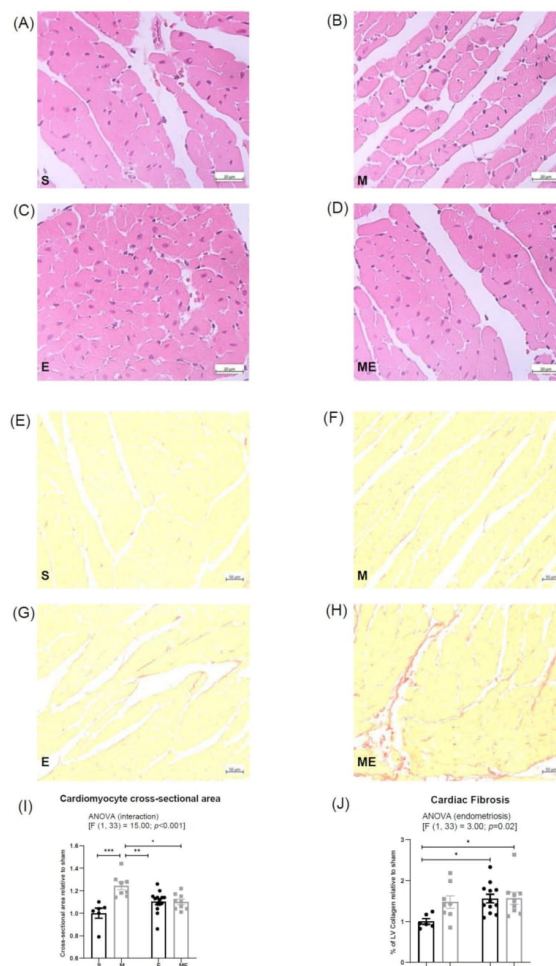


Figure 1. Representative images of heart sections stained with Hematoxylin–Eosin to assess cardiomyocyte area, groups S (A), M (B), E (C) and ME (D), and with Picrosirius Red to evaluate fibrosis, groups S (E), M (F), E (G) and ME (H). Graphical representation of cardiomyocytes cross-sectional surface area (I) and percentage of collagen on left ventricle (J) are shown. In each graphic, results in groups M, E and ME are displayed as fold increase relative to S group (represented by an arbitrary value of 1). $n(S) = 6$, $n(M) = 8$, $n(E) = 12$ –14 and $n(ME) = 9$. Regular two-way ANOVA followed by *post-hoc* with Tukey’s test was employed. E—endometriosis, M—metformin, ME—metformin/endometriosis and S—Sham. Values displayed as mean $\pm$ SEM. * p value < 0.05 , ** p value < 0.01 , *** p value < 0.001 .

Moreover, regarding cardiac fibrosis computer-assisted analysis (Figure 1e–h,j), the two-way ANOVA showed that endometriosis had a significant effect on the ratio of the fibrotic area ($F(1, 33) = 3.00$, $p = 0.02$), considering that both groups with endometriosis, E and ME, revealed a 56% ($p = 0.03$) and 57% ($p = 0.04$) higher ratio of the fibrotic area than the S group. Interestingly, mice treated with metformin showed a tendency to increase fibrosis comparatively with controls ($p = 0.12$).

3.2. ET-1 and VEGF Are Expressed in the Vascular Smooth Muscle Cells and VEGFR-2, eNOS and iNOS in the Endothelium

Figure 2 depicts the dual immunolabeling of iNOS and ET-1, eNOS and α -smooth muscle actin (α -SMA), a marker of smooth muscle cells (SMC) and VEGF and VEGFR-2, in heart sections of mice from the different experimental groups and respective negative controls (Figure 2m,n). ET-1 (red) expression was detected in the cytoplasm of vascular SMC in all groups (Figure 2a–d). Low co-localization of ET-1 and iNOS (green) was observed in vascular SMC (yellow) (Figure 2a–d); iNOS was almost exclusively detected in the endothelium as a thin labeled layer surrounding the vascular spaces. An apparent increase in iNOS labeling was observed in the heart of mice treated with metformin independently of endometriosis (Figure 2b,d). The endothelial isoform of NOS, eNOS (green), was identified in endothelial cells and endocardium of all experimental groups, with an apparent higher labeling than the observed for iNOS (Figure 2e–h). No co-localization with vascular SMC (red) was verified.

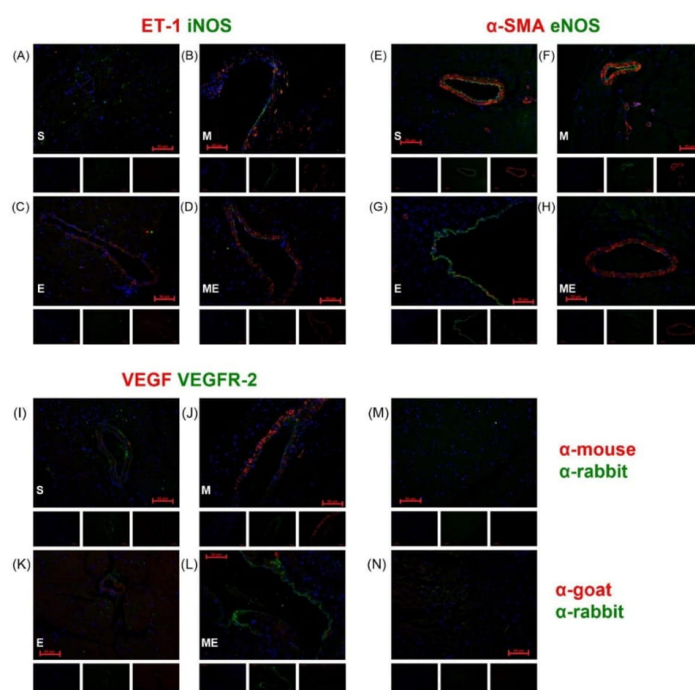


Figure 2. Representative images of dual-immunolabeling of ET-1(red) plus iNOS (green) in heart sections of groups S (A), M (B), E (C) and ME (D), of α -SMA (red) plus eNOS (green) in groups S (E), M (F), E (G), ME (H) and of VEGF (red) and VEGFR-2 (green) in groups S (I), M (J), E (K) and ME (L). Negative controls were prepared by omission of primary antibody (M,N). Nuclei were labeled blue (DAPI). The isolated blue, green and red channels are shown for all images. E—endometriosis, M—metformin, ME—metformin/endometriosis and S—Sham. DAPI—4',6-diamidino-2-phenylindole, eNOS—endothelial nitric oxide synthase, ET-1—endothelin-1, iNOS—inducible nitric oxide synthase, α -SMA—alfa smooth muscle actin, VEGF—vascular endothelial growth factor and VEGFR-2—vascular endothelial growth factor receptor 2.

VEGF (red) was detected in the cytoplasm of vascular SMC and myocardium adjacent to the endocardium in every group (Figure 2i–l), with an apparent higher expression in

group M (Figure 2j). On the other hand, the VEGF main receptor, VEGFR-2, was identified in the endothelium, endocardium and some vascular SMC without apparent differences among experimental groups.

3.3. Endometriosis Increases NF- κ B Expression in the Heart and Metformin Attenuates Endothelial Dysfunction through ET-1 Downregulation and eNOS Upregulation

The expression of ET-1, eNOS, VEGF, NF- κ B, I κ B α and KEAP-1 was evaluated by Western blotting. For each protein, a band with the expected molecular weight was identified: ET-1—24 kDa, eNOS—140 kDa, VEGF—20–25 kDa, NF- κ B—65 kDa, I κ B α —39 kDa and KEAP-1—60–64 kDa. Figure 3 shows representative blots for each protein (Figure 3a) and the graphical representation of protein expression relative to the sham group (Figure 3b–g). Respective membranes stained with Ponceau S are displayed below the blots (Figure 3a).

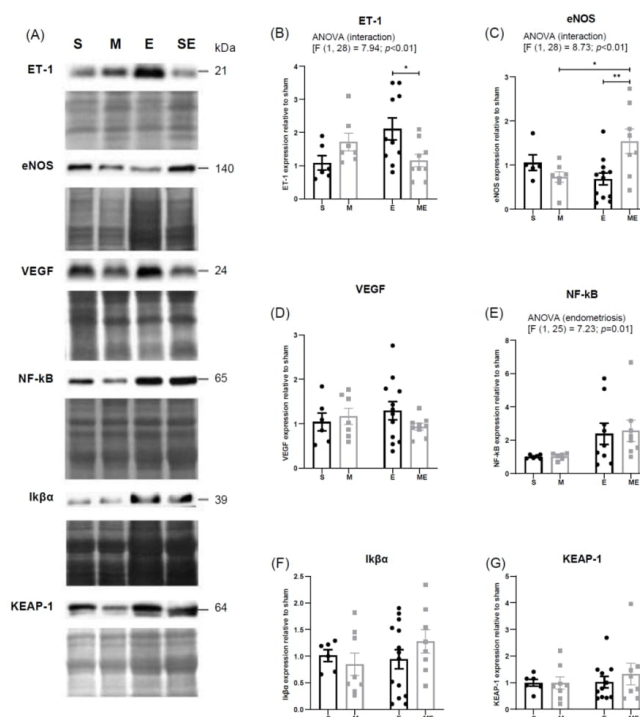


Figure 3. Representative bands of proteins obtained by Western blotting and respective Ponceau S staining (A). Graphical representation of expression levels of ET-1 (B), eNOS (C), VEGF (D), NF- κ B (E), I κ B α (F) and KEAP-1 (G). Expression in groups M, E and ME is displayed as fold increase relative to S group (represented by an arbitrary value of 1). Values are displayed as mean $\pm$ SEM. * p -value below 0.05 and ** below 0.01. n(S) = 5–6, n(M) = 6–8, n(E) = 9–13 and n(ME) = 8–9. Regular two-way ANOVA followed by *post-hoc* with Tukey's test and multiple *t*-tests per row with correction by the Holm–Sidak method was applied. E—endometriosis, M—metformin, ME—metformin/endometriosis and S—Sham. eNOS—endothelial nitric oxide synthase, ET-1—endothelin-1, I κ B α —kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, KEAP-1—Kelch-like ECH associated protein 1, NF- κ B—nuclear factor kappa-light-chain-enhancer of activated B cells, VEGF—vascular endothelial growth factor.

A two-way ANOVA analysis evidenced a statistically significant effect of the interaction of endometriosis and metformin treatment on ET-1 expression [$F(1,28) = 7.94$, $p < 0.01$]; when performing multiple t -tests per row, a decrease in ET-1 expression in group ME comparatively with group E ($p = 0.03$) was found. Group E showed a trend of increased expression relative to group sham ($p = 0.07$). No differences between groups S vs. M ($p = 0.50$) nor M vs. ME ($p = 0.50$) were observed, reinforcing the absence of effect on ET-1 expression when metformin treatment or endometriosis were considered alone.

Likewise, a statistically significant interaction between endometriosis and metformin treatment was found regarding eNOS expression ($F(1,28) = 8.73$, $p < 0.01$); an increase was found in group ME, relative to groups E ($p < 0.01$) and M ($p = 0.03$). No differences in groups E and M relative to S were observed ($p = 0.70$ and $p = 0.50$, respectively).

VEGF expression presented a decreasing trend in group ME compared to E ($p = 0.14$).

An increase in NF- κ B expression was verified in both experimental groups with endometriosis, E and ME [$F(1,25) = 7.23$, $p = 0.01$]; no differences were observed between M and S groups ($p > 0.99$), neither between ME and E groups ($p > 0.99$), supporting the absence of metformin effect. The expression of the NF- κ B inhibitor, I κ B α , had an increasing tendency in group ME relative to group E ($p = 0.16$), but the two-way ANOVA analysis failed to show an effect of both factors, either isolated or associated.

KEAP-1 expression did not vary between groups.

3.4. Endometriosis Downregulates Expression of Cardioprotective MIR199a, MIR16-1 and MIR18a

The expression of MIR199a, MIR16-1, MIR18a, MIR20a, MIR155, MIR342, MIR200a, MIR24-1 and MIR320, assessed by RT-PCR and relativized to the expression of group S (1 arbitrary unit), is depicted in Figure 4.

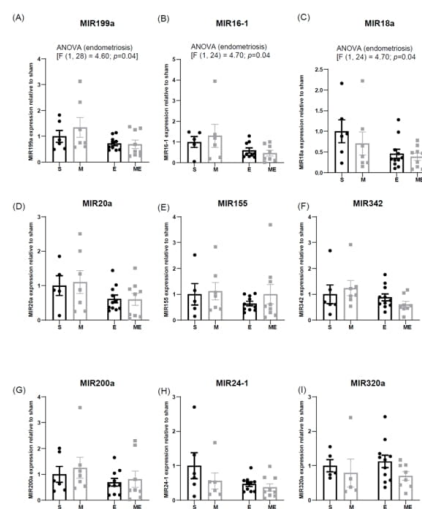


Figure 4. Graphical representation of expression levels of MIR199a (A), MIR16-1 (B), MIR18a (C), MIR20a (D), MIR155 (E), MIR-342 (F), MIR200a (G), MIR24-1 (H) and MIR320 (I) assessed by real-time qPCR. After employing the formula, $2^{-(CT \text{ target miR} - CT \text{ RNU})}$, levels were normalized in groups M, E and ME relative to the expression of S group (represented by an expression value of 1). Values are displayed as mean $\pm$ SEM. n(S) = 5–6, n(M) = 5–7, n(E) = 9–11 and n(ME) = 8–9. Regular two-way ANOVA followed by *post-hoc* with Tukey's test was employed. E—endometriosis, M—metformin, ME—metformin/endometriosis and S—Sham.

Among the studied MIRs, expression of MIR199a, MIR16-1 and MIR18a were decreased in the heart of mice with endometriosis: MIR199a ($F(1,28) = 4.60, p = 0.04$), MIR16-1 ($F(1,24) = 4.70, p = 0.04$), MIR18a ($F(1,29) = 5.80, p = 0.02$); MIR20a almost reached a significant decrease ($F(1,28) = 4.20, p = 0.05$) (Figure 4a–d).

The two-way ANOVA analysis did not demonstrate variation in MIR155, MIR342, MIR200a and MIR24 expression levels, neither in endometriosis nor after metformin treatment (Figure 4e–h). However, expression levels of those MIRs presented a decreasing tendency in groups E and ME, comparatively with groups of mice without endometriosis. MIR320a expression showed a tendency to decrease in both treated groups, M and ME (Figure 4i).

4. Discussion

Until recently, endometriosis had been considered a strictly pelvic disease, but now it is evident that endometriosis is a systemic condition that affects multiple organs [3] and that endometriosis-associated systemic inflammation increases CVD risk [16].

Overt CVD is often reported in women with endometriosis later in their lives [45]. In younger women, there is no evidence of structural vessel alterations, but endothelial dysfunction (ED), which antedates the development of atherosclerosis progressing to CVD, has been recognized as an early manifestation of endometriosis [45].

Reduced expression of eNOS and enhanced activity of ET-1 are important changes in ED; ET-1 is a potent vasoconstrictor and platelet activation inducer, whereas decrement in the expression of eNOS results in NO production inhibition. Together, they contribute to endothelial damage and subsequent CVD [11].

In the current heart study, when endometriosis animals were compared to sham counterparts, an antiparallel variation in the expression of ET-1 and eNOS was observed, suggesting that such mechanisms of endothelial damage are operating in endometriosis [13]. Interestingly, the circumstance that heart changes result from a process originating in a distant (pelvic) location provides support for an extended, systemic nature of endometriosis.

In addition, our data show the upregulation of inflammatory pathways in the experimental condition. When comparing groups E and S, there is an increase in NF- κ B expression in the heart of mice with surgically induced endometriosis. NF- κ B is an important mediator of metabolic and age-induced myocardial disease and a contributor to myocardial inflammation [14] and to the onset of CVD. Its involvement in endometriosis-related inflammation is thus likely, and since these are long-term conditions, the inflammatory process favors the establishment of local structural changes.

In line with NF- κ B expression variation, endometriosis led to increased cardiomyocyte cross-sectional area and heart fibrosis. While the changes may be part of tissue repairing in the beginning, in which cell hypertrophy could be the outcome of a compensatory process, later, tissue fibrosis indicates fibroblast activation, impairment of cardiac function and further contribution to the pathophysiology of CVD [46].

These findings suggesting harmful effects on endothelial cells and on the cardiomyocytes may be the underlying mechanism of endometriosis-associated CVD risk, loss of cardiac function and actual CVD observed in women with endometriosis.

In this setting, one reasonable hypothesis is that the anti-inflammatory known effects of metformin might be useful by playing a protective role on CVD risk in endometriosis. In the current study, in endometriosis animals, metformin administration reversed ET-1 and eNOS expression in the heart in a statistically significant fashion. This is a most interesting finding because it suggests that upon metformin administration, early vascular changes are likely to be prevented, and the CVD-prone condition of endometriosis may be stalled.

However, metformin failed to reduce NF- κ B levels in the whole heart extracts of mice with endometriosis since no differences were observed between groups E and ME. Furthermore, no differences between mice treated with metformin (M) and controls (S) were seen. It was not an expected result, considering the reported anti-inflammatory effect of metformin, recently reviewed by Bai and Chen [47], including the downregulation of

NF- κ B expression in the liver of mice [37] and TNF- α -dependent NF- κ B inflammatory signaling in cultured hepatocytes [37]. The absence of the effect of metformin on NF- κ B expression does not preclude its modulatory effect in the heart. In fact, in cultured breast cancer cells, metformin did not change total NF- κ B levels, although a decrement in its nuclear localization and DNA binding ability was noticed [48].

However, such changes in cell localization or the likelihood of sex- or tissue-specific modulation may underlie the unexpected observation that metformin administration to the healthy animals (M) resulted in a significant increase in the size of cardiomyocytes and a slight increase in fibrosis, possibly related to the hypertrophic effect of the treatment [49]. In fact, cardiac hypertrophy can be a physiological or pathological response that depends on the stimulus and on the signaling mechanisms activated by the cardiac stressor [50].

Sustained activation of Akt/mTOR pathways leads to pathological hypertrophy, but AKT1 seems to also be crucial for a physiological response [50]. As an inhibitor of the Akt/mTOR pathway, we expected metformin to reduce cardiac hypertrophy, as previously reported in both mice and humans [51,52]. In line, novel evidence indicates that metformin mitigates cardiac hypertrophy through epigenetic modification and energy regulation [53,54], but that was not the case. We should consider, however, the fact that metformin reduces estrogen secretion in the ovary [39] and that estrogen confers cardioprotection and delays cardiomyocyte hypertrophy [49]. Most of the studies are conducted in male rodents, but the assessment of cardiac metabolism and longevity in female mice demonstrated a reduction in longevity and cardiac fibrosis in metformin-treated animals compared with controls [55]. In order to elucidate the role of metformin in hypertrophic response, including sex-specificity and dose-dependent patterns, additional studies of cardiac contractility and molecular pathways involved in hypertrophic response ought to be performed.

Similarly to the data related to NF- κ B expression, we found that although endometriosis increased heart fibrosis, metformin treatment failed again to mitigate those changes. Our hypothesis is that the endometriosis-associated structural changes in cardiac muscle are irreversible or, at most, take a longer time to revert.

The growth of endometriotic tissue depends on angiogenesis [17], and the formation of new vessels from pre-existent vasculature. VEGF, whose levels increase not only in endometriotic tissue but also in peritoneal fluid of women with endometriosis, is an essential contributor to angiogenesis [56]. The role of angiogenesis in CVD is unclear, considering that the neo-formation of vessels is important in post-ischemic lesion recovery but also plays a role in the development of atherosclerosis plaques [18]. We did not see differences in heart VEGF expression among experimental groups. The absence of differences was an unexpected finding, considering that endometriosis is a pro-angiogenic condition and that metformin was appointed as a medication that mitigates the pro-angiogenic environment [36]. However, VEGF expression has never been studied in the cardiac tissue of mice with endometriosis. Thus, the importance of VEGF in the progression and treatment of CVD in mice with endometriosis remains to be clarified [18].

The overproduction of reactive oxygen species (ROS) is also part of the pathogenesis of endometriosis and of ischemic heart disease. The tissue responses to the increase in oxidative stress are complex and involve several molecular pathways. Among those pathways, activation of Nrf2, a transcription factor that regulates anti-oxidative defenses, confers protection against CVD in animal models; however, when overactivated, it halts ROS physiological functions [57]. We evaluated the expression of protein KEAP-1 (Kelch-like ECH-associated protein 1), a sensor of oxidative stress that binds itself to Nrf2, promoting its degradation [58], and no differences in the cardiac tissue among experimental groups were found. Despite the absence of changes in the expression of KEAP-1, we cannot exclude oxidative imbalance. In order to elucidate whether endometriosis and metformin intervene in oxidative stress response in the heart, additional analysis, including evaluation of anti-oxidative enzymes expression and activity, will be necessary.

By aiming to elucidate the regulation of the expression of the studied molecules in the heart of mice and to identify putative markers of CVD risk in endometriosis, a selection of MIRs recognized to intervene in such processes was quantified.

MIR18a, MIR199 and MIR342 were appointed as serum markers of endometriosis [20,21,23]. In the heart of mice with endometriosis, however, the expression of MIR18a and of MIR199a was significantly lower, while MIR342 showed a decreasing trend, comparatively with groups S and M. These miRNAs intervene in cardiac cell survival mechanisms, and heart regenerative processes [24,25,27] and their reduced expression in mice with endometriosis indicates that the disease compromises the cardiac regenerative processes, which is supported by the increased cardiac fibrosis observed in such animals. In line with the fibrosis analysis data, metformin treatment had no effect on the expression of MIR18a, MIR199a and MIR342. The only MIR appointed as a serum marker of endometriosis [22] that showed a trend to increase in the heart with endometriosis was MIR320a. MIR320a contained in cardiomyocyte-released exosomes negatively affects cardiac repair and induces cardiomyocyte death [26].

Endometriosis downregulated the expression of MIR16-1, as evidenced in both groups E and ME relative to healthy animals. The MIR16 cluster family targets proangiogenic signaling pathways, including VEGF and VEGFR-2 gene expression, reducing local angiogenesis [27]. VEGF expression showed a trend to increase in animals with endometriosis, which aligns with the lessening of MIR16-1 expression.

Upregulation of MIR24-1, MIR200a and MIR20a is associated with cardioprotective mechanisms, mainly relating to apoptosis and fibrosis pathways regulation [25,28,29]. The reducing trend in the heart of mice with endometriosis may relate to poorer cardiac health. Overall, endometriosis seems to downregulate the expression of the cardioprotective MIRs, and metformin does not revert this condition.

On the contrary, expression of MIR155 that promotes cardiac hypertrophy by targeting calcium signaling [59] and inhibits angiogenesis [27] showed a slight trend to increase in metformin-treated relative to non-treated groups, which aligns with the higher cardiomyocyte areas found in group M.

The present study has some limitations regarding the translation of the results to the clinical setting. While the surgically induced mice model of endometriosis is a valuable tool to investigate pelvic and systemic features of endometriosis, rodents do not spontaneously develop the disease or menstruate.

5. Conclusions

To our understanding, this is the first study on a mice model that aims to elucidate cardiac morphologic alterations associated with endometriosis and the effect of metformin treatment. In summary, whilst treatment with metformin diminished endometriosis-associated endothelial dysfunction, it failed to mitigate NF- κ B-associated inflammation and cardiac fibrosis. In addition, metformin-induced hypertrophy of the cardiomyocytes of mice. In order to fully understand whether the effect of metformin in the cardiac tissue of young mice with endometriosis is globally protected or otherwise deleterious, additional studies are necessary.

Author Contributions: A.F.M. carried out the experiments concerning microscopy and molecular analysis and wrote the draft of the manuscript. A.C.N. created the animal model and performed experiments on animals. A.R.R. and A.M.G. supervised the experiments of miRNAs and protein analysis. S.M.O., C.S.-M. and A.L.-M. conducted the computer-assisted histologic study of the heart. A.F.M., A.C.N., H.A. and D.N. designed the study. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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PUBLICATION III

“Anti-oxidant effect of metformin through AMPK/SIRT1/PGC-1 α /SIRT3- independent
GPx1 expression in the heart of mice with endometriosis”

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Original Article

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Anti-oxidant effect of metformin through AMPK/SIRT1/PGC-1 α /SIRT3– independent GPx1 expression in the heart of mice with endometriosis

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Abstract

Objectives: Endometriosis is a gynecological disease associated with an imbalance between oxidative species production and anti-oxidative defenses. In women, endometriosis has been reported to associate with increased incidence of cardiovascular events. As such, this study aimed to analyze the oxidation-responsive AMPK/SIRT1/PGC-1 α /SIRT3 pathway in the heart of a mouse model of endometriosis. The effect of metformin, an insulin-sensitizing and anti-oxidative drug with already shown positive results in endometriotic tissue was studied.

Methods: Thirty-six female B6CBA/F1 mice were divided into 4 groups (Control-C, Surgery-induced Endometriosis and Metformin-EM (50 mg/kg/day orally administrated for 3 months), Endometriosis-E and Metformin-M). Immunofluorescent labelling of SIRT1 and SIRT3 was performed in the heart tissue. Assessment of expression of AMPK α , SIRT1, PGC-1 α , SIRT3, SOD2, and GPx1 was performed by Western Blotting. The quantification of microRNA(miR)-34a, miR-195, miR-217, miR-155 and miR-421, involved in

the regulation of expression of SIRT1 and SIRT3, was performed by Real-Time PCR.

Results: Data showed an increase in phospho-AMPK α and in GPx1 expression in the EM group when compared to the C group, but not in the total AMPK, SIRT1, PGC-1 α , SIRT3 and SOD2, suggesting a GPx1 expression increase independently of the AMPK/SIRT1/PGC-1 α /SIRT3 pathway. MicroRNAs, excepting miR-217, showed a consistent trend of increase in the M group.

Conclusions: Our study showed that endometriosis does not significantly affect the expression of the components of the AMPK/SIRT1/PGC-1 α /SIRT3 pathway in the heart. However, it indicates that an oxidative condition underlying endometriosis is required for metformin to evidence an increment in the expression of the anti-oxidative enzyme GPx1.

Keywords: AMPK; cardiovascular disease; endometriosis; glutathione peroxidase 1; metformin.

Introduction

Endometriosis is a benign gynecological disease characterized by the presence of ectopic endometrial tissue [1, 2]. It associates with increased incidence of cardiovascular diseases [2, 3], supporting the view that endometriosis is a systemic disease with metabolic involvement.

Considering the lower concentrations and/or activity of antioxidant enzymes associated with deleterious profiles of reactive oxygen species (ROS) production observed in peritoneal fluid and tissues from women with endometriosis, a possible role has been proposed for oxidative stress, defined as the imbalance between the oxidative species production and the anti-oxidative defenses, in the onset and progression of endometriosis [4–6].

In an attempt to identify alternatives to the currently recommended medication for endometriosis [7], we tested

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the *N,N*-dimethylbiguanide-metformin in a mice model of endometriosis. Metformin not only reduces oxidative stress, but also reduces inflammation and regulates insulin sensitivity while lowering glycemia [8, 9]. These changes culminate in the activation of 5'-adenosine monophosphate-activated protein kinase (AMPK), an important cellular nutrient sensor activated by fasting [9] and by ROS increase [10]. Activity of AMPK decreases the nicotinamide adenine dinucleotide (NAD⁺) to NADH conversion rate [11], which increases NAD⁺ levels and activates Sirtuin (SIRT)1, a member of the mammalian NAD⁺ dependent deacetylases family of sirtuins. AMPK and SIRT1 act together in the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) [12, 13], a transcription factor that upregulates expression of SIRT3, an additional member of the family of sirtuins directly involved in the activation of mitochondrial biogenesis/function genes [14, 15]. Moreover, SIRT3 activates Foxo3a that promotes protection against ROS through increase in Mn-dependent superoxide dismutase 2 (SOD2) expression and activity [16, 17]. SOD2 catalyzes the conversion of superoxide radicals into hydrogen peroxide [18, 19], then converted to water by the action of enzymes, such as Glutathione Peroxidase-1 (GPx1) [20]. In response to oxidative stress, GPx1 expression increased concurrently with PGC-1 α through a mechanism mediated by nuclear factor erythroid 2-related (NRF2) [21, 22].

The regulation of the expression of the proteins of the AMPK/SIRT1/PGC1- α /SIRT3 pathway depends on specific microRNA(miR)s. Among those, miR-34a inhibits the expression of SIRT1 and its suppression increases the expression of various antioxidant enzymes [23, 24]. MiR-195 and miR-217 also target SIRT1, which increases ROS and apoptosis in cardiomyocytes and induces senescence in human endothelial cells, respectively [25, 26]. SIRT3 was identified as a target of miR-421 that suppresses its expression, with ensuing increment in ROS in liver cells, by down-regulation of SOD2 and catalase [27]. An increase in SIRT3 was demonstrated in parallel with a suppression of miR-155, a potential oncogene involved in inflammatory response [28].

Supporting the beneficial effect of metformin in endometriosis, regression of endometriotic implants and local increment in SOD expression has been demonstrated in rats [29]. This evidences the importance of mitigation of oxidative stress in the control of the disease. Thus, we hypothesize that metformin, owing to its anti-oxidative properties, mitigates the endometriosis-associated cardiovascular disturbances.

In the present study, we address the role of metformin in the AMPK/SIRT1/PGC-1 α /SIRT3 oxidative stress-responsive pathway in the heart of mice with endometriosis. As well, we aimed to identify the miRNAs involved in the regulation of this pathway.

Methods

Mouse model and endometriosis induction

All animal procedures were undertaken according to the European Community Guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals and were approved by the i3S Animal Ethics Committee (internal reference-2018/27) and the Portuguese Competent Authority (DGAV–Direção-Geral de Alimentação e Veterinária). All investigations in this study adhere to the ARRIVE 2.0 guidelines.

Female B6CBA/F1 mice, the first-generation progeny of a cross between male C57BL/6J and female CBA/CaCrI (Charles River Laboratories, Les Oncins, France) resistant to the after-effects of surgical procedures [30, 31], were bred and housed at the i3S Animal Facility under pathogen-free conditions. All animal handling was performed by in-house personnel under veterinarian supervision and the research team was blinded, receiving only relevant experimental data and biologic materials. During experiments, animals were maintained under a 12 h light/dark cycle in a temperature- and humidity-regulated room ($22 \pm 2^\circ\text{C}$, $55 \pm 5\%$, respectively) with free access to water and standard rodent chow. A total of 36 mice were randomly divided into 4 groups: Control (C; n=6), Endometriosis with Metformin (EM; n=9), Endometriosis (E; n=14) and Metformin (M; n=7). This ensured a minimum of 4–6 mice/group for quantification procedures, a number obtained after application of a simplified formula for sample size calculation in animal studies [32]. Endometriosis was surgically induced [33] in animals of groups E and EM at 6–8 weeks of age under isoflurane volatile anesthesia (5% v/v for induction and 2.5–3% for maintenance), by heterologous transplantation of endometrium from one donor onto the peritoneum of receptors from the same strain, according to a previously described protocol [34]. The endometriosis-like phenotype was confirmed by ultrasound visualization of the implants. Animals from groups C and M were subject to a sham operation. Metformin (Alfa Aesar Chemicals, Massachusetts, USA) was administered orally in drinking water, after 4 weeks of recovery, in a dosage of 50 mg/kg/day for 3 months to mice from groups M and EM. After this time, animals were euthanized, and the hearts were collected and divided in two equivalent halves. One half of each whole heart, longitudinally sectioned, was immediately placed into liquid nitrogen and then stored at -80°C for molecular analysis. The other half was fixed with 4%-buffered formaldehyde, dehydrated, and embedded in paraffin to use in microscopy studies. Equivalent regions of the mice's hearts were used for each experiment.

Immunofluorescence

Five μm sections were cut from two formalin-fixed, paraffin-embedded (FFPE) blocks of each experimental group and adhered onto glass slides. Deparaffinization was conducted by warming the slides for 30 min within a stove at 36°C , followed by a 10 min 100% xylene incubation. Afterwards, sequential 5 min incubations in 100, 90 and 70% ethanol v/v aqueous solutions, were carried out, with a final submersion in water. This was followed by an incubation in a 1% w/v Sudan Black solution in 70% ethanol v/v aqueous solution for 20 min, to attenuate autofluorescence previously noted in heart sections observations. The slides were then incubated for 10 min twice in a phosphate-buffered saline (PBS) and twice in PBS

supplemented with 0.02% Tween 20 (PBST). The slides were incubated for 30 min in a 1 M HCl solution for antigen retrieval, followed by acid neutralization in a 2% w/v sodium tetraborate solution, and washes for 5 min in water and 10 min twice in PBS. Sections were then blocked with a 5% w/v bovine serum albumin (BSA) (Nzytech, Lisboa, Portugal) solution in PBS for 1 h, followed by an overnight incubation in a humidity chamber at 4 °C with the primary antibodies diluted in the same blocking solution: rabbit anti-SIRT1 (ProteinTech, Rosemont, Illinois, USA) 1/100 v/v or rabbit anti-SIRT3 1/300 v/v (Cell Signaling Technology, Devers, MA, USA) diluted with mouse anti- α -actin, a marker of smooth muscle cells, 1/300 v/v (Millipore, Billerica, MA, USA). Negative controls were prepared with primary antibodies omission. After the incubation, the slides were washed twice in PBS for 5 min and twice in PBS containing 1% v/v Triton X-100 (PBSTx) for 5 min. Then, sections were incubated with the appropriated secondary antibodies, anti-mouse conjugated with Alexa Fluor® A568 (Molecular Probes, Leiden, Netherlands) - red and anti-rabbit conjugated with Alexa Fluor® A488 (Molecular Probes) - green, diluted in PBSTx for 1 h at room temperature. The slides were washed twice for 5 min with PBSTx solution, followed by two washes 5 min each in PBS. Finally, nuclei were stained blue with a 1% w/v 4,6-diamidino-2-phenylindole (DAPI) solution (Sigma-Aldrich, St Louis, MO, USA) for 5 min, followed by a rapid PBS wash. The slides were mounted with cover slips using a 50% v/v glycerol-PBS solution and analyzed under an ApoTome fluorescence microscope (Imager Z1, Carl Zeiss AG, Oberkochen, Germany). Images were captured utilizing the AxioVision Software (Carl Zeiss AG).

Western blotting

Tissue fragments were homogenized in lysis buffer solution (0.25% v/v Triton X-100, 50 mM Tris, 10 mM NaCl, 5 mM EDTA, 1 mM β -glycerophosphate, pH 7.6), supplemented with phosphatase and protease inhibitors (phosphatase inhibitor cocktail 2, ref. P5726, 0.4% v/v; phosphatase inhibitor cocktail 3, ref. P0044, 0.4% v/v; protease inhibitor cocktail, ref. P8340, 0.5% v/v, Sigma-Aldrich) with magnetic BulkBeads (Pierce, Montigny-le Bretonneux, France) using the MagNA Lyser Instrument (Roche, Mannheim, Germany). Homogenization followed a protocol of 2 $\times$ 30 s cycles at 6,500 rpm. Then, each sample was ultrasonicated for 15 min and centrifuged at 15 000g for 5 min. All the procedures were carried out at 4 °C. After the centrifugation, the supernatant of each sample was collected into a new tube and preserved at -20 °C. The concentration of protein in each sample was quantified using the Bradford Reagent colorimetric assay (Bio-Rad, Hercules, CA, USA). Twenty-five μ g of total protein per sample were diluted in gel loading buffer (50 mM Tris pH 8.8, 2 mM EDTA, 10% v/v glycerol, 2% w/v sodium dodecyl sulphate (SDS), 0.0125% w/v bromophenol blue) containing 10% v/v dithiothreitol and denatured through gentle shaking for 15 min at 65 °C, followed by 10 min at 95 °C, and centrifugation at 12000 g for 5 min at room temperature. Proteins were then separated in a 12% acrylamide resolving gel within an electrophoresis system applying a constant current of 20 mA/gel until all protein was stacked, followed by a constant current of 25 mA/gel (Thermo Fischer Scientific, Waltham, MA, USA) and transferred to a nitrocellulose membrane (Bio-Rad) in a blotting system (Bio-Rad) at a constant voltage of 30 V for 2 h. After protein transfer, nitrocellulose membranes were stained with Ponceau S and images were acquired in a ChemiDoc XRS + system (Bio-Rad), using ImageLab software, for loading control on the semi-quantification of proteins. Membranes

Table 1: Western-blot antibodies.

Primary antibodies	Concentration v/v
GPx1 – abcam ref. ab22604	1:1,250
pAMPK α – Cell signaling technologies ref. Thr172	1:1,000
PGC-1 α – ProteinTech ref. 66369-1-Ig	1:1,000
SIRT1 – ProteinTech ref. 13161-1-AP	1:500
SIRT3 – Cell signaling technologies ref. D22A3	1:1,000
SOD2 – Santa Cruz biotechnology, Dallas, TX, USA ref. sc-30080	1:10,000
AMPK α (total) – Cell signaling technologies ref. 23A3	1:1,000
Secondary antibodies	Concentration v/v
Anti-mouse – Santa Cruz biotechnology ref. sc-2005	1:10,000
Anti-rabbit – Jackson ImmunoResearch Europe Ltd (Cambridgeshire, United Kingdom) ref. 711-035-152	1:5,000

were blocked with a 5% w/v nonfat dry milk (Molico®) solution diluted in Tris-buffered saline supplemented with 0.1% v/v Tween 20 (TBST) for 1 h, with gentle shaking at room temperature. Primary antibodies (Table 1) were diluted in a 5% w/v BSA solution in TBST containing 1% w/v sodium azide and each membrane was incubated for a minimum of 2 overnights, at 4 °C. Following this incubation, membranes were thoroughly washed for three times 10 min with TBST and incubated for 1 h at room temperature with appropriated secondary antibodies (Table 1), diluted in a 5% w/v nonfat dry milk in TBST. Membranes were then washed with TBST three times 15 min. Semi-quantification of pAMPK α was carried out relatively to total AMPK α , which implies that membranes allocated for the analysis of the former were reutilized for AMPK α analysis by undergoing an antibody stripping protocol, consisting of a thorough TBST wash, followed by a 30 min incubation in a 10% w/v SDS solution and further TBST wash. Afterwards, membranes were again blocked with nonfat dry milk and incubated with the anti-total AMPK α antibody and respective secondary antibody according to previous description.

For detection of protein bands in the immunoblots, the Clarity Western ECL Substrate (Bio-Rad) was used following the manufacturer instructions. Labelled protein band images were acquired using the ChemiDoc XRS + system (Bio-Rad) and the ImageLab software (Bio-Rad).

Semi-quantification of each protein band in the different experimental groups was obtained utilizing the respective Ponceau S lane staining value as a loading control. Afterwards, differences in

Table 2: PCR miRNA Primers.

MiRNA	Reference
RNU	Hs_RNU1A_11 miScript primer assay, ref. MS00013986
miR-34a	Hs_miR-34a_1 miScript primer assay, ref. MS00003318
miR-195	Mm_miR-195_1 miScript primer assay, ref. MS00001792
miR-217	Mm_miR-217_2 miScript primer assay, ref. MS00007966
miR-421	Hs_miR-421_1 miScript primer assay, ref. MS00004165
miR-155	Mm_miR-155_1 miScript primer assay, ref. MS00001701

exposure times that could exist between membranes were corrected by using the average value of samples from control group present in each membrane as a control reference for all the samples at that membrane. Each experiment was repeated 3 times and the repeated values for the same sample were averaged into a single value.

Real Time–Polymerase Chain Reaction (RT-PCR)

Five 15 µm sections were cut from FFPE blocks (n=5/group) using a fresh microtome blade for each sample and placed in RNase-free tubes. Excess paraffin was trimmed, and samples were deparaffinized in 100% xylene, washed with 100% ethanol, and incubated with protease for 2 h at 50 °C, followed by 15 min at 75 °C to allow sample mixtures to clarify. RNA extraction was conducted utilizing the RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fischer Scientific), according to manufacturer instructions. 7 µL of total RNA from each sample were used to generate cDNA using the MysticCq microRNA cDNA synthesis Mix (Sigma-Aldrich Co). [35]. According to the manufacturer's instructions, RT-PCR was conducted by adding 1 µL of cDNA, to 6 µL of PowerUp SYBR® Green Master Mix (Thermo Fischer Scientific), 0.25 µL of Universal Primer and 1.2 µL of miRNA-specific primer (miScript Primer Assay, Qiagen, Hilden, Germany; Table 2). Primers for RNU1a, miR-34a and miR-421 were previously identified identical between human and mouse. RT-PCR was conducted using the StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using 96-thin-well PCR plates. The procedure consisted of a 10 min initial denaturation at 95 °C, followed by 45 cycles of 15 s denaturation at 95 °C, 30 s annealing at 55 °C and 30 s extension at 60 °C. Two control reactions were conducted for each PCR, one excluding Reverse Transcriptase and another excluding poly(A) polymerase. Amplification data were analyzed using StepOne Software (Applied Biosystems). Each PCR was normalized with an internal control using the RNU1a primer assay (Qiagen) and employing the formula $2^{-CT_{\text{target}} - CT_{\text{RNU1a}}}$.

Statistical analysis

The protein expression values for each animal were organized into the respective experimental groups (n=5–13/group) and analyzed with GraphPad Prism 6 Software, version 6.01 (GraphPad Software, Inc). Statistical analysis of protein expression was conducted using a one-way Analysis of Variance (ANOVA) test, followed by a multiple comparisons test utilizing Bonferroni's Correction. Results are presented as mean ± SEM, with p value <0.05 considered statistically significant.

Results

Dual immunolabeling of SIRT1 and SIRT3 in the heart

The results of Immunofluorescence labelling of SIRT1, SIRT3 and α-actin in the heart tissue are visible in Figure 1. SIRT1 (green) was detected in all groups (Figure 1a), apparently more intense in endothelial cells (arrow) in groups E and EM, although some mild dispersed fluorescence is visible within

the myocardium. The marker of smooth muscle cells, α-actin (red), was observed in the vascular muscle layer. Co-localization of both proteins (yellow) was detected, particularly in the EM group. Despite this, overall differences in IF labelling among experimental groups were mild.

Regarding SIRT3, expression was evident in all experimental groups (Figure 1b). Labelling was restricted to endothelial cells (green) without co-localization with α-actin (red) that was observed exclusively in cardiac blood vessels.

Western blotting semi-quantification of pAMPKα, AMPKα, SIRT1, SIRT3, PGC-1α, SOD2 and GPx1

To understand whether endometriosis and/or metformin have an impact on the AMPK/SIRT1/PGC1-α/SIRT3 oxidative stress-responsive pathway in the heart, we conducted a semi-quantification of the protein expression levels through western blotting. Bands of expected molecular weight were identified for each protein: AMPKα-62kDa, SIRT1-140kDa, SIRT3-28kDa, PGC-1α-105kDa, SOD2-25kDa, GPx1-22kDa, representative bands and Ponceau S staining of proteins for the experimental groups were depicted in Figure 2a. Graphical representation of the quantitative variation in expression of each protein among experimental groups is displayed in Figure 2(b–h), where data are presented as fold change relative to control group (1 arbitrary unit).

A 2.5-fold increase in the expression of pAMPKα in the EM group, comparatively with C (One-Way ANOVA p=0.0292, Bonferroni comparison between C and EM p=0.021) was observed (Figure 2b). An increasing tendency that did not reach statistical significance was observed in EM relatively to E and M groups. Expression levels of total AMPKα did not show difference among groups (Figure 2c), despite an equivalent trend to the variation observed for the phosphorylated form of the enzyme.

SIRT1 expression levels showed no difference among the groups (Figure 2d), though a slight increasing trend in E and EM groups congruent with immunofluorescence results is present. Regarding SIRT3 (Figure 2e), PGC-1α (Figure 2f) and SOD2 (Figure 2g), no statistical differences between the groups were observed.

GPx1 expression was significantly higher in EM group compared to C (2.5-fold increase, One-Way ANOVA p=0.0292, Bonferroni comparison between C and EM p=0.021) (Figure 2h). Despite the absence of statistical significance, an equivalent variation trend among groups, relatively to that observed for pAMPK1α (Figure 2b) and AMPK1α (Figure 2c) was observed for GPx1 α.

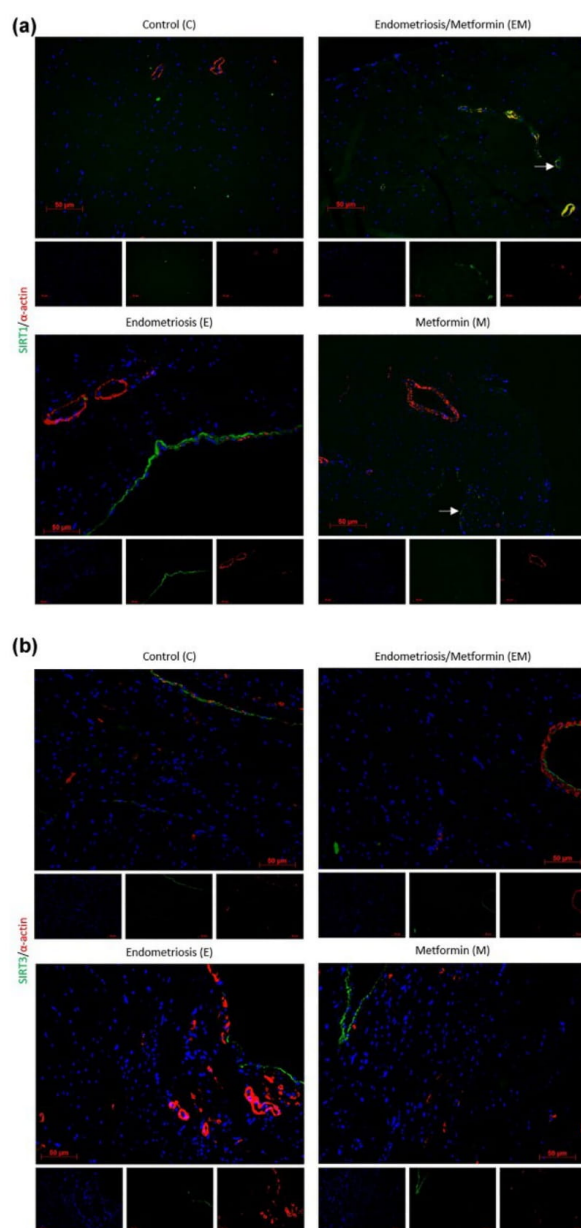


Figure 1: Representative images of dual-immunolabeling of SIRT1/α-actin and SIRT3/α-actin in heart from C:Control, EM:Endometriosis/Metformin, E:Endometriosis and M:Metformin groups. SIRT1 (green) is evident in endothelial cells (arrow) whereas α-actin (red) is marked in vascular smooth muscle cells (a). Co-localization of SIRT1 with α-actin resulted in yellow labeling. SIRT3 (green) was detected exclusively in endothelial cells, without colocalization with α-actin (b). Nuclei were labeled blue (DAPI). Isolated channels are displayed below the corresponding composite images.

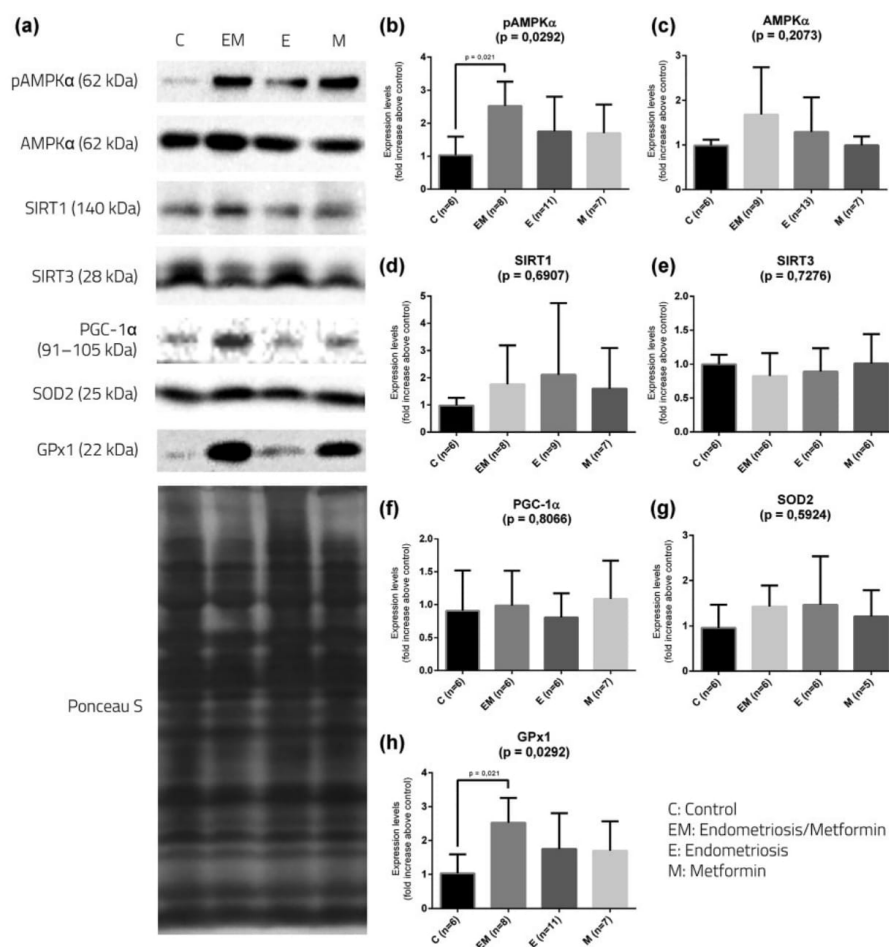


Figure 2: Western-blotting semi-quantification of phospho-AMPKα, AMPKα, SIRT1, SIRT3, PGC-1α, SOD2 and GPx1 levels in mouse heart. (a) Representative blots and of Ponceau S-stained membrane. (b-h) graphical representation of protein band densitometric expression relative to the respective ponceau S band. Expression values in EM: Endometriosis/Metformin, E: Endometriosis and M: Metformin groups are displayed in arbitrary units representing fold increase comparatively to the C:Control group (n=5-13/group). Bars represent the standard error of the mean.

Quantification of expression of miR-34a, miR-195, miR-217, miR-155 and miR421 by Real-Time PCR

RT-PCR results for quantification of the miR-34a, miR-195, miR-217, miR-155 and miR421 are visible in Figure 3. None of these miRNAs showed statistically

significant differences in expression between any groups. MiR-34a (Figure 3a), miR-195 (Figure 3b), miR-155 (Figure 3d) and miR-421 (Figure 3e), all displayed a consistent trend of increase in the M group, while miR-217 (Figure 3c) displayed an opposite trend, with the C group having the highest levels and the EM and M groups the lowest levels.

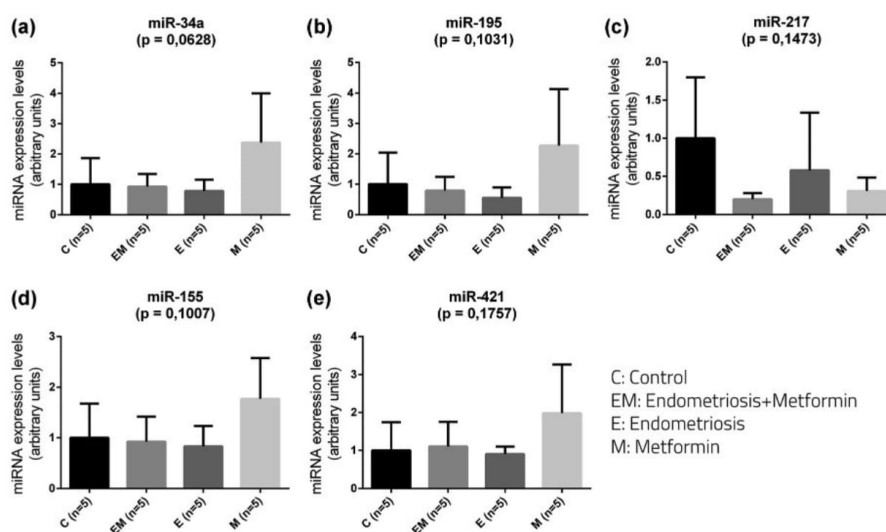


Figure 3: Expression levels of miR-34a (a), miR-195 (b), miR-217 (c), miR-155 (d) and miR-421 (e) by real-time PCR in EM: Endometriosis/Metformin, E: Endometriosis and M: Metformin groups relatively to C:Control. Bars represent the standard error of the mean.

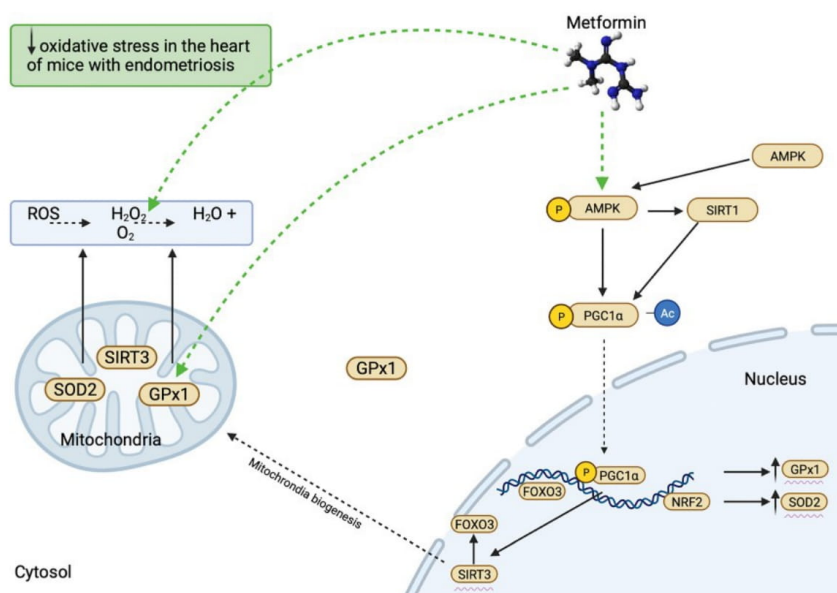


Figure 4: Metformin anti-oxidative effect in the heart of mice with endometriosis, through the increment in phospho-AMPK α and GPx1 (green arrows). The increase in GPx1 accelerates conversion of hydrogen peroxide to water and oxygen, which mitigates oxidative stress.

Discussion

Endometriosis is a chronic condition that increases the risk of developing cardiovascular disorders in women. Additionally, patients tend to manifest subclinical atherosclerosis, resulting in a higher risk of developing disease without overt structural atherosclerotic changes [38]. For over a decade, metformin has been known to have a positive effect on heart diseases, with studies demonstrating an improvement of cardiovascular endpoints and overall mortality in patients undergoing treatment with this biguanide [39, 40]. The positive effect of metformin has been attributed, at least partially, to its intervention on the intracellular ROS balance and in the anti-oxidative response [9]. Despite the exact contribution of oxidative stress in endometriosis having yet to be elucidated, its increase in this disease is well documented [5, 36, 37]. However, to the best of our knowledge, the effect of metformin in the prevention or mitigation of endometriosis-related cardiovascular diseases remains to be investigated. Therefore, in this study, we assessed the effect of the disease and concurrently of the metformin treatment in the expression of the elements of the AMPK/SIRT1/PGC1- α /SIRT3 oxidative stress-responsive pathway in the heart of mice with surgically induced endometriosis.

We found a significant increase in pAMPK levels in the cardiac tissue of mice with endometriosis treated with metformin comparatively with controls. This finding corroborates previous demonstrations that metformin activates AMPK by phosphorylation [41]. However, such increase was not evident in healthy metformin-treated group, nor in untreated animals with endometriosis. Parallel tendencies were visible in AMPK relatively to pAMPK expressions among the experimental groups, with an increase in groups E and M relatively to C, strengthened in EM group. The absence of increment in pAMPK in M group was an unexpected finding, considering prior studies have determined a therapeutic potential for metformin regarding endometriosis through its action on AMPK and ensuing suppression of CYP19A1 expression, confirmed by nullifying its effects upon AMPK silencing in cultured endometriotic stromal cells [42]. However, when compared with the control group, metformin had a differential effect in the phosphorylation of AMPK in mice with endometriosis. This could suggest that metformin is more efficient in the activation of heart anti-oxidative defenses of endometriosis individuals.

Others have demonstrated that the increment in AMPK activation increases activity of SIRT1 through NAD⁺ and phosphorylation of PGC-1 α [12, 13]. Our results showed no difference in either of these

proteins in any experimental group. Additional studies concerning the evaluation of the activity of SIRT1 and the deacetylation and phosphorylation status of PGC-1 α will be necessary to clarify if the verified increase in pAMPK in EM group causes an increment in PGC-1 α activation. According to previous evidence, the activation of PGC-1 α should result in an increment of SIRT3 expression and of SOD2 [14], but the assessment of their expression levels demonstrated that neither SIRT3 nor SOD2 were increased in mice with endometriosis treated with metformin. Taken together, these findings suggest that SOD2 activation is not a primary antioxidative defense in the heart in endometriosis, treated or not with metformin. Consistent with this hypothesis is the absence of differences among groups in the expression of miR-34a and miR-195 that target SIRT1 mRNA and result in decreased expression of antioxidant enzymes [23–25]. As well, miR-155 and miR-421 that act together in the regulation of expression of SIRT3 [27, 28] failed to reveal differences in expression among experimental groups. Interestingly, these four miRs share a trend of increase in the M group, comparatively with other groups. The expression of miR-217, however, shows a trend of decrease when compared with C group. This trend was more evident in groups treated with metformin. This finding only partially matches protein analysis results of SIRT1 expression, raising questions on possible interactions with additional miRs.

GPx1 presented a significant increase in the group of mice with endometriosis treated with metformin (EM) when compared to controls, which does not conflict with previous reports. A prior study in rats evidenced the regression of endometriotic implants concomitantly with increase of GPx1 [43], with a putative involvement of AMPK and SIRT1 in this response; in contrast, another study reported a lower level of antioxidant enzymes in patients with endometriosis, when compared with healthy counterparts [44]. Our findings, in the heart of mice with endometriosis displayed an increase in GPx1 without statistical significance, which is likely to reflect tissue specificity. Indeed, metformin use was shown to increase GPx1 in non-cardiac cells [45].

The increment in GPx1 in EM group relatively to controls is consistent with the differences found in the phosphorylation of AMPK, indicating that metformin is more efficient in the activation of anti-oxidative defenses in the heart of endometriosis individuals than in healthy counterparts. Also, several of the molecular mechanisms activated by metformin are AMPK-independent [46] and further analysis of other molecular pathways will be necessary to fully clarify its effects on the heart of mice with endometriosis.

Our data present some limitations, particularly in what concerns the translation to patients with endometriosis. The surgery-induced rodent model of endometriosis is a valuable tool to test the effect of medication and to identify the systemic impact of endometriosis in mice, but rodents do not spontaneously develop this disease nor exhibit menstruation, which is appointed as a probable cause of endometriosis in women when it occurs retrogradely [47].

This study, on the other hand, strongly indicates that metformin could be beneficial in the control of the endometriosis-associated cardiovascular disease in women. Endometriosis is typically diagnosed several years after the onset of symptoms, and the risk of cardiovascular disease in these patients is frequently underestimated. However, further studies clarifying the effects of metformin in the mitigation of the oxidative stress-related damage are required in order to understand the potential therapeutic value of metformin in patients with endometriosis.

Conclusion

This study investigated for the first time the expression of the elements of the AMPK/SIRT1/PGC1- α /SIRT3 pathway in the heart of an endometriosis-induced mice model treated with metformin. Although the impact is not clear-cut, in this model, the heart responds to metformin by upregulating antioxidant enzymes, as GPx1, and pAMPK (Figure 4). The finding indicates that metformin is cardioprotective through mitigation of oxidative stress and that it could be a valuable medication in the control of cardiovascular diseases in women with this gynecological condition.

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Author contributions: Rodrigo Felgueiras: Data obtaining and curation, Writing – Original draft preparation. A Catarina Neto: Conceptualization and preparation of the animal model. Data curation. Adriana R Rodrigues: Data curation. Alexandra M Gouveia: Writing – Reviewing. Henrique Almeida: Writing-Reviewing and Supervision. Delminda Neves: Writing – Reviewing and Supervision. **Competing interests:** Authors state no conflict of interest. **Informed consent:** Not applicable.

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PUBLICATION IV

“Identification of Proteins and MicroRNAs with Prognostic Value for Assisted Reproduction
Technology Outcomes in Follicular Fluid of Women with Endometriosis: A Pilot Study”

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Article

Identification of Proteins and MicroRNAs with Prognostic Value for Assisted Reproduction Technology Outcomes in Follicular Fluid of Women with Endometriosis: A Pilot Study

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Abstract

This study aims to identify molecular markers with prognostic value for biochemical pregnancy in follicular fluid (FF) samples from women with endometriosis after assisted reproductive technology (ART) intervention. Levels of growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15), and anti-Mullerian hormone (AMH) proteins were semi-quantified by Western blotting and microRNAs 20a_1, 145_1, 320a_1, 125b-5p, 212-3p, and 199_a by qPCR in FF samples from women submitted to ART with a previous diagnosis of endometriosis (n = 20) or male factor infertility (controls) (n = 44). An increase in GDF-9 and BMP-15 and a decrease in AMH mature protein were observed, as well as an increase in miR20a_1 ($p = 0.04$), miR145_1 ($p = 0.003$), and miR320a_1 ($p = 0.006$) in FF samples collected from women with endometriosis compared with controls. A reduction was observed in miR125b-5p ($p = 0.004$) and 212-3p ($p = 0.02$) in endometriosis. Receiver operating characteristic (ROC) curve analysis indicated that miR125b-5p, miR212-3p, and miR-145_1 are potential predictors of endometriosis, and miR145_1 and miR320a_1 of biochemical pregnancy in controls. Although limited by a small sample size, the current study demonstrated alterations in AMH, BMP-15, GDF-9, and specific miRNA levels in FF samples harvested from women with endometriosis, emphasizing their potential roles in endometriosis-related infertility. These microRNAs, dysregulated in women with endometriosis, unveil their biomarker properties and their predictive value for ART success.

Keywords: anti-Mullerian hormone; assisted reproductive techniques; biochemical pregnancy; bone morphogenetic protein-15; endometriosis; growth differentiation factor-9; human follicular fluid; infertility; microRNAs



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1. Introduction

Infertility affects approximately 15% of couples in Western societies [1]. Among the female causes of infertility, endometriosis presents a major contribution, considering that it globally afflicts 5–15% of women of reproductive age and up to 40% of infertile women [2].

Endometriosis is characterized by ectopic presence of endometrial-like glands and stroma that cause pelvic pain and anatomic distortion [3]. The pathophysiology of the disease remains poorly understood, but it is known that endometriosis comprises a complex inflammatory response induced by pro-oxidative conditions, cellular proliferation, migration and invasion, and angiogenesis [4,5].

Patients with endometriosis-associated infertility often require assisted reproductive technology (ART) treatments [6,7]. During oocyte harvesting for in vitro fertilization or intra-cytoplasmic sperm injection, follicular fluid (FF) is aspirated and usually discarded. Because FF composition parallels composition of the serum as a result of molecular diffusion, it presents a high potential for use in the detection of markers with prognostic value, despite rarely being analyzed in the setting of ART treatments. Indeed, FF contains a multitude of hormones, metabolites, polysaccharides, growth factors [8], and microRNAs (miRNAs/miRs) (small, non-coding RNAs approximately 22–25 nucleotides in length) [9,10], mostly secreted by theca and granulosa cells (GCs), that reflect metabolic activity and follicular development phase, influence oocyte quality and success of fertilization, and ultimately embryo development [11].

Growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) are two oocyte-secreted factors (OSFs) that exhibit greater homology between themselves than with the remaining members of the transforming growth factor β (TGF- β) family [12]. Those OSFs appear to intervene synergistically in cellular mechanisms and are crucial for folliculogenesis and female fertility [13]. However, while GDF-9 silencing in mice results in decreased GC proliferation, abnormal oocyte growth, and follicular development failure [14] because GDF-9 inhibits follicular atresia and GC apoptosis [15], BMP-15 induces proliferation of those cells [16].

In contrast to GDF-9 and BMP-15, anti-Müllerian hormone (AMH), also a member of the TGF- β family, is produced by GCs from 36 weeks of gestation until menopause and considered a marker of oocyte reserve [17,18]. In endometriosis patients, AMH levels are diminished in peritoneal fluid, even when serum levels are normal, suggesting that AMH may have a role in the disease [19]. The discrepancy between circulating and local levels suggest differential regulation of AMH synthesis or disposal in endometriosis. In this setting, miRNAs are potential contributors on account of their recognized role as post-transcriptional regulators of gene expression [20]. MicroRNAs are concentrated in exosomes and secreted into body fluids, such as blood and FF, which makes them putative prognostic biomarkers of ART outcome in endometriosis, owing to their easy accessibility and stability under experimental conditions [10,21,22]. Several miRNAs have been implicated in endometriosis-related infertility (Table 1). In particular, miR-125b-5p is upregulated in serum and plasma of women with endometriosis, being considered a putative marker of endometriosis [10] and contributing to systemic inflammation and potentially altering the follicular and oocyte environment through PI3K/Akt (phosphatidylinositol 3-kinase (PI3K)/protein kinase B) and MAPK (mitogen-activated protein kinase) pathways [23,24]. In FF, miR-212-3p and miR-320a have been associated with embryo developmental potential and oocyte and embryo quality, respectively [25–27]. These miRs correlate with fertilization success and embryo quality in in vitro fertilization (IVF) [25], processes that may be compromised in endometriosis [28], with lower levels of both linked to impaired fertilization outcomes [26,29]. miR199a, miR20a, and miR145 are involved in TGF- β signalling [30–32], impacting crucial endometriosis-associated mechanisms, such as hypoxia, angiogenesis,

inflammation, and cell proliferation [33,34]. While miR-20a is frequently dysregulated in endometriosis tissue and serum, which links to disease recurrence, implantation failure, follicular dysfunction, angiogenesis, and cell proliferation [35–37], miR-145 has been shown to suppress GC proliferation in mice [38]. Functionally, overexpression of miR-145 in human endometriotic cell lines (12Z) inhibits proliferation, invasion, and stem-like traits by repressing pluripotency factors and cytoskeletal proteins expression [39]. Moreover, miR-199a levels are also altered in the serum of patients, with lower levels linked to disease severity according to the endometriosis fertility index (EFI) and poorer fertility outcomes, possibly due to miR-199a impact in extracellular matrix remodelling, inflammation, and lesion invasiveness [40–43].

Table 1. MiRNA specific roles on endometriosis-related infertility.

miRNA	Role in Endometriosis-Related Infertility	Reference(s)
miR-125b-5p	Upregulated in endometriosis. Implicated in lesion invasion, inflammation, and alterations of the oocyte/follicular environment. Pathways: PI3K/Akt, MAPK, and inflammation-related signalling.	[10,23,24,40]
miR-212-3p	Higher levels are associated with improved embryo development. Pathways: Regulation of folliculogenesis and oocyte competence (specific targets not consistently identified).	[25,26]
miR-320a_1	Upregulated in serum exosomes of endometriosis patients. Associated with oocyte and embryo quality and IVF outcomes. Lower or altered expression linked to poorer embryo development. Pathways: Regulation of energy metabolism, oxidative stress, and embryo development genes.	[26,29,44]
miR-199a_1	Upregulated in serum of endometriosis patients. Considered in the EFI and related to disease severity. Lower levels correlate with worse EFI and poor fertility prognosis. Pathways: ECM remodelling, inflammation, and lesion invasiveness.	[40–42]
miR-20a_1	Dysregulated in endometriosis. Associated with disease recurrence and lesion biology. Influences implantation and follicular function, impacting fertility. Pathways: TGF- β signalling, cell proliferation, and angiogenesis.	[35–37]
miR-145_1	Downregulated in endometriosis (serum and lesions). Functional studies show regulation of granulosa cell proliferation. Impacts follicular cell behaviour, potentially reducing oocyte quality and ovarian function. Pathways: Cell-cycle regulation and expression of cytoskeletal-related genes.	[38,39]

Abbreviations: PI3K/Akt—phosphatidylinositol 3-kinase (PI3K)/protein kinase B; MAPK—mitogen-activated protein kinase; IVF—in vitro fertilization; EFI—endometriosis fertility index; ECM—extracellular matrix; TGF- β —transforming growth factor β .

To identify biomarkers with prognostic value for ART outcomes in women with endometriosis, AMH, GDF-9, BMP-15, and miRNAs 20a_1, 145_1, 320a_1, 125-b-5p, 212-3p, and 199_a, were quantified in FF samples harvested from endometriosis patients that undertook ART procedures [27].

2. Results

2.1. Population Characteristics

Data relative to women enrolled in this study are summarized in Table 2. The average age of women from whom FF samples were collected, which impacts the expression of AMH and the quality of oocytes, was equivalent between groups: both in those employed for protein analysis (35 and 36.1 years in male factor and endometriosis groups, respectively, $p = 0.5$) and for miRNAs analysis (34.8 and 34.6 years in male factor and endometriosis groups, respectively, $p = 0.8$). As well, no differences were observed in body mass index or parity between women included in the two groups ($p = 0.34$). Neither the mean number of type A embryos observed at day 3 ($p = 0.19$), nor the percentage of transfers at day 3 or day 5, differ between patients with endometriosis and controls.

Table 2. Resume of the characteristics of the women enrolled in the study.

	Age (Years)	Body Mass Index (Kg/m ²)	Parity (Number of Live Births/% of Women)	Type A embryos at 3rd day (Average Number)	3rd Day Transfer (% of Cases)	5th Day Transfer (% of Cases)
Male factor (n = 34)	26–42	23.5	0/88 1/8 2/4	1.81	47.1	52.9
Endometriosis (n = 15)	27–41	21.9	0/67 1/33	2.63	46.7	53.3

2.2. AMH Decreases and GDF-9 and BMP-15 Increases in FF Samples of Women with Endometriosis

A single band with an apparent molecular weight of 60 kDa corresponding to AMH protein was identified in all samples from both groups of women (Figure 1a). A decrease in AMH levels in FF samples of endometriosis patients was found relative to the controls ($p = 0.009$) (Figure 1b).

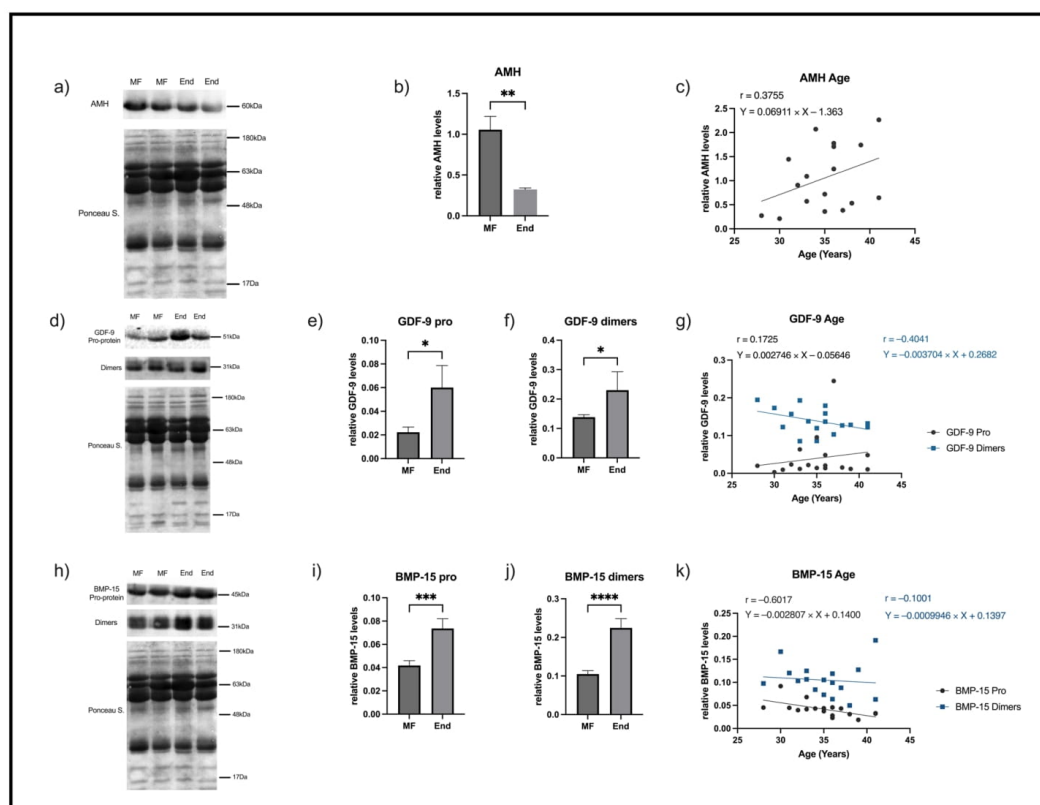


Figure 1. Representative blots and graphical depictions of AMH, GDF-9, and BMP-15 analyses in FF samples from 2 different patients of male factor group (MF) and 2 different patients with endometriosis (End).

(a) A single band corresponding to AMH was detected with a molecular weight of 60 kDa. (b) AMH levels in MF/controls and endometriosis. (c) The correlation between AMH levels and age in controls. (d) Representative blots of GDF-9 pro-protein and homo/heterodimers in FF that show, respectively, a 51 kDa pro-protein single band (e) or 31 kDa dimers (f) in controls and endometriosis. (g) Representation of correlation between GDF-9 and age in controls. (h) Representative blots of FF BMP-15 pro-protein and homo/heterodimers showing, respectively, a single band with an apparent molecular weight of 45 kDa (i) and 31 kDa dimers (j) in controls and End. (k) Graphical representation of the correlation between BMP-15 levels and age in controls. For loading control of immunoblots the respective Ponceau S-stained membrane was used. AMH—anti Mullerian hormone, BMP-15—bone morphogenetic protein 15, End—endometriosis, FF—follicular fluid, GDF-9—growth differentiation factor 9, MF—male factor/controls (n = 17); endometriosis patients (End, n = 7). * $p < 0.05$ compared to MF, ** $p < 0.01$ compared to MF. *** $p < 0.001$ compared with MF, **** $p < 0.0001$ compared with MF.

No correlation of AMH levels in FF with age was found within the group of women without disease through the Pearson correlation test [45] (Figure 1c).

GDF-9 was detected in all the studied FF samples, and three bands with different molecular weight were identified: the pro-protein (51 kDa) (Figure 1d) and the mature protein present in homodimeric/heterodimeric forms presenting two bands with very close molecular weight (about 31 kDa) (Figure 1d). An increase in expression of both the pro-protein (Figure 1e) and dimeric forms (Figure 1f) of GDF-9 in patients with endometriosis relative to the controls was found ($p = 0.01$ and $p = 0.036$, respectively). No Pearson correlation for GDF-9 levels and age [45] in the male factor group of women was found (Figure 1g).

BMP-15 was also identified by Western blotting, and, similarly to what was observed for GDF-9, three bands were detected in all FF samples. A pro-protein (45 kDa) (Figure 1h) more expressed in the group of patients with endometriosis was identified ($p = 0.0009$) (Figure 1i), and in line with this the dual band referring to protein present in homodimeric and heterodimeric complexes (about 31 kDa) (Figure 1j) was also significantly increased in this group ($p < 0.0001$) (Figure 1j). A negative correlation between the pro-form of BMP-15 levels and age ($p = 0.01$) in male factor group of women was found (Figure 1k). No correlation was found for BMP-15 dimer levels and age in those women.

2.3. Levels of miR125b-5p and miR212-3p Decrease and of miR20a_1, miR145_1, and miR320a_1 Increase in FF Samples of Women with Endometriosis

The levels of miR125b-5p, miR212-3p, miR20a_1, miR145_1, miR199a_1, and miR320a_1 assessed by RT-qPCR are depicted in Figure 2.

Levels of miR125b-5p (Figure 2a) and miR212-3p (Figure 2b) were decreased in the FF samples of women with endometriosis when compared with controls ($p = 0.004$ and $p = 0.017$, respectively). On the other hand, levels of miR20a_1 (Figure 2c), miR145_1 (Figure 2d), and miR320a_1 (Figure 2f) were increased in the FF samples of women with endometriosis relative to those without disease ($p = 0.04$, $p = 0.003$ and $p = 0.006$, respectively). No difference was found in miR199a_1 level between the groups (Figure 2e).

To identify whether women's age interferes in the FF levels of miRs, a Pearson correlation between the studied miRNAs and age in patients and controls was carried out separately for each miRNA (Figure 2g–l). Among those analyses, no correlations were found in neither endometriosis patients nor controls.

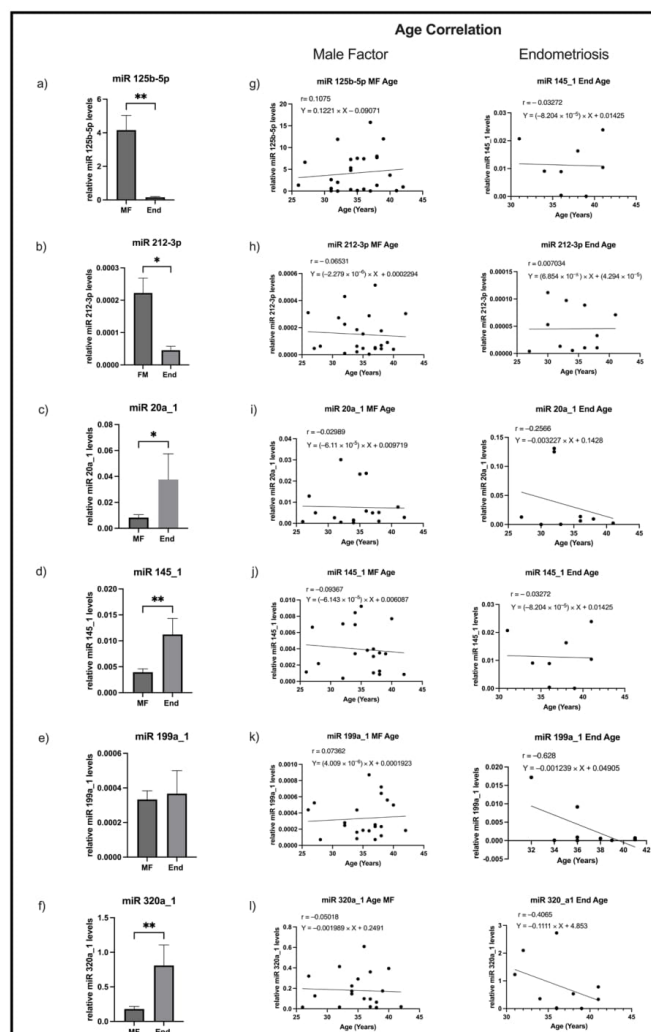


Figure 2. MiRNAs analyses. Graphical representation of expression levels of (a) miR125b-5p, (b) miR212-3p, (c) miR20a_1, (d) miR145_1, (e) miR199a_1, and (f) miR320a_1 assessed by RT-qPCR in FF samples of women with male factor/controls infertility (MF) and with endometriosis (End). (g–l) Graphical representation of correlation between miRs levels and age in male factor/controls (n = 32) and endometriosis women (n = 20). * $p < 0.05$ compared to MF, ** $p < 0.01$ compared to MF. End—endometriosis, MF—male factor.

2.4. MiR125b-5p, miR212-3p, and miR145_1 Levels in FF Are Putatively Predictors of Endometriosis and miR145_1 and miR320a_1 of Biochemical Pregnancy

To assess whether the studied miRNAs could be valuable markers of endometriosis, ROC analysis was performed. Among the studied miRNAs, miR125b-5p (area under the curve—AUC: 0.84; $p = 0.0009$) (Figure 3a), miR-212-3p (AUC: 0.73; $p = 0.03$) (Figure 3b), and miR-145_1 (AUC: 0.74; $p = 0.049$) (Figure 3d) were shown to be putative predictors of endometriosis.

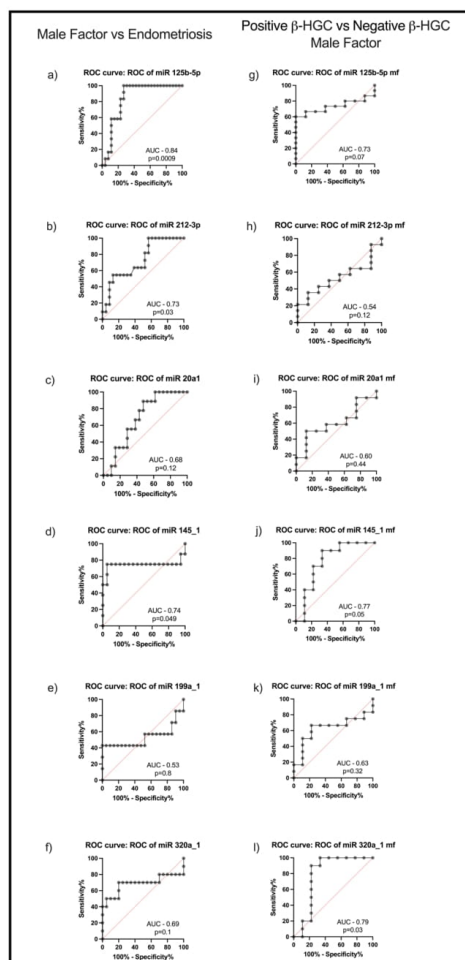


Figure 3. ROC analysis for miRNAs based on the real-time qPCR data. Male factor/controls vs. endometriosis for (a) miR125b-5p, (b) miR212-3p, (c) miR20a_1, (d) miR145_1, (e) miR199a_1, and (f) miR320a_1. Positive β-hCG vs. negative β-hCG in male factor group (g–l). AUC—area under the curve; β-hCG—human chorionic gonadotropin, ROC—receiver operating characteristic.

In addition, ROC analysis was also employed to verify whether studied miRNAs could be used as predictors for biochemical pregnancy achievement (β-human chorionic gonadotropin—hCG-positive test). In the control group of women, both miR-145_1 (AUC: 0.77; $p = 0.05$) (Figure 3j) and miR-320a_1 (AUC: 0.79; $p = 0.03$) (Figure 3l) levels in FF were shown to be indicators of increased probability of biochemical pregnancy achievement. Regarding miR125b-5p levels in FF (AUC: 0.73; $p = 0.07$) (Figure 3g), a strong parallel tendency was observed; however, it did not reach statistical significance.

3. Discussion

Infertility is a public health problem that leads to ART treatments for a growing number of couples. Research on the molecular mechanisms that underlie infertility has

gained increasing importance, and analysis of biological samples, collected when oocytes are retrieved for ART interventions, has been identified as an important approach to predict the success of treatment [46,47]. In particular, FF is a valuable source for studying infertility causes since FF directly contacts and receives the molecules secreted by the oocyte, thus reflecting the microenvironment of the female gametes in ovaries. In the present study, the levels of AMH, GDF-9, BMP-15, and specific miRNAs in FF samples collected from patients with endometriosis were assessed in an attempt to identify molecular markers with prognostic value for biochemical pregnancy.

Our findings show that FF of women with endometriosis presented lower levels of AMH when compared with controls, indicating reduced ovarian reserve and supporting the impaired fertility often observed in endometriosis patients [17]. This result aligns with previous studies showing parallel decrease in AMH levels in FF and serum in women with endometriosis [48,49]. Additionally, Kitajima et al. reported a reduction in AMH levels in the peritoneal fluid, but not in serum, of women with endometriosis, further suggesting a local, pelvic, effect of AMH decline in a reduced fertility disease [19].

The decrease in AMH correlates with the increase in miRs targeting its mRNA, as reported by Belguith et al. who detected an upregulation of miR199a in the plasma of infertile women with dysregulated AMH levels [50]. We did not observe such a difference in miR199a levels in FF samples of endometriosis patients (possibly due to the different sample types here analyzed), but we found an increase in miR20a_1 and miR145_1, which have been reported as intervenients in AMH gene expression regulation through TGF- β signalling modulation [31,32,51,52].

The proteins GDF-9 and BMP-15, primarily secreted by the oocyte [53], are crucial for follicular development and oocyte quality [11,54]. Both proteins belong to the TGF- β family and form homo- and heterodimers [55], detectable in the FF [27,53]. In cumulus oophorus cells, when acting as homodimers or as heterodimers (known as cumulin) [56], they enhance oocyte maturation in vitro and improve embryo development and fetal survival in several animal models [56–61]. Levels of the monomeric form of GDF-9 protein, albeit rarely detected in FF, positively correlate with oocyte maturation and embryo development [27,62]. As well, high BMP-15 levels in FF, particularly in younger women, improve pregnancy chances [62,63]. In agreement with this observation, in the current study FF BMP-15 pro-protein levels in older women were lower when compared to younger women and with all the other studied peptides. This odd finding provides additional support to the view that BMP-15 has a major role in the follicle development of mono-ovulatory species such as humans [64,65] and sheep [66], in contrast to poly-ovulatory species [67], and it is interesting to verify that cumulus cell receptors for BMP-15 in humans show age-related decrements too [68].

In this study, an increase in BMP-15 and GDF-9 levels was found in FF samples of patients with endometriosis, both in pro-proteins and mature proteins in dimers, considering the 31kDa double band identified in blots when BMP-15 or GDF-9 were detected. That increase appears amidst conflicting GDF-9 results which show a decrease or no difference when women with severe endometriosis are compared to women without the disease [69–72]. The isolated assessment of GDF-9 pro-protein, compared to the simultaneous immature and mature protein assay of the present study, may underlie such differences. In addition, this discrepancy may also be attributed to the type of analysis, as some results were obtained from serum or granulosa cells, while others reflect mRNA expression rather than protein levels [70,72]. In contrast to GDF-9, BMP-15 levels agree with a previous observation [71], which provides additional consistency to BMP-15 relevance in human follicle growth and development. As a whole, the consistent increase in both GDF-9 and BMP-15 proteins, and the probable formation of homo- and heterodimers found in FF

samples of women with endometriosis, favour their role as an intrinsic compensatory mechanism to enhance oocyte quality.

The analysis of miRNA levels in FF provides further insight into the molecular landscape of endometriosis. We found a decrease in miR125b-5p levels in FF of patients with endometriosis that contrasts with previous reports showing its upregulation in serum [40]. MiR125b-5p acts as a tumour suppressor, inhibiting cell proliferation and promoting apoptosis [73–75], processes that are dysregulated in endometriosis. Indeed, the decrease in miR-125b-5p in FF locally favours the inflammatory and proliferative nature of endometriosis [76]. Interestingly, the inflammatory response modulator miR212-3p [77,78] is also downregulated, which aligns with the disrupted immune environment in endometriosis. The potential contribution of miR125b-5p and miR212-3p to endometriosis establishment is supported by their significant predictive value for endometriosis found by ROC analysis, anticipating the clinical utility of both miRNAs as diagnostic tools. Besides miR125b-5p and miR212-3p, miR145_1 levels in FF also showed predictive value for endometriosis, distinguishing women with endometriosis-related infertility from fertile women undergoing ART due to male factors, which agrees with previous studies that highlight the role of specific miRNAs in endometriosis diagnosis and pathogenesis [37,79,80]. In addition, levels of miR145_1 and of miR320a_1 were found to be strong predictors of biochemical pregnancy success in women with male factor infertility, reinforcing that miRNAs in FF not only influence endometriosis, but may also impact fertility outcomes. Unfortunately, information relative to biochemical pregnancy outcomes in women with endometriosis is scarce, which impedes us concluding whether those miRs present equivalent prognostic value in those patients and constitutes a limitation of this study.

Levels of miR20a_1, miR145_1, and miR320a_1 were increased in FF of women with endometriosis. According to Andreas et al., the overexpression of miR20a_1, differently expressed in cumulus cells (higher) and oocyte (lower), accelerated maturation of oocytes in vitro [81]. MiR20a_1 directly targets phosphatase and tensin homologue (PTEN) and bone morphogenetic protein receptor type 2 (BMPR2) to regulate their expression both at the mRNA and protein levels in cultured GCs [82] and takes part in TGF- β signalling [31]. In fact, overexpression of both miR20a_1 and miR145_1 [32,51] inhibits TGF- β signalling and suppresses cell migration and invasion [83]. Moreover, in line with our results, miR320a_1 levels were significantly upregulated in serum exosomes from patients with endometriosis [44]. This miRNA presents tumour suppressor properties [84–86] and modulates migration of endometrial stromal cells [87], which might result in a balanced condition when confronted with miR-125. It is interesting to note that miR320a_1 in FF had been associated with high-quality embryos [29], a finding consistent with the correlation we found for miR320a_1 levels in FFs of control women and biochemical pregnancy success. Together with data relative to GDF-9 and BMP-15, the increase in miR20a_1, miR145_1, and miR320a_1 levels in FFs of women with endometriosis supports an adaptive response to increase oocyte quality and mitigate endometriosis progression in patients.

Considering that pregnancy outcome in ART strongly depends on women age, Pearson's correlation between levels of every analyzed molecule in FF and age were assessed. In what concerns OSFs, only a negative correlation for BMP-15 pro-protein and age was found, which agrees with the overall oocyte quality decrement along time [88]. We failed to identify a correlation between miRNA levels and women's age, although previous studies evidenced variation in specific miRNAs with age in serum and in airways [89,90]. Although miRNAs are considered key regulators of reproductive processes [91], we hypothesize that miRNA levels in FF are more likely to be influenced by disease state than by patient age, reinforcing their potential as disease biomarkers.

However, the present study presents limitations. The main is the low sample size, particularly of women with diagnosed endometriosis. In addition, considering that FF analysis is restricted to women that are undertaking ART interventions, it will be of interest to assess levels of miRNAs with potential prognostic value in blood.

4. Materials and Methods

4.1. Follicular Fluid Collection and Processing

FF samples obtained from women aged 26–41 years submitted for ART after informed consent from 2008 to 2020 were assigned by CETI (Centro de Estudo e Tratamento de Infertilidade), Porto. The use of biological samples discarded during processing in ART was approved by the local Ethics Committee (Unidade de Bioética, Biodireito e Economia da Saúde DCSS—Faculdade de Medicina da Universidade do Porto). Rules and directives of the Helsinki Declaration were fully adopted. The harvest of the FF was conducted during the follicular puncture with a 17-gauge needle (Vitrolife, Göteborg, Sweden) on the largest follicle (diameter greater than 17 mm, which predicts higher oocyte maturation and competence—both of which may serve as indirect indicators of oocyte quality) [92] identified by ultrasound at the most reachable ovary in each patient.

For this study, only samples from women who completed their ART treatment were selected. Completion of the treatment cycle was defined as undergoing embryo transfer, regardless of whether it was performed on the 3rd or 5th day of embryo development. The decision regarding the day for embryo transfer was based on the number of oocytes retrieved, fertilization rate, and the number of grade A embryos at the cleavage stage on days 2 and 3 of development. Embryo classification during the cleavage stage was based on three main morphological criteria: cleavage symmetry, blastomere size uniformity, and the extent of cytoplasmic fragmentation [93]. For instance, an embryo at approximately 68 h post-fertilization (corresponding to day 3 of development) is expected to present around eight blastomeres. Ideally, these cells should be symmetrical. Additionally, high-quality embryos (classified as Grade A) typically exhibit $\leq 25\%$ cytoplasmic fragmentation. These characteristics are indicative of optimal developmental. In certain cases, previous endometrial maturation assessments and hormonal analyses on the day of the trigger for oocyte pick-up were performed to facilitate not only the decision on the transfer day but also on whether to proceed with a freeze-all strategy. The therapeutic indications and the execution of the most appropriate technique, either conventional IVF or intracytoplasmic sperm injection (ICSI), were followed to complete the respective cycle of treatment. Following the retrieval of cumulus-oocyte complexes, the FF of each woman were collected into conical-bottom tubes and centrifuged at $200\times g$ for 6 min. After centrifugation, the supernatant was collected, aliquoted into 1 mL fractions, and stored at $-20\text{ }^{\circ}\text{C}$ for subsequent analysis.

Immediately after ovarian follicular puncture, FF samples were anonymized and classified according to the age of the patient and clinical diagnosis. According to infertility origin, FF samples were divided into two groups: male factor (control group) ($n = 44$) and endometriosis ($n = 20$). Data relative to women enrolled in this study are summarized in Table 2. All patients with infertility were evaluated for suspected endometriosis, starting with a clinical history and physical examination, followed by imaging techniques, such as ultrasound and, when indicated, magnetic resonance imaging. Laparoscopy was performed in patients presenting with symptoms strongly suggestive of endometriosis. In our dataset, we included patients with a history of previous surgery, those who underwent adapted hormonal suppression prior to ovarian stimulation, or both. Patients who presented comorbidities, such as diabetes, arterial hypertension, asthma, depression, dyslipidemia, polycystic ovary syndrome, tubal issues, or endometriosis associated with other infertility cause, were excluded from the study. All the patients included in the control group were

evaluated by a gynecologist, independently of the identification of male cause of infertility, to confirm the unlikelihood of endometriosis or exclude additional causes of infertility.

4.2. Semi-Quantification of AMH, BMP-15, and GDF-9 in FF by Western Blotting

Analysis of proteins in FF requires the prior removal of albumin, with the ALBUMINOUT™ kit (G Biosciences, St. Louis, MO, USA) according to the manufacturer's instructions. After this procedure, total protein in samples was quantified by the colorimetric method of Bradford [94]. Then, electrophoretic separation of 15 µg of total protein of each FF sample loaded on a 12% sodium dodecylsulfate–polyacrylamide resolution gel, was performed in a discontinuous buffer system (Biorad Laboratories, Hercules, CA, USA) [95] for approximately 1h at room temperature under a constant electric current of 25 mA per gel.

After electrophoretic separation, proteins were transferred to a nitrocellulose membrane 0.45 µm (Biorad Laboratories), applying a potential difference of 30V for 90 min. After staining with Ponceau S for 2 min, membrane images were captured in Chemidoc TM XRS equipment (BioRad Laboratories). Then, membranes were incubated for 1h at room temperature with blocking solution (5% *w/v* non-fat powdered milk Molico® in TBS with 0.1% *v/v* Tween-20 (TBS.T)), or alternatively 5% *w/v* bovine serum albumin (BSA) in TBS.T for membranes used for the detection of BMP-15, and then with primary antibodies, rabbit anti-AMH (Abcam, Cambridge, UK) diluted 1: 7500 *v/v* or goat anti-BMP-15 (R & D, Minneapolis, MN, USA) diluted 1: 1000 *v/v* in blocking solution, overnight at 4 °C and with stirring. GDF-9 was detected with a rabbit anti-GDF-9 antibody (Abcam) diluted 1:500 *v/v* or 1:250 *v/v* (for hetero- or homodimers, respectively) in blocking solution for two nights at 4 °C. Membranes were then washed with TBS.T and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase diluted in blocking solution for 1 h at room temperature with stirring. Immunoreaction of the membrane was performed through application of chemiluminescent substrate for peroxidase Clarity Western ECL SUBS (BioRad Laboratories) for 5 min, followed by signal detection in Chemidoc TM XRS equipment (BioRad Laboratories). Normalization of the studied proteins was carried out with the corresponding lane stained with Ponceau S. Each experiment was repeated three times.

4.3. Reverse Transcription qPCR

MiRNAs were extracted from FF samples using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Purified RNAs were converted to cDNA using miRCURY LNA RT kit (Qiagen). Then, miRNAs were analyzed in 384-well plates after mixing 1 mL of cDNA with 1 mL of miRNA-specific primer (miScript Primer Assay/miRCURY LNA miRNA assay) (Table 3), 3.5 mL of 2x miRCURY SYBR Green Master mix (Qiagen), and 4.5 mL of MQ water. Real-time polymerase chain reaction (qPCR) was conducted on a CFX Opus 384 real-time PCR system (BioRad Laboratories) and started with denaturation (10 min) at 95 °C, followed by 50 cycles of 15 s denaturation at 95 °C, 30 s annealing at 55 °C, and 30 s extension at 60 °C. PCR data were normalized with a control, RNU5G, using the formula $2^{-(CT_{\text{target}} - CT_{\text{control}})}$ to quantify miRNA's expression levels. RNU5G, a small nucleolar RNA, was selected as the reference gene for miRNA normalization due to its stability, abundance, size similarity, and comparable biological and technical properties to target miRNAs, as well as its high conservation across species. One negative control per experiment was prepared by omission of reverse transcriptase.

Table 3. List of primers used in miRNAs quantification by reverse transcription qPCR.

MiRNA	Reference
RNU5G	hsa_miR-RNU5G miRCURY LNA miRNA PCR Assay (YP00203908)
miR-125b-5p	hsa_miR-125b-5p miRCURY LNA miRNA PCR Assay (YP00205713)
miR-212-3p_1	Mm_miR-212-3p_1 miScript Primer Assay (MS00024570)
miR-20a_1	Hs_miR-20a_1 miScript Primer Assay (MS00003199)
miR-199a_1	Hs_miR-199a_1 miScript Primer Assay (MS0006741)
miR-145_1	Hs_miR-145_1 miScript Primer Assay (MS00003528)
miR-320a_1	Hs_miR-320a_1 miScript Primer Assay (MS00014707)

4.4. Pregnancy Assessment

All participants were instructed to undergo biochemical pregnancy assessment 12 days post-embryo transfer. This involved the collection of peripheral blood for the quantification of β -hCG levels by quimioluminescence analysis. In cases of a positive β -hCG result, clinical pregnancy was confirmed approximately 15 days later via transvaginal ultrasound.

All data were systematically recorded by associating the respective sample code with the corresponding pregnancy follow-up outcomes.

4.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 10.3.1 (GraphPad Software Inc. San Diego, CA, USA). Results are presented as mean $\pm$ standard error of the mean (SEM). Comparative analysis of the proteins studied was carried out by Student's *t* test. The analysis of miRNAs was performed using a receiver operating characteristic (ROC) curve. Probability values of $p < 0.05$ were considered statistically significant. For the correlation analysis of the studied protein levels the Pearson correlation test was employed [45]. Considering that this is an exploratory study carried out in a small group of women, in which data are collected with an objective but not with a prespecified key hypothesis, multiple test adjustments, such as Bonferroni correction, were not performed [96].

5. Conclusions

In sum, our pilot study reveals significant alterations in AMH, BMP-15, GDF-9, and specific miRNAs in FF samples from women with endometriosis. While the increase in BMP-15 and GDF-9 may represent a compensatory mechanism to increase oocyte quality, miRNA dysregulation highlights their role as biomarkers and potential therapeutic targets in both endometriosis and ART management. These findings set the stage for further investigations into the molecular pathways influencing fertility in women with endometriosis.

Author Contributions: A.C.N. conceived the research and wrote the original draft, A.C.N. and A.R.R. obtained and analyzed data, C.F. diagnosed patients, Â.R. collected the samples during A.R.R. techniques, H.A. and D.N. supervised and wrote and revised the manuscript, and J.L.S.-C. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The use of biological samples discarded along processing in ART was approved on 2 April 2013 (the ethical code has not been attributed to the approval document) by the local Ethics Committee (Unidade de Bioética, Biodireito e Economia da Saúde DCSS—Faculdade de Medicina da Universidade do Porto). Rules and directives of the Helsinki Declaration were fully adopted.

Informed Consent Statement: Informed consent was obtained from all women submitted to ART involved in the study.

Data Availability Statement: The data is not publicly available due to privacy and ethical restrictions but are available from the corresponding author by reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AUC	Area Under the Curve
AMH	Anti-Mullerian hormone
ART	Assisted Reproductive Technology
BMP-15	Bone morphogenetic protein-15
BSA	Bovine Serum Albumin
CETI	Centro de Estudo e Tratamento de Infertilidade
END	Endometriosis
FF	Follicular fluid
GC	Granulosa cells
GDF-9	Growth differentiation factor-9
β-hCG	Human Chorionic Gonadotropin
ICSI	Intracytoplasmic Sperm Injection
IVF	In vitro fertilization
MF	Male Factor
miR	MicroRNA
MiRNA	MicroRNA
OSFs	Oocyte-Secreted Factors
ROC	Receiver Operating Characteristic
TBS	Tris-buffer Saline
TGF-β	Transforming Growth Factor β

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PUBLICATION V

“Follicular Fluid redox involvement for ovarian follicle growth”

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REVIEW

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Follicular Fluid redox involvement for ovarian follicle growth

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Abstract

As the human ovarian follicle enlarges in the course of a regular cycle or following controlled ovarian stimulation, the changes in its structure reveal the oocyte environment composed of cumulus oophorus cells and the follicular fluid (FF). In contrast to the dynamic nature of cells, the fluid compartment appears as a reservoir rich in biomolecules. In some aspects, it is similar to the plasma, but it also exhibits differences that likely relate to its specific localization around the oocyte. The chemical composition indicates that the follicular fluid is able to detect and buffer excessive amounts of reactive oxygen species, employing a variety of antioxidants, some of them components of the intracellular milieu. An important part is played by albumin through specific cysteine residues. But the fluid contains other molecules whose cysteine residues may be involved in sensing and buffering the local oxidative conditions. How these molecules are recruited and regulated to intervene such process is unknown but it is a critical issue in reproduction. In fact, important proteins in the FF, that regulate follicle growth and oocyte quality, exhibit cysteine residues at specific points, whose untoward oxidation would result in functional loss. Therefore, preservation of controlled oxidative conditions in the FF is a requirement for the fine-tuned oocyte maturation process. In contrast, its disturbance enhances the susceptibility to the establishment of reproductive disorders that would require the intervention of reproductive medicine technology.

Keywords: *Cumulus Oophorus* cells, Follicular fluid, Oocyte maturation, Oxidative stress

Background

The ovarian follicle is the center of a complex network of systemic and local signaling pathways [1–3] whereby the follicle grows and the oocyte acquires maturity, which is necessary for fertilization to occur and for a viable embryo to develop.

This is a long process that in its early course does not exhibit striking structural features, apart from enlargement of oocyte volume and an increase in the number of its surrounding cells. Then, a distinct cellular and liquid environment is established and encircles the evolving oocyte. It consists mainly of *cumulus oophorus* (c.o.) cells and the follicular fluid (FF), that occupies the antrum (Fig. 1a, b).

The complexity of the structural and functional changes observed in this tissue, that comprises cells with different lineages, and the remarkable mixture of biomolecules present in the FF is by itself surprising. But, how the regulation is carried out in order to result in ovulation of a good quality oocyte, is extraordinary. The purpose of this review is to focus on a part of this regulation.

The follicular microenvironment – *Cumulus oophorus* cells

C.o. cells are a structural feature of the antral follicle, easily identifiable because they immediately envelope the oocyte. Despite the difference in cell lineage, their anatomical proximity and the mutual, paracrine actions they perform [4, 5] have contributed to the establishment of the concept of a single entity, designated cumulus oocyte complex (COC).

The c.o. cells originate from the structurally homogeneous granulosa cell layers of the pre-antral follicles; however, during follicle growth, they become functionally distinct from the mural granulosa cell counterparts

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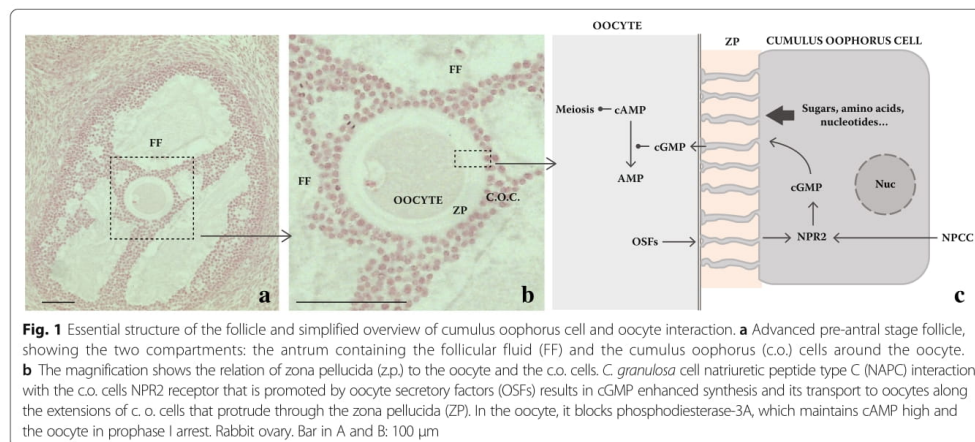
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as they exhibit different proliferative and steroid secretory abilities, in an oocyte dependent manner [6]. In addition, c.o. cells have cytoplasmic extensions, whose tips, upon protruding through the zona pellucida, make direct contact with the oocyte cell membrane by way of gap junctions [4]. In their molecular organization, small calibre molecular channels connect both cytoplasmic across the intercellular space; however, while the channels limit the passage of large molecules, they allow the small ones to flow and exert actions in the target cell. As consequence, c.o. cells become the source of small nutrients to the oocyte, as sugars, amino acids and nucleotides [7], and they are also the origin of signal molecules that regulate the progression of meiosis. Moreover, c.o. cells supply the oocyte with reduced glutathione, which is a major intracellular antioxidant [8, 9], and with cysteine for additional glutathione synthesis [10]. C.o. cells thus afford protection to the oocyte, notably from oxidative stress [11, 12].

The communication c.o. cells/oocyte is not a one-way communication. Indeed, the structural organization of the COC allows transportation of molecules in both directions, implying that signals originated in the oocyte may interact with appropriate receptors on c.o. cells and modulate their function. In fact, it was reported that while removal of the oocyte from the COCs holds proliferative and secretory abilities of c.o. cells, resuming the contact with the oocyte resulted in the regaining of those properties [6]. These and other previous findings favoured the existence of diffusible oocyte secretory factors (OSFs) that act on the c.o. cells and modulate their proliferative and secretory abilities [13, 14]. OSFs behave as growth factors and include the growth differentiation factor 9, GDF9, and the structurally related bone

morphogenetic protein 15 (BMP15, former GDF-9B), members of the Transforming Growth Factor Beta (TGF β), superfamily [15]. Usually, this group of proteins exhibits a set of seven conserved cysteine residues at the mature region but both GDF-9 and BMP-15 have only six residues of the seven-cysteine set; moreover, BMP15 contains two additional cysteine residues upstream of the first conserved cysteine [16]. While, the absence of one cysteine in GDF9 and BMP15 restrains their ability to establish covalent binding and dimerize, their non-covalent binding still results in functional homo or heterodimers [15]. Interestingly, the heterodimer arrangement shows stronger biological activity compared to the homodimers and appears to promote a different Type 1 and Type 2 TGF β receptor assembly [17, 18].

The importance of these factors for normal follicle development is high. The ovaries of *Gdf9* knockout mice have normal structure but the follicles do not develop beyond the primary follicle stage [19] and granulosa cells proliferative response is weaker [20]. *Bmp15* knockout female mice have ovulation disturbances and smaller COCs [21].

Upon OSFs interaction with c.o. cell membrane receptors, a transductive pathway is initiated, involving SMAD factors [15, 22]. Following their translocation to the nucleus, they function as transcription factors and activate genes involved in c.o. cell proliferation [13] and glycolysis for pyruvate production [23], extracellular matrix expansion [24], cholesterol synthesis [25] and intra-oocyte glutathione level regulation [26].

OSFs are thought to be determinant for the establishment of c.o. cells lineage, with implications to c.o. cells proliferation and metabolism [27]. However, recent evidence favors the existence of other determinants of c.o.

cells distinct features as cell response may differ according to the size of the follicle [28].

An important effect of OSFs is to promote c.o. cells NPR2 receptor expression which, when activated by the granulosa cell Natriuretic peptide type C, enhances c.o. cells cyclic Guanosine Monophosphate (cGMP) synthesis; in turn, cGMP is transported across the gap junctions to the oocyte where it inhibits phosphodiesterase 3A and thus prevents cyclic Adenosine Monophosphate (cAMP) hydrolysis. While cAMP level is high, the oocyte is arrested in prophase I [29]; however, when LH surge ensues, cGMP flow to the oocyte is blocked, which results in intra-oocyte drop of cAMP level and release of the break on meiosis (Fig. 1c).

These fine-tuned c.o. cells actions, whose end-point is successful fertilization, suggest that a change in their structural arrangement around the oocyte or in their function might be related to infertility. Indeed, the description of c.o. cells structural properties and the assignment of a quantitative score thereof, have been used to predict fertilization ability [30] although the subjective nature of the interpretation has posed natural accuracy concerns [31].

The follicular microenvironment – Follicular Fluid

The presence of FF, the other component of the oocyte microenvironment, is noticed early in the pre-antral stage of the ovarian follicle when small, apparently empty spaces appear between granulosa cells (Fig. 1a, b). During the following months, as the follicles grow, to reach 20 mm of diameter and more in the case the dominant follicle stage is attained [1], the FF occupies a single compartment that enlarges progressively until it surrounds most of the c.o. cells and the oocyte.

The FF contains a complex mixture of steroids, metabolites, polysaccharides, proteins and small peptides, reactive oxygen species (ROS) and antioxidant enzymes [32, 33]. Probably, apart from acting directly as cellular signals, the mutual interaction of many of these FF molecules, or their absence, will also contribute to appropriate follicular growth and oocyte maturation.

The identification of FF peptides and related proteins has been the subject of intense research. In the last two decades, the emergence of powerful techniques for protein analysis, as 2D PAGE combined with MALDI-MS or liquid chromatography MS/MS, opened wide perspectives for the study of the oocyte liquid environment by identifying a large number of FF proteins.

Nearly 50% of FF proteins have extracellular origin; most of the remaining proteins can be related to organelles or other cell features; regarding their biological activities, about 20% are enzymes, 14% are part of the extracellular matrix or are involved in cell adhesion, and another 16% include regulatory proteins as growth

factors, protease inhibitors, transcription factors and other signaling related molecules [32]. Therefore, the FF compartment has a remarkable diversity of biomolecules; however, the fact that many of the proteins are enzymes, suggests that the compartment is not a mere reservoir but also supports intense metabolic activity, with impact on c.o. cells functioning and oocyte fate.

Angelucci et al. reported 695 different FF proteins or peptides, of which 625 were common to plasma and 27 FF additional proteins were considered absent from the plasma [34]; these figures were enlarged to over 3700 peptides but only 480 proteins received firm identification [32]. When these findings were combined with previous data [34, 35] an impressive list of 789 different FF proteins was generated that included 64 FF specific proteins [32].

Many of the identified proteins relate to biological processes as acute response signaling, inflammation, coagulation and complement cascades. In this respect, plasma and FF seem indistinguishable [34–36], indicating that although they are localized in distinct compartments, interchange between them exists. This is not unexpected because there is a rich vascular network around the growing follicle [37] that favors plasma proteins movement across the vessel walls into the FF compartment [38]. Whether some selectivity exists is controversial; while some suggest it [38], others argue that there is no control on the passage of molecules when ovulation approaches [39], supporting the view that the process has similarities with an inflammatory reaction [40].

The blood compartment is not the sole source of biomolecules to the FF because proteins also originate in mural granulosa cells, the c.o. cells and the oocyte [7, 32]. Assuming that identification of new FF molecules will continue, it is likely that other, low weight or low concentrated peptides will be identified in the future and, eventually, be ascribed to reproductive disorders.

The delicate nature of molecular interactions that fine tune the COC functioning, as long as the follicle grows until ovulation, requires an appropriate environment that is thus provided by the local organization and interaction of c.o. cells and the FF. However, these players have a profound difference: while cells are efficient and adaptable entities, albeit small, the FF compartment is a reservoir with limited versatility. So, to maintain a balance in a complex mixture of biomolecules, it is reasonable to admit that important local chemical buffering systems are required.

Redox reactions and their regulators are likely to fit into this requirement. On the one hand, redox reactions are at the foundation of cell metabolism and, therefore, are a basic condition for life existence; in addition, their regulated intervention is important for a variety of reproductive processes and their dysregulation seems to

contribute to reproductive disorders [41]. Furthermore, important players involved in redox balance were identified in FF composition [34].

Oxidation and effects of oxidants on biomolecules.

Antioxidant balance

Among the oxidants that affect cell functioning, ROS are compounds inevitably produced in the course of cell metabolism. The primary oxidant is superoxide, $O_2^{\cdot-}$ (Fig. 2), that is rapidly dismutated by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2). Either by itself or upon conversion into more reactive species, their continued production imposes an oxidative burden on cells and harmful consequences in biomolecules as sugars, nucleic acids, lipids and proteins.

Briefly, polysaccharide oxidation results in polymerisation loss [42], the formation of advanced-glycation end products (AGEs) when reacting with amino groups, and further oxidation when AGEs interact with their receptors [43]. In nucleic acids, ROS target their sugar moieties, purines and pyrimidines, resulting in strand breaks, base deamination or loss, and adduct formation. Together with the failure of repair mechanisms, DNA may be irreversibly damaged due to the establishment of mutations and result in cell death. In lipids, the C = C double bonds of unsaturated fatty acids are important ROS targets; by continuously forming peroxy radicals, they extend the damage to nearby lipids, produce more hydroperoxides and affect membrane structure and functional properties.

Proteins are another category of biomolecules subjected to oxidation. The effect of ROS on them is diverse, and relates to the intensity of the challenge, the oxidized amino acids and the type of protein involved [44]. Cysteine and methionine are very sensitive to oxidation, but are amenable to revert on account of reductive enzymes [45]. A particularly important effect is carbonylation which causes irreversible damage by exposing hydrophobic residues previously buried in the protein structure. All these changes lead to the unwanted state of oxidative stress, in which there are too many ROS in relation to the available antioxidants [43].

Fortunately, organisms are endowed with intracellular and extracellular antioxidants whose effect is to delay, prevent or remove oxidative damage to a target molecule [46]. They may be enzymes, the most important of which are SOD isoforms, catalase (CAT) and glutathione peroxidase (GPx). The metalloenzyme SOD promotes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 and is considered the first defense line against ROS by way of three mammalian isoforms: the cytosolic copper zinc SOD (CuZnSOD or SOD1) [47], the mitochondrial manganese SOD (MnSOD or SOD2) [48] and the extracellular copper and zinc containing SOD (EC-SOD or SOD3) [49]. The action of SOD enzymes results in enhanced H_2O_2 production, controllable by CAT and GPx activity [50]. CAT localizes mainly to peroxisomes, where they convert H_2O_2 to water and molecular oxygen [51]. In turn, there are four human GPx isoenzymes: the GPx1, cytosolic and mitochondrial, the cytosolic GPx2, the extracellular GPx3 and the phospholipid hydroperoxide

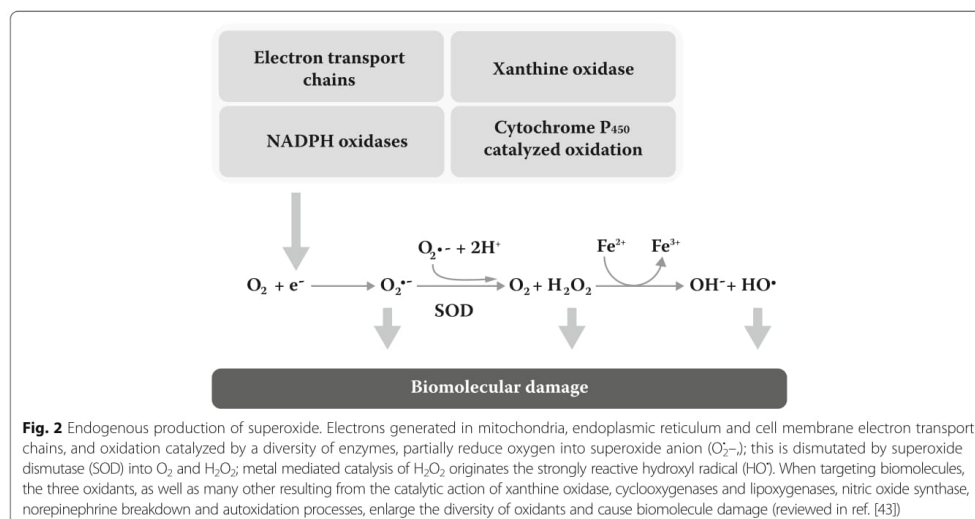


Fig. 2 Endogenous production of superoxide. Electrons generated in mitochondria, endoplasmic reticulum and cell membrane electron transport chains, and oxidation catalyzed by a diversity of enzymes, partially reduce oxygen into superoxide anion ($O_2^{\cdot-}$); this is dismutated by superoxide dismutase (SOD) into O_2 and H_2O_2 ; metal mediated catalysis of H_2O_2 originates the strongly reactive hydroxyl radical ($HO^{\cdot}$). When targeting biomolecules, the three oxidants, as well as many other resulting from the catalytic action of xanthine oxidase, cyclooxygenases and lipoxygenases, nitric oxide synthase, norepinephrine breakdown and autooxidation processes, enlarge the diversity of oxidants and cause biomolecule damage (reviewed in ref. [43])

GPx4 [52]. They neutralize H_2O_2 or organic peroxides to water or alcohol, while converting reduced glutathione (GSH) to oxidized glutathione (GSSG) [53].

In addition, other non-enzymatic antioxidant compounds are endogenously synthesized or acquired from the diet. Coenzyme Q10 (CoQ), uric acid (UA) and GSH are examples of endogenous antioxidants. CoQ, which also plays a central role in the mitochondrial respiratory chain, is an antioxidant that prevents or neutralizes lipid peroxyl radicals and regenerates vitamin E [54]. In humans, UA acts as a strong scavenger of singlet oxygen and hydroxyl radicals and is oxidized mainly to allantoin [55]. Apart from its participation in enzymatic reactions, GSH is a direct, potent scavenger of free radicals; it is oxidized to GSSG and is subsequently reduced to GSH by glutathione reductase, thus maintaining cellular supply of GSH [56]. Nutrient antioxidants include ascorbic acid (vitamin C), naturally present in fresh vegetables and in some mammals. Primates lack the enzyme that catalyzes the last step in ascorbate synthesis [57]. The biologically active form, L-ascorbic acid, is a strong reducing agent that readily neutralizes free radicals and harmful ROS, such as the hydroxyl radical, H_2O_2 and singlet oxygen [58]. The fat-soluble alpha-tocopherol (vitamin E) has high antioxidant potency, particularly as cell membrane protector. It prevents lipid peroxidation by scavenging peroxyl, oxygen and superoxide anion radicals while converting itself to the alpha-tocopherol radical that may be reduced back to its original form by ascorbic acid, CoQ or other cellular reducing compounds [59]. Folate is another important free radical scavenger with antioxidant properties [60]. UA, ascorbate and tocopherol plasma levels are reflected in the frequently used assay of the total antioxidant capacity, TAC [61].

Follicular Fluid redox activity involvement in reproductive modulation

ROS are usually considered harmful agents because their effects modify the structure and functioning of biomolecules but some ROS are also useful signalling agents.

Hydrogen peroxide is at the centre of such processes. Following early reports showing that H_2O_2 is required for growth factor signal transduction [62], the scope of ROS involvement in signalling increased. The multifaceted reproductive system is an example of the expansion of ROS physiological and regulatory roles to folliculogenesis, oocyte maturation, luteal regression and fertilization [41]. In fact, H_2O_2 appears to intermediate crucial changes as is c. o. enlargement, that occurs during the pre-ovulatory LH surge, which can be mimicked by the addition of H_2O_2 and is amenable to inhibition by antioxidants [63]. Less clear is the limit between signalling and unwanted or harmful consequences. In a

selected process as oocyte maturation, it was estimated that 60 ng of ROS/oocyte maintains it in diplotene arrest, whereas a moderate increase in ROS production to 80 ng/oocyte is able to resume meiosis [64].

It has been recognized that important targets of signalling ROS are thiol groups of «free» cysteine residues, not involved in the establishment of disulphide bonds [65]; these are widespread at the surface of proteins and may be oxidized rapidly, but reversibly, thus contributing to attenuate the oxidative insult and to prevent its spread along the entire protein [65]. The diversity of modifications exhibited by oxidized cysteine thiol group [66] is evidence of its versatility and ability to acquire unique functional properties [67]. Such modifications, apart from the simple disulfide bond formation, include the glutathionylation and nitrosylation and other forms of increasing thiol level of oxidation as are the sulfenic, the sulfinic and the sulfonic acid modifications [66, 67].

Not all cysteine residues are regulatory targets [65, 68]. However, there is evidence that different categories of proteins are susceptible to oxidation through cysteine thiol groups and it is likely that different experimental conditions will produce specific sets of intracellular or secreted modified proteins [66, 67]. Susceptible proteins include tyrosine phosphatases, proteases as caspases, adaptors and chaperones as heat shock proteins, transcription factors as the bacterial OxyR protein, p53, hypoxia-inducible factor, redox modulator proteins, as thioredoxin and peroxiredoxin, and other intracellular and extracellular proteins as albumin, transthyretin, high-mobility group protein box 1 (HMGB1), profilin and vimentin [66, 67]. As even non-active residues, may also be targeted by oxidation, provided they are localized in a susceptible position, it was estimated that >500 proteins are in such condition [69], thus making the oxidation of cysteine thiols a process with the potential to affect a variety of biological functions.

In contrast to the intracellular compartment, extracellular spaces such as plasma and FF compartments are oxidizing environments that affect their contents and, in particular, contribute to the diminished ratio of reduced cysteine. The condition results in near absence of free cysteine [66] and in the decrement of free thiols of the FF compartment, which appears critical for the redox balance and has strong implication to the reproductive outcome [70].

The exposure to an oxidative environment of the OSFs BMP15 and GDF9 present in the FF suggests that the thiols from cysteine residues at the pro-region are susceptible to oxidize. Although this event may prevent the oxidation of the remaining molecule, it would do so at the expense of the OSFs ability to dimerize or heterodimerize [17] and interact with their c.o. cells receptors, which is a necessary step to promote COC development and oocyte maturity.

FF samples obtained from women upon controlled ovarian stimulation contain common antioxidants as SOD isoforms 1, 2 and 3, GPx, CAT, glutathione S-transferase (GST), peroxiredoxins (Prx) and glutathione reductase [32, 34, 35]. Beyond their presence, enzyme activity was also detected [71, 72], favouring the existence of an ongoing antioxidation in the FF, eventually intensified close to ovulation when follicles enlarge considerably [73]. Other, important antioxidants as GSH, vitamin E and SOD were assayed in the FF and their levels were found to parallel serum levels [74]. These findings further support the view that an effective communication between FF and plasma compartments exists and that understanding the redox regulatory mechanisms in plasma will improve the knowledge on similar mechanisms operating in FF.

Albumin, a major component of both the serum and the FF, is well known for its role in osmotic regulation. In addition, albumin has antioxidant properties. In fact, its structure includes a cysteine in position 34, not engaged in disulfide bonding, whose thiol group is able to react reversibly with oxidizing species; moreover, because of its abundance, albumin behaves as antioxidant and oxidation sensor [65]. Interestingly, although most serum and FF albumin is detected in its reduced form, the relative concentration of reduced albumin is higher in FF compared to the serum [75] suggesting that FF albumin stands as an important means to buffer oxidative conditions.

The level of reduced albumin in FF has positive reproductive impact as antioxidant. In fact, FF samples associated to viable oocytes, exhibited significantly higher level of reduced albumin, compared to FF samples surrounding structurally degenerated oocytes, whose level of oxidized albumin was higher [75]. Later in development, oxidation in the FF maintains its relevance; it was reported that oocytes resulting in low quality embryos, had been retrieved from FF containing higher H_2O_2 level, compared to oocytes that resulted in high quality embryos [76]. Moreover, oxidized methionine and ubiquitinated proteins were reported to be increased in FF from women whose pregnancy was unsuccessful [77], further emphasizing the irreversibility of the damaging effects of continued oxidation [78] and the functional disability therein. Therefore, enhanced oxidation in the FF results in lesser reproductive success and, in fact, lower level of ROS and higher total antioxidant capacity (TAC) were recognized as pregnancy predictors for intracytoplasmic sperm injection [79].

Although much of the stress is attributed to increased ROS production [80, 81], it is also consequent to local decrement of antioxidant level. Higher FF concentrations of vitamin E and beta carotene (and ascorbic acid to a lesser extent) were associated with better pregnancy outcome [82], whereas the level of antioxidants was smaller in unexplained infertility and

other female reproductive disorders, when compared to their healthy counterparts [82].

The reduction of plasma and FF level of vitamin E and of beta carotene that accompanies the less successful reproductive outcome, parallels the reduced antioxidant scavenging properties reported in ageing. In the FF of reproductively aged women, the SOD activity increased, catalase and GST decreased and both GPx and GSSG-Reductase exhibited a slight increase [72]; in addition, in older women, there was a drop in the FF concentration of hemopexin, which binds and clears the iron containing heme [83] and some evidence indicates that the response to enhanced local oxidation is sluggish compared to younger women [84]. Not surprisingly, culturing bovine oocytes with FF from older animals, and containing enhanced AGEs level, resulted in accelerated oocyte nuclear maturation, enhanced ROS production and abnormal fertilization [85].

So, in parallel to the plasma, the complex mixture of biomolecules present in the FF is indicative of their involvement in multifaceted biological processes. We are convinced that a part of them modulates the local oxidative balance by way of the properties of their cysteine residues. How the modulation is exerted or how the molecules involved are recruited is essentially unknown but is likely to aim at the provision of appropriate conditions for oocytes to be fertilized. It is interesting that when sensing environmental oxidative challenges, cells activate thiol production mechanisms to buffer the imbalance and regain homeostasis [84].

Reproductive disorders and perspective of antioxidant intervention

An important part of the discussion about ROS effects on cells relate to their ill-separated dual, harmful or signaling, properties. Also difficult to verify is whether the harmful effects of oxidation antedate well-known disorders or a previously established disorder provides a favorable field for oxidative harmful consequences. This difficulty may underlie the controversies on the effects of oxidation in the reproductive success [86–88].

As oxidation is harmful, it is reasonable to anticipate that the administration of antioxidants will provide protection from excessive oxidation and health improvement. Actually, while the provision of some antioxidants as nutritional supplements to fulfil the recommended dietary allowance (RDA) is beneficial in specific conditions as childhood, pregnancy and perimenopause, their administration in amount above RDA does not support, and sometimes contradicts, what was reasonably expected.

In fact, consistent results indicate that mortality is significantly increased when Beta-carotene, vitamin A and vitamin E are administered in amount above the RDA [89]. In addition, antioxidant supplementation with

vitamin C, vitamin E or beta carotene did not offer overall benefits for the primary prevention of cancer incidence or mortality [90] and, instead, they may have a role in the establishment of colorectal cancer [91].

In the area of reproduction, polycystic ovarian syndrome (PCOS) and endometriosis, two major disorders that limit reproductive outcome, have been related to local unbalanced oxidation.

In c.o. cells from patients with PCOS, the ratios of NAD⁺/NADH and NADP⁺/NADPH were higher, the ratio of GSH/GSSH was lower and the mitochondrial ROS production, assessed by the fluorescence emission of 2',7'-Dichlorofluorescein was higher, all suggesting enhanced oxidative stress [92].

Endometriosis is another condition where, apart from some controversy [93], enhanced oxidation was recognized [41, 94–96]. In endometriosis, under the action of the cyclical hormonal changes, an inflammatory reaction is triggered in the proximity of ectopic endometrial cell implants, resulting in attraction of inflammatory cells that activate ROS production by NADPH oxidases. In time, because the inflammatory reaction is cyclical, local structural and functional abnormalities ensue.

In endometriosis, both the elevated ROS production and the reduction of antioxidants were reported. In fact, SOD and vitamin E levels in FF from women with endometriosis were lower, and ROS were higher, compared to controls [97]. In addition, the reduced GSH concentration found in endometriosis FF, compared to controls, was also associated to inferior embryo quality [70].

Moreover, when FF samples from women with endometriosis, who had unsuccessful implantation, were compared with FF samples from women whose implantation was successful, increased concentration of ROS, nitric oxide and malonaldehyde was verified, in contrast to other molecules involved in oxidative stress alleviation [98]. Interestingly, FF albumin and hemopexin, both implicated in oxidative burden mitigation, were identified as distinct proteins in women with endometriosis who conceived, compared to women with endometriosis who did not conceive [99]. These findings indicate that the FF of endometriosis patients has insufficient antioxidant capacity, likely as consequence of extensive FF thiol groups oxidation [70].

Similarly, women whose diagnosis was tubal obstruction or mild tubal disorders and who had successful pregnancies, showed lower concentration of oxidant molecules in the FF when compared with cases of unsuccessful pregnancy [98].

In view of the association of reduced FF antioxidant capacity with diminished reproductive success, and experimental data favoring beneficial effects of antioxidant administration [100], some studies addressed the effects of antioxidant supplementation in humans.

It was reported that the administration of a micronutrient mixture (including vitamins C and E and folic acid,) prior to FF collection, resulted in reduced oxidation of FF components [101] and even increased FF TAC, free thiol residues and the number of viable oocytes [83]. In addition, vitamin C and E intake within the RDA range, compared to intake below the RDA, resulted in amelioration of fertilization rate and improvement of early embryo structural fitness [102] and also in a drop of FF myeloperoxidase assessed in women diagnosed with severe endometriosis [103]. Administration of other compounds with antioxidant properties as folate [104] or with D-chiro-inositol phosphoglycan [105] resulted in decrement of FF homocysteine level and in increased free thiol groups of FF proteins, respectively; in both, improvement of oocyte quality was found. A similar change in FF free thiols level was noticed in reproductively aged women upon administration of a micronutrient supplementation containing vitamins C and E and folate [106].

In general, these results favor the existence of a beneficial effect from the use of antioxidant supplementation. However, limitations remain because of the number of subjects enrolled in the studies and the difficulty to verify the precise amount of administered antioxidant.

Conclusions

Along approximately 3 months, local and systemic signals promote the growth of the ovarian follicle and the establishment of a biomolecule rich fluid compartment.

The structural organization of the COC endows the FF with the ability to supply the oocyte and c.o. cells with a variety of compounds. Among these, the presence and activity of important enzymatic and non-enzymatic antioxidants reflects FF involvement in harmful ROS effects prevention. Less clear is the role of ROS in local biological processes regulation but, the FF volume and rich composition in molecules that modulate redox reactions, that is noticed in pre-ovulatory follicles, suggest that they have a relevant intervention in oocyte growth and maturation.

Current data indicates that an important part of antioxidant buffering effect occurs upon engagement of specific cysteine residues that oxidize rapidly; in this sense, such proteins behave as redox sensors [65], a view that could be extended to albumin in FF because of its abundance and its cysteine mediated antioxidant properties. For other, less concentrated FF proteins, one may speculate that their cysteine residues exert similar roles for self-protection and for local antioxidantation. Thus, similarly to the proposed contribution of the intracellular highly concentrated actin [107], cysteines in FF proteins might work as sensors and then as buffers of oxidative stress.

This is an exciting and growing area of research [108, 109]. In the particular field of reproduction, it will provide additional knowledge of a critically regulated set of steps, as is the oocyte maturation process, whose main intervening growth factors are the cysteine rich mature oocyte secretory factors BMP15 and GDF9.

Abbreviations

AGEs: Advanced glycation end products; BMP15: Bone morphogenetic protein 15; c.o.: *Cumulus oophorus*; CAT: Catalase; cGMP: Cyclic Guanosine Monophosphate; cAMP: Cyclic Adenosine Monophosphate; COC: Cumulus oocyte complex; CoQ: Coenzyme Q10; FF: Follicular fluid; GDF9: Growth differentiation factor 9; GPx: Glutathione peroxidase; GSH: Reduced Glutathione; GSSG: Oxidized Glutathione; GST: Glutathione S-transferase; LH: Luteinizing Hormone; OSFs: Oocyte secreted factors; PCOS: Polycystic ovarian syndrome; Pox: Peroxiredoxins; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TAC: Total antioxidant capacity; TGFβ: Transforming Growth Factor Beta; UA: Uric acid

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Authors' contributions

CF and ACN contribute equally. CF suggested the review and organized the clinical contribution, ACN suggested the review and organized the Follicular Fluid section, LM and ES wrote the text related to oxidation and redox activity, AR organized the text of the *cumulus oophorus* section, JLSC designed the review and reviewed the clinical aspects, HA designed the review and integrated the contributions of all. All authors reviewed the text. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Consent for publication

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PUBLICATION VI

“The Unmet Needs for Studying Chronic Pelvic/Visceral Pain Using Animal Models”

Biomedicines 2023



Review

The Unmet Needs for Studying Chronic Pelvic/Visceral Pain Using Animal Models

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Abstract: The different definitions of chronic pelvic/visceral pain used by international societies have changed over the years. These differences have a great impact on the way researchers study chronic pelvic/visceral pain. Recently, the role of systemic changes, including the role of the central nervous system, in the perpetuation and chronification of pelvic/visceral pain has gained weight. Consequently, researchers are using animal models that resemble those systemic changes rather than using models that are organ- or tissue-specific. In this review, we discuss the advantages and disadvantages of using bladder-centric and systemic models, enumerating some of the central nervous system changes and pain-related behaviors occurring in each model. We also present some drawbacks when using animal models and pain-related behavior tests and raise questions about possible, yet to be demonstrated, investigator-related bias. We also suggest new approaches to study chronic pelvic/visceral pain by refining existing animal models or using new ones.

Keywords: endometriosis pain syndrome; chronic primary bladder pain syndrome; animal model



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1. The Implications of the Definitions and Terminologies of Chronic Pelvic and Visceral Pain

According to the International Association for the Study of Pain (IASP), chronic primary pain is defined as a persistent pain lasting for 3 months that is associated with emotional and/or functional impairment and cannot be associated with another condition [1]. If pain is associated with the head/neck viscera, thoracic viscera, abdominal visceral, or pelvic viscera with referral pain patterns from specific visceral organs, it is called chronic primary visceral pain (CPVP) [1]. If pain is felt in the pelvic region with referral pain patterns from specific pelvic organs without a diagnosed cause, after excluding all other causes it is called chronic primary pelvic pain syndrome (CPPPS) [1]. Endometriosis pain syndrome (EPS) is an example of CPPPS and should not be confused with endometriosis, whose lower abdominal pain is related to ectopic endometrium and, therefore, considered a chronic secondary visceral pain syndrome caused by persistent inflammation/vascular mechanisms (Table 1) [1–3]. However, the American Association of Gynecologic Laparoscopists, the European Society for Gynecological Endoscopy, the European Society of Human Reproduction and Embryology, and the World Endometriosis Society do not distinguish the chronic pain associated with EPS and endometriosis, assembling the patients under an umbrella term of endometriosis [4,5]. According to the IASP, if the pain is felt in the urinary bladder and there is at least one lower urinary tract symptom, without apparent cause or bladder inflammation, it is called chronic primary bladder pain

syndrome (CPBPS), formerly known as interstitial cystitis, painful bladder syndrome, and PBS/IC or BPS/IC (Table 1) [1]. The 2022 European Association of Urology Guidelines on Chronic Pelvic Pain followed the IASP definitions and nomenclature [6]. The 2022 American Urology Association Guidelines did not approach the chronic pelvic/visceral pain terminology issue [7].

According to the International Continence Society (ICS) definitions, chronic pelvic pain (CPP) is a persistent, recurrent, or continuous pain for 6 months that is related to the pelvic/abdominal region and is often associated with gynecological and lower urinary tract symptoms. If central sensitization and other chronic pain mechanisms are identified, the definition of chronic pelvic pain also applies, even if the general rule of six months is yet to be confirmed [8]. According to the ICS, whenever chronic pelvic pain cannot be associated with a well-defined pathology, it is called chronic pelvic pain syndrome (CPPS) [9]. Bladder pain syndrome (BPS) is an example of CPPS (Table 1). The European Society for the Study of IC/BPS (ESSIC) follows the same definition as the ICS [10]. According to the ICS, if chronic pain is associated with visceral organ damage, inflammation, or pressure and is detected by visceral organ nociceptors, it is called chronic visceral pain (CVP) [8]. Endometriosis is an example of CVP with an organic cause, specifically with an inflammatory origin (Table 1) [8]. The ICS does not have a definition for EPS.

Table 1. Summary of IASP and ICS classifications of pain related to endometriosis, endometriosis pain syndrome (EPS), and bladder pain syndrome (BPS) and chronic primary bladder pain syndrome (CPBPS).

	IASP/EAU	ICS/ESSIC
Endometriosis	Chronic secondary visceral pain syndrome	Chronic visceral pain
EPS	Chronic primary visceral pain—chronic primary pelvic pain syndrome	-
CPBPS/BPS	Chronic primary visceral pain	Chronic pelvic pain
Interstitial cystitis	-	Chronic pelvic pain

Both the IASP/EAU and ICS/ESSIC classifications consider that endometriosis is associated with visceral pain. However, while in the IASP/EAU definition pain is due to the possible extra-pelvic location of the lesion [1,2,6] and the term secondary indicates that it is associated with tissue inflammation, in the ICS/ESSIC definition pain results from the tissue inflammation detected by nociceptors [9].

The ICS/ESSIC do not have a definition for EPS. The ICS classification is based on symptoms, and therefore it may not distinguish endometriosis from EPS [9]. Hence, endometriosis may have been used to define EPS in many clinical studies and reports. Accordingly, the ICS/ESSIC pain classification also does not distinguish bladder pain syndrome from interstitial cystitis, as patients may present similar pain symptoms, although it approaches them as two different conditions. The evolution of the bladder pain syndrome nomenclature showed that in the past, interstitial cystitis was used to define bladder pain syndrome in clinical studies and reports.

Although EPS patients may have extra-pelvic lesions and/or may report extra-pelvic pain, the IASP/EAU define it as pelvic pain. On the other hand, CPBPS is not considered a pelvic pain syndrome by the IASP/EAU, probably because the location of pain, although difficult to identify, is mostly (but not exclusively [11]) found in the visceral region. In the ICS/ESSIC definition, however, the pelvic classification is due to the lack of connection of the pain to a visceral organ impairment [9].

The different criteria used by the IASP/EAU (origin and location) and the ICS/ESSIC (functional/symptoms) definitions of pain related to endometriosis and bladder pain syndrome influence the approaches to study these conditions. While using the IASP/EAU classification, the three identified conditions are endometriosis, EPS, and CPBPS (without IC). Using the ICS/ESSIC classification, the three identified conditions are endometriosis, BPS, and IC.

2. The Implications of Symptom Identification in the Study of Chronic Pelvic/Visceral Pain

One of the characteristics of EPS is that it was previously diagnosed as endometriosis, which justifies the origin of the pelvic pain [1,2]. However, after the surgical removal of the ectopic endometrium or its hormonal treatment, the pain may remain [1]. Therefore, the existence of ectopic endometrium in EPS patients may be, in some cases, irrelevant to the pain phenotype [1]. CPBPS, on the other hand, does not require the existence of inflammation and tissue damage in its diagnostic, although some patients might present inflammatory cells in the urinary bladder wall [1].

One approach to understanding if the mechanisms of chronic pain associated with EPS and CPBPS overlap is to identify the common features of these conditions. One common aspect is the location of pain described by patients. Most of the work published concerning the mapping of pain in endometriosis was performed pre-surgery and complemented by post-surgery diagnosis. Hence, it is not clear which pain locations were related to endometriosis and which were related to EPS or even if locations were related to EPS and changed after the removal of the lesions. Nevertheless, there was an extensive overlap in the mapping of the pain of endometriosis and CPBPS, as shown in Figure 1.



Figure 1. Illustrative image of pain mapping in women with endometriosis (grey dots, adapted from [12]) and CPBPS (black dots, adapted from [11]), regardless of the reported intensity of the pain.

Another common aspect in both syndromes is that patients present cognitive, behavioral, sexual, and/or emotional modifications, which are reflected in comorbidities such as depression and anxiety [1,13–15]. Moreover, the same patient may be diagnosed simultaneously with EPS and CPBPS [16]. It is not surprising, therefore, that both syndromes are associated with changes in the central nervous system [16], with the insula and the thalamus being the most commonly altered brain structures in both syndromes [17–20]. It is also noteworthy that both EPS and CPBPS present changes in the hypothalamic–pituitary–adrenal (HPA) axis, altering the stress-mediated response to noxious stimuli [16,21,22]. In fact, changes in cortisol levels have been associated with pain intensity (negative correlation) in these conditions [22,23]. Concurrent with HPA-axis hormonal changes, patients with both syndromes simultaneously present changes in the autonomic nervous system [24,25]. Therefore, both EPS and CPBPS patients present several phenotypes, which may explain why the available therapies are not pan-effective. As a corollary, many clinicians strongly support clustering patients by clinical and histological findings and by their responses to specific therapies or even to individualized management.

3. Back-translation of Patient Symptoms According to Their Associations with Chronic Pelvic/Visceral Pain

The clustering of patients according to their symptoms and signs is a complex issue. The multiplicity of phenotypes presented by patients and the multiplicity of scientific organizations make the definition of a worldwide accepted cluster very complex. Never-

theless, some attempts have been made. The UPOINT system proposed by Nickel and co-workers for phenotyping CPBPS patients is a good example of a simple tool to phenotype patients [26]. In the UPOINT algorithm, symptoms and signs are not exclusively related to the urinary bladder [26,27]. Rather, UPOINT is an acronym for the different domains observed in or reported by patients, which include urinary symptoms, psychosocial dysfunction, organ-specific findings, infection, neurologic/systemic comorbidities, and tenderness (related to pain) [26]. By broadening the urinary symptoms concept into functional symptoms, UPOINT may help to cluster patients with EPS, CPBPS, and even other chronic pelvic/visceral pain syndromes. The UPOINT score has shown strong positive correlations with the score obtained using the Interstitial Cystitis Symptom Index (ICSI) questionnaire and with the pain score obtained using a visual analogue scale (VAS) [26,27]. UPOINT has evolved to include other symptoms, including sexual dysfunction [28], and has already been used to define the therapeutic approach according to the patient's phenotype [29,30]. That is, the presence and severity of the symptoms observed in each subdomain of UPOINT determine if a single or a multimodal therapy should be offered to the patient [29].

Back-translation of the UPOINT may help researchers to choose and refine the animal models used to study chronic visceral/pelvic pain. Some aspects of EPS and CPBPS are more easily back-translated than others, either due to methodological limitations or due to the pain characteristics of individual subjects. Taking the organ-specific findings as an example, in EPS one of the most common organ-specific findings is the presence of endometrial lesions. Nonetheless, the number of lesions in patients does not correlate with the intensity of pain [31]. Moreover, the presence of lesions in patients is not a necessary condition for the development of pain [32,33]. This is an important concept, as back-translated data are generally supported by clinical studies that use patients with chronic pain without endometrial lesions as controls for patients that present endometrial lesions.

Recent works have suggested that, independent of being related to endometriotic lesions, the pain associated with EPS results from changes in the way it is integrated in the central nervous system (CNS) [32]. Hence, when using the induction of lesions in an EPS animal model, those lesions should induce changes in the same way that the patients' CNSs integrate pain. Moreover, to better replicate what is observed in EPS patients, the CNS changes should persist after the removal of those lesions (as occurs during EPS) to ensure that the observed animal pain behavior is not a consequence of the inflammation generated by the lesions (as occurs during endometriosis). However, this is methodologically complex, time-consuming, and, to our knowledge, has never been performed. Hence, the finding of specific markers for these CNS changes will help to surpass this methodological drawback.

In CPBPS, one of the most common organ-specific findings is urothelium functional impairment (not denudation, as in the case of IC). The urothelium barrier impairment has been associated with submucosa inflammation of the urinary bladder in patients with CPBPS and other bladder pathologies [34,35]. The urothelial barrier impairment is thought to be the mechanism by which the urine content activates bladder nociceptors that, in turn, induce neurogenic inflammation locally and promote CNS changes that lead to pain chronification [36]. This hypothesis, however, is difficult to test using bladder-centric CPBPS animal models, not only because of the technical complexity but also because of the difficulty in defining if the animal pain-like behavior is due to neurogenic inflammation (as might predominate in IC) or due to the changes induced in the CNS (as happens during CPBPS). Hence, the identification of which/how symptoms correlate with the etiology of chronic pelvic/visceral pain phenotypes is difficult in animals, especially when it is apparent that different insults/events may lead to similar chronic pelvic/visceral pain phenotypes.

4. Systemic Symptoms Prompt Systemic Models for the Study of Chronic Pelvic/Visceral Pain

EPS and CPBPS patients commonly present systemic symptoms, potentially reflecting the existent comorbidities and CNS changes that translate into the multiple pain sites ob-

served in Figure 1. Hence, animal models with CNS alterations that lead to pain behaviors and/or contribute to the development of symptoms of comorbidities (changes in organ activity and/or sensitivity as well as depression and/or anxiety symptoms, among others) may potentially provide more information about the chronic pain associated with EPS (white, Table 2) and CPBPS (grey, Table 2) than organ-centered models. In Table 2, the most used complex rodent models of EPS/endometriosis and CPBPS [37] and how they relate to UPOINT are summarized.

Table 2. Rodent models used recently to study EPS (white background) and CPBPS (grey background). The animals' characteristics are organized according to their similarity to patients' symptoms and signs in each UPOINT subdomain.

Model	U	P	O	I	N	T	References
Retrograde menstruation						+	[38]
Autologous transplantation	+	+	+		+	+	[39–63]
Allogenic transplantation						+	[39]
Autologous transplantation to the sciatic nerve					+	+	[64–70]
Autologous transplantation to muscle						+	[71–75]
Syngeneic transplantation	+		+		+	+	[76,77]
Xenograft transplantation						+	[78]
Intraperitoneal injection of endometrial tissue						+	[79–81]
Peritonitis					+	+	[82,83]
Colonic instillation of TNBS	+		+		+	+	[84–89]
Uterine pain					+		[90]
Autoimmune	+		+		+	+	[91–96]
Prostatic inflammation	+			+	+		[97–100]
Pseudorabies virus			+	+	+	+	[101–108]
Systemic administration of molecules	+				+	+	[109–111]
Restraint stress	+		+		+		[112–115]
Water avoidance stress	+	+	+		+	+	[116–121]
Chronic variable stress	+		+				[122]
Cold stress	+						[123,124]
Social stress	+		+		+		[125–127]
Foot shock stress		+			+	+	[128,129]
Early-life stress by odor-shock conditioning			+		+	+	[130,131]
Early-life stress by neonatal maternal deprivation	+	+	+		+		[132–134]

Among the EPS/endometriosis and CPBPS/BPS/IC models in use, those that more similarly back-translate the systemic symptoms are those that present urinary/functional symptoms, psychosocial dysfunction, and neurologic/systemic (comorbidities) impairments. This is the case of the autologous transplantation, water avoidance stress (WAS), and neonatal maternal deprivation (NMD) models. However, even these models need further refinements if the goal is to unveil the pathophysiological mechanism or find new treatments for EPS and CPBPS.

Considering the autologous transplantation model (ATM), endometrial tissue transplantation must be performed because the rodents do not have a menstrual cycle and consequently do not spontaneously develop endometrial lesions. The only exception is the spiny mouse [135]. Nevertheless, as in any other rodent species, the spiny mouse also possesses an ovarian bursa that encapsulates the ovaries and the oviducts, not allowing the endometrium tissue to escape to the peritoneal cavity. Only recently, an ovarian endometriosis model was developed in spiny mice via bursectomy [136], which provides a more physiologic endometrial peritoneal lesion model for investigation. The most used ATM, normally induced in BALB/c mice, offers the possibility, within its limitations, to study the chronic pain associated with endometriosis once the transplantation is performed and the ectopic lesions are formed [135]. However, as stated before, the pain induced in this model may be of inflammatory/neurogenic origin and may not reflect the CNS

changes observed in patients with EPS. In fact, variations (species and lesion location, among others) in ATM model induction may be enough to induce different modulations of the brain activity, which are then reflected in distinct pain-related behavior. For instance, the use of the ATM in Sprague-Dawley rats (implantation in the peritoneal wall) induces increased regional homogeneity of the anterior cingulate cortex, in the CA1 field of the hippocampus, in the thalamus, and in the brainstem, showing that these regions have abnormal neuronal activity [137]. A high number of neuronal cells show the activation of the apoptosis cascade [137]. Apart from the thalamus, the overactive neurons found in these nuclei show higher TRPV1 mRNA levels [137]. In addition, the overactive neurons from the hippocampus present increased levels of NMDA receptor mRNA [137]. On the other hand, there is a decrease in the regional homogeneity of the basomedial nucleus of the amygdala and in the primary motor cortex [137], providing evidence of altered basal brain activity. All these changes are concomitant with the development of thermal hyperalgesia and mechanical allodynia but not anxious behavior [137]. The ATM induced in C57BL/6 mice (implantation in the peritoneal wall) presents some similarities to the induction of the ATM in Sprague-Dawley rats (such as changes in hippocampus activity) but presents other important differences, such as changes in the activity of different brain areas, namely the insula [138]. Moreover, the observed changes in neuronal activity in the amygdala may differ, as in C57BL/6 mice, considering that the ATM increased the expression of the emotional regulation gene *Lcn2*, which may explain the increased anxiety induced by the ATM in these animals [138]. From a behavior phenotype point of view, the ATM induces thermal hyperalgesia in C57BL/6 mice but decreases locomotor activity [138]. On the other hand, this animal model presents changes in the thalamus and anterior cingulate cortex that are concomitant with anxiety behavior and mechanical allodynia [77].

Another model is the water avoidance stress (WAS) model. In this model, pain induction involves the activation of the peripheral adrenergic system [116]. How long does the pain phenotype induced by stress last? WAS carried out for 10 consecutive days was shown to induce a pain-related visceromotor response that lasted, in some animals, for 40 days but induced somatic nociception, which faded away much sooner. Is a 10-day WAS protocol sufficient to make an imprint in the CNS? When studying chronic pelvic/visceral pain using animal models of EPS and CPBPS, CNS pain integration changes may need more time to occur/establish than the other symptoms commonly associated with these syndromes. The great advantage of the neonatal maternal deprivation (NMD) model is that the pain-related phenotype occurs and is studied in adulthood, when the early-life insult is long gone. However, NMD is not free of drawbacks. The spontaneous pain behavior may not be easily observed, although the changes in the CNS pain integration pathway are already provoked.

Which test should be used to study chronic pelvic/visceral pain? What endpoint should be looked for? Many reviews have brought to light the advantages and disadvantages of using spontaneous and induced pain behavior tests [139,140]. The induced pain behavior tests, such as von Frey filament stimulation or the hot plate test, are normally based on the reflexive and conscious withdrawal of body parts submitted to a stimulus [139]. Therefore, their principal endpoints, based on nocifensive behaviors, are normally the latency time to a response or equivalent to an LD50 threshold [139]. Although these tests are indisputably associated with a bothersome/painful sensation, the focal location of such a sensation is hard to determine in the case of chronic pelvic/visceral pain. Additionally, they do not allow the spontaneous pain felt by the animals to be measured. Hence, it is imperative to find adjusted endpoints to study chronic pelvic/visceral pain that may overcome the drawbacks of the classically used tests, including the concomitant use of behavior tests with other techniques, such as neuroimaging or functional/electrophysiological measurements. In fact, tests that are based on pain-related spontaneous and/or cognitive behaviors (psychosocial dysfunction and the neurologic/systemic aspect of the UPOINT approach) associated with animal quality of life have been used more recently [139]. However, such tests are more susceptible to some pitfalls that are not directly associated with the tests

per se, which may endanger their interlaboratory comparison and impair their validity. These pitfalls are related to the animals' species-specific characteristics, animal husbandry and manipulation conditions, and/or data acquisition requirements, which are often overlooked and therefore not included in standard operating protocols. Additionally, the use of social cues also raises the question of the impact of genetic/epigenetics, which are known to be implicated in the balance of the HPA axis, in the study of chronic pelvic/visceral pain. The induction of WAS for 7 days is known to induce changes in the expression of glucocorticoid receptor and corticotropin-releasing factor genes of neuronal cells of the amygdala of male F-344 rats that are intimately related with increases in pain-related visceromotor responses through epigenetic mechanisms [141]. Therefore, we should evaluate if, for example, outliers should be disregarded. Moreover, investigators should not expect all animals to develop pain phenotypes after the induction in each experimental model, a piece of evidence that raises the question of how to handle the groups. Should we use different strains in the same protocol? What is the real impact of litter balancing in the study of chronic pelvic/visceral pain?

It is clear that the animal models of EPS and CPBPS require better characterization before deciding which tests should be used in the evaluation of the pathophysiological mechanisms of chronic pain or even pain treatments. For NMD, it should be elucidated which symptoms and signs NMD replicates and which pathways induce the NMD pain/sensitization pathway and associated behavior changes, such as anxiety. For instance, NMD is known to increase perigenital sensitivity and increase the number of voiding episodes in adult male mice [133]. NMD also influences the sensitivity of the colon, as it increases the visceromotor response to colorectal balloon distention in adult male rats [142], a fact that may accelerate the progression of endometrial lesions in female mice [143]. NMD causes mastocytosis in the bladder and prostate in adult male mice [133,144]. NMD also promotes the impairment of hippocampal neurogenesis and alters stress-related gene expression within the hypothalamic–pituitary–adrenal axis [145]. Moreover, it was recently postulated that NMD may prime a glial response that is reflected in adulthood in the development of pelvic/visceral pain, similar to what is observed in somatic pain [146].

Besides the rodent models, non-human primates (NHP) and domestic cats have been used to study EPS and CPBPS, respectively [147–150]. NHPs share similar reproductive system anatomy and endocrinology with humans. Baboons and some macaca species (such as rhesus, pigtail, and cynomolgus) menstruate and may develop spontaneous endometriosis [151–153]. The EPS in macaques is very similar to the condition in humans, as they present pain behavior, and the functional magnetic resonance imaging of their brains revealed a sensitized insula and thalamus [154]. Moreover, their endometrium and endometriosis tissues present mitochondrial impairment, which may be related to oxidative stress [155], a feature also observed in patients. As happens with some endometriosis patients, some macaques are anemic [156]. Furthermore, it is possible to find other species that spontaneously develop endometriosis in non-menstruating animals such as gorillas, horses, dogs, and guinea pigs, among others [157–159]. Despite the straightforward phylogenetic gap between humans, rodents are, at the end of the day, animal models with a high level of physiological homology. The ethical problems associated with the use of many other species, the maintenance expenses, and the decreased number of animals available for experimentation when compared to rodents make non-rodent models difficult to use routinely. The same holds true for CPBPS. Cats may develop feline interstitial cystitis (FIC), which is a similar form of CPBPS [160]. These cats exhibit flare-like symptoms, lower urinary tract abnormalities, and central nervous changes, and in some cases they present comorbidities such as behavioral, endocrine, cardiovascular, and gastrointestinal alterations [150]. However, the low availability of cats with FIC and the high cost necessary for their maintenance when compared with rodents make this FIC model less attractive [160,161].

5. Going beyond the Existing Models to Study the Chronic Pelvic/Visceral Pain Associated with EPS and CPBPS

To study EPS, the ATM can be combined with procedures/protocols that alter the way the CNS processes pain (Figure 2). For instance, the combination of the ATM with stress models (known to alter the HPA axis and/or induce CNS changes [141,162]) or with autoimmune models (known to induce CNS sensitization due to prolonged inflammation and/or tissue damage [163]) may help to refine the ATM to study EPS. In fact, female Balb/C mice submitted to NMD presented depression-like and anxiety-like symptoms in adulthood [143]. However, it was only after the induction of endometrial lesions in these animals that it was possible to observe endometriosis-associated generalized hyperalgesia [143]. Another putative approach is the use of longer ATM protocols to understand if, at longer time points, the CNS changes that lead to chronic pain are already observed.

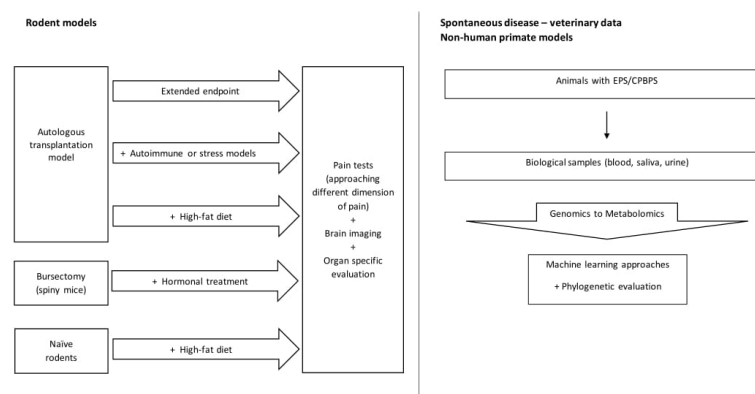


Figure 2. Possible refinement strategies to improve the study of chronic pelvic/visceral pain using animal models or animals that naturally develop EPS or CPBPS.

A frequently disregarded aspect regarding chronic pelvic/visceral pain is the role of visceral fat. EPS/endometriosis are related to an imbalance in the levels of estrogen, a hormone known to modulate the distribution and amount of fat tissue in the body [164,165]. Moreover, the lesions commonly observed in EPS/endometriosis occur in close proximity to the visceral fat present in the pelvic cavity [166–169]. The visceral fat is known to secrete pro-inflammatory molecules, which may promote tissue inflammation, even in the absence of endometrial lesions. In a study involving 3026 patients with osteoarthritis, visceral fat was associated with an increased pain phenotype, regardless of BMI [170], showing that the systemic inflammation induced by visceral fat promotes changes in the pain phenotype. In a study with 795 participants, it was observed that visceral fat was associated with sympathetic system activity [171]. Accordingly, visceral fat innervation is known to develop crosstalk with brain centers, namely the paraventricular nucleus (PVN) of the hypothalamus [172]. The hyperactivation of the PVN nucleus, as occurs in stressful situations, is thought to have a role in endometriosis/EPS, as it promotes HPA axis activation, modulating cortisol release concomitantly with endometrial dysfunction [170,173]. Therefore, combining a high-fat diet with lesion induction might provide a valid approach for studying EPS (Figure 2), as shown by Heard and colleagues in 2016 [174]. Moreover, changes in PVN activity, namely the increased release of corticotropin-releasing hormone (CRH), might also have a role in CPBPS, as these patients present urothelial expression changes in those hormone receptors [175]. The change in CRH receptors is intimately related to pain scores and markers in those patients [175]. Hence, high-fat-diet animal models may provide the chance to study features that are overlapped in EPS and CPBPS.

Besides the induction of chronic pelvic pain in animal models, a more exhaustive study of biological samples (ectopic endometrial tissue, blood, saliva, and urine) taken from animals that naturally develop EPS or CPBPS, together with other veterinary analyses that might be performed on those animals, should be considered. Important lessons can be taken, such as the possibility of performing a phylogenetic evaluation of the disease. An example of this is the recent genetic comparison between humans and the rhesus macaque that unveiled the importance of the coding variants in neuropeptide S receptor 1 in the pain treatment associated with endometriosis [158]. The phylogenetic evaluation of the disease may provide important clues based not only on the similarities but also on the most noticeable differences. It should be recalled that FIC affects both genders in a relatively equal way [150,160], a characteristic that may help investigators to understand CPBPS in the male population.

6. Conclusions

Altogether, the refinement of the translational chronic pelvic/visceral pain model to better understand the similarities with human conditions is essential to perform more meaningful studies. In addition, the study of specific cellular and subcellular mechanisms in the peripheral organs and the central nervous system nuclei will be fundamental to understanding the pathophysiology of EPS and CPBPS and to developing more effective treatments in a systemic setting.

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III DISCUSSION

Endometriosis-related infertility constitutes a serious clinical challenge, affecting 30% to 50% of women with endometriosis (Bonavina & Taylor, 2022; Leone Roberti Maggiore et al., 2024). The pathophysiological mechanisms underlying this association are multifactorial, involving inflammatory, hormonal and immunological dysfunction, and ultimately anatomical distortions. Importantly, therapeutic strategies currently available for alleviation of endometriosis symptoms are largely based on hormonal suppression, which is contraindicated and need to be interrupted in patients actively attempting to conceive (Rafique & Decherney, 2017; Taylor et al., 2021). Not rarely, those patients suffer an intensification of endometriosis-associated symptoms while the disease progresses during the treatment suspension. The therapeutic gap for women that pretend to conceive, highlights a pressing need for effective non-hormonal interventions capable of managing endometriosis while preserving reproductive potential.

1. Metformin Attenuates Lesion Growth in Endometriosis

In light of these considerations, metformin, a well-established anti-diabetic medication and a pharmacological mimetic of energy restriction, was hypothesized to offer therapeutic benefits in the context of endometriosis. Its pleiotropic properties, including anti-inflammatory, antioxidant, anti-proliferative, and anti-fibrotic effects, made it a compelling candidate for addressing the multifaceted pathogenesis of the disease.

Metformin's properties were approached in the experimental studies presented in **Publications I, II, and III**, which comprehensively evaluated the effects of a low-dose metformin regimen (70 mg/kg/day) in a murine model of surgically-induced endometriosis. This investigation spanned a broad spectrum of disease features,

encompassing not only implanted lesion growth and molecular characterization, but also reproductive function and cardiovascular parameters.

Despite the inherent limitations of studies on rodent models—particularly the absence of spontaneous menstruation—they have been widely validated and employed to study disease mechanisms, particularly in what regards systemic impact of the disease, and to assess therapeutic candidates for endometriosis (Bilotas et al., 2015; Cohen et al., 2015; Greaves et al., 2014). In my study, endometriosis was surgically induced through allogeneic peritoneal implantation of uterus fragments, and disease establishment confirmed via ultrasound and laparotomy. Notably, the employed metformin dosage, considering the equivalence between species, was lower than standard therapeutic doses in humans (Nair & Jacob, 2016) and demonstrated no significant effects in blood glucose or lipid levels, reaffirming its safety profile (Ainuddin et al., 2015; Beyuo et al., 2015).

However, supporting our hypothesis, metformin treatment was found to significantly attenuate implanted lesion development, as evidenced by reduced growth along time and altered lesion morphology. While untreated animals exhibited implanted structures reminiscent of clinical endometriomas, typically characterized by thick-walled cysts filled with dark hemolyzed blood, metformin-treated animals showed less vascularized and less proliferative lesions. These morphological observations were corroborated by molecular analyses, which revealed reduced expression of PCNA, a well-recognized marker of cellular proliferation, comparatively with implants from non-treated mice.

A downregulation of CYP17a1 was also observed in both eutopic and ectopic endometrial tissues, aligning with previous findings indicating metformin's capacity to suppress local estrogen biosynthesis (Burris-Hiday & Scott, 2021; Jing Cheng et al., 2022;

J. Zhang et al., 2017). Given the estrogen-dependence of endometriosis (Rizner, 2009), these results suggest that metformin may mitigate lesion growth by disrupting local hormonal support.

2. Metformin is Involved in Immune and Anti-Oxidative Responses and Fibrosis in Endometriosis

Also, the local immune environment of endometriotic lesions was modulated by metformin. While low expression of immune markers such as galectin-3, NFkB, and F4/80 in untreated animals may reflect immune tolerance or a resolution-phase inflammatory response, which favors implant growth in the setting of endometriosis, metformin treatment led to increased expression of these markers. This finding indicates that metformin may help restore local immune surveillance by counteracting the defective inflammatory and immune responses typically observed in endometriosis (Capobianco & Rovere-Querini, 2013; Yamashita et al., 2022). On the other hand, endometrium fibrosis, that ensues chronic inflammatory processes and is a key feature of endometriotic lesions (Itoga et al., 2003), was increased in mice with endometriosis and alleviated by metformin administration. These anti-fibrotic effects, consistent with prior observations in reproductive tissues (Wu et al., 2021; Zhang et al., 2013), appeared to be tissue-specific, because while metformin mitigated fibrosis in proliferative ectopic endometrium, it did not affect cardiac fibrosis or alter the inflammatory markers analyzed in the heart (**Publication II**). In fact, metformin treatment did not attenuate NFkB or change its inhibitor Ikba expression on myocardium, which correlates with the absence of mitigation of fibrosis. Animals with endometriosis exhibited hallmark features of cardiac remodeling, including increased myocardial fibrosis, and evidenced endothelial dysfunction with reduced

expression of eNOS, and elevated expression of ET-1, modifications that align with the increased risk for cardiovascular disease that patients with endometriosis present along life (Poeta do Couto et al., 2023). Metformin administration that we could expect to confer cardiovascular protection (Schernthaner et al., 2022), effectively improved endothelial function and restored eNOS expression in mice with induced endometriosis; however, while it did not attenuate myocardial inflammation or fibrosis, unexpectedly it induced hypertrophy of cardiomyocytes. These findings indicate a peculiar effect of metformin on cardiovascular remodeling, whose impact in women is unknown.

Besides inflammation, oxidative stress, also impacted by metformin treatment, is a well-recognized contributor to endometriosis pathogenesis (Donnez et al., 2016). Elevated oxidative markers and reduced antioxidant capacity have been consistently reported in affected women, reflecting a persistent redox imbalance (Clower et al., 2022; Santulli et al., 2014; Turkyilmaz et al., 2016). The peritoneal pro-oxidative microenvironment enhances inflammation and angiogenesis, supporting survival and implantation of ectopic endometrial cells (Scutiero et al., 2017), neovascularization and further lesion growth (Nanda et al., 2020). Additionally, oxidative stress contributes to chronic inflammation, fibrosis and peripheral nerve sensitization, causing pain and ultimately sustaining lesion persistence (Cacciottola et al., 2021; Clower et al., 2022; Samimi et al., 2019). These changes were impacted by metformin treatment. In fact, in the ectopic endometrium, metformin enhanced the expression of antioxidant enzymes, including HO-1 and GPX-1, indicating activation of antioxidant pathways (Kapoor et al., 2019; Lubos et al., 2011). In addition, in the heart of endometriosis animals, metformin also increased GPX-1 expression (**Publication III**). When we analysed metformin-dependent molecular pathways, we verified that AMPK (pAMPK) was upregulated exclusively in metformin-

treated endometriosis animals, suggesting a disease-dependent modulation of energy-sensing pathways. On the other hand, the downstream pathway AMPK/sirtuin 1/PGC1- α /sirtuin 3 did not evidence activation, which led us to conclude that an AMPK-independent mechanism was involved in the activation of GPX-1 expression in the heart of those animals. Together, these findings support the hypothesis that metformin attenuates global oxidative damage, including in the endometriotic tissues.

3. Metformin Restores Fertility in Endometriosis

Given the significant impact of endometriosis on fertility, evaluating the effects of metformin during gestation in animal models of the disease is particularly relevant. Understanding how metformin modulates the inflammatory, oxidative, and endocrine environment throughout pregnancy may provide key mechanistic insights. This approach also carries strong translational value, as it mirrors clinical situations in which women with endometriosis may benefit from therapeutic support before and during pregnancy. Ultimately, the findings may help position metformin as a safe and effective strategy to improve reproductive and obstetric outcomes in this population.

In my study, pregnancy itself exerted regulatory effects on several endometriosis markers. Compared with non-pregnant, pregnant animals demonstrated increased serum estradiol and decreased cholesterol levels, along with attenuation of lesion markers of cell proliferation, estrogen synthesis, inflammation, and immune activity. These findings are consistent with the well-described immunosuppressive and anti-inflammatory milieu of gestation, which promotes maternal tolerance toward the fetus and reduces excessive immune activation (Oală et al., 2024; Williams, 2012). When metformin treatment was administered in pregnancy, the regulatory effects on disease markers were maintained. In

fact, pregnant animals treated with metformin exhibited equivalent levels of expression of lesion markers associated with cell proliferation, estrogen synthesis, inflammation, and immune activity. Moreover, it is important to note that metformin has a well-established safety profile in the management of gestational diabetes and polycystic ovary syndrome. Furthermore, emerging evidence indicates that it may confer additional benefits in preventing pregnancy complications (D'Souza et al., 2025; Nassif et al., 2024; Nie et al., 2025; Paschou et al., 2024; Saadati et al., 2025).

A particularly compelling finding was the complete restoration of fertility in metformin-treated animals with endometriosis. These animals achieved pregnancy rates and fetuses numbers comparable to healthy controls, a result not previously demonstrated for any other pharmacological agent except oleuropein, whose safety for use in pregnant women has not yet been established (Cohen et al., 2015; Park et al., 2022).

These findings provide robust evidence that metformin not only reduces lesion growth and modifies molecular hallmarks of endometriosis, but also effectively restores reproductive competence in a preclinical model. Its ability to simultaneously modulate inflammation, oxidative stress, steroidogenesis, and endometrium fibrosis supports its potential as a multitarget therapeutic agent. The favorable safety profile of metformin during pregnancy and its positive systemic metabolic impact further strengthen its repurpose as a fertility-friendly treatment. These findings position metformin as a unique non-hormonal therapeutic option, approved and safe to use during pregnancy, capable of reversing endometriosis-associated infertility even in a sub-clinical dose. Our findings are summarized in table 1 and figure 3. While translational and clinical studies are needed to confirm these results in humans, this work represents a significant advancement toward

non-hormonal, personalized therapies for endometriosis-associated infertility and systemic comorbidities.

4. Biomarkers of Follicular Fluid are Predictors of Endometriosis and ART Outcomes

The reliance on ART in women with endometriosis underscores the persistent challenges of infertility in this population (Prefumo & Rossi, 2018). This reinforces the importance of identifying reliable biomarkers that can accurately predict outcomes in ART. Such biomarkers would not only provide insights into oocyte and embryo quality but could also guide individualized treatment strategies, enabling clinicians to optimize protocols and improve the likelihood of successful conception. Ultimately, the discovery of predictive biomarkers has the potential to enhance reproductive success and offer a more personalized approach to managing infertility in women with endometriosis. Follicular Fluid provides a valuable resource to analyse ovarian microenvironment, encompassing biochemical and molecular factors that directly influence oocyte quality and embryo development (Nagy et al., 2019; Templeton, 2000). In this context, **Publication IV** offers novel insights by simultaneously analysing AMH, BMP-15, GDF-9, and key miRNAs in FF, highlighting potential biomarkers and therapeutic targets for ART.

Among the analysed molecules and according to Falconer *et al.* (serum and FF) and Yin *et al.* (serum) (Falconer et al., 2009; Yin et al., 2024), we observed a significant decrease in AMH levels in FF from women with endometriosis compared to controls, indicating a reduction in ovarian reserve in those patients that may contribute to impaired fertility (La Marca et al., 2010; Lemos et al., 2008; Yoo et al., 2011). Interestingly, AMH levels in FF did not correlate with age in the cohort of controls, suggesting that disease-specific mechanisms, rather than chronological aging, led to the marked reduction of AMH

levels in FF. The decrease in AMH in endometriosis patients aligns with the upregulation of miR20a_1 and miR145_1, known regulators of AMH via TGF- β signalling (Fu et al., 2020; Lutful Kabir et al., 2018; Melling et al., 2018; Zhao et al., 2015), suggesting a molecular mechanism linking miRNA dysregulation to diminished follicular reserve. Local inflammation, oxidative stress, and altered granulosa cell signalling in endometriosis may further contribute to this reduction. Concomitantly with AMH decrement, both GDF-9 and BMP-15 were significantly increased in FF from endometriosis patients, in both pro-protein and mature dimeric forms. These oocyte-secreted factors are essential for follicular development, cumulus expansion, and embryo competence (Da Broi et al., 2018; Gode et al., 2011; McNatty et al., 2004; Mottershead et al., 2015; Xie et al., 2023). Their increase may reflect an adaptive or compensatory mechanism aimed at preserving oocyte quality in the context of endometriosis-induced alterations in the follicular microenvironment. Notably, BMP-15 proprotein levels decreased with age in controls, which is in line with the age-dependent decay of mechanisms that intervene in follicle maturation and oocyte competence (Di Pasquale et al., 2004; Ribeiro et al., 2017). These findings highlight the potential of BMP-15 and GDF-9 as indicators of oocyte competence, reflecting the ovarian microenvironment in endometriosis, but further investigation into their functional roles could inform strategies to support follicular development and improve ART outcomes.

Aiming to identify novel biomarkers of disease with potential prognostic value for ART success, we proceeded to the analysis of specific miRNAs in FF (**publication IV**). Data revealed a decrease in miR125b-5p and miR212-3p, concomitantly with an increase in miR20a_1, miR145_1, and miR320a_1 in women with endometriosis. The alterations in FF miRNAs observed in women with endometriosis suggest a complex regulatory network that may simultaneously reflect disease progression and influence oocyte competence. The

decrease in both miR-125b-5p and miR-212-3p, which act as tumour suppressors and modulators of apoptosis and immune responses, indicate locally promotion of inflammation and pro-proliferative ovarian environment, despite others demonstrated upregulation in those miRNAs in serum of patients with endometriosis (Chen et al., 2017; Feng et al., 2015; Hua et al., 2019). Conversely, the upregulation of miR-320a_1 in FF may represent adaptive or protective mechanisms aimed at supporting oocyte maturation and embryo quality, potentially counteracting the endometriosis-induced disturbances in the follicular microenvironment (Andreas et al., 2021; Feng et al., 2015; Lutful Kabir et al., 2018; Melling et al., 2018). Also, the upregulation of miR-20a_1 and miR-145_1 may play equivalent function, despite its probable effect on AMH expression mitigation. It is known that each miRNA may target multiple genes and pathways, and in the case of miR-20a_1, miR-145_1, and miR-320a_1, TGF- β signalling, PTEN, and BMPR2 are common targets (Bartel, 2009; Fu et al., 2020; Mendell & Olson, 2012). In fact, the combinatorial regulation of miRNAs may account for some of the variability we have observed in FF, reflecting a dynamic balance between disease-promoting and protective mechanisms. In our study it was verified that miR-320a_1, previously associated with high-quality embryos (Feng et al., 2015), was elevated in FF from women with endometriosis compared to controls. In addition, in control women, FF miR-320a_1 levels correlated with successful biochemical pregnancy, further supporting its role in promoting oocyte and embryo quality. This finding indicates that miR-320a_1 increase in endometriosis is one example of a compensatory response aimed at preserving oocyte competence.

Importantly, the concomitant increase in BMP-15 and GDF-9, together with the observed miRNA changes, supports the presence of coordinated adaptive mechanisms that preserve oocyte quality while mitigating the deleterious ovarian environment in

endometriosis. Nevertheless, the predictive value of these miRNAs for biochemical pregnancy in women with endometriosis remains to be fully determined, highlighting the limitations of the current study. In fact, the small sample size, particularly for women with diagnosed endometriosis, is a limitation of our pilot study that reduces statistical power in data analysis. In particular, biochemical pregnancy outcomes in the endometriosis group were limited, preventing full evaluation of the predictive value of miRNAs quantified in FF in this group of women. Our data, however, underscores the need for further investigation. Future studies should investigate FF markers in larger cohorts of women to explore their functional and prognostic significance. This approach will be of paramount importance for the development of targeted interventions, such as individualized molecular assessment to identify optimal oocytes or guide embryo selection or even miRNA-based therapies to optimize ART success in the population of women with endometriosis-associated infertility.

Together, the findings obtained in our pilot study brought new insights in what concerns ART outcomes prediction, considering that it was demonstrated that miRNAs in FF may serve as predictive markers for both endometriosis diagnosis and ART success. Our study provides a comprehensive molecular characterization of FF in women with endometriosis, integrating protein and miRNA analyses to identify markers with both diagnostic and prognostic potential. However, analysis of relevant markers in blood in a higher number of patients will be valuable to strengthen the appliance of those quantifications in the clinical practice.

The combined evaluation of AMH, BMP-15, GDF-9, and miRNAs in FF offers a novel molecular panel that could enhance ART decision-making. Table 2 and figure 3 underlie the main results obtained in **publication IV**.

In summary, our findings highlight disease-specific alterations in proteins and miRNAs secreted in the FF, that enhance understanding of endometriosis pathophysiology and offer novel biomarkers and mechanistic targets for ART optimization, bridging molecular insights with clinical application.

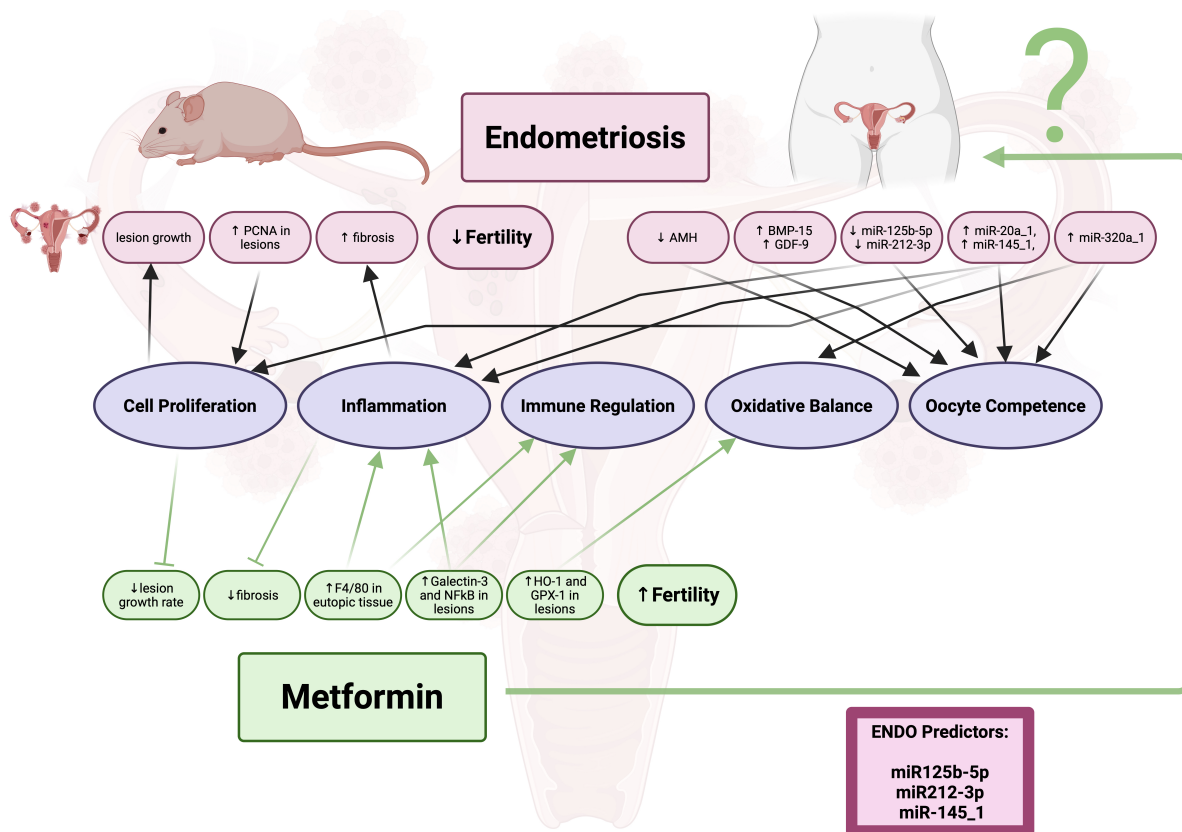


Figure 3 - Endometriosis and fertility: human evidence and experimental metformin insights from murine models.

Endometriosis exerts systemic effects that extend beyond the pelvis, encompassing alterations in cellular proliferation, inflammation, immune regulation, oxidative balance, and oocyte competence.

In the experimental mouse model of endometriosis, increased lesion growth, elevated PCNA expression, and enhanced fibrosis were associated with inflammatory processes, culminating in reduced fertility.

Metformin treatment mitigated several pathological features by reducing lesion growth and fibrosis, increasing macrophage presence ($\uparrow$ F4/80), and modulating inflammatory mediators such as Galectin-3 and NFkB. It also enhanced antioxidant defences ($\uparrow$ HO-1 and GPX-1), collectively resulting in improved fertility outcomes in the mouse model.

In women, alterations in follicular fluid markers ($\downarrow$ AMH, $\uparrow$ BMP-15, $\uparrow$ GDF-9) and dysregulated microRNA profiles ($\downarrow$ miR-125b-5p, $\downarrow$ miR-212-3p, $\uparrow$ miR-20a-1, $\uparrow$ miR-145-1, $\uparrow$ miR-320a-1) further indicate impaired oocyte competence and reproductive potential. Importantly, microRNAs do not act as discrete layers but, rather as part of interconnected regulatory networks. Notably, our results showed that miR-125b-5p, miR-212-3p, and miR-145-1 displayed predictive value for endometriosis. A specific panel of differentially expressed miRNAs may serve as predictive biomarkers for endometriosis and oocyte competence, ultimately influencing pregnancy potential and improving ART outcomes.

Although the direct effects of metformin on female fertility in endometriosis remain to be fully elucidated, this integrative model supports the hypothesis that metformin confers beneficial reproductive effects by attenuating inflammation, oxidative stress, and immune dysregulation, thereby promoting improved oocyte quality and fertility potential.

5. Take-Home Message

My work reinforces that endometriosis is a systemic and multifactorial disease, extending beyond localized lesions to a profound affection of the ovarian microenvironment and reproductive function. Within this framework, low-dose metformin emerges as a novel and safe non-hormonal therapeutic approach, capable of attenuating lesion activity, restoring immune and oxidative balance, and fully rescuing fertility in a preclinical model. These findings expand the therapeutic potential of metformin beyond its traditional metabolic context, positioning it as a fertility-preserving agent in endometriosis.

In parallel, the comprehensive characterization of FF revealed a distinctive molecular landscape, identifying proteins and miRNA signatures with both endometriosis-diagnostic and prognostic relevance for ART outcomes. The integration of these biomarkers into clinical practice could provide valuable insights into oocyte competence assessment, enabling molecule-guided embryo selection and personalized reproductive strategies.

Altogether, this study contributes to a paradigm shift in the understanding and management of endometriosis, highlighting the potential of combining systemic therapeutic interventions such as metformin with molecular profiling of FF to promote fertility preservation and personalized reproductive care in women affected by this complex disease.

DISCUSSION

Table 3 - Effects of Metformin on Endometriosis in a Murine Model and Implications for Fertility and Systemic Features

	Demonstrated Metformin Effect	Proposed Mechanism For Metformin	Implication For Fertility / Systemic Features
Lesion Growth & Morphology	↓ proliferation; ↓ vascularization; halting of lesion growth; ↓ PCNA expression	Anti-proliferative, anti-angiogenic effects	Attenuates endometriosis severity; potential improvement in oocyte environment
Local Estrogen Synthesis	↓ CYP17a1 expression in ectopic and eutopic endometrium	Inhibition of local estrogen biosynthesis	Limits estrogen-dependent lesion growth
Immune Markers	↑ expression GALECTIN-3, NFκB, F4/80	Restoration of immune surveillance; modulation of defective inflammatory response	Supports immune-mediated control of lesion progression
Endometrium Fibrosis	↓ fibrosis in lesions	Anti-fibrotic effect; tissue-specific action	Improves endometrial receptivity and fertility potential
Oxidative Stress Markers in Endometrium	↑ expression HO-1, GPX-1	Activation of antioxidant/cytoprotective pathways	Reduces local oxidative damage; contributes to systemic antioxidant effects
Cardiac Remodelling	Mixed effects: improved endothelial function - ↑ eNOS expression; ↑ AMPK-independent GPX-1 expression; hypertrophy of cardiomyocytes	Disease-dependent modulation of AMPK activation and GPX-1 expression	Partial cardiovascular protection; uncertain impact on myocardial fibrosis and hypertrophy
Fertility Outcomes	Complete restoration of pregnancy rates and fetus numbers	Multitarget modulation of proliferation, inflammation, angiogenesis, oxidative stress, and steroidogenesis	Positions metformin as a non-hormonal therapy capable of reversing endometriosis-associated infertility
Safety Profile	No significant effects on blood glucose or lipids at studied dose; generally safe in pregnancy (caution advised)	Physiological effects for low-dose regimen	Feasible non-hormonal therapy for reproductive-age women, including potential use in pregnancy

List of Abbreviations: **AMPK** – AMP-activated protein kinase; **CYP17A1** – Cytochrome P450 17a1; **eNOS** – Endothelial nitric oxide synthase; **GPX-1** – Glutathione peroxidase 1; **HO-1** – Heme oxygenase 1; **NFκB** – Nuclear factor kappa B; **PCNA** – Proliferating cell nuclear antigen

Table 4 - Molecular Alterations in Follicular Fluid of Women with Endometriosis and ART Implications

Marker	Observed Change in FF	Proposed Mechanism	Potential Implication in ART
AMH	↓ Decreased	Local ovarian stress, inflammation, oxidative stress; miR20a_1 and miR145_1 upregulation suppresses AMH via TGF-β signalling	Indicator of diminished ovarian reserve; may guide ovarian stimulation protocols
BMP-15	↑ Increased (pro- and mature forms)	Compensatory upregulation to maintain follicular development and oocyte competence under endometriosis-associated dysregulation	Potential target for supplementation or modulation to improve oocyte quality
GDF-9	↑ Increased (pro- and mature forms)	Similar compensatory mechanism as BMP-15; supports cumulus cells expansion and follicular maturation	Biomarker for oocyte competence; potential target for follicle optimization
miR125b-5P / miR212-3P	↓ Decreased	Dysregulation of apoptosis and immune signalling; contributes to inflammatory ovarian environment	Potential predictive marker for ovarian microenvironment health; may guide therapeutic interventions
miR20A_1 / miR145_1	↑ Increased	Modulation of TGF-β signalling, suppression of cell migration; compensatory support for oocyte maturation	Could inform embryo selection; potential target for molecular therapy
miR320A_1	↑ Increased	Associated with high-quality embryos	Predictive marker for embryo competence and biochemical pregnancy success

List of Abbreviations: **AMH** – Anti-Müllerian hormone; **BMP-15** – Bone morphogenetic protein 15; **GDF-9** – Growth differentiation factor 9; **TGF-β** – Transforming growth factor beta

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