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ENDOMETRIOMICS: *IN SILICO* DATA MINING OF OMICS STUDIES IN ENDOMETRIOSIS

SEARCH FOR BIOMARKERS IN ENDOMETRIOSIS

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ENDOMETRIOMICS:

IN SILICO DATA MINING OF OMICS STUDIES IN ENDOMETRIOSIS

Thesis for Doctoral Degree (Ph.D.)

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A mis padres, Ricardo e Isabel

ABSTRACT

Endometriomics -the study of endometriosis using high-throughput omics techniques- is a big area of research in understanding this complex disease. However, the big amount of data derived from the omics analyses is accumulating and often remaining under-explored. With this thesis, we aimed to make our contribution to the research in the endometriomics area using *in silico* data mining approaches with the purpose of gaining knowledge in the mechanisms leading to endometriosis and identifying candidate biomarkers of the disease. For that, three studies are presented: **Study I** summarises the main advances in reproductomics and presents examples of analysis of omics data for guiding students, researchers and clinicians in the field in understanding and performing omics studies; in **Study II**, a systematic review of the literature on endometriosis and related comorbidities is presented together with an *in silico* approach, which allowed us to identify a subgroup of diseases that we named as Endometrial Sibling Disorders. Further analysis of this subset of comorbidities using microarray and gene variants data analysis pointed to *BDNF*, *ESR1*, *IL10*, *MMP9*, and *PGR* as putative biomarkers of endometriosis; in **Study III**, the endometrial mid-secretory transcriptome in endometriosis was evaluated to identify a potential dys-regulation that could contribute to endometriosis-associated infertility. The systematic review of the literature and the subsequent meta-analysis of data using the robust rank aggregation method led us to detect a dys-regulation of complex pathways previously linked to endometriosis. The study of endometrial receptivity-specific genes retrieved from commercial receptivity tests in the meta-analysed data led us to identify *C4BPA*, *MAOA*, and *PAEP* as recurrently dys-regulated in endometriosis. In conclusion, this thesis provides deeper understanding of the genes and molecular pathways underlying endometriosis through the use of data resulting from the application of omics technologies.

PUBLICATIONS INCLUDED IN THE THESIS

Book chapters

1) Vargas E, Esteban FJ, Altmäe S. Computational approaches in Reproductomics. Reproductomics: The -omics revolution and its impact on human reproductive medicine. Academic Press. 2018; 347-383.

Scientific papers

1) Vargas E, Aghajanova L, Gemzell-Danielsson K, Altmäe S, Esteban FJ. Cross-disorder analysis of endometriosis and its comorbid diseases reveals shared genes and molecular pathways and proposes putative biomarkers of endometriosis. Reprod Biomed Online. 2020; 40(2): 305-318.

2) Vargas E, García-Moreno E, Aghajanova L, Salumets A, Horcajadas JA, Esteban FJ, Altmäe S. The mid-secretory endometrial landscape in endometriosis: a meta-analysis. 2021; Submitted to Human Reproduction Open.

ADDITIONAL PUBLICATIONS NOT INCLUDED IN THE THESIS

Vargas E, Díaz-Beltrán L, Esteban FJ. Integración y análisis de datos mediante biología de sistemas. In Biología molecular y celular. Volumen I. Técnicas y fundamentos. Editorial de la Universidad de Jaén. 2019; 43-72.

Molina NM, Plaza-Díaz J, Vilchez-Vargas R, Sola-Leyva A, Vargas E, Mendoza-Tesarik R, Galán-Lázaro M, Mendoza-Ladrón de Guevara N, Tesarik J, Altmäe S. Assessing the testicular sperm microbiome: a low-biomass site with abundant contamination. Under review in Reprod Biomed Online. 2021; Ms. Ref. No.: RBMO-D-21-00144R1.

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LIST OF ABBREVIATIONS

AGTR1:	Angiotensin II Receptor Type 1
APEX1:	Apurinic/Apyrimidinic Endodeoxyribonuclease 1
ART:	Assisted Reproductive Technologies
BDNF:	Brain-Derived Neurotrophic Factor
C3:	Complement C3
C4BPA:	Complement Component 4 Binding Protein Alpha
CA-125:	Cancer Antigen-125
CCL2:	C-C Motif Chemokine Ligand 2
CCR2:	C-C Motif Chemokine Receptor 2
CD40:	Cluster of Differentiation 40
COS:	Controlled Ovarian Stimulation
CYP17A1:	Cytochrome P450 Family 17 Subfamily A Member 1
CYP1A1:	Cytochrome P450 Family 1 Subfamily A Member 1
CYP1B1:	Cytochrome P450 Family 1 Subfamily B Member 1
DAVID:	Database for Annotation, Visualisation and Integrated Discovery
ERG:	Endometrial Receptivity Genes
ESD:	Endometriosis Sibling Disorders
ESR1:	Oestrogen Receptor 1
ESR2:	Oestrogen Receptor 2
ERA:	Endometrial Receptivity Array
FDR:	False Discovery Rate
FOXP3:	Forkhead Box P3
FSH:	Follicle-Stimulating Hormone
GEO:	Gene Expression Omnibus
GO:	Gene Ontology
GSEA:	Gene Set Enrichment Analysis
GWAS:	Genome-Wide Association Studies
HCW:	Healthy Control Women
ICAM1:	Intercellular Adhesion Molecule 1

ICD-10:	International Classification of Diseases, version 10
IGF1:	Insulin Like Growth Factor 1
IGF2:	Insulin Like Growth Factor 2
IGF1R:	Insulin Like Growth Factor 1 Receptor
IL10:	Interleukin 10
IL16:	Interleukin 16
IL1A:	Interleukin 1 Alpha
IVF:	*in vitro* Fertilisation
KEGG:	Kyoto Encyclopaedia of Genes and Genomes
KRAS:	KRAS Proto-Oncogene, GTPase
LH:	Luteinising Hormone
MAOA:	Monoamine Oxidase A
MeSH:	Medical Subject Headings
miRNAs:	microRNAs
MMP1:	Matrix Metallopeptidase 1
MMP7:	Matrix Metallopeptidase 7
MMP9:	Matrix Metallopeptidase 9
NOS3:	Nitric Oxide Synthase 3
PAEP:	Progestogen-Associated Endometrial Protein
PCOS:	Polycystic Ovary Syndrome
PGR:	Progesterone Receptor
PIK3CA:	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PPARG:	Peroxisome Proliferator Activated Receptor Gamma
PPI:	Protein-Protein Interactions
PRISMA-P:	Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols
PTPN22:	Protein Tyrosine Phosphatase Non-Receptor Type 22
REAC:	Reactome
RIF:	Recurrent Implantation Failure
RNA-seq:	RNA-sequencing
RRA:	Robust Rank Aggregation

SERPINE1:	Serpin Family E Member 1
SNP:	Single Nucleotide Polymorphism
SOD2:	Superoxide Dismutase 2
TIMP2:	Tissue Inhibitor of Metalloproteinases 2
TNF:	Tumour Necrosis Factor
TP53:	Tumour Protein P53
VEGFA:	Vascular Endothelial Growth Factor A
WOI:	Window Of Implantation
WP:	WikiPathways
WWE:	Women With Endometriosis
XRCC1:	X-Ray Repair Cross Complementing 1

PHD THESIS REPORT

1. INTRODUCTION

Endometriosis is one of the most common and heterogeneous reproductive disorders affecting women. Endometriosis is defined as the presence of endometrial-like tissue outside of the uterine cavity, that can affect different anatomical sites, mainly the ovaries and the peritoneum (Akter *et al*., 2020; Giudice, 2010). All forms of endometriosis cause varying degrees of pelvic pain, dysmenorrhea, dyspareunia, painful defecation, and/or infertility. This benign condition is estimated to affect around 176 million women worldwide (Horne *et al*., 2019), which represents around 10% of reproductive aged women (Taylor *et al*., 2021), and is believed to affect up to 50% of infertile women. However, the true prevalence of endometriosis is uncertain, since definitive diagnosis and treatment after symptoms onset requires invasive surgical protocols, which usually lead to a delay of around 5-10 years (Soliman *et al.*, 2017; Zondervan *et al.*, 2020). Furthermore, the classic therapy has not changed in a century, despite the chronic, recurrent nature of this condition. Given the chronic character and the difficulties derived of endometriosis, it is considered a major medical, social, and economic problem (Smolarz *et al*., 2020).

Reducing the time to diagnosis would not only improve the field of endometriosis management, but also enhance the quality of life of affected women. There is strong international recognition that accurate non-invasive/minimally-invasive diagnostic markers and more effective disease modifying agents are needed for this commonly occurring disease (Rogers *et al.*, 2017). Extensive research in the endometriosis field during the past 30 years has identified a myriad of markers associated with disease, such as inflammatory, immunologic, adhesion, and migration markers; genetic polymorphisms; oxidative stress; RNA transcripts and microRNAs; hormonal receptors; leukocytes; and circulating cells (Domínguez, 2018). Yet, none of them has proven to be unequivocally useful for clinical diagnosis.

This PhD aims to provide more understanding into the molecular mechanisms underlying the pathology of endometriosis and to identify possible molecular biomarkers by applying computational data mining tools.

1.1. The human endometrium

The human endometrium is a complex and intriguing tissue that constitutes the inner layer of the uterus. Histologically, it is a unique mucosa with ability of self-regeneration in each menstrual cycle composed of a surface epithelium overlying glands with accompanying stroma and immune cells (Nikolakopoulou and Turco, 2021). This fascinating tissue has received much research attention over the last decades, with big efforts deposited in the understanding of its biology, as it is well understood that it is the place that harbours the first stages of embryo development (Pawar *et al*., 2014).

The activity in the endometrium is highly controlled by the influence of the ovarian hormones, whose metabolism follows a precise cycle that can be temporally defined into phases (Figure 1). Regarding hormonal levels, while the determining hormone of the proliferative phase is oestrogen, progesterone is the dominant hormone during the secretory phase (Strowitzki *et al*., 2006). Therefore, the endometrium undergoes cyclical and distinguished phases where changes in its growth and differentiation happen at a histological and functional level (Ozturk and Demir, 2010).

Despite the ability of the incipient blastocyst to implant in different human tissues, this process does not take place in a non-receptive endometrium (Achache and Revel, 2006). The window of implantation (WOI) -term introduced for the first time by Psychoyos at 1973-, is defined as the delimited span accurately coordinated by hormone signalling in which endometrium becomes receptive for embryo to implant (Psychoyos, 1973). During this period of time, the maternal endometrium acquires a prime morphological and functional state that on one hand removes all the sources of hostility that normally

compromise the embryo attachment, and on the other hand provides the signalling factors required to nourish the incipient embryo during the first few weeks of pregnancy in case of fertilisation takes place (Evans *et al*., 2016; Katzorke *et al*., 2016; Miravet-Valenciano *et al.,* 2015; Paria *et al.*, 2002).

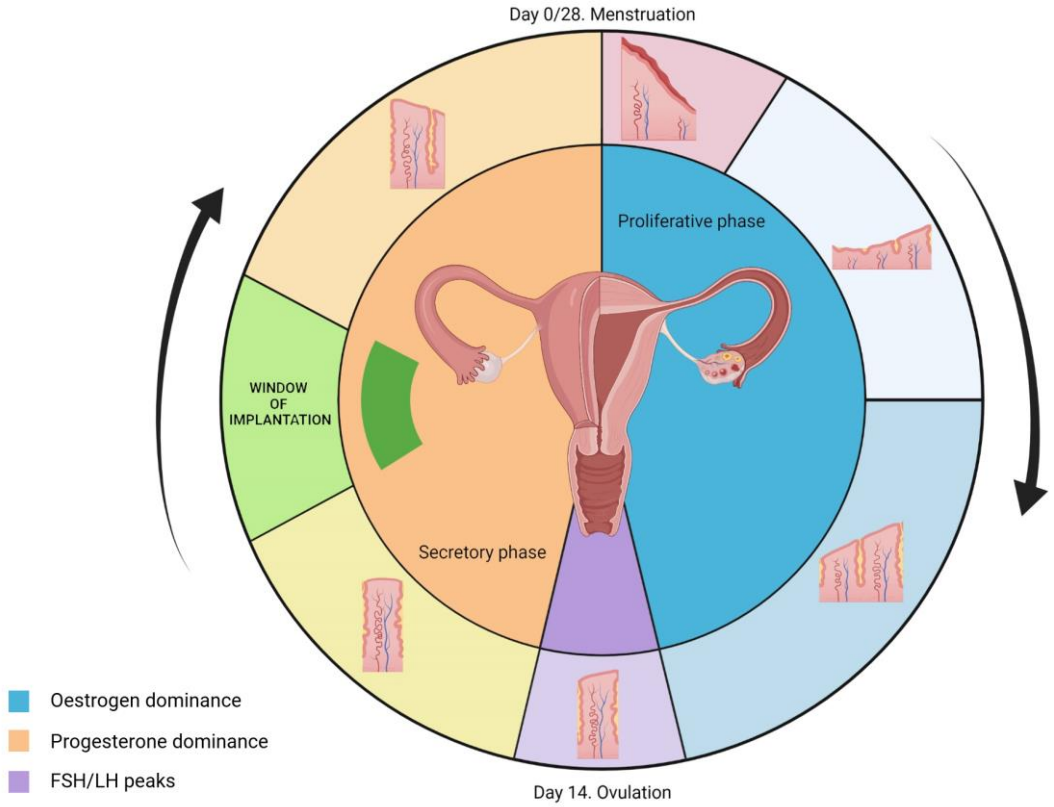

Figure 1. Endometrial cycle phases in a typical 28-days cycle. The main phases in the uterine cycle are described in the centre of the image (proliferative and secretory). The external part of the figure shows endometrial transformation across the cycle. Window of implantation (green) is restricted to a period of 48-72h during the mid-secretory phase. Colours denote subphases in the endometrial cycle (pale blue: early proliferative; blue sky: advanced proliferative; purple: ovulation; yellow: early secretory; green: mid-secretory; orange: late-secretory; pale red: menstruation). Inner circle layers denote hormone predominance in each phase: oestrogens (blue) in proliferative phase; progesterone (orange) in secretory phase; Follicle-Stimulating Hormone (FSH) and Luteinising Hormone (LH) peaks in ovulation. Figure was created using BioRender.

In normally-cycling women, the WOI is narrowly delimited and restricted to the mid-secretory phase of the menstrual cycle. Classically, this time frame is considered to occur 8 to 10 days after the luteinising hormone (LH) surge, that induces ovulation, and is expected to last around 2 or 3 days (Katzorke *et al*., 2016; Messaoudi *et al.*, 2019). However, the displacement of the WOI has been widely reported in many patients and proposed as one of the endometrial origins of embryo implantation failure (He *et al*., 2021; Li *et al*., 2020; Mahajan *et al*., 2018).

The harmonisation of three major players is required in the game of successful embryo implantation in the maternal endometrium: a viable blastocyst, a receptive endometrium and a perfectly synchronised molecular dialogue between them (Aghajanova *et al.*, 2008). In such a complex triad, the most investigated element is the embryo, with research focussing on the understanding and optimisation of its quality (Katzorke *et al*., 2016). In contrast, despite the advances in Assisted Reproductive Technologies (ART) that allow to obtain good quality embryos under clinical and culture conditions, optimised endometrial receptivity still remains a big barrier, often neglected and lagged behind the biology of the blastocyst (Demiral *et al.*, 2015). Indeed, major reasons behind failures in the establishment of pregnancy lie on impaired uterine receptivity once it is assumed that the transferred embryo is of high-quality (Messaoudi *et al*., 2019). In terms of percentages, inadequate endometrial receptivity is responsible for around two-thirds of implantation failures in *in vitro* fertilisation (IVF), while the rest of failures may be due to the quality of the embryo itself (Achache and Revel, 2006; Devesa-Peiro *et al.*, 2021; Messaoudi *et al.*, 2019; Somigliana *et al*., 2018). Thus, there is an urgent need for filling the gap of knowledge to understand the dynamics in the transition from a non-receptive to a receptive endometrium and to determine the causes leading to a suboptimal endometrial status.

Altogether, the complex modifications that take place in the human endometrium have boosted research on the field to the identification of informative biomarkers of

endometrial receptivity (Gómez *et al*., 2015). However, current knowledge of human endometrial biology is nascent, based on decades of studies using endometrial tissue, as well as *in vivo* and *in vitro* models (Nikolakopoulou and Turco, 2021). To date, a consensus on single specific histological or molecular biomarkers as objective and reliable indicators of endometrial receptivity has not been reached (Katzorke *et al.*, 2016). In 1950, Noyes criteria based on histological dating of endometrial biopsies emerged as a revolutionary technique for the assessment of endometrial receptivity on fertility status (Noyes *et al.*, 1950). However, its utility has been continuously called into question in multiple studies (Cohen *et al*., 2020; Garrido-Gómez *et al*., 2013; Murray *et al*., 2004), with much criticism in the intra- and inter-observer variability associated (Bourgain, 2004). Alternatively, some parameters such as endometrial morphology or subendothelial blood flow have been interrogated with no success in prognosing receptivity status (Aghajanova *et al.*, 2008; Demiral *et al*., 2015). Subsequently, research was concentrated on the discovery of biochemical markers able to assess the endometrial status (Katzorke *et al*., 2016). These potential molecular biomarkers include growth factors, cytokines, chemokines, lipids, or adhesion molecules (Achache and Revel, 2006), however, none of them has been implemented in clinical practice so far (Messaoudi *et al*., 2019). Thus, the lack of accuracy and reproducibility of these classical methods has shifted the focus towards more *in silico* data mining approaches that take advantage of the high-throughput omics technologies (i.e., genomics, transcriptomics, metabolomics…), whose development has been noticeably boosted by the advances in molecular biology techniques. This myriad of resources aims to diagnose endometrial receptivity objectively. Indeed, commercially available endometrial receptivity tests based on analysis of transcriptome data have been developed (Altmäe *et al*., 2017; Díaz-Gimeno *et al.*, 2011; Enciso *et al*., 2018) and are successfully used in clinical practice.

1.2. Endometriosis

Endometriosis affects 10% of women, is also present in 20-30% of women with subfertility, and 40-60% of women with chronic pelvic pain and infertility (Akter *et al*., 2020). Even though the maximum incidence of endometriosis is observed between the 25th and the 45th years of life, diagnosis of cases in both postmenopausal and young women are reported sporadically and sometimes remain under-recognised (Benagiano *et al*., 2021; Cope *et al*., 2020; Naem *et al*., 2020; Smolarz *et al*., 2020). Indeed, among the symptomatic adolescent population, estimates range from 49% to even 75% for those with pain unresponsive to medical treatment (Zondervan *et al.*, 2020).

Endometriosis can adversely impact women's daily life, as many aspects of social, family, sexual, educational, and occupational activity can be deteriorated (Smolarz *et al*., 2020). The presence of endometriosis implies a societal burden as well, as women suffering from incapacitating pain due to this condition are impeded to prosper personally, professionally, and financially (Filby *et al*., 2020).

It is estimated that the annual costs of endometriosis treatment in Europe may reach 12.5 billion euros, and are comparable with the costs of therapy of other chronic diseases, such as type 2 diabetes or rheumatoid arthritis, among others (Simoens *et al.*, 2012; Smolarz *et al.*, 2020; Zondervan *et al*., 2020). Further, the costs of care to manage common symptoms as chronic pelvic pain, dysmenorrhea, deep dyspareunia, dysuria, dyschezia, or fatigue, are even greater due to the collateral impact of these symptoms in physical, mental, sexual, occupational and social aspects of life (Zondervan *et al.*, 2020). Moreover, endometriosis is also a major cause of infertility -about the half of affected women have problems to conceive-, with the subsequent substantial negative effect that the inability to give birth involves, and often are encouraged to undergo surgery to remove lesions in order to overcome the pain and increase the chances of pregnancy (Filby *et al*., 2020).

Furthermore, endometriosis is believed to alter the course or pregnancy, with a higher incidence of obstetric complications such as miscarriage, preterm delivery, or placental abruption reported in patients with endometriosis (Porpora *et al*., 2020). Indeed, higher incidence of certain serious adverse outcomes at maternal, foetal and neonatal levels in women with endometriosis has been suggested (Lalani *et al*., 2018). Regardless of the considerable impact of endometriosis on women and their environment, public and professional awareness of the disease still remains insufficient (Missmer *et al*., 2021; Zondervan *et al*., 2020).

1.2.1. Causes and origin of endometriosis

The precise causes of endometriosis are still unknown. Nevertheless, diverse theories with a strong scientific basis have been postulated. The most widespread and accepted hypothesis is the retrograde menstruation, whereby the reflux of endometrial cells and tissue fragments to the fallopian tubes is observed (Filby *et al.*, 2020; Yang *et al*., 2017). However, this theory would not fully explain the origin of endometriosis, as the prevalence of retrograde menstruation is estimated to be 90% in cycling women, whereas the prevalence of endometriosis remains around 10% (Vallvé-Juanico *et al*., 2019). The cellular debris shed during menstruation would initiate a neuroangiogenic and proinflammatory response that would progressively evolve to endometriotic lesions (Filby *et al*., 2020). Other popular and reasonable theories that partially explain the origin of endometriosis comprise coelomic metaplasia, whereby peritoneal mesothelium transforms into glandular endometrium; lymphovascular metastasis, that involves transport of endometrial cells through lymphatic and blood vessels; and the theory of neonatal uterine bleeding, a phenomenon that may explain early-onset endometriosis (Brosens and Benagiano, 2013; Dekker *et al*., 2021; Filby *et al*., 2020; Poli-Neto *et al.*, 2020; Zondervan *et al.*, 2020). However, given the complex and multisystemic nature of the disorder, these theories neither are able to fully explain the origin and evolution of

the disease, and it is claimed that genetic susceptibility, endocrine, immunological factors, or even the presence of microbiological contamination should be additionally considered (Yang *et al.*, 2017; Poli-Neto *et al.*, 2020). However, it is not clear whether these factors are a cause or a consequence of the disease and therefore more research is required to explain the complex mechanisms that underlie the origin and development of endometriosis.

1.2.2. Diagnosis and biomarkers of endometriosis

There is still no cure for endometriosis; however, the most common symptom -associated pelvic pain- can be managed with the use of hormonal therapy directed towards the suppression of oestrogen production, and to the inhibition of tissue proliferation and inflammation (Zondervan *et al*., 2020). Furthermore, the removal of the lesions with surgery represents a partial solution with reduced effectiveness, as 30-60% of cases exhibit recurrence within 12 months (Filby *et al.*, 2020).

Regarding the diagnosis, the majority of cases are detected in women between menarche and menopause (Smolarz *et al*., 2020). The current gold standard for the diagnosis of the disease consists on the direct visualisation of endometriotic lesions after laparoscopy surgery. It is evident that a delay in the diagnosis and misdiagnosis are frequently observed in the clinical practice (Taylor *et al.*, 2021). Despite the necessity of early diagnosis to prevent women from suffering the progression of the disease and its sequelae (Akter *et al*., 2020), the multiple presentations, the absence of evident clinical symptoms, signs or biomarkers that are good predictors of the disease, the lack of awareness and education among the community and healthcare providers, and the requirement of surgical procedures with invasive nature lead to a considerable delay in diagnosis, which is presently estimated in 7 years after onset of symptoms (Filby *et al*., 2020; Poli-Neto *et al*., 2020; Taylor, 2019; Zondervan *et al*., 2020).

During the last decade, there is a big interest in the identification of potential biomarkers of endometriosis that may help to detect the presence of the disease or its activity (May *et al.*, 2011). Indeed, biomarker research has been defined as a research priority by the World Endometriosis Society and World Endometriosis Research Foundation (Horne *et al*., 2017). Despite extensive research, no biomarker has been validated for clinical use so far (Gupta *et al*., 2016; Nisenblat *et al*., 2016; O *et al*., 2018), as the main focus has been into the discovery instead of reproducibility and validation (D'Hooghe *et al.*, 2019).

The strategies in the discovery of biomarkers of endometriosis can be divided in two groups: "hypothesis-driven" and "hypothesis-generating" approaches (O *et al.*, 2018). The first may be seen as a classical approach, in which one or a few biomarkers are interrogated upon their role in the pathogenesis of the disease. In contrast, the development of the high-throughput omics technologies has led to adopt the second strategy, in which general differences between patients are expected to be detected. The major advantage of this last approach is that data collection does not necessarily need a starting hypothesis neither a primary research question (Saare *et al.*, 2017).

A plethora of molecules have been investigated as potential biomarkers of endometriosis through the study of endometrial tissue, blood, serum and urine samples. Among the studied biomarkers related to endometriosis are different hormones, cytokines, glycoproteins, chemokines, prostaglandins, angiogenic factors, markers of apoptosis, oxidative stress markers, cell adhesion molecules, matrix-related proteins, mRNAs, microRNAs, and immune system proteins (Table 1). However, the results of evaluating the potential biomarkers are often controversial, and even if they conclude that certain molecules may have a diagnostic value, more research starting with validation is warranted (Akter *et al*., 2020). In a recent review, Anastasiu *et al.* aimed to evaluate the effectiveness of putative biomarkers of endometriosis at serum and urine and concluded that none of the studies in the field has been able to date to identify a

single biomolecule or a panel of biomarkers with enough sensitivity and specificity in endometriosis (Anastasiu *et al.*, 2020).

High-throughput technologies have provided new insights into the complexity of endometriosis (Saare *et al*., 2017), giving hope to the challenge of finding reliable biomarkers of the disease (Tian *et al*., 2020). Definitely, they might represent promising substitutes of invasive techniques and therefore offer a novel perspective of non-invasive biomarkers discovery strategy (Anastasiu *et al*., 2020).

As endometriosis is believed to hold a strong genetic background (Mirza and Abdel-dayem, 2020), omics studies assessing the presence of genetic variants and the risk of disease are strongly informative (Saare *et al*., 2017). Genome-wide association studies (GWAS) in endometriosis have resulted in identification of some variants that seem to be promising candidates to explain the origin of the disease (Matalliotakis *et al*., 2017; Rahmioglu *et al.*, 2014; Smolarz *et al.*, 2020).

Table 1. Potential biomarkers of endometriosis. WWE: women with endometriosis. HCW: healthy control women. Please note that some molecules could belong to various groups.

	Biomarker	Evidence of dys-regulation in endometriosis or of its utility as diagnostic biomarker of endometriosis	Reference(s)
hormones	GREB1	Increased expression in endometriotic lesions of the peritoneum *vs.* eutopic endometrium. Involved in oestrogen-dependent development of endometriosis	(Smolarz *et al*., 2020)
	CYP19, ESR2	Involved in the biosynthesis of oestrogens. The presence of polymorphisms in these genes has been associated with a higher risk for the occurrence of endometriosis	(Smolarz *et al*., 2020)
cytokines	IL-6	Higher levels in blood, PF and ectopic endometriosis stromal cells of WWE	(Li *et al.*, 2021)
	IL-13, IL-15	Higher expression in the peritoneal fluid of WWE compared with fertile controls	(Chegini *et al*., 2003)
	TNF-α	Increased endometrial expression during the menstrual phase of the cycle in WWE compared with controls	(Kyama *et al*., 2006)
	IL2RG, LOXL1	Significantly differently expressed among a panel of mRNAs in peripheral blood lymphocytes of WWE *vs.* HCW	(Flores *et al.*, 2006)
glycoproteins	CA-125	Most commonly described biomarker of endometriosis; elevated in advanced endometriosis stages	(Nisenblat *et al.*, 2016)
	CA-19-9	Higher expression levels in WWE compared with HCW; positive correlation with the severity of the disease	(Anastasiu *et al*., 2020)
	Glycodelin	Significantly increased concentrations of glycodelin-A in the serum of WWE compared to HCW	(Kocbek *et al*., 2015)
growth factors	EGF	Significantly increased in the peritoneal fluid of WWE compared with HCW	(Rakhila *et al*., 2016)
	VEGF	Involved in angiogenic processes, it has been investigated endometriosis blood biomarker	(Tian *et al.,* 2020)
	NGF	Decreased expression in eutopic endometrium of women with peritoneal endometriosis, useful for early diagnosis	(Szubert *et al*., 2021)
markers of apoptosis	Bcl-2	Immunohistochemistry studies confirmed increased expression in WWE, while other studies showed no changes	(May *et al.*, 2011)
	survivin	mRNA levels of this apoptosis inhibitor were detected in the serum of WWE in a higher proportion than in HCW	(O *et al.*, 2018)
	CASP8	Elevated levels in women with endometriotic ovarian cysts *vs.* HCW may represent an early sign of tissue injury	(Di Nisio *et al.,* 2019)

chemokines	CXCL8	A systematic review identified it as the best chemokine as marker for endometriosis	(Borrelli *et al*., 2014)
	CCL2	Higher levels during the secretory phase of the menstrual cycle in WWE compared to healthy controls	(Nirgianakis *et al*., 2020)
	RANTES	The first chemokine evaluated in association with endometriosis and the third most assessed as a biomarker	(Borrelli *et al*., 2014)
	CCR1	Measurement in peripheral blood leukocytes in combination with MCP-1 and CA-125 proteins as a promising diagnostic test with high sensitivity and specificity	(Agic *et al*., 2008)
matrix-related proteins	MMP2	Overexpression in WWE compared to HCW consistently reported across studies	(Zahrah *et al*., 2021)
	MMP9	Increase expression in ectopic and eutopic samples from WWE compared to HCW	(Barbe *et al.*, 2020)
	TIMP-1	Lower levels in serum samples of WWE compared with control women with tubal factor infertility	(Nanda *et al.*, 2020)
prostaglandins		Released by macrophages in endometriosis, which contributes to the inflammatory character of the disease	(Anastasiu *et al*., 2020)
oxidative stress markers	SOD, GPx	The combination of these two biomarkers could be used as preoperative biomarker for endometriosis given the changes in their concentration in the blood of WWE diagnosis	(Ekarattanawong *et al*., 2017)
	eNOS	Increased levels in the glandular and luminal epithelium of infertile WWE *vs.* infertile women	(May *et al.*, 2011)
	MPO	Higher levels in the follicular fluid of WWE (severe); proposed as a potential target in WWE-associated infertility	(Santanam *et al*., 2017)
cell adhesion molecules	β3 integrin	Reduced expression in WWE compared with HCW and women with other causes of infertility. Contradictory results with other studies that detected increased expression during menses of WWE	(May *et al.,* 2011)
	E-cadherin	Higher levels during the mid-secretory phase of the menstrual cycle of WWE, lower levels in late secretory phase	(Matsuzaki *et al.*, 2010)
	VCAM-1	Serum levels proposed as promising marker in WWE; urine levels showed no difference between WWE and HCW	(Proestling *et al.*, 2020)
microRNAs	miR-200a	From a panel of miRNAs, it was the one with the best discriminative power to differentiate WWE from HCW	(Rekker *et al*., 2015)
	miR-200c, miR-34a-5p	Significantly dys-regulated (miR-200c up-, miR-34-5p down-regulated) in WWE compared to HCW; serum levels show high sensitivity (>78% miR-34a-5p and 100% miR-200c) and specificity (>49% miR-34a-5p and 100% miR-200c)	(Misir *et al.*, 2021)
	miR-125b	>10-fold up-regulation in microarray analysis of WWE *vs.* HWC; highest specificity (96%) and sensitivity (100%)	(Cosar *et al.*, 2016)

1.3. Endometriosis-associated infertility

Up to 50% of women with endometriosis are suffering infertility (Bulletti *et al*., 2010). Infertility is a common health problem that affects couples worldwide, and is defined as the incapacity to conceive after 12 months of regular, unprotected sexual intercourse (Vander Borght and Wyns, 2018). Infertility is recognised as a public health priority; however, its prevalence rates are difficult to determine. It is estimated that around 48 million couples and 186 million individuals suffer from infertility globally (Rutstein and Shah, 2004), and these values seem to be increasing, which constitutes a real matter of concern (Sun *et al.*, 2019). The percentage of couples that remain involuntarily childless represents around 10-15%, with a subsequent social and emotional burden that goes beyond the boundaries of the healthcare system. Over the last four decades, the development of ART has helped many infertile couples with the chance to conceive. However, and despite the extensive research and efforts in improving the techniques employed, there are still many attempts to become pregnant that do not result in a successful conception (Messaoudi *et al.*, 2019).

Endometriosis has an impact on fertility, with reduced pregnancy chances commonly associated to the disease (Nisenblat *et al*., 2016). Fecundity rates among couples with the woman partner suffering from endometriosis ranges between 2-10%, whilst endometriosis-free controls achieve 15-20% (Ghosh *et al*., 2020). Despite the strong evidence of connection between endometriosis and infertility, it is not possible to prove the existence of a causal relation, and the precise mechanisms leading to infertility remain still elusive (Pirtea *et al*., 2021; Porpora *et al*., 2020).

Endometriosis infertility may respond to a combination of distorted pelvic anatomy due to the inflammatory environment created by the disease itself, effects on oocyte and sperm quality and embryo development, and unwelcoming conditions for embryo nidation (Ghosh *et al*., 2020; Pirtea *et al*., 2021; Vallvé-Juanico *et al*., 2019). To control

for these compromising factors in the reproductive cascade may help to improve the fertility rates in women with endometriosis.

Regarding the above-exposed causes, it seems that the inflammatory processes associated to endometriosis contribute to the development of an inhospitable environment that affects the survival of either gametes and/or embryos, thereby reducing pregnancy outcomes (Pirtea *et al*., 2021). However, when this factor is controlled and equal quality embryos are transferred to women with endometriosis and couples with an uterine factor presumed to be normal, lower implantation rates are observed in the disease group, suggesting an alteration of the endometrial function in women with endometriosis (Blank *et al*., 2021). In fact, altered endometrial receptivity at a molecular level among women with endometriosis has been detected (Prašnikar *et al*., 2020). Moreover, molecular changes in the eutopic endometrium of patients with endometriosis have been described (Vallvé-Juanico *et al*., 2020), and some omics-based studies have reported different regulation at molecular level when the endometrial tissue of patients with endometriosis is compared with that from healthy women (Altmäe and Aghajanova, 2015). Nevertheless, despite of the extensive research in the field, there is no consensus whether women with endometriosis expose dys-regulated endometrial receptivity or not when compared to women without the disease.

1.4. Reproductomics: a nascent area of research and biomarker discovery

The term ‘reproductomics’ encompasses the strategies based on high-throughput omics technologies that are employed with application in the field of reproductive medicine (Bellver *et al*., 2012). Over the last two decades, the advances in molecular biology techniques have boosted the emergence of studies with reproductive tissues, with a particular explosive growth in the number of omics studies involving the human endometrium (Altmäe *et al*., 2014). The omics studies applied in studying endometriosis

could be termed as endometriomics. It is believed that the integration of omics data at multiple levels (i.e., multi-omics approach) would provide powerful insights into complex diseases, including unravelling pathophysiological mechanisms of endometrium (Figure 2) (Prašnikar *et al*., 2020).

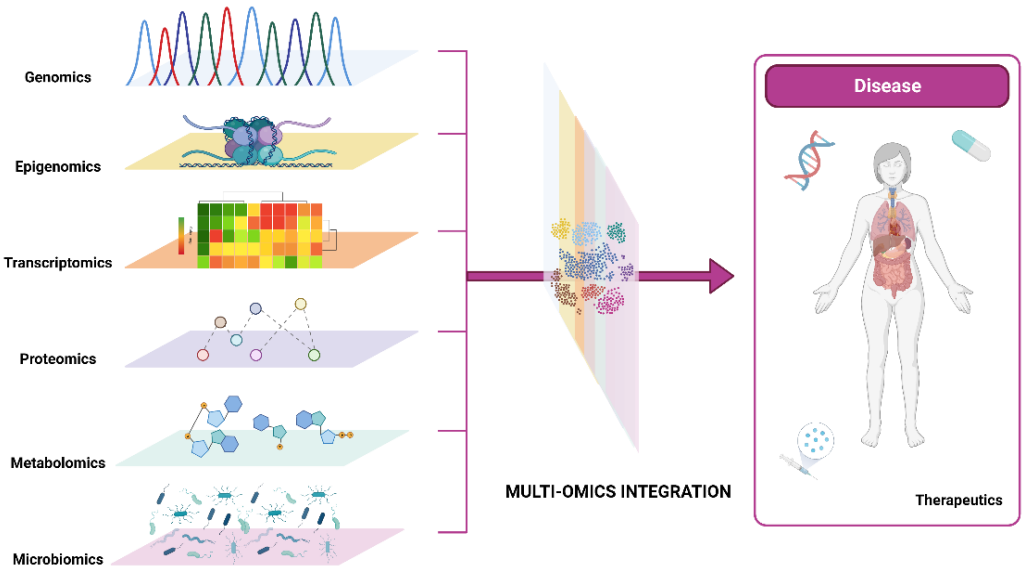

Figure 2. Integration of omics data at multiple levels: genomics, epigenomics, transcriptomics, proteomics, metabolomics, microbiomics with the ultimate goal to understand complex diseases. Figure was created using BioRender.

Transcriptomics studies have been the most popular within reproductomics research. For instance, differential gene expression patterns have been observed during different phases of the menstrual cycle, including the WOI (Katzorke *et al*., 2016); in fact, transcriptomics paved the way to the investigation of the optimal gene expression profile representative of endometrial receptivity and necessary for embryo implantation. In particular, the endometrial receptivity array (ERA) was published in 2011 after years of basic and translational research (Díaz-Gimeno *et al*., 2011). Recently, the first transcriptomic analysis of the human endometrium at a single-cell level has provided a precise characterisation of the different phases of human endometrial transformation

across the menstrual cycle, as well as the main cell types, changing some of the traditional conceptions about the tissue (Wang W *et al.*, 2020). Regarding endometriomics, the impact of endometriosis in the transcriptome has been assessed through numerous microarray and RNA-seq studies (Colón-Caraballo *et al.*, 2019; Da Broi *et al.*, 2019; Fassbender *et al*., 2012; Matsuzaki, 2011), that have yielded interesting results and many candidate genes to explore, although the number of consistently up- and down-regulated transcripts has remained insufficient (Altmäe *et al.,* 2014; Saare *et al*., 2017).

Although transcriptomic studies have hold the majority of the research, other omics fields such as genomics, epigenomics, proteomics, metabolomics and microbiomics are also applied in reproductomics in general, and in endometriomics in particular.

Given the strong genetic background classically attributed to endometriosis, genomic studies have been popular in endometriomics research, especially in the search of loci associated with disease risk (Zondervan *et al*., 2016). However, studies analysing the eutopic endometria of women with endometriosis have been rare (Saare *et al*., 2017).

Epigenomics studies in the endometrium have demonstrated how aberrant expression profiles at miRNA levels may lead to gynaecological conditions such as endometriosis (Altmäe *et al*., 2014). Moreover, epigenomic studies have evidenced a dys-regulation of the DNA-methylation profiles of the endometrium of women with endometriosis when compared with healthy control women (Houshdaran *et al.*, 2016; Rahmioglu *et al*., 2017).

Proteomics has been used to identify differences between receptive and non-receptive samples (Garrido-Gómez *et al.*, 2014; Guo, Li and Chen, 2021) and to test uterine receptivity in uterine fluid with minimal invasiveness (Kasvandik *et al*., 2020). Moreover, omics have found an interesting application in the research of endometrial pathology, such as repeated implantation failure (RIF) (Wang *et al*., 2021) and

endometriosis (Poliness *et al*., 2004; Saare *et al*., 2017). In the last decade, proteomics applied to endometriosis evidenced the differential expression of a number of proteins among healthy controls and women with endometriosis that were proposed as candidate diagnostic biomarkers (Cho *et al*., 2012; Hwang *et al*., 2014, 2013; Wang *et al*., 2014).

Metabolomics is a promising tool to explore diagnostic biomarkers of non-invasive/minimally invasive nature. In endometriosis, the use of metabolomics techniques has provided the identification of potential biomarkers for the diagnosis of endometriosis from plasma (Dutta *et al*., 2012; Vouk *et al*., 2012) and eutopic endometrium (Li J *et al*., 2018). However, it is claimed that the use of metabolomics as non-invasive biomarker for the diagnosis of endometriosis needs thorough research before being translated into the clinical practice (Anastasiu *et al*., 2020).

Regarding the microbiome, recent studies have deep sequenced a hypervariable region of 16S ribosomal RNA gene and have identified a unique uterine microbiota that differs from that of the vagina (Chen *et al*., 2017; Franasiak *et al*., 2016; Moreno *et al*., 2016; Verstraelen *et al*., 2016). As less than 1% of microbes can grow and form colonies on agar plates, analysis of the genomes of the microorganisms overcomes two limitations of the traditional culture-based microbe characteristics: non-culturability and genomic diversity (Giudice, 2016). The presence of bacteria in the uterus has been associated with poor reproductive outcomes and endometriosis; however, a cause and effect relationship has not been clearly established (Molina *et al*., 2020). These preliminary studies open a new era of research in medicine for gynaecological diseases and human reproduction, and evidence the necessity of new studies that allow to understand the development and aetiology of endometriosis and the role of the microbiome in the reproductive success/failure.

Nowadays, and independently of the strategy, a challenging task that omics have to face is the identification of biomarkers through the use of non-invasive/minimally invasive techniques (Domínguez *et al*., 2017; Irungu *et al*., 2019; Katzorke *et al*., 2016).

Despite the wide range of advantages derived from the use of omics technologies, the huge amount of data generated represents a challenge, and the high variability of the omics profiles make difficult to validate the use, reproducibility and interpretation of the data (Altmäe *et al*., 2014). Furthermore, an important lag exists between the generation and the analysis, integration and interpretation of the big data (Alyass *et al*., 2015), thus the vast majority of data generated remains underexplored.

The need of highly powered studies and integrated approaches was manifested as a future path for research (Altmäe *et al*., 2014), since it is believed that multi-omics approaches would help to unblock the bottleneck in accumulating data and gain insight into the investigation of complex phenotypes (Goulielmos *et al*., 2020; Subramanian *et al*., 2020). Thus, the use of high-throughput omics integrative approaches in combination with computational tools (i.e., systems biology) would improve considerably our understanding of the complex gynaecological disease such as endometriosis.

2. AIMS

The aim of this thesis is to bring more understanding into the complex gynaecological disease as is endometriosis in applying *in silico* data mining approaches that would enable to identify potential biomarkers and molecular pathways involved in the disease.

The specific aims of the thesis include:

Study I: to summarise the main computational methods that are frequently used in the field of omics with application in reproductive biomedicine and to set out a pipeline for omics data analysis that could be easily replicated.

Study II: to determine the most common conditions that are comorbid to endometriosis and to detect a possible overlap between them that could explain their co-occurrence in order to identify candidate biomarkers of endometriosis.

Study III: to meta-analyse endometrial transcriptome profiles in women with endometriosis during the mid-secretory phase of the menstrual cycle in comparison to healthy control women in order to identify potential biomarkers and molecular processes that are dys-regulated in endometriosis and could explain the endometriosis-associated infertility.

3. MATERIALS AND METHODS

This section summarises the key considerations and methods used during the development of this PhD thesis. Detailed descriptions of the protocols can be found in the respective studies. The overview of the main steps of analysis for each study are highlighted in Figure 3.

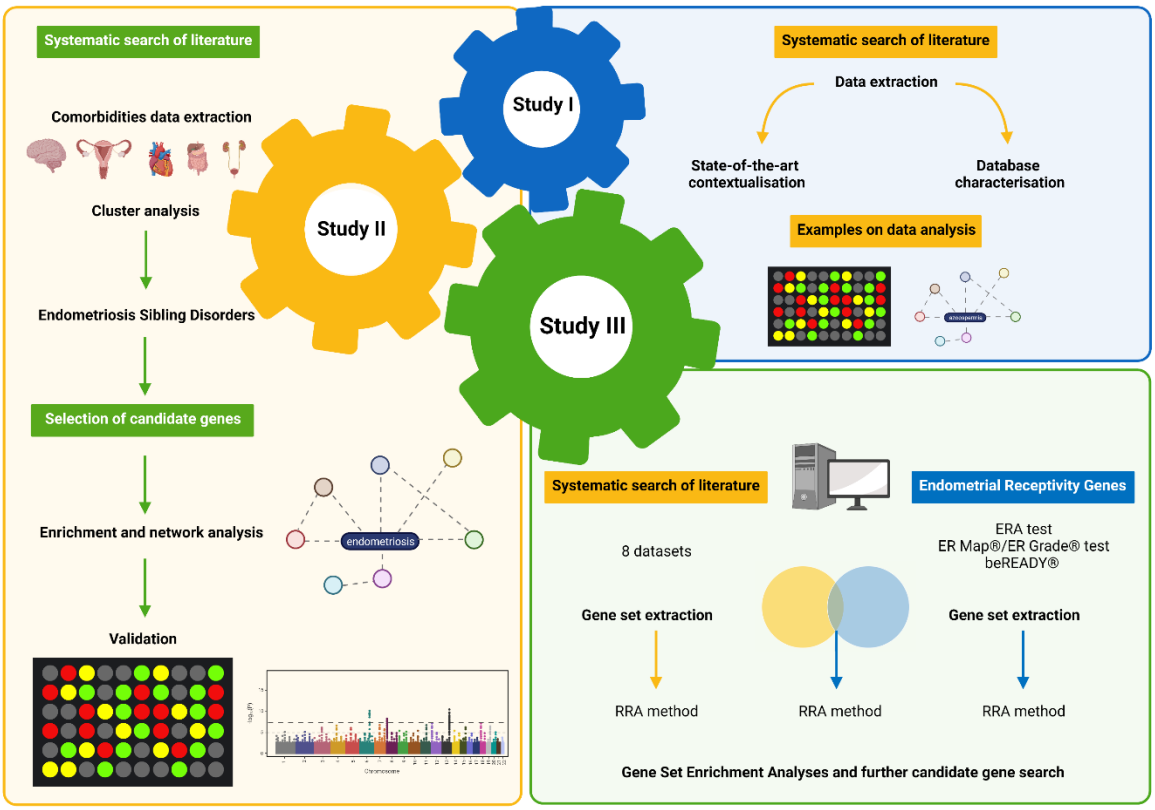

Figure 3. Synthesis of the main steps of analysis followed in the studies integrating this thesis. Figure was created using BioRender.

3.1. Materials

Given the computational nature of this PhD project, the majority of the materials used was data originating from different public sources. Figure 4 summarises the main tools that have been used in the different studies comprising this PhD thesis and that will be commented across this section.

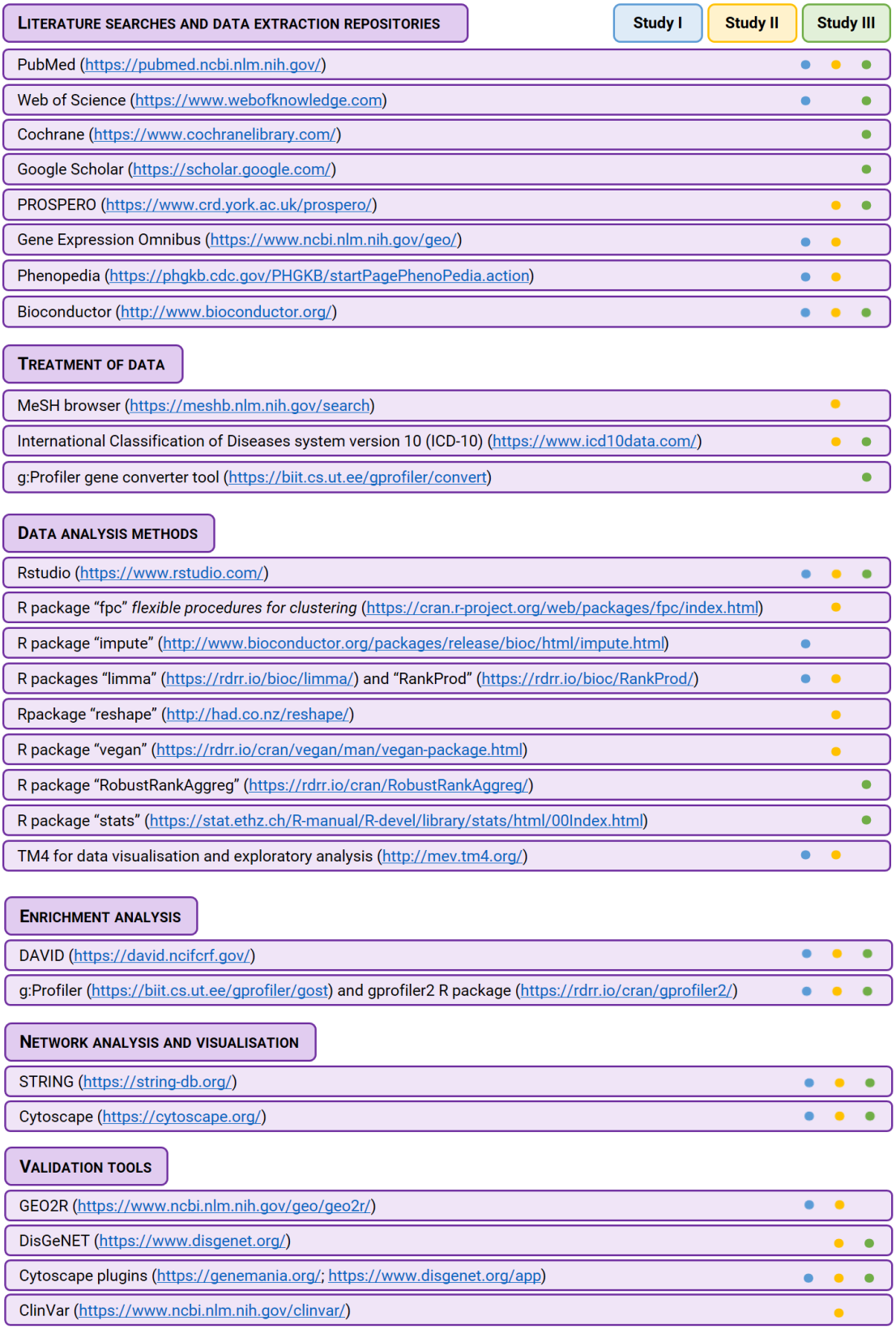

Figure 4. Overview of the main tools for in silico analysis that have been suitable of use in the studies integrating this PhD thesis. Please note that despite this list pretends to be exhaustive, only the most important tools for the achievement of the results presented are shown.

In **Study I**, a comprehensive revision of the literature was performed in order to consolidate the information about the orthodox approaches in computational and systems biology applied to the reproductive field. Data was retrieved from public literature repositories (PubMed and Web of Science), and practical examples on how to perform omics analyses in reproduction were presented as well. Specifically, public data sources used to create the presented examples consisted in: 1) access through the repository Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) to set out a pipeline of analysis of microarray data in an example model of Polycystic Ovary Syndrome (PCOS); and 2) use of Phenopedia (https://phgkb.cdc.gov/PHGKB/startPagePhenoPedia.action), a web-based tool providing disease-centred views of GWAS (Yu *et al*., 2010), to explore the main genes that have been reported in association to a given MeSH term (in our example, "azoospermia"), and subsequently a network analysis using the specific network software STRING and Cytoscape was performed.

Study II and **Study III** started with a systematic literature search for identifying the required data. In **Study II**, the systematic search was focussed on the extraction of clinical conditions that had been previously reported in comorbidity with endometriosis. This data was codified into Medical Subject Heading (MeSH) terms, that were subsequently processed in order to obtain significant overlaps among co-occurring diseases. Data from microarray experiments were additionally accessed to collect records that served as validation datasets.

In **Study III**, we aimed to compile all publications involving the transcriptomic analysis of the endometrial tissue of women with endometriosis in the receptive phase of the menstrual cycle in comparison with healthy control women to generate a pool of differentially expressed genes suitable for meta-analysis. Gene expression levels at the mid-secretory phase were selected for assessing the molecular differences in the receptive phase endometrium.

3.2. Methods

3.2.1. Literature searches and data extraction

The extensive revision of the literature constitutes an initial step in our analyses, as it corresponds to the first source of information for data selection and accession. In **Study I**, review of the literature was performed searching for the relevant studies available in public repositories and the information was selected and classified according to the structure planned for the book chapter. For the systematic literature searches conducted in **Studies II** and **III**, literature databases (Cochrane, PubMed, and Web of Science) and Google Scholar were consulted. Schemes of the searches is summarised in Table 2.

Table 2. Summary of the criteria employed in the literature search for each study included in this thesis. Data extraction represents the data of interest that were processed for further analyses.

	Databases	Search criteria	Data extraction
S1	PubMed Web of Science	Relevant papers on the field of Reproductomics, i.e., application of omics in reproductive medicine according to the structure planned for the book chapter	Information of interest on advances and application of omics approaches in reproductive medicine
S2	PubMed	"Endometriosis" AND "comorbidity"/ "comorbidities"/ "coexistence"/ "disease"/ "disorder"	• List or endometriosis comorbidities • List of genes associated to each comorbid disorder
S3	PubMed Web of Science Cochrane Google Scholar	"Endometriosis" AND "transcriptomics"/ "transcriptome"/ "gene expression"/ "RNA-seq"/ "sequencing"/ "array"	Differentially expressed genes in the eutopic endometrium of women with endometriosis during the mid-secretory phase

The systematic reviews for **Studies II** and **III** were carried out following the guidelines established in the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) statement (Moher *et al*., 2015; Page *et al*., 2021; Shamseer *et al*., 2015) and conveniently recorded in the International prospective register of systematic reviews PROSPERO database (https://www.crd.york.ac.uk/prospero/). This method was used to extract the original data, which were: **Study II)** a list of clinical

conditions comorbid to endometriosis, and **Study III)** a list of differentially expressed genes in the endometrium of women with endometriosis during the mid-secretory phase of the menstrual cycle. Initial data was conveniently prepared for subsequent analyses.

3.2.2. Treatment of data

Personalised scripts for the use of packages of analysis in R language implemented in RStudio software were designed to treat the data (R Core Team, 2020).

In **Study I**, the dataset GSE5090 was downloaded from GEO repository to proceed with the creation of the first example of analysis. The dataset reflected the expression profile of nine PCOS samples *vs.* eight control samples (Cortón *et al*., 2007). For data analysis, preprocessing and normalisation were performed using R packages *impute* and *limma*, respectively. Further, advanced analysis of microarray data was performed using *TM4* software (http://mev.tm4.org/; Saeed *et al.*, 2006). For the second example, genomic information on azoospermia was retrieved from Phenopedia (https://phgkb.cdc.gov/PHGKB/startPagePhenoPedia.action; Yu *et al*., 2010), and interaction networks were subsequently constructed.

In **Study II**, the clinical conditions comorbid to endometriosis were codified into MeSH terms using the 10th version of the International Classification of Diseases system (ICD-10) and this data was used to access specific gene expression profiles for each term using Phenopedia (https://phgkb.cdc.gov/PHGKB/startPagePhenoPedia.action), that retrieves lists of genes that have been predicted in association with a given MeSH term based on the results of genetic association studies (Yu *et al*., 2010). Thereafter, clustering methods were applied to select a subset of diseases that were considered as closer to endometriosis in terms of gene expression profile similarities, and finally subsequent analyses were performed to investigate the implications of these findings at a functional level.

In **Study III**, adapted computational scripts run in RStudio software were used to perform a meta-analysis on the differentially expressed genes in the endometrium of women with endometriosis during the receptive phase of the cycle using the Robust Rank Aggregation (RRA) method. The RRA is a powerful method for the integration of ranked lists that is applicable to data from high-throughput omics experiments. It combines multiple lists into a single ranking to detect a meaningful combination from different data sources (Kolde *et al*., 2012). With this approach we aimed to identify candidate biomarkers of the disease and gain knowledge about the biology of the endometrium during the WOI in women with endometriosis.

3.2.3. Enrichment analyses

In order to understand the biological complexity, functional enrichment analyses were performed. During the development of this thesis, web-based tools as the Database for Annotation, Visualisation and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) (Jiao *et al*., 2012) and g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) (Raudvere *et al*., 2019) were used in **Studies I-III**. In brief, enrichment analysis tools use statistical approaches to identify groups of molecules that might be over-represented in a large set of molecules and try to decipher their possible association with known disease phenotypes. Among the main sources of evidence, Gene Ontology (GO) was used for discovery of Biological Processes (BP), Molecular Functions (MF) and Cellular Components (CC) in combination with the Kyoto Encyclopaedia of Genes and Genomes (KEGG), WikiPathways (WP) and Reactome (REAC) databases.

3.2.4. Visualisation

The understanding of the biological complexity could be enhanced through the use of visualisation tools. Network analyses provide users with integrative tools that allow

them to evaluate the results obtained from a system perspective. During the development of the studies presented, two main network analysis tools were used: STRING (https://string-db.org/) (Szklarczyk *et al*., 2019) and Cytoscape (https://cytoscape.org/) (Shannon *et al*., 2003), with the purpose of going in depth with the relationship among the elements conforming our system of study. Specifically, in **Study I**, STRING and Cytoscape were used to compose the examples that illustrate the practical section of the book chapter. In particular, regarding Cytoscape analyses, both baseline default software and the plugin GeneMANIA were utilised (Montojo *et al*., 2010). In **Study II**, STRING was used to investigate the interactions among the set of genes that were proposed as biomarkers of endometriosis and its comorbidities. Further, Cytoscape was utilised to construct interaction networks based on the information on protein-protein interactions (PPI) provided by STRING. Finally, in **Study III**, STRING was used to obtain the PPI among the proposed candidate biomarkers, Cytoscape plugin GeneMANIA was used to predict the function of gene sets of interest (Montojo *et al*., 2010), and DisGeNET plugin was employed to visualise variant-disease and gene-disease networks (Piñero *et al*., 2021).

3.2.5. Validation

After the application of all the strategies directed to the search of putative biomarkers of endometriosis through computational approaches, the results generated in **Study II** were subjected to validation techniques. In particular, data from microarray studies in women with endometriosis was accessed through the public repository GEO and analysed with the GEO2R interactive tool implemented in the GEO web server to search for the possible dys-regulation of the proposed candidates at the gene expression level. A total of 9 studies were included in the validation analysis, whose main characteristics are summarised in Table 3.

Other techniques for gene validation and candidate biomarker discovery were utilised during the development of this thesis such as: 1) DisGeNET, 2) ClinVar, and 3) GeneMANIA. Some of them have been previously mentioned as visualisation tools, however their extended functionality deserves mentioning apart.

Table 3. Characteristics of the transcriptome studies utilised for validation of the results obtained in Study II. WWE: women with endometriosis.

Geo Number	Platform	Participants/samples
GSE5108	GE Healthcare/Amersham Biosciences CodeLink Human Whole Genome Bioarray	Ectopic endometrium of WWE (n=11) *vs.* matched eutopic endometrium (n=11)
GSE7305	Affymetrix Human Genome U133 Plus 2.0 Array	Endometrial samples from WWE (n=10) *vs.* endometrial samples from healthy women (n=10)
GSE7846	Affymetrix Human Genome U133 Plus 2.0 Array	Eutopic endometrium from WWE (n=5) *vs.* eutopic endometrium from patients without endometriosis (n=5)
GSE11691	Affymetrix Human Genome U133A Array	Ectopic endometrium of WWE (n=9) *vs.* matched eutopic endometrium (n=9)
GSE12768	Institut Cochin HG18 60mer expression array 47K	Ectopic endometrium of WWE (n=2) *vs.* matched eutopic endometrium (n=2)
GSE23339	Illumina human-6 v2.0 expression beadchip	Patients undergoing surgery for endometriomas (n=10) Non-endometriotic control endometrium (n=9)
GSE25628	Affymetrix Human Genome U133A 2.0 Array	Ectopic endometrium of WWE in the proliferative phase (n=8) Eutopic endometrium of WWE in the proliferative phase (n=8) Endometrium of normal health donors in the proliferative phase (n=6)
GSE35287	Affymetrix Human Gene 1.0 ST Array	Human endometrial stromal fibroblasts from WWE (n=40) Human endometrial stromal fibroblasts from WWoutE (n=40)
GSE58178	Illumina HumanHT-12 V3.0 expression beadchip	Stromal cells from women with ovarian endometriosis (n=6) *vs.* stromal cells from normal endometrial tissues (n=6)

DisGeNET is one of the largest repositories of genes and variants associated to human diseases (Piñero *et al*., 2020). This discovery platform integrates data from a variety of sources and provides metrics that are helpful to assist in the gene prioritisation process. Among its main virtues, the possibility of searching for gene-variant and gene-disease associations was of particular help for our studies on candidate genes generated

after running the corresponding analyses. In particular, **Studies II** and **III** benefited from the information retrieved in this database, both in its web browser (https://www.disgenet.org/) and Cytoscape plugin versions (Piñero *et al*., 2021).

ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) is a catalogue of the already known gene variants associated with a given phenotype. It was particularly helpful for the complementary analyses carried out in **Study II**, where it allowed us to get information about the gene variants described for our set of candidate genes.

GeneMANIA (http://www.genemania.org; Warde-Farley *et al*., 2010) is a web interface for the prediction and analysis at a functional level of a given gene or set of genes and that may be implemented in Cytoscape through the use of a specific plugin (Montojo *et al*., 2010). This initial set is then combined with omics data that help to extend the list with genes with a similar function. This predictive algorithm was used in **Studies I** and **III** to shed light in the predicted functions of groups of genes of interest.

In addition, tools for creation and visualisation of Euler diagrams were used. The main platforms used to this purpose were Venny (https://bioinfogp.cnb.csic.es/tools/venny/; Oliveros *et al*., 2015) and Interactivenn (http://www.interactivenn.net/; Heberle *et al*., 2015). The detection of overlap among different data sets was employed in all the **Studies I-II** of the thesis.

4. RESULTS AND DISCUSSION

Research on human endometrium has awaken an increased interest over the years, as the key role of this intriguing tissue in the establishment and development of pregnancy is evident. The presence of endometrial tissue outside the uterus, known as endometriosis, constitutes a benign gynaecological disorder whose incidence over the decades is rising. Endometriosis involves a wide spectrum of symptoms and situations that commonly result in negative consequences for women. Indeed, the burden of endometriosis goes beyond the clinical symptoms, with notable impact arising at socioeconomic level. The origin of endometriosis has been postulated as a confluence of genetic and environmental causes, but the precise aetiology and aetiopathogenesis remain still undecipherable due to the complexity of the disease.

Endometriosis has a complex genetic architecture, resulting in high variability among different studies. These circumstances often compromise the diagnosis, with a current delay of ~7 years and that is likely to be solved through the discovery of non-invasive and reliable biomarkers that might make possible earlier therapeutic interventions. This challenging task has been addressed over the last decades thanks to the emergence of high-throughput omics technologies, with a huge amount of information generated and often poorly explored. Research on the topic has evidenced that systems biology approaches constitute a useful way to embrace the challenge of biomarker discovery through the application of computational tools that are able to answer questions with a biological meaning.

In this Doctoral Thesis, three publications (one book chapter and two manuscripts) have been developed with the aim to bring more understanding into the complex disease of endometriosis. In the following sections, a summary of the key results achieved is presented, as well as a brief discussion of the main findings.

STUDY I: computational tools in reproductomics

In **Study I**, a contextualisation of the computational tools for the analysis of omics data in reproduction was presented. First, a brief overview emphasising the impact of the development of omics technologies was summarised, with special focus on the reproductive field. Next, the analysis of complex diseases under systems biology approaches was described exposing how they were applied to the study of endometriosis, where data mining and correlation techniques have been useful to determine the role of certain genes in the aetiopathogenesis of the disease. One the most representative strategies in omics data analyses is the meta-analysis. Although it will be described in depth in **Study III**, through the revision in **Study I** we aimed to bring to light the main studies using meta-analysis as an approach to unravel the complexity of both endometrial receptivity and endometriosis, with high impact studies from which important aspects that led to a better understanding of these phenomena were derived from. Also, in other section of the chapter, and continuing with the contextualisation, systems biology and systems medicine studies of the reproductive field were highlighted. The first part of the chapter concluded with major advances in interactomics, which was presented as an interesting area of discovery in reproduction, as implantation process is known to harbour important molecular interactions required for the successful completion of the process.

Further, the most technical part of the chapter concerning computational tools of analysis for omics in reproduction was divided into sections that could be summarised as: databases, computational tools, and a presentation of an orthodox protocol of analysis with the main steps involved.

Finally, to integrate the two first parts of the chapter, a section composed by two examples of analysis was presented: in the first one, entitled "*Microarray data analysis of female infertility due to PCOS*", a typical workflow of analysis for transcriptomic data was presented, showing its main steps: 1) preprocessing, 2) normalisation, 3)

exploratory analysis, 4) differential gene expression, and 5) enrichment analysis. In the second approach, entitled "*Network construction applied to the study of male infertility due to azoospermia*" STRING is used to obtain PPI among the genes that have been predicted in association to azoospermia via Phenopedia database. This information was subsequently analysed through Cytoscape to perform: 1) a classical network approach in which topology parameters were calculated to determine the importance of the nodes in the system, and 2) an alternative analysis using GeneMANIA plugin to predict the function of the gene set. The aim of these examples was to provide the reader with an overview of feasible approaches using public databases with low computational cost that could be useful to apply to complement current research strategies.

In summary, **Study I** serves as an introductory part to this thesis project, as much of the software presented is used as a tool of analysis in the subsequent studies integrating this book with the aim to guide students, scientists and clinicians in the field of reproduction in understanding and performing omics studies.

STUDY II: cross-disorder comparative analysis of endometriosis and its comorbidities

Women with endometriosis are more likely to possess comorbidity with other chronic conditions than women without endometriosis (Zondervan *et al*., 2020). In **Study II**, a cross-disorder analysis was developed to gather information about endometriosis through the study of its comorbid conditions, i.e., clinical conditions that often coexist with endometriosis. For that purpose, a systematic review of the literature was carried out to extract the most significant papers to date reporting the comorbidity between endometriosis and some other disorders. After the assessment for eligibility of 163 full-text articles, 50 studies were suitable for data extraction. After exclusion of duplicates and removal of all potentially non eligible studies, a list of 197 conditions comorbid with

endometriosis was generated. These items were subsequently transformed into MeSH terms, which reduced the list to 99 comorbid conditions (Figure 5).

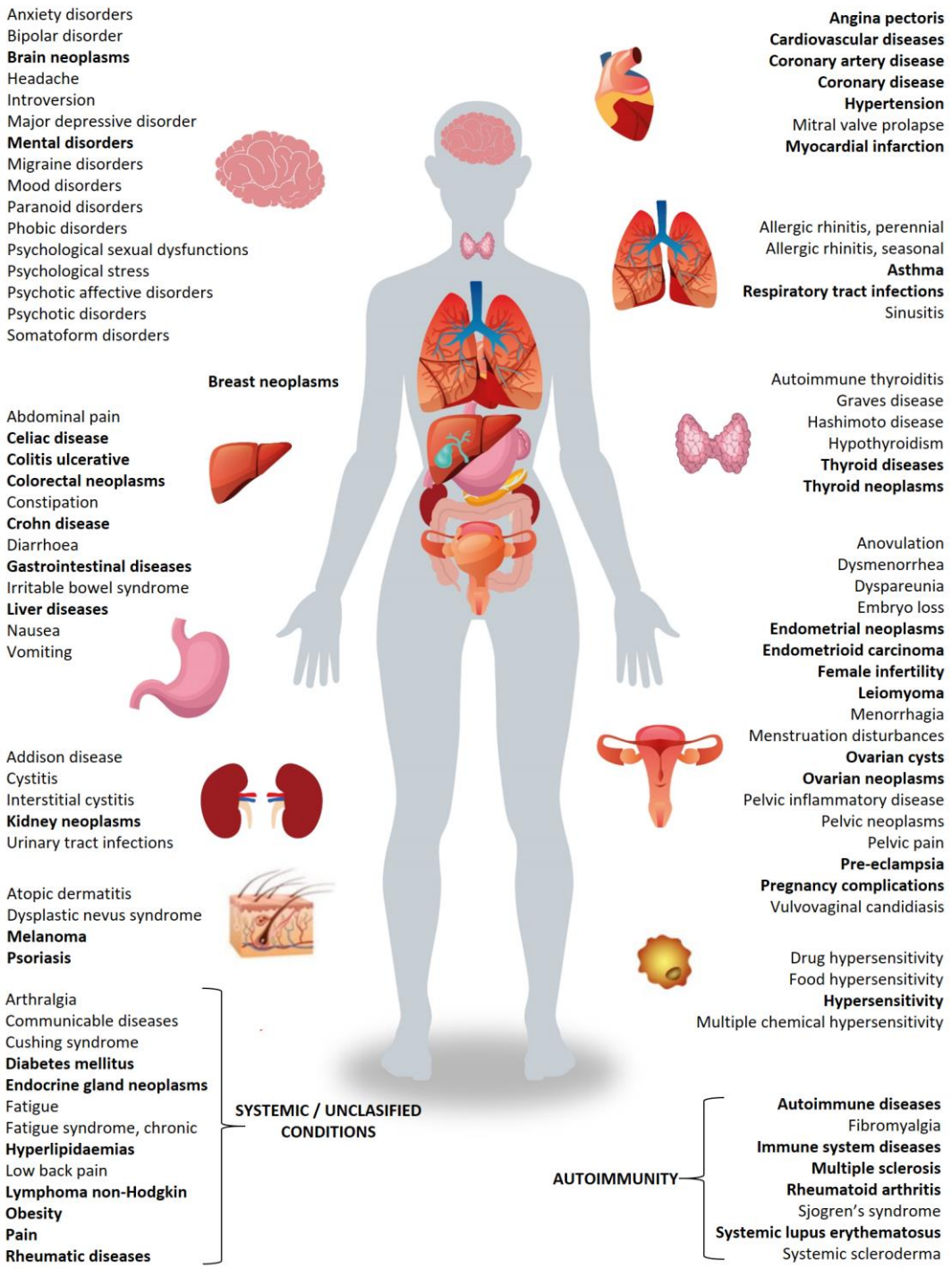

Figure 5. Comprehensive results of the characterisation of endometriosis comorbidities, where 99 disorders are shown. Comorbid diseases are grouped depending on the main organ or system involved. Disorders highlighted in bold correspond to the 43 conditions in the Endometriosis Sibling Disorders (ESD) group. Reproduced with permission (Vargas et al., 2020).

Different studies have reviewed exhaustively the co-existence of endometriosis with other common conditions such as autoimmune diseases (Eisenberg *et al*., 2012; Kvaskoff *et al*., 2015; Shigesi *et al*., 2019), allergies and asthma (Kvaskoff *et al*., 2015; Matalliotakis *et al*., 2012), some types of cancer (Gemmill *et al*., 2010; Kvaskoff *et al*., 2015), mental health problems (Laganà *et al*., 2017; Pope *et al*., 2015; Vannuccini *et al*., 2018), gastrointestinal problems (Parazzini *et al*., 2017), cardiovascular diseases (Kvaskoff *et al*., 2015; Parazzini *et al*., 2017; Teng *et al*., 2016) and gynaecological conditions (Pan *et al*., 2017; Tanmahasamut *et al*., 2014). However, the authors usually focus on a group of diseases with a common basis (i.e., affecting the same organ or system). To the best of our knowledge, **Study II** constitutes the most comprehensive revision of the literature covering the gap in the integration of all the spectra of diseases described in comorbidity with endometriosis into a unique list. The initial hypothesis set in our study was that the coexistence of diseases may be explained from the molecular point of view, and that alterations in common molecular roots may lead to the disease state. To search for candidate genes that may be contributing to this disease state, we investigated whether there was any overlap between the genes reported in association with endometriosis and its comorbid diseases. We detected a group of 43 diseases that were close to endometriosis in terms of shared genes and we determined them as Endometriosis Sibling Disorders (ESD) (highlighted in bold in Figure 5). From this reduced group of diseases covering around 12,000 genes in total, we selected a set of 127 genes for further studies (ESD genes), as these were the genes predicted in association with endometriosis according to the epidemiological repository Phenopedia (Yu *et al*., 2010). When these genes were interrogated for Gene Set Enrichment Analysis (GSEA), important hallmarks of endometriosis such as immune response and inflammatory response arose, confirming thereby the dys-regulation of some biological pathways not only in endometriosis but also in the closest comorbid conditions. Next, the ESD genes were subjected to validation through the analysis of nine independent public datasets comparing endometrial eutopic *vs.* ectopic tissues, which led us to identify a

total of 16 genes (*AGTR1*, *BDNF*, *C3*, *CCL2*, *CD40*, *CYP17A1*, *ESR1*, *IGF1*, *IGF2*, *IL10*, *MMP1*, *MMP7*, *MMP9*, *PGR*, *SERPINE1*, and *TIMP2*) as significantly dys-regulated in endometriosis samples (Figure 6). Another strategy of analysis involved the intersection among the ESD genes and the Single Nucleotide Polymorphisms (SNPs) previously described in association with endometriosis according to the data registered in DisGeNET database (Piñero *et al*., 2020). This approach led us to identify a total of 26 genes in the ESD group (*APEX1*, *BDNF*, *CCR2*, *CYP1A1*, *CYP1B1*, *CYP19A1*, *ESR1*, *ESR2*, *FOXP3*, *ICAM1*, *IGF1R*, *IL1A*, *IL10*, *IL16*, *KRAS*, *MMP9*, *NOS3*, *PGR*, *PIK3CA*, *PPARG*, *PTPN22*, *SOD2*, *TNF*, *TP53*, *VEGFA*, and *XRCC1*) as genes harbouring polymorphisms described in association with endometriosis. Interestingly, 5 out of these genes (*BDNF*, *ESR1*, *IL10*, *MMP9*, and *PGR*) overlapped among the two approaches carried out in our study.

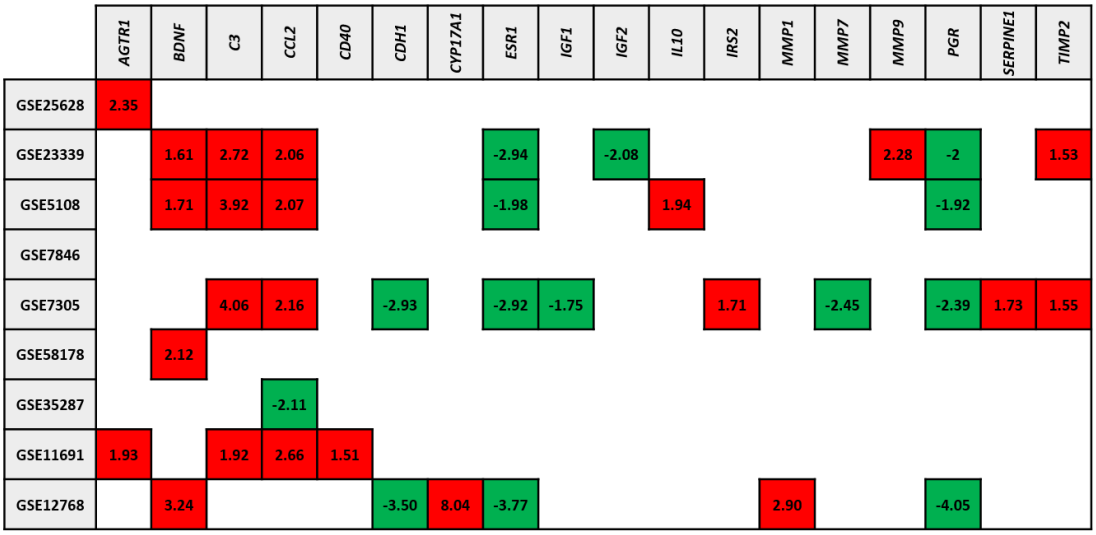

Figure 6. Validation of the 16 genes (columns) significantly dys-regulated (false discovery rate [FDR<0.05]) in endometriosis samples after validation in nine independent datasets. Fold changes (FC) for each gene are shown inside each box. Rows denote the Gene Expression Omnibus (GEO) codes for each dataset. Red cells represent overexpression (FC>1.5), while green cells represent down-regulation (FC<-1.5). Characteristics of the datasets included are presented in Table 3. Reproduced with permission (Vargas et al., 2020).

Brain-derived neurotrophic factor (*BDNF*). Endometriotic lesions exhibited higher levels of the BDNF protein (Browne *et al*., 2012; Wessels *et al*., 2016), while others report that BDNF overexpression in endometriosis is not necessarily an indicative biomarker of the disease state (Perricos *et al*., 2018; Rocha *et al*., 2017). The latest studies in the field, however, pinpoint BDNF serum levels as a useful tool to assess the clinical stage of endometriosis (Liang *et al*., 2020), and with an important potential power as candidate biomarker given its role in uterine physiology (Chow *et al*., 2020).

Oestrogen receptor 1 (*ESR1*). The role of ESR1 in endometriosis has been considered a highly potential target due to its influence in sex steroid hormone pathways. One previous study pointed to genomic regions in or near this gene as harbouring endometriosis risk loci (Sapkota *et al*., 2017). However, a recent rigorous meta-analysis where the only polymorphism selected for study of its potential link with endometriosis was rs2234693, showed a lack of association with the disease (Méar *et al*., 2020) which was confirmed in parallel by other study (Wang L *et al.*, 2020). A recent study assessing the effect of endometriosis risk variants on the expression of genes in the region close to the *ESR1* locus was not able to evidence a direct regulation of the gene by the risk variants, and therefore the necessity of deeper studies identifying the functional effects of these variants on endometriosis risk are needed (Marla *et al*., 2021).

Interleukin 10 (*IL10*). Endometriosis pathogenesis is believed to rely on the dys-regulation of the immune system. Thus, the influence of molecules such as cytokines has been widely postulated. IL10 belongs to this group of molecules, and its levels have been examined in women with endometriosis, exhibiting a differential expression in serum, peritoneal fluid and ectopic lesions (Zhou *et al*., 2019). In fact, some authors have suggested that higher levels of inflammatory factor such as *IL10* might underlie the infertility of the patients with endometriosis (Wang *et al*., 2018).

Matrix Metallopeptidase 9 (*MMP9*). MMP9 is the most studied biomarker of endometriosis among our list of five candidates. The role of metalloproteinases in the

development and pathogenesis of endometriosis has been widely investigated, given their activity in remodelling of the extracellular matrix underlining lesion formation (Luddi *et al*., 2020). In fact, it is believed that the angiogenesis and invasion of ectopic implants in endometriosis might respond to an over-expression of *MMP9* (Arablou *et al*., 2021). Recently, MMP9 levels in the ectopic and eutopic endometrium of women with endometriosis have shown to be significantly higher than those in the samples of patients without endometriosis, suggesting it could act as a possible diagnostic value to predict endometriosis (Barbe *et al*., 2020; Madjid *et al*., 2020).

Progesterone receptor (*PGR*). PGR possible involvement is not surprising given the extensively described progesterone resistance observed in patients with endometriosis (Aghajanova *et al*., 2010; Altmäe *et al*., 2016; Brown *et al*., 2018; Bulun *et al*., 2010; Chen *et al*., 2020; Mousazadeh *et al*., 2019). This fact may be partially explained by a reduction in the expression of the intracellular progesterone receptor in the ectopic endometrium (Vázquez-Martínez *et al*., 2020), which is in line with our findings. Behind the causes leading to this diminished expression, epigenetic mechanisms involving changes in the DNA methylation pattern have been proposed (Meyer *et al*., 2014; Rocha-Junior *et al*., 2019; Wu *et al*., 2006). Moreover, the presence of two polymorphisms in *PGR* that could be associated to endometriosis has been recently described (Méar *et al*., 2020).

In conclusion, comorbidity of endometriosis with 99 disorders was detected, with special relevance of a group of 43 closer diseases in terms of gene expression similarities that we aimed to identify as Endometrial Sibling Disorders (ESD). Further, five potential biomarkers of endometriosis *BDNF*, *ESR1*, *IL10*, *MMP9* and *PGR* were proposed after our *in silico* data mining analyses.

STUDY III: endometrial mid-secretory transcriptome landscape in endometriosis

Women with endometriosis have higher incidence of infertility, with up to 50% reporting difficulties in achieving pregnancy (Navarro, 2019; The Practice Committee of the American Society for Reproductive Medicine, 2012). It is shown that embryo quality can be impaired in women with endometriosis due to the inflammatory environment created by the disease (Simopoulou *et al*., 2021), but also endometrial quality might have its role in the establishment of successful pregnancy as many other studies have shown (Lessey and Young, 2019). It is claimed that women with endometriosis have reduced implantation rates (Blank *et al*., 2021; Cakmak and Taylor, 2011), but whether endometrial receptivity is dys-regulated in endometriosis is an open debate.

For that purpose, in **Study III**, a meta-analysis was performed to investigate whether the endometrial molecular profile during the mid-secretory phase of the menstrual cycle in women with endometriosis differ from women without the disease and could explain the endometriosis-associated infertility. To proceed with data extraction, a systematic review of the literature was carried out. The revision of 130 full-text articles led us to 12 studies assessing the endometrial transcriptome of women with endometriosis and controls at the receptive phase that were selected as suitable for meta-analysis. However, only 8 out of them remained suitable for quantitative analysis due to data unavailability. A total number of 3496 up- and 4283 down-regulated genes was retrieved, which were subjected to the meta-analysis using the Robust Rank Aggregation method.

74 mid-secretory-specific genes were consistently dys-regulated in endometriosis according to the results generated by the RRA method, but no transcript remained significant after False Discovery Rate (FDR) correction. The gene set enrichment analysis detected four biological processes such as regulation of molecular function, chemotaxis, taxis, and locomotion as significantly enriched among women with endometriosis. The role of some of these processes in the etiopathogenesis of

endometriosis has been previously reported. Chemotaxis is modulated in part by the action of oestrogens and progestogens. This pathway has shown to be perturbed in women with endometriosis, contributing to known hallmarks of the disease as inflammatory responses or abnormal tissue remodelling, among others (Reis *et al*., 2013). Another study using mice with induced endometriosis as animal model evaluated the changes in the gene expression of a panel of genes in several regions of the brain. Interestingly, some of the genes that exhibited a significant differential expression were involved in locomotion. All these evidences suggested a possible effect of endometriosis on the brain that may underlie some of the classical clinical manifestations reported in women with the disease (Li T *et al*., 2018).

The impact of endometriosis on endometrial receptivity has been widely discussed (Lessey and Kim, 2017; Miravet-Valenciano *et al*., 2017). It is well known that women with endometriosis often suffer from infertility, however the molecular causes leading to the incapacity of achieving pregnancy remain largely unknown. In parallel, basic research has promoted the development of devices that are able to assess the endometrial receptivity status. These tools are designed under the premise of detecting accurately the moment in which the quality of the endometrium is optimal for successful embryo implantation. The commercially available endometrial receptivity tests developed over the course of the last decade, the Endometrial Receptivity Analysis (ERA ®), the Endometrial Receptivity Map (ER Map ®) (Díaz-Gimeno *et al*., 2011; Enciso *et al*., 2018), and the beREADY® test (based on a meta-analysis by Altmäe *et al*., 2017) were used for further data mining. In brief, we set out to investigate whether the changes in the expression level of the list of meta-analysed genes may highlight receptivity-specific dys-regulated genes in endometriosis. For that, we created a customised list composing of 260 genes based on the three commercial endometrial receptivity tests that we named as Endometrial Receptivity Genes (ERG) and determined the recurrent dys-regulation of these genes among our meta-analysed datasets. Interestingly, we evidenced a recurrent

dys-regulation of 3 genes: *C4BPA*, *MAOA*, and *PAEP* which exhibited significance after FDR correction.

Complement Component 4 Binding Protein Alpha (*C4BPA*). Although there is not much literature reporting its relationship with endometriosis, decreased levels of *C4BPA* have been detected in the endometrium of women with endometriosis, implantation failure and unexplained recurrent abortion during the mid-secretory phase (Altmäe *et al.*, 2017; Herington *et al*., 2016; Kao *et al*., 2003; Tapia *et al.*, 2008), which may suggest a possible influence in the mechanisms leading to successful embryo implantation. Further, its diagnostic utility as candidate target marker in such a well-known comorbidity of endometriosis as ovarian clear carcinoma has been described (Mikami *et al*., 2015).

Monoamine oxidase A (*MAOA*). Literature regarding the possible link between this gene, endometriosis and infertility is scarce. However, in the meta-analysis that gave rise to the development of the beREADY® endometrial receptivity test used in our study and that aimed to identify specific biomarkers of the receptive-phase endometrium, *MAOA* was one of the highest-scored transcripts (Altmäe *et al*., 2017). Further, the recent single-cell transcriptome study characterising the endometrial cells detected the participation of *MAOA* during the abrupt transcriptional activation that takes place during the WOI (Wang W *et al*., 2020).

Progestogen-Associated Endometrial Protein (*PAEP*). *PAEP* has been presented as an important gene shown to take part in endometrial receptivity (Altmäe *et al*., 2017; Herington *et al*., 2016; Kao *et al*., 2003; Oehninger *et al*., 1995; Seppälä *et al*., 1998; Tapia *et al*., 2008; Vargas *et al*., 2012; Wang W *et al*., 2020) and in many aspects of endometriosis (O *et al*., 2018). Altered expression profiles of PAEP in endometriosis have been largely described (Kämäräinen *et al*., 1993; Koninckx *et al*., 1992; Nirgianakis *et al*., 2020), and the relationship among its dys-regulation and the impaired endometrial receptivity observed in some women has been also suggested (Focarelli *et al*., 2018).

The subsequent analysis of these three genes through the search of gene-disease associations using DisGeNET evidenced their implication in multiple diseases (Figure 7). Interestingly, we were able to detect a predicted association between *MAOA* gene and endometriosis. Further, the association between our set of genes and some disorders in which endometrial receptivity is compromised including miscarriage, early pregnancy loss, and spontaneous abortion was revealed.

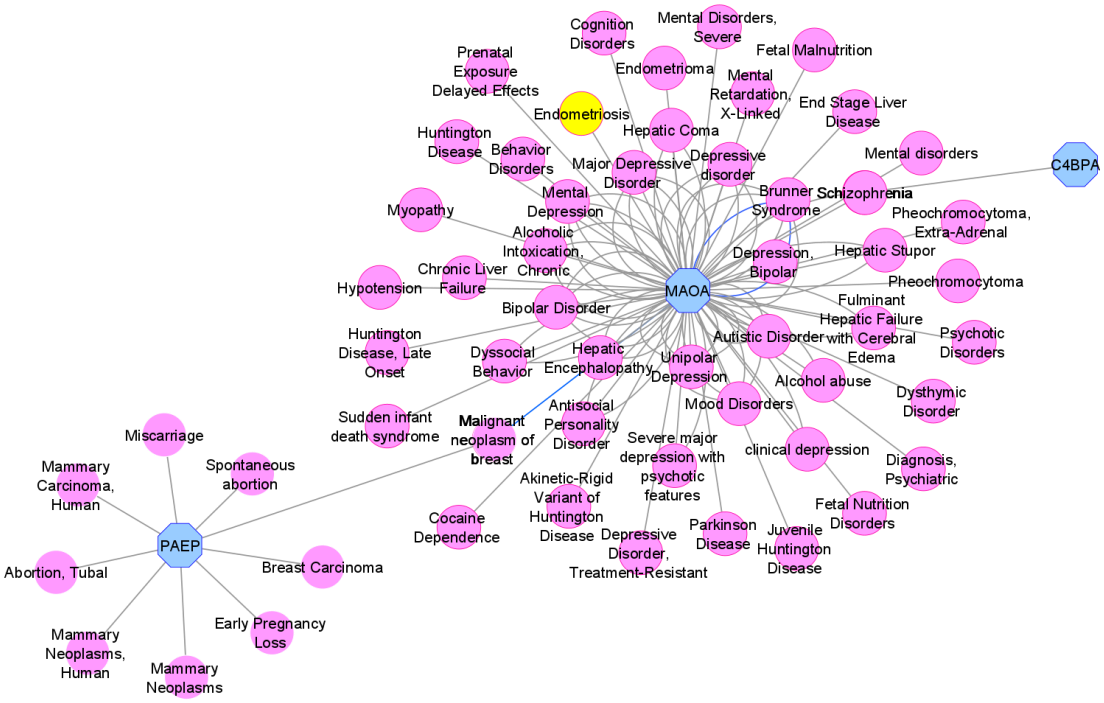

Figure 7. Network showing the evidence of association between our set of genes (blue nodes) and predicted disorders (pink nodes). Endometriosis is highlighted in yellow.

In conclusion, when focussing on genes expressed in the mid-secretory phase of the endometrium, no individual genes were dys-regulated in women with endometriosis, while the transcriptome profile altogether seemed to alter in molecular pathways involved in regulation of molecular function, chemotaxis, taxis, and locomotion. Further, *in silico* data mining focussing on endometrial receptivity genes from commercial endometrial receptivity assessment platforms identified aberrant expression of *C4BPA*, *PAEP* and

MAOA genes, which could serve as future endometrial receptivity-specific markers in women with endometriosis.

5. CONCLUSIONS

The developments in the high-throughput omics technologies have generated a big amount of data whose analysis would give valuable information about the molecular events in complex biological systems as is the human endometrium and its pathologies. 'Endometriomics' (i.e., the application of omics technologies in studies of endometriosis), is an active area of research which is generating a big amount of data that need to be carefully analysed in order to extract biologically relevant conclusions. Altogether, this thesis brings more knowledge into the complex disease of endometriosis by highlighting genes and molecular pathways involved in this common gynaecological disease.

The main findings of the thesis are:

- A summary of the major milestones in reproductomics and guidelines for students, scientists and clinicians for using omics data for *in silico* data mining of a reproductive disorder was presented.
- The systematic review of the literature revealed that women with endometriosis often have other comorbid diseases. In the current thesis, 197 different comorbid conditions occurring together with endometriosis were identified, from which a total of 43 were identified as Endometriosis Sibling Disorders.
- The treatment of the data obtained after the cross-disorder comparative analysis led us to the identification of *BDNF*, *ESR1*, *IL10*, *MMP9*, and *PGR* as potential candidate biomarkers of endometriosis.
- The systematic review of the literature and the subsequent meta-analysis using the robust rank aggregation method led us to conclude that women with endometriosis have dys-regulated endometrial transcriptome profile at the mid-secretory phase when compared to women with no disease.
- Endometrial receptivity-associated genes *C4BPA*, *MAOA*, and *PAEP* could serve as potential endometrial biomarkers in endometriosis-associated infertility.

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CHAPTER I:

COMPUTATIONAL APPROACHES IN REPRODUCTOMICS

Vargas, E., Esteban, F.J., Altmäe, S., 2018. Computational approaches in Reproductomics. In: Reproductomics: The omics revolution and its impact on human reproductive medicine. Horcajadas, J.A., Gosalvez, J. (Eds.). Elsevier Academic Press, 347-383.

Abstract

The development of the omics high-throughput technologies has led to a better understanding of the molecular basis underlying many physiological and pathological processes. However, the large amount of information derived from its application needs to be carefully analyzed in order to extract as much biological meaningful information as possible. For this purpose, several computational tools and methods have been designed to adequately manage the analysis of complex high-throughput omics data. In the current chapter, we provide an overview of the different computational approaches/options for analyzing the generated omics data, present developed databases and analysis tools in the field, and finalize the chapter with an example of analysis application in reproductomics.

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CHAPTER II:

CROSS DISORDER ANALYSIS OF ENDOMETRIOSIS AND ITS COMORBID DISEASES REVEALS SHARED GENES AND MOLECULAR PATHWAYS AND PROPOSES PUTATIVE BIOMARKERS OF ENDOMETRIOSIS

Vargas, E., Aghajanova, L., Gemzell-Danielsson, K., Altmäe, S., Esteban, F.J., 2020. Cross-disorder analysis of endometriosis and its comorbid diseases reveals shared genes and molecular pathways and proposes putative biomarkers of endometriosis. *Reprod. Biomed. Online*. 40(2):305-318.

Abstract

Research question: Women with endometriosis are considered to be at higher risk of several chronic diseases, such as autoimmune disorders, gynaecological cancers, asthma/atopic diseases and cardiovascular and inflammatory bowel diseases. Could the study of endometriosis-associated comorbidities help to identify potential biomarkers and target pathways of endometriosis?

Design: A systematic review was performed to identify all possible endometriosis-associated comorbid conditions. Next, this list of disorders was coded into MeSH terms, and the gene expression profiles were downloaded from the Phenopedia database and subsequently analysed following a systems biology approach.

Results: The results identified a group of 127 candidate genes that were recurrently expressed in endometriosis and its closest comorbidities and that were defined as 'endometriosis sibling disorders' (ESD). The enrichment analysis showed that these candidate genes are principally involved in immune and drug responses, hormone metabolism and cell proliferation, which are well-known hallmarks of endometriosis. The expression of ESD genes was then validated on independent sample cohorts (n = 207 samples), in which the involvement of 16 genes (AGTR1, BDNF, C3, CCL2, CD40, CYP17A1, ESR1, IGF1, IGF2, IL10, MMP1, MMP7, MMP9, PGR, SERPINE1 and TIMP2) in endometriosis was confirmed. Several of these genes harbour polymorphisms that associate to either endometriosis or its comorbid conditions.

Conclusions: The study results highlight the molecular processes underlying the aetiopathogenesis of endometriosis and its comorbid conditions, and identify putative endometriosis biomarkers.

Keywords: Biomarker; Comorbidity; Endometriosis; Gene set enrichment; Systems biology.

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CHAPTER III:

THE MID-SECRETORY ENDOMETRIAL LANDSCAPE IN ENDOMETRIOSIS: A META-ANALYSIS

Vargas, E., García-Moreno, E., Aghajanova, L., Salumets, A., Horcajadas, J.A., Esteban, F.J., Altmäe, S., 2022. The mid-secretory endometrial transcriptomic landscape in endometriosis: a meta-analysis. *Hum. Reprod. Open*, 2022(2):hoac016.

Abstract

Study question: Do women with endometriosis have a different endometrial gene expression profile at the time of embryo implantation than women without endometriosis?

Summary answer: The endometrial gene expression profile of women with endometriosis differs from that of women without endometriosis at the mid-secretory phase, although the differences are small.

What is known already: About 50% of women with endometriosis suffer infertility. Several molecular studies have suggested impaired endometrial receptivity in women with endometriosis, while others have detected no dysregulation of endometrial receptivity. Nevertheless, the previous endometrial transcriptome studies comparing women with and without endometriosis have been performed in small sample size with limited statistical power. We set out to systematically search and compile data of endometrial gene expression signatures at the receptive phase in women with endometriosis versus control women. Based on the obtained data, we conducted a meta-analysis of differentially expressed genes in order to raise the power of the analysis for identifying the molecular profiles of receptive phase endometria in endometriosis.

Study design size duration: A systematic literature search was conducted up to February 2022 following PRISMA criteria and included PubMed, Cochrane and Web of Science databases. For the systematic search, the term 'endometriosis' was paired with the terms 'transcriptomics', 'transcriptome', 'gene expression', 'RNA-seq', 'sequencing' and 'array', by using the Boolean operator 'AND' to connect them. Articles written in English were screened and interrogated for data extraction.

Participants/materials setting methods: A meta-analysis was performed on the selected studies to extract the differentially expressed genes described at the mid-secretory phase in women with endometriosis versus women without endometriosis in natural cycles, using the robust rank aggregation method. In total, transcriptome data of 125 women (78 patients and 47 controls) were meta-analysed, with a special focus on endometrial receptivity-specific genes based on commercial endometrial receptivity tests.

Main results and the role of chance: In total, 8 studies were eligible for the quantitative meta-analysis, gathering transcriptome data from the mid-secretory phase endometria of 125 women. A total of 7779 differentially expressed transcripts between the study groups were retrieved (3496 up-regulated and 4283 down-regulated) and were meta-analysed. After stringent multiple correction, there was no differential expression of any single molecule in the endometrium of women with endometriosis versus controls, while enrichment analysis detected that the pathways of chemotaxis and locomotion are dysregulated in endometriosis. Further analysis of endometrial receptivity-specific genes highlighted dysregulation of C4BPA, MAOA and PAEP and enrichment of immune and defence pathways in women with endometriosis.

Limitations reasons for caution: Most of the studies included into the meta-analysis were relatively small and had different study designs, which might have contributed to a bias.

Wider implications of the findings: The current meta-analysis supports the hypothesis that endometrial receptivity is altered in women with endometriosis, although the changes are small. The molecules and pathways identified could serve as future biomarkers and therapeutical targets in detecting and treating endometriosis-associated infertility.

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